Latin American Journal of Aquatic Research, 53(3): 488-502, 2025
DOI: 10.3856/vol53-issue3-fulltext-3341

Research Article

Optimizing aquaculture wastewater treatment by marine microalgae cultivation with a circular bioeconomy approach

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ABSTRACT. The urgency of optimizing wastewater treatment highlights the need for a sustainable circular bioeconomy. One promising strategy involves using microalgae to treat wastewater, producing both clean water and valuable biomass-an approach that minimizes the environmental impact of the aquaculture industry. The culture medium's nitrogen/phosphorus (N/P) ratio plays a crucial role in microalgal productivity and nutrient removal due to species-specific variations in adaptability and cell composition. Since wastewater N/P ratios fluctuate depending on the source, optimizing microalgae-based treatment requires carefully adjusting these nutrient levels. This study investigated the growth of three indigenous marine microalgae strains belonging to two species, *Nannochloris* (NRRE-1) and *Nannochloropsis* (NSRE-1, NSRE-2) in an aquaculture wastewater-based medium under varying N/P ratios (2, 4, 6, 9, 12, 14, 18, and 20). Results indicated that biomass productivity was significantly higher at N/P ratios of 9, 12, and 14 than at lower (2, 4, 6) and higher (16, 18, 20) ratios. The highest recorded biomass productivity (~10 mg L^{-1} d^{-1}) was accompanied by relatively low nitrogen removal (~20%) across all tested conditions. To enhance wastewater treatment efficiency, phosphorus supplementation was applied to achieve specific N/P ratios (9, 12, or 14). This optimization led to a 350-470% increase in biomass productivity and improved nutrient removal efficiency (~90%). These findings suggest that strategic phosphorus supplementation can significantly enhance microalgae-based wastewater treatment in aquaculture systems.

Keywords: wastewater treatment optimization; circular bioeconomy; phosphorus supplementation; indigenous microalgae; N/P ratio

INTRODUCTION

Aquaculture has emerged as the leading sector in global food production, growing at an annual rate of 3.2%. In recent decades, it has become essential for meeting the rising global demand for animal protein, accounting for 67.7% of total fisheries production in 2017 (Nie et al. 2020). However, its rapid expansion has led to significant wastewater generation, causing severe contamination of surrounding water bodies and negatively impacting the environment (Ottinger et al. 2016).

Aquaculture wastewater contains high concentrations of pollutants, including suspended solids, nutrients such as nitrogen (N) and phosphorus (P), chemicals, and organic compounds (Rico & Van den Brink 2014). Additionally, biological activity within aquaculture systems contributes to substantial waste accumulation. To mitigate these environmental impacts, a circular bioeconomy strategy has been proposed.

A circular bioeconomy views waste as a valuable resource, promoting the recovery of biomolecules for

Associate Editor: Walisson de Souza

applications in bioproducts such as cosmetics, pharmaceuticals, biofertilizers, and biofuels. It also supports the development of integrated multiproduct aquaculture systems. This approach revalorizes aquaculture by-products, delivers economic benefits, reduces greenhouse gas emissions, and contributes to climate change mitigation, offering a sustainable path forward for industry (Lange et al. 2021).

Microalgae cultivation in aquaculture wastewater presents a promising circular bioeconomy approach, enabling biomass production without competing for limited freshwater resources while reducing nutrient costs. Additionally, microalgae can sequester atmospheric CO_2 and convert wastewater pollutants into valuable biomass, which can be utilized for fish meal, biofuels, or raw materials for high-value products. However, despite extensive research on microalgae-based wastewater treatment, the commercial viability of microalgal biomass remains uncertain. This challenge stems from limitations in upstream processes, including low nutrient availability in the medium and insufficient biomass productivity (Mutanda et al. 2020).

Furthermore, several techno-economic studies highlight that microalgal cell density (g L^{-1}) is a key factor in determining the feasibility of large-scale microalgae production alongside wastewater treatment. Schipper et al. (2021) reported that a biomass concentration between 4-16 g L^{-1} is necessary to sustain microalgae-based products during cultivation. Therefore, developing an efficient and scalable microalgae cultivation process that effectively treats wastewater is crucial for enhancing the viability of a circular aquaculture economy.

