Journal of Global Ecology and Environment

Volume 20, Issue 4, Page 169-179, 2024; Article no.JOGEE.12518
ISSN: 2454-2644

Utilizing *Tetradesmus obliquus* for Phycoremediation Enhanced Nitrogen and Phosphorus Removal in Urban Wastewater Treatment

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: https://doi.org/10.56557/jogee/2024/v20i48955

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://prh.ikprress.org/review-history/12518

Original Research Article

Received: 14/09/2024
Accepted: 16/11/2024
Published: 22/11/2024

ABSTRACT

Microalgae can scavenge pollutants through a process called phycoremediation, which involves the removal or biotransformation of pollutants in wastewater and gaseous media. Algae must grow rapidly, tolerate seasonal and diurnal variations, and form aggregates easily. In this study, the phosphorus and nitrogen removal capacity of the microalga *Tetradesmus obliquus*, isolated from urban water bodies, was evaluated and its cultivation was proposed as an alternative for tertiary treatment of urban effluents. The inoculum was placed in a bioreactor at 25 ºC with natural lighting for 21 days in modified Bold Basal Medium (BBM). The experiment included a control group (BBM) and a treatment with distilled water (AD) and increasing concentrations of urban effluent (12.5%,

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Cite as: *Dana, Muñoz Carabajal, Daruich, Griselda Jorgelina, and Pedranzani, Hilda Elizabeth. 2024. "Utilizing Tetradesmus Obliquus for Phycoremediation Enhanced Nitrogen and Phosphorus Removal in Urban Wastewater Treatment". Journal of Global Ecology and Environment 20 (4):169-79. https://doi.org/10.56557/jogee/2024/v20i48955.*

25%, 50%, and 100%), inoculated with 8.8x10^5 cells/ml of biomass. The treatments were carried out in triplicate for 15 days. pH, dissolved oxygen (DO), conductivity, NO^{3-} and PO_4^{-3} concentrations, and biological variables such as cell count and optical density were determined. *Tetradesmus obliquus* showed efficiency in removing phosphates and nitrates. Maximum efficiency was shown after 15 days of treatment with 100% effluent, removing 22.92% of phosphates and 65.66% of nitrates.

Keywords: Microalgae; phycoremediation; wastewater.

1. INTRODUCTION

Phytoplankton belong to micoalgae, a diverse community of organisms that live suspended in a body of water. Most microalgae are photosynthetic and unicellular, and represent the key producing community in aquatic ecosystems as they support the upper trophic levels [1]. Seasonal changes in the availability of nutrients, especially nitrogen and phosphorus, can increase primary productivity, but also alter the structure of the phytoplankton community, leading one species to dominance by exclusion of the others [2].

Urban development and expansion, industrial activities, agriculture, livestock farming, etc., generate effluents and wastewater that require proper management. When discharged into natural bodies of water, they generate problems of contamination and/or eutrophication, affecting the natural purification processes of these aquatic ecosystems with loss of biodiversity and destruction of these ecosystems [3,4,5]. Eutrophication is a process of deterioration in the quality of water bodies. It is caused by the enrichment of nutrients, mainly nitrogen and phosphorus, generating major ecological, health and economic impacts on a regional scale [6].

The microalgae species used in phycoremediation must meet certain conditions: they must have a high growth rate, high tolerance to seasonal and diurnal variations, and a good capacity to form aggregates for later separation [7]. Some algae suitable for removal belong to the genera *Scenedesmus* sp., *Chlorella* sp., *Chlamydomonas* sp. and *Desmodesmus* sp. [8,9]. Another advantage of using microalgae for wastewater remediation is that the biomass produced contains high-value molecules that can be used in different industrial fields, such as biofuels, fodder, fertilizers, etc. [10].