Efficient microalgae biomass cultivation requires sunlight and essential nutrients, including N, P, carbon (C), sulfur (S), potassium (K), and iron (Fe). Among these, N and P are critical as they are primary limiting factors for productivity and must be supplied in appropriate ratios (Villar-Argaiz & Sterner 2002). P is vital in cellular function as a key component of nucleic acids, phospholipids, and phosphorylated sugars. It also regulates protein activity and cellular energy metabolism. Meanwhile, N availability is crucial for microalgae growth, as it supports protein and nucleic acid synthesis and is essential for chlorophyll formation (Maltsev et al. 2021).

The interplay between N and P concentrations in a culture medium is complex, with the N/P ratio playing a crucial role in determining nutrient removal efficiency and microalgae growth. This ratio provides essential insights into cultivation duration for optimal biomass productivity and helps schedule nutrient supplementation or harvesting (Singh et al. 2023). The N/P ratio significantly influences microalgal growth rate, photosynthetic efficiency, biochemical composition, and nutritional value (Åkerström et al. 2014). Numerous studies highlight the importance of maintaining a balanced N/P ratio to enhance biomass production and improve nutrient removal from wastewater. This necessity arises due to species-specific variations in nutrient uptake efficiency, adaptability, and cellular composition.

Additionally, wastewater N/P ratios fluctuate depending on the source and environmental conditions. As a result, recent research has shifted toward optimizing microalgae-based wastewater treatment by ensuring a well-balanced nutrient composition in the culture medium. Jakhwal et al. (2024) explored the nutrient removal potential and microalgal biomass production of *Nannochloropsis oculata*, *Pavlova gyrans*, *Tetraselmis suecica*, and *Phaeodactylum tricornutum* in a water recirculating aquaculture system with low phosphate concentrations, supplemented with vitamins. Their findings revealed that vitamin supplementation significantly enhanced *T. suecica* growth from 0.16 to 0.33 g L^{-1}. Additionally, *T. suecica* improved nitrate removal efficiency from 80.88 ± 2.08 to 83.82 ± 2.08%.

In a separate study, Gupta et al. (2024) optimized nutrient removal and biomass production of *Scenedesmus acutus* and *Tetradesmus* by mixing untreated wastewater with anaerobically digested wastewater to balance nutrient content. The optimal treatment used an inoculum concentration of 0.2 optical density and a 3:1 ratio of wastewater to anaerobically digested wastewater, achieving a 10.5:1 C/N ratio. Biomass productivity increased by 35-89%, with *Tetradesmus* sp. exhibiting the highest productivity at 77.9 mg L^{-1} d^{-1}, which was accompanied by pollutant removal efficiencies of 95% total N, 99% N-NH_3, and 68% P-PO_4^{3-}.

Literature reports indicate that maintaining an appropriate nutrient balance in wastewater can enhance the circular bioeconomy by promoting the valorization of by-products and residue streams (Zilia et al. 2021). Therefore, optimizing microalgae-based wastewater treatment is essential to improving both microalgae production and nutrient removal, thereby strengthening the viability of an aquaculture circular bioeconomy.

This study focuses on optimizing aquaculture wastewater treatment using three native marine microalgae. It was conducted in two stages to develop a sustainable and efficient treatment process. In the first stage, the impact of different N/P ratios (2, 4, 6, 9, 12, 14, 16, 18, and 20) on the growth of *Nannochloropsis* and *Nannochloris* species, as well as nutrient removal efficiency, was evaluated in an aquaculture wastewater-based medium. The second stage examined P supplementation at N/P ratios of 9, 12, and 14 -conditions demonstrating the highest biomass productivity and nutrient removal- to further optimize microalgae-based wastewater treatment.

MATERIALS AND METHODS

Marine microalgae

The three marine microalgae used in this study were previously isolated and identified by López-Rosales et al. (2019). Samples were collected at depths of 0.0, 0.5, and 1.0 m using a Van Dorn bottle (1120-G45, Wildco, Yulee, FL, USA) in the marine-coastal waters of Puerto Progreso, Yucatán. The collected samples were sedimented at 4°C for 3-4 h, after which the biomass was combined and analyzed for microalgae isolation. The isolated species were then cultured in Guillard f/2 medium (20% seawater) and genetically identified as *Nannochloris* (NRRE-1) and *Nannochloropsis* (NSRE-1 and NSRE-2).

Aquaculture wastewater

The wastewater effluent from *Octopus maya* cultivation was collected at the UNAM-Sisal Academic Unit in Sisal, Yucatán, Mexico. The physicochemical characterization of the wastewater was as follows: 100.34 ± 0.058 mg L^{-1} of N-NO_3^- (nitrogen-nitrate), 5.22 ± 0.11 mg L^{-1} of P-PO_4^{3-} (phosphorus-phosphate), 1.25 ± 0.31 mg L^{-1} of N-NO_2^- (nitrogen-nitrite), a pH of 7.5, and a salinity of 38 ± 0.81.

Microalgae cultivation in culture medium

Microalgae cultivation in modified Guillard f/2 medium served as a blank. The experiments were conducted in 500 mL Erlenmeyer flasks containing 170 mL of culture medium and 30 mL of inoculum (~0.3 g L^{-1}). The modified Guillard f/2 medium used for cultivation contained (per liter): 600 mg $NaNO_3$, 40 mg $NaH_2PO_4 \cdot H_2O$, 4.36 mg Na_2EDTA, 3.15 mg $FeCl_3 \cdot 6H_2O$, 0.02 mg $MnCl_2 \cdot 4H_2O$, 0.02 mg $ZnSO_4 \cdot 7H_2O$, 0.01 mg $CoCl_2 \cdot 6H_2O$, 0.01 mg $CuSO_4 \cdot 5H_2O$, 0.006 mg $Na_2MoO_4 \cdot 2H_2O$, 30 mg Na_2SiO_3, 0.2 mg thiamine HCl, 0.01 mg vitamin B_{12}, and 0.1 mg biotin (Guillard & Ryther 1962). The cultures were maintained under continuous illumination at 90 µmol m^{-2} s^{-1} provided by 30 W white light at 25 ± 2°C. At the stationary phase, biomass was harvested via flocculation by adjusting the pH with 0.5 N NaOH, following Rojo-Cebreros et al. (2016). The biomass was centrifuged at 4,000 rpm for 10 min at 4°C (HERMLE Model Z 206 A). The supernatant was discarded, and the pellet was washed with distilled water until residual salts were removed. The biomass was freeze-dried (LABCONCO FREEZONE) at -56°C and 0.100 mBar for 72 h. The lyophilized biomass was stored at 4°C until lipid extraction.

Microalgae aquaculture wastewater adaptation and cultivation

Different wastewater-to-distilled water ratios (10:90, 20:80, 30:70, 40:60, 50:50, and 60:40) were evaluated to adapt microalgae to aquaculture wastewater. Experiments were conducted in 500 mL Erlenmeyer flasks containing 170 mL of the wastewater-distilled water mixture and 30 mL of inoculum (~0.3 g L^{-1}). The cultures were maintained under continuous illumination at 90 µmol m^{-2} s^{-1} using 30 W white light at 25 ± 2°C. Optimal marine microalgae growth was achieved at a 50:50 wastewater-to-distilled water ratio, with a composition of 49 ± 0.38 mg L^{-1} N-NO_3^-, 2.32 ± 0.12 mg L^{-1} P-PO_4^{3-}, a pH of 7.56, and a salinity of 19.36 ± 0.25. Consequently, all subsequent experiments utilized this ratio as the aquaculture wastewater-based medium.

Further experiments in 500 mL Erlenmeyer flasks focused on adjusting the aquaculture wastewater-based medium's N/P ratio. Nine N/P levels (2, 4, 6, 9, 12, 14, 16, 18, and 20) were evaluated, with adjustments based on the initial N/P ratio (21.12). For example, to achieve an N/P ratio of 2, a final concentration of 21.18 mg L^{-1} P-PO_4^{3-} was required, necessitating the addition of 4.23 mL of a 1,000 mg L^{-1} P-PO_4^{3-} solution to 200 mL of microalgae culture. Phosphate (P-PO_4^{3-}) was supplemented as KH_2PO_4 (Sigma-Aldrich, San Luis, Missouri, USA), with no significant changes observed in medium pH after its addition. The culture remained at continuous light (90 µmol m^{-2} s^{-1} from a 30 W white light source) and 25 ± 2°C. Biomass was harvested at the stationary phase via flocculation, as described in section microalgae cultivation in culture medium.