Tetradesmus obliquus is a green algae species of the family Scenedesmaceae. It is commonly known by its synonym, *Scenedesmus obliquus*. It is a common species found in a variety of freshwater habitats [11,12]. *Tetradesmus obliquus* forms colonies of two or four (occasionally eight) cells in a single row; in culture, solitary cells are often present. Cells are spindle-shaped, (4–)6–15(–25) µm long and 2.2–9.6(–11) µm wide; cells taper to acute apices and are sometimes slightly asymmetrical [13]. Cells contain a single chloroplast filling the cell, with a pyrenoid present in the center. The objective of the present study was to evaluate the nitrogen and phosphorus removal capacity of the native microalga *Tetradesmus obliquus* cultivated in urban effluents.

2. MATERIALS AND METHODS

2.1. Sampling and Isolation of Microalgae

The sample was obtained from an artificial pond with a phytoplankton net, 10µm mesh size. In order to separate algal populations standard plating methods were used with media BBM (Bold Basal Medium) segúnel CPCC (Canadian Phycological culture centre) added with 1.5% agar-agar, and incubated at 25 ± 2°C in a humid chamber with continuous illumination of ~3000 lux for 16 days. Following the isolation, the individual colonies were transferred to liquid media and incubated in the conditions before mentioned. The cell count was determined using a haemocytometer (Neubauer). Then, inocula of 3,4x10^4 cell/ml were transferred to batch liquid cultures that were hand shaken twice daily.

2.2 Obtaining Biomass for the Tests

To obtain biomass, a bioreactor was designed consisting of a 2 L Erlenmeyer connected to a Rs Electrical Rs 16000 brand aerator for air injection. This system was maintained for 21 days at a temperature of 25 ºC ±1 ºC and natural lighting, with the addition of 150 mL distilled water and 150 mL algal medium until obtaining 2L of unialgal culture. BBM medium was prepared following the

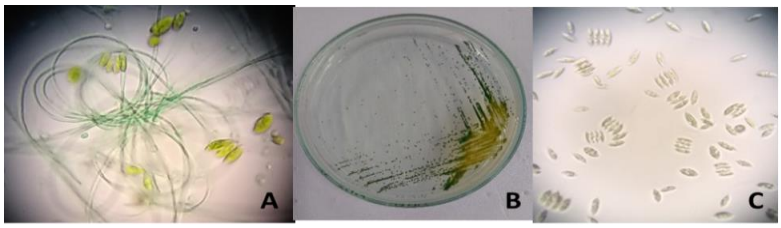

Fig. 1. A: artificial pond sample, B: solid medium isolation, C. isolation unialgae*T. obliquus*

CPCC (Canadian Phycological Culture Centre). Modified BBM contains (g/L): NaNO3 (0.25 g), CaCl2 (0.025 g), MgSO4 (0.035 g), K2HPO4 (0.075 g), NaCl (0.025 g), KH2PO4 (0.175 g), KOH (0.031 g), EDTA (0.05 g), FeSO4.7H2O (0.0049 g), H3BO3 (0.01142 g), ZnSO4 (0.00495 g), MnCl2.4H2O (0.00144 g), MoO3 (0.00071 g), CuSO4.5H2O (0.00157 g), CoCl2.6H2O (0.00049 g), H2SO4 (0.0001 mL). The CPCC says Trace Metal Solution 0.075 but it was changed to 0.035.

2.3 Variables Were Determined in the Wastewater

2.3.1 pH:

A potentiometric method was used based on measuring the potential difference between a glass membrane indicator electrode sensitive to the concentration of H+ and a reference electrode (calomel or Ag, AgCl) immersed in the same solution. This physical-chemical variable was determined using a combined glass electrode brand ORION and a portable potentiometer brand ORION Model 290 A. The electrode was calibrated using pH buffer solutions of 4, 7 and 9, reference solutions established by IUPAC (International Union of Pure and Applied Chemistry) and NIST (National Institute of Standards and Technology).

2.3.2 Conductivity

(µS/cm): An electroanalytical method was used. Conductivity was measured with an OAKTON conductivity meter, which consists of a platinized platinum parallel plate conductometric cell with a built-in temperature compensator. The conductivity cell was calibrated with a KCl solution of the exact concentration. It was expressed in µS cm^{-1}.

2.3.3 Biochemical oxygen demand - BOD5 (mg/L O2)

It was determined by aerating a sample 20 times and filling two airtight Winkler flasks with it. To the first flask was added 1 mL of SO_2Mn_4 and shaken, then 1 mL of alkali-iodide-azide reagent and shaken, then 1 mL of SO4H2 to dissolve the precipitate and shake. A 200 mL aliquot of sample was taken, placed in an Erlenmeyer flask and titrated with 0.025N $Na_2S_2O_3$until pale yellow coloration, then 1 mL of starch was added and titration continued until the blue color disappeared. The second flask was incubated at a set temperature for 5 days and then the same procedure was performed for the determination of final dissolved oxygen. The difference in measurement between the dissolved oxygen concentration before and after the incubation period is known as biological oxygen demand. It was expressed in mg L-1 of O2.

2.3.4 Dissolved Oxygen - DO (mg/L O2)

DO was determined by an electrometric method using a membrane electrode. The electrometric method is based on the diffusion of molecular oxygen through an oxygen-permeable plastic membrane, which covers the sensitive element of an electrode and acts at the same time as a diffusion barrier against many impurities that interfere with other methods for determining DO. Under regular conditions, the “diffusion current” is linear and directly proportional to the DO concentration. It was expressed in mg/L of O_2.

2.3.5 Nitrate - NO_3^- (mg/L)

It was determined spectrometrically, using a cadmium salt that reduced the nitrates in the sample to nitrites; this nitrite ion reacted in an acidic medium with $C_6H_7NO_3$Sto form a diazonium salt (R−N2+X–) as an intermediate product. This salt was coupled to gentisic acid to give an amber-colored product, the intensity of which was measured in a HACH DR/2000 spectrophotometer at a wavelength of 400 nm. It was expressed in mg L-1 of NO_3^-

2.3.6 Phosphate - PO_3^- (mg/L)

In this method, ammonium molybdate and potassium antimony tartrate react in an acidic medium with orthophosphate to form an

ammonium phosphomolybdate complex. This complex is reduced by ascorbic acid to an intensely colored molybdenum blue complex with a maximum absorbance at a wavelength of 880 nm, which is proportional to the concentration of phosphorus present. The absorbance values were measured in a HACH DR/2000 spectrophotometer and the values obtained were compared with a calibration curve prepared with absorbances of phosphate solutions of known concentrations. It was expressed in mg L-1 of P.

2.4 Experimental Design

An experimental design was carried out with a control group (BBM), a group with distilled water (AD) and treatments with different increasing concentrations of urban effluent 12.5%, 25%, 50% and 100%, which were inoculated with 125 mL of unialgal culture, with a concentration of 8.83 x 10^5 ± 0.5 x 10^5 cells/ml. Controls and treatments were carried out in triplicate and incubated for 15 days, according to [7, 8]. The tests were carried out in open containers in the laboratory, with natural lighting, in the absence of sterility (non-axenic) and daily agitation with an orbital shaker for 2 hours [7]. At 0, 5, 10 and 15 days, samples were taken and the physicochemical variables were determined: pH, dissolved oxygen (DO), conductivity, phosphorus and nitrate concentration, using the same techniques used to characterize the effluent.

2.5 Qualitative and Quantitative Analysis of microalgae

Qualitative analysis was carried out through direct observation of the treatments, with photographic follow-up and description of what was observed with the naked eye according to Lucero [7]. Quantitative analysis included counting cells on 1, 5, 10 and 15 days using a haemocytometer (Neubauer) (Fig 3) and measuring the optical density of the culture at 580 nm in a spectrophotometer.