Analytical methods

Dry cell mass, measured as total suspended solids (TSS), was determined gravimetrically following standard method 2540 D (APHA 2005) using a drying chamber (Binder ED 56, Tuttlingen, DEU). To remove residual salts, the filter containing the retained biomass was washed with twice the volume of the filtered sample using distilled water.

The concentrations of N-NO_3^- and P-PO_4^{3-} in the filtered samples were analyzed according to APHA standard methods using a UV-Vis spectrophotometer (Agilent Cary 60, Santa Clara, CA, USA). Nitrate (N-NO_3^-) was quantified following standard method 4500-NO_3^--B (APHA 2005), with KNO_3 (Sigma-Aldrich, San Luis, Missouri, USA) used to prepare the nitrate standard solution. Phosphate (P-PO_4^{3-}) analysis was conducted using standard method 4500-P E, utilizing an ascorbic acid reagent prepared with ammonium molybdate, sulfuric acid, potassium antimony tartrate (J.T. Baker, Phillipsburg, NJ, USA), and ascorbic acid (Sigma-Aldrich, San Luis, Missouri, USA). KH_2PO_4 (Sigma-Aldrich, San Luis, Missouri, USA) was used as the phosphate standard solution (APHA 2005).

Lipid extraction

Lipid extraction was performed at least in duplicate following the method described by Folch et al. (1957). Briefly, 38 mg of dry biomass was mixed with a chloroform-methanol solution (2:1, v/v) and incubated at 40°C with constant shaking at 150 rpm for 3 h. The residual biomass was separated from the extract using a 125 mL separating funnel, and the liquid phase was collected and evaporated.

The lipid extract was dissolved in chloroform and transferred into pre-weighed vials. Chloroform evaporation was carried out inside a fume hood for 24 h. Once the solvent had completely evaporated, the vials were weighed to determine lipid content (Folch et al. 1957).

Data analysis

Each experiment was repeated at least three times. Two samplings were conducted to assess growth, N-NO_3^-, and P-PO_4^{3-} concentrations. Graphical representations and statistical analyses were performed using OriginPro 2018 (OriginLab Corporation).

Data are presented as mean ± standard deviation. Microalgae productivity results were analyzed using a one-way analysis of variance (ANOVA) to evaluate the effect of the N/P ratio, followed by Tukey's *post hoc* multiple comparison test to identify significant differences between N/P ratios (Ferrão-Filho et al. 2003). Before ANOVA, all data were tested for homogeneity and independence using Levene's test to ensure the assumptions of ANOVA were met. Statistical significance was set at $P < 0.05$.

RESULTS

Microalgae cultivation in culture medium

The native marine microalgae used in this study, based on theoretical estimations from triacylglycerides (TAG) and free fatty acids (FFA) composition, can produce biodiesels that meet six of the seven estimated properties (ASTM D6751 and EN 14214). Furthermore, they synthesize other precursors for renewable fuels, such as hydrocarbons and terpenes (López-Rosales et al. 2019).

The growth curves for NRRE-1, NSRE-1, and NSRE-2 in a modified Guillard f/2 medium are shown (Fig. 1, Table 1). The three microalgae strains showed similar growth curves with a short lag phase (2-4 days). The incipient exponential phase began on day 4 and was sustained until day 16, when the stationary phase began. The biomass productivity of microalgae was 8.23 ± 1.17, 8.21 ± 1.19, and 9.36 ± 1.39 mg L^{-1} d^{-1} for NRRE-1, NSRE-1, and NSRE-2, respectively. The three strains also showed N and P removal, which was more evident from day 7 of cultivation than from days 1 to 7. From day 8 to 16, N and P consumption maintenance is observed. The removal efficiency of N-NO_3^- within 21 days cultivation was 18.33 ± 0.56, 17.77 ± 1.68, and 17.49 ± 0.69% for NRRE-1, NSRE-1, and NSRE-2, respectively. The removal efficiency of P-PO_4^{3-} for NRRE-1, NSRE-1, and NSRE-2 within 21 days cultivation was 65.56 ± 2.17, 64.38 ± 2.42, and 64.67 ± 1.64%, respectively. The three strains achieved a biomass concentration between 180 and 200 mg L^{-1} in this period.