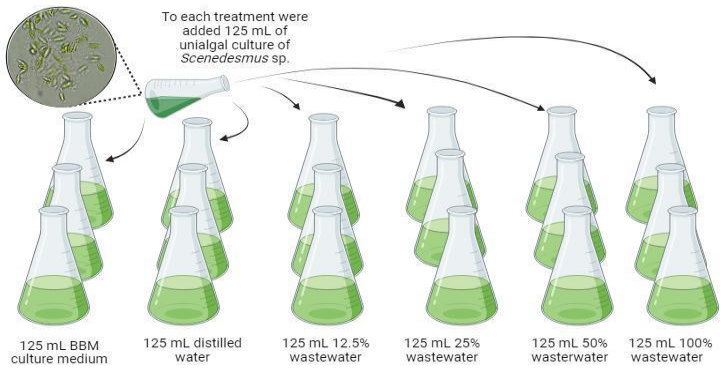

Fig. 2. This figure shows the experimental design. It consisted of 4 dilutions of the residual water: 12.5%; 25%; 50% and 100% plus a control with distilled water and another with culture medium. 125 mL, in triplicate. 125 mL of unialgal culture of *Tetradesmus obliquus* were added to all treatments and controls. The trial continued for 15 days

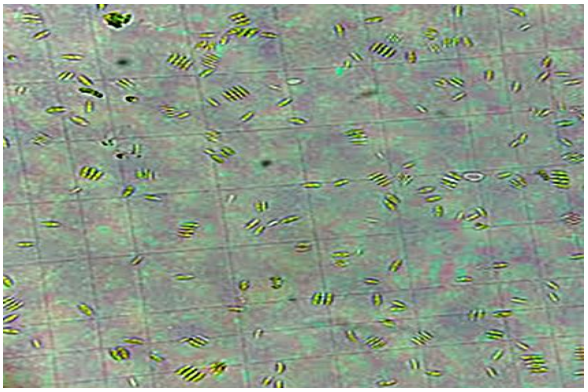

Fig. 3. Neubauer chamber count of *T. obliquus*

2.6 Nutrient Removal Efficiency Analysis

The initial concentration was taken on the day before phycoremediation and the final concentrations were taken after 15 days. The percentage removal efficiency of the algae was calculated using the formula:

$$\% \, Removal = \frac{(C_0 - C_f)}{C_0} \times 100$$

Where C_0= initial concentration, Cf =final concentration

3. RESULTS

3.1 Dissolved Oxygen (DO), pH and Conductivity in Culture Medium with Different Percentage of Effluents

Fig. 4 shows a decrease in dissolved oxygen (DO) concentration between the first day and the fifth day after the start of the treatment. From this day on, the DO starts to increase due to the photosynthetic activity of the algae. The culture media that showed the highest DO were those with 50% effluent that reached 12.8 mg/L O2 and 100% effluent that reached 14.08 mg/LO2.

Fig. 5 shows an increase in pH from day 5 of all culture media with different proportions of effluent. The pH also showed higher values in the culture media that had 50 and 100% effluent, whose values were 9.17 and 9.28.

Fig. 6 shows the variation in conductivity over time. A decrease in conductivity is observed in the 100% treatment. The conductivity levels for the culture media with 12.5, 25 and 50 % effluent remained between 275 µS/cm and 354 µS/cm over time. In the 100% treatment, the conductivity decreased from 505 µS/cm to 402 µS/cm at the end of the test.

3.2 Nitrate and Phosphate Values in Culture Media with Different Effluent Proportions

Fig. 7 shows the variation in nitrate concentration throughout the test. The trend shows that the culture medium with 100% and 50% effluent decreased by 60% compared to the media without effluent after 15 days of culture. Fig. 5 b shows that the phosphorus content does not vary with any percentage of effluent over 15 days of measurements.

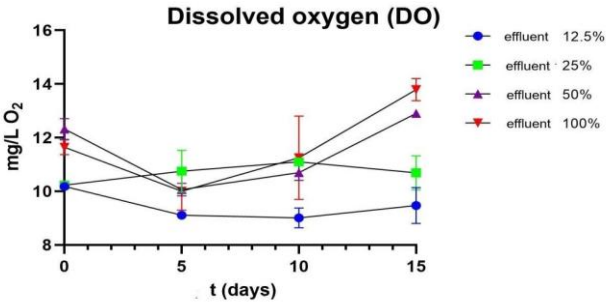

Fig. 4. Variation of the DO over time in the media with effluent concentrations of 12.5%, 25%, 50%, 100%

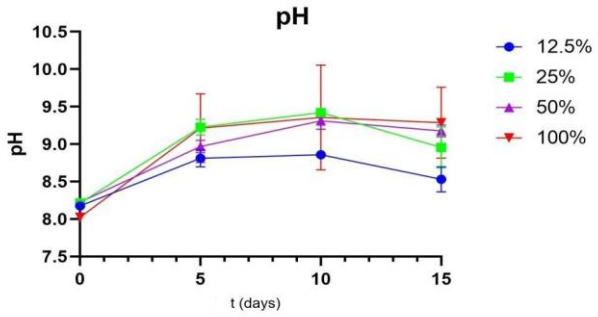

Fig. 5. Variation of the pH of the algae culture, over time in the media with effluent concentrations of 12.5%, 25%, 50%, 100%

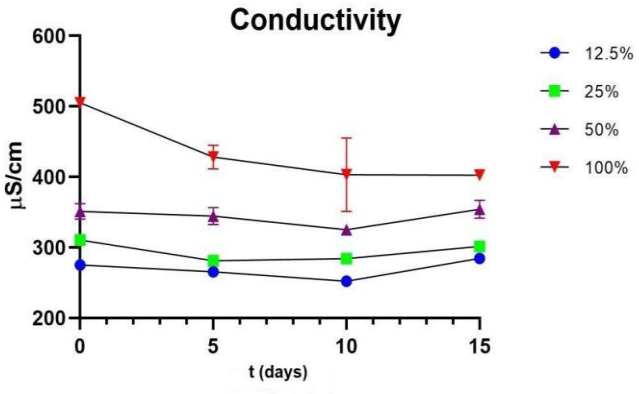

Fig. 6. Variation of the conductivity of the algae culture, over time in the media with effluent concentrations of 12.5%, 25%, 50%, 100%

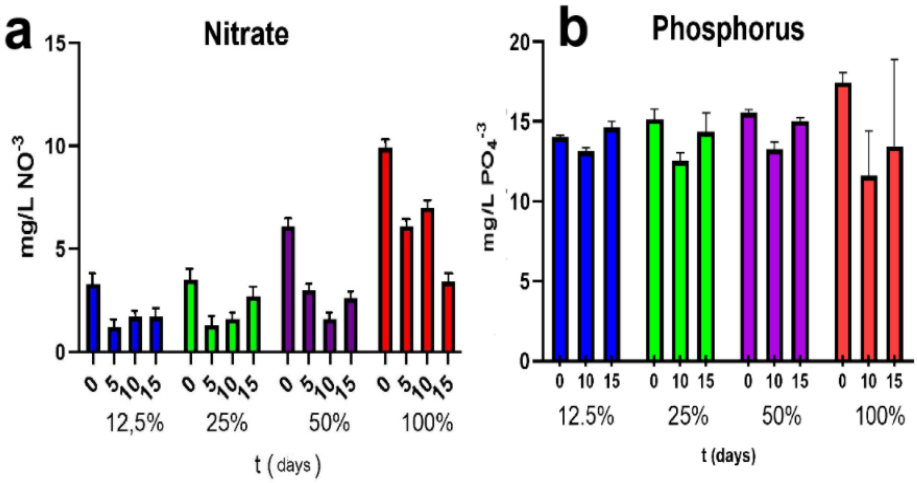

Fig. 7. Variation of the Nitrate (a) and Phosphorus (b) of the algae culture, over time in the media with effluent concentrations of 12.5%, 25%, 50%, 100%