The three strains studied showed low N and P removal efficiency. The low removal efficiency can be explained since the initial N/P ratio in the culture medium was ~10. Nevertheless, the N/P ratio on day 16 was ~25. The microalgae growth decreased at a 25 N/P ratio since the P concentration in the culture was low compared to the N concentration. In modified Guillard f/2 medium, the NSRE-2 strain showed the highest growth rate (9.36 ± 1.39 mg L^{-1} d^{-1}), despite the NRRE-1 strain showing the highest nutrient (N and P) removal (18.33 ± 0.56 and 65.56 ± 2.17%, respectively).

N/P ratio effect on growth profiles of microalgae cultivated on aquaculture wastewater-based medium

The evaluated N/P ratios showed a similar effect on NRRE-1, NSRE-1, and NSRE-2 (Fig. 2, Tables 2-4) strains. 2, 4, and 6 N/P ratios caused a 6-day adaptation phase, followed by a death phase in which biomass concentration declines. Therefore, 2, 4, and 6 N/P ratios caused cellular death after 8 days of cultivation. Nine, 12, 14, and 16 N/P ratios in the medium induced a 4-day adaptation phase in which biomass productivity was low. From days 4 to 14, the microalgae entered a fast-growth stage for the three strains with an accelerated increase in biomass concentration. During