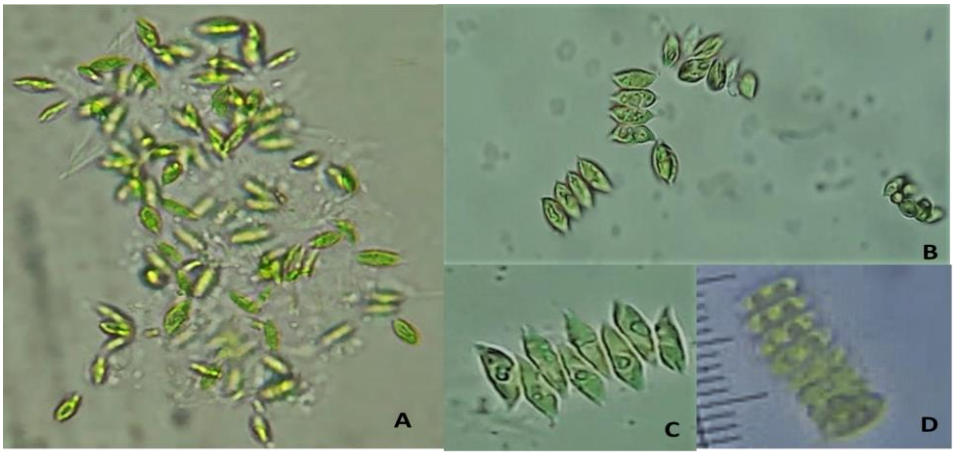

Fig. 8. A-B. Macroscopic and microscopic monitoring of algal growth in the culture medium with different effluent concentrations; B: A Unicells on day 0.B Cenobia of 4 cells on day 15. C Cenobium of 8 cells in 25% treatment day 10 and D Cenobium of 8 cells in 100% treatment day 15

3.3 Qualitative and Quantitative Analysis of Microalgae

Fig. 8 shows microscopic monitoring of the different treatments and controls over time. A: Al inicio de todos los tratamientos, las algas se encontraban como unicélulas. B: Cenobios de 4 células encontrados a partir del día 5 (tratamiento 25%). C: Cenobio de 8 células encontrado en el tratamiento 25% en el día 10. D: cenobio de 8 células encontrado en el tratamieto 100% en el día 15.

Fig. 9 (a) shows the trend of the OD 580 nm increase over the 15 days measured and Fig. 9 (b) illustrates the variation of the number of cells over time for each treatment. In all treatments, an increase in the number of cells/mL was observed as the effluent concentration increased. In those with a higher concentration of nutrients (50% and 100%) a greater amount of biomass was observed towards the end of the test coinciding with the cell count, the trend shows an increase in the amount of biomass over time. The microalgae quickly adapted to the change in nutrient concentrations in the different treatments, suggesting its ability to use organic and inorganic compounds present in the effluent as a nutritional substrate.

3.4 Analysis of Nutrient Removal Efficiency (phycoremediation)

Fig. 10 a shows the percentages of nitrate removal by *T. obliquus in* the different treatments. On the 10th day of sampling, NO_3^- removal increased from 48.48% with 12.5% effluent to 54.28% with 25% effluent, reaching a maximum removal of 73.77% with 50% effluent. When there was 100% effluent, NO_3^-removal decreased to a percentage of 29.29%. On the 15th day of experiment, with 12.5% effluent, there was a removal percentage of 48.48, then it decreased when there was 25% effluent and with 50% effluent it reached 57.57% removal and reached its maximum percentage of NO_3^-removal

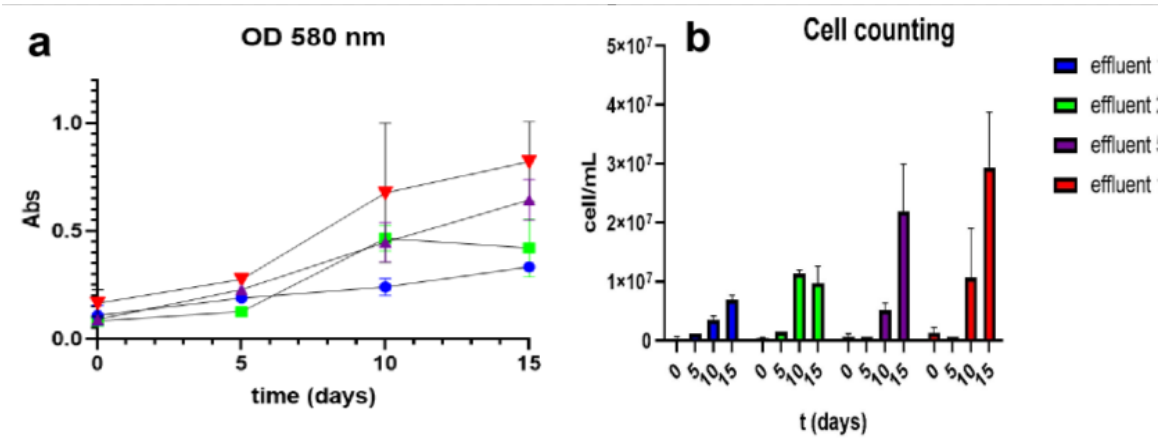

Fig. 9. Algal growth is measured by optical density (a) and by cell quantity/mL over time in different media with effluent proportions of 12.5%, 25%, 50% and 100% (b).

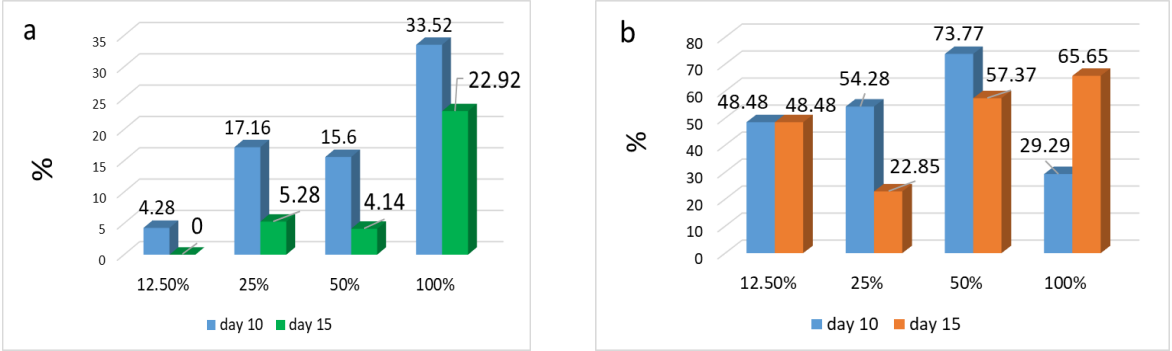

Fig. 10. Percentage of removal of PO_4^{3-} (a) and NO_3^- (b) of the algae culture, over 10 and 15 days in the media with effluent concentration

(65.65), with 100% effluent. Fig. 10 b shows the percentages of phosphate removal by *T.obliqus* in different treatments. On the 10th day of sampling, PO_4^{-3} removal increased from 4,28 % with 12.5% effluent to 17,16% with 25% effluent, reaching a maximum removal of 33,52% with 100% effluent. On the 15th day of sampling, with 12.5% effluent, there was a removal minimum, then it increased to 5,28% with 25% effluent and 4,1% with 50% effluent, it reached at 22,92%PO_4^{-3}removal with 100% effluent**s** of 12.5%, 25%, 50%, 100%.

4. DISCUSSION AND CONCLUSION

The photosynthetic activity of the algae variety throughout the test, in the first days it decreased associated with the decrease in DO concentration, Some authors say that the decrease in the concentration of DO is due to consumption by bacteria present in the effluent and from the fifth day onwards, it increased. [9], had similar results and considered that the decrease in photosynthetic activity is due to consumption by heterotrophic microorganisms (mainly aerobic bacteria).

The increase in the number of cell density and the optical density (OD) was associated with the increase in biomass in all treatments, especially those with the highest nutrient concentration (50% and 100%). The microalgae quickly adapted to the change in nutrient concentrations, suggesting its ability to use organic and inorganic compounds present in the effluent as nutritional substrate. A relevant characteristic during nutrient uptake and photosynthetic CO2 fixation in microalgae cultivation is that the process tends to increase the pH of the medium [14], where alkaline values have been indicated to have the highest photosynthetic activity [15]. The considerable increase in pH observed in our experience could be largely explained by the consumption of HCO3– ions, whose dissociation to CO2 provides the carbon necessary for the growth of microalgae, while also promoting the accumulation of OH– which causes a gradual increase in pH due to the following reaction [16].