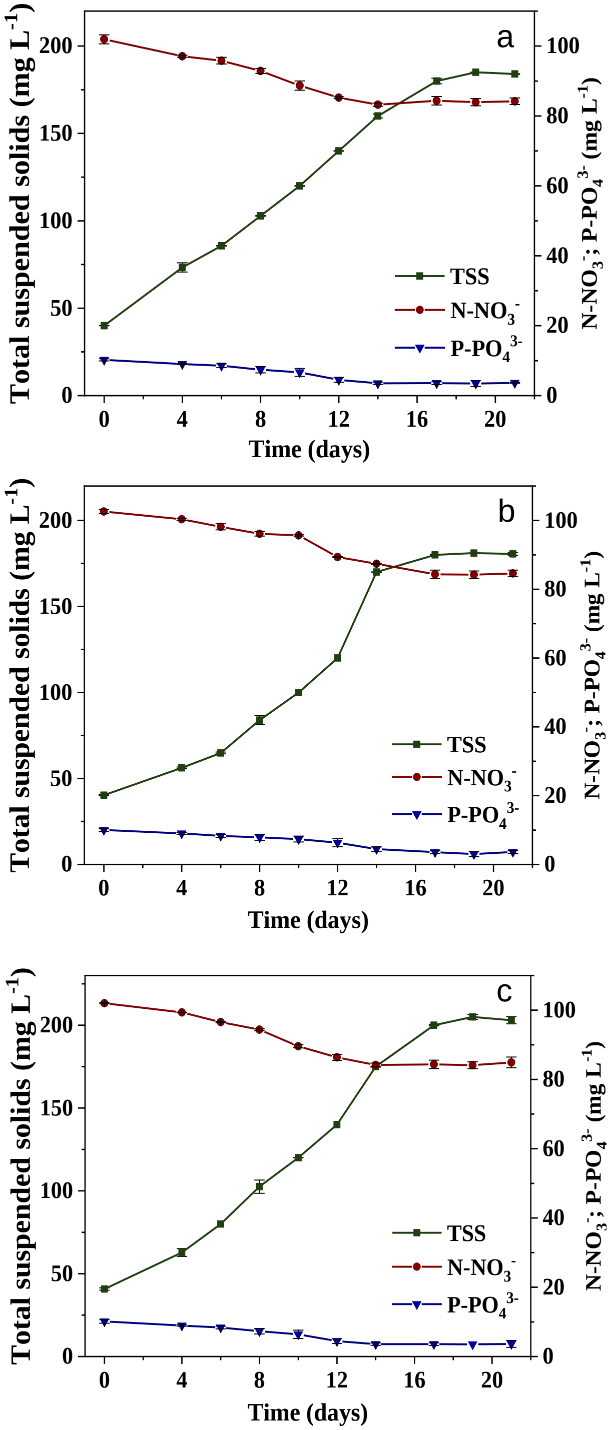

Figure 1. Microalgae growth curves for a) *Nannochloris* (NRRE-1), b) *Nannochloropsis* 1 (NSRE-1), and c) *Nannochloropsis* 2 (NSRE-2) cultivated in modified Guillard f/2 medium.

this phase, the concentration of microalgae showed a consistent increase, while the nutrient concentration decreased correspondingly. Finally, microalgae entered a slow-growth phase from day 14 to day 16. During this phase, the growth rate begins to decline because of the ongoing consumption of nutrients (mainly P), despite the biomass concentration of the microalgae continuing to rise. Finally, 18 and 20 N/P ratios caused a 9-day adaptation phase followed by a gradual decline in microalgae growth, which ends in a death phase after 10 days of cultivation. After 10 days of cultivation, microalgae biomass productivity starts to decline.

On the other hand, the evaluated N/P ratios caused a diverse effect on the biomass productivity of the three strains studied. NRRE-1 biomass productivity was significantly higher at 9, 12, and 14 N/P ratios than at 2, 4, 6, 16, 18, and 20 N/P ratios ($P < 0.05$). NSRE-1 biomass productivity was significantly higher at 9, 12, 14, and 16 N/P ratios, and NSRE-2 biomass productivity was significantly higher at 9, 12, 14, 16, and 18 N/P ratios ($P < 0.05$). NRRE-1 showed the highest biomass productivity (10.05 mg L^{-1} d^{-1}) at the 12 N/P ratio. At this N/P ratio, N-NO_3^- and P-PO_4^{3-} removal efficiency was 41.83 ± 0.39 and 64.54 ± 0.01%, respectively (Fig. 3, Tables 2-4). NSRE-1 showed similar results with the highest biomass productivity (10.14 mg L^{-1} d^{-1}) at 12 N/P ratio. At these experimental conditions (12 N/P ratio), N-NO_3^- and P-PO_4^{3-} removal efficiency was 22.09 ± 0.64 and 44.66 ± 0.23%, respectively (Fig. 3, Tables 2-4). Finally, NSRE-2 showed the highest biomass productivity (10.68 mg L^{-1} d^{-1}) at a 9 N/P ratio. At this 9 N/P ratio, the N-NO_3^- and P-PO_4^{3-} removal efficiency was 22.54 ± 1.78 and 99.43 ± 0.02%, respectively (Fig. 3, Tables 2-4).

Aquaculture wastewater treatment optimization by P supplementation

P supplementation to the aquaculture wastewater-based medium is derived from optimization of wastewater treatment by increasing nutrient removal efficiency and maximum productivity and biomass concentration (Fig. 4, Table 5). The three strains studied showed a similar lifecycle in which three phases were observed. The adaptation phase (days 0 to 6) exhibited low microalgae productivity. At the end of the adaptation phase, P supplementation began since approximately 40% of the initial P-PO_4^{3-} was removed. P supplementation was carried out every 72 h after P-PO_4^3 concentration analysis. The fast-growth stage generally started on day 8 and ended on day 30. During this period, a rapid increase in biomass concentration was observed. From days 30 to 33, a slow-growth phase was observed. During this phase, biomass concentration increased slowly because P supplementation was paused since

Table 1. Biomass productivity and $N\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$ removal efficiency of *Nannochloris* (NRRE-1), *Nannochloropsis* 1 (NSRE-1), and *Nannochloropsis* 2 (NSRE-2) cultivated in modified Guillard f/2 medium. Data are presented as mean ± standard deviation (n = 3).

Microalgae strain	Biomass productivity (mg