The removal of phosphate is associated with NO_3^-removal through their respective roles in cellular metabolism [17]. Nitrogen is the second most important nutrient for microalgae after carbon, accounting for 10% of microalgae biomass [18]. Phosphorus is another essential macronutrient for microalgae growth, which is assimilated as PO4-3. Treatments with higher nutrient concentrations (50% and 100%) did not show loss of green pigments (chlorosis). It has been reported that phosphorus deficiency causes a decrease in the synthesis of nucleic acids, ATP and chlorophyll; under nitrogen deficiency conditions, the content of photosynthetic pigments and the rate of photosynthesis are reduced [19]. The low availability of these nutrients could be causing chlorosis in treatments with lower effluent concentration (12.5% and 25%).

As they grow, microalgae consume carbon and nitrogen from the culture medium, thus modifying the pH, increasing it to values of 10 and above. High pH values adversely affect microalgae, limiting their performance and their growth rate, and therefore their capacity to remove contaminants from the medium. The injection of flu glasses, or other residual gasses, is a good alternative [20,21]; nonetheless, this has to be performed carefully to avoid inefficient CO_2 use, leading to subsequent CO_2 release into the environment [22]. The best strategy for supplying CO_2 into microalgae cultures is by on-demand injection of flu gasses, the higher the flue gas CO_2 content, the lower the gas flow required; indeed, no adverse effects were observed in microalgae cultures even when using pure CO_2 [23].

Sánchez [23] observed the phenomenon known as “chlorosis” in cyanobacteria in which the microalgae changed from their characteristic blue-green color to a greenish-yellow in culture media where nutrient limitation was very high from the beginning of the culture. It is also important to note that microalgae pigments exhibit variations that provide information about changes that occur in response to environmental stress and physiological conditions. Therefore, pigment content can be used as a bioindicator of the environmental conditions to which these microalgal communities are exposed [24].

In the present investigation, *Tetradesmus obliquus* obtained the highest nitrate removal (73.77%) in the 50% effluent treatment after 10 days of testing. The highest phosphorus removal (33.52%) was obtained in the 100% treatment after 10 days of testing associated with big biomass, coinciding with [25], who studied the growth kinetics of three strains of *Scenedesmus* sp, they observed the highest microalgal biomass. Among the stages of preliminary, primary and secondary treatment in wastewater, secondary effluents are considered the most

suitable medium for the cultivation of microalgae due to their lower concentration of heavy metals, organic carbon and competition for nutrients with the bacterial load [26].

Roa [27] *Scenedesmus incrassatulus* immobilized in calcium alginate as a new technological alternative for removal. On day 8, a removal of 60% of the initial amount of nitrates was achieved, while phosphates decreased by 47%. In our research, *Tetradesmus obliquus* showed optimal growth when 100% effluents were added, for this reason it can be suggested to replace the conventional BBM medium with urban effluents. [28] agree that the growth of microalgae in wastewater is equivalent to an alternative substrate for economic growth and will allow the production of biomass for biotechnological and industrial purposes. The developments of microalgae-based wastewater treatment processes are to reduce greenhouse gas emissions, improve the energy balance of the process, and recover nutrients. At the same time, however, a positive economic balance also has to be demonstrated in order to convince the end users to adopt these technologies; hence, the valorization of the biomass produced is highly relevant [29].

We concluded that *T. obliquus* showed differential efficiency in the removal of phosphates and nitrates. The highest efficiency was achieved on day 15 in the 100%. Effluent treatment since no water was used for dilution, reducing economic costs, obtaining a removal of 22.92% of phosphates and 65.66% of nitrates. The highest biomass production was obtained in the 100% effluent treatment. The use of the native microalgae *T. obliquus* as a nutrient (N and P) purifying agent in the treatment of domestic effluents is a feasible and promising technological alternative.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

ACKNOWLEDGMENTS

This work was financially supported by the Secretary of Science and Technology, and CONICET. Faculty of Chemistry, Biochemistry and Pharmacy. National University of San Luis. San Luis, Argentina.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
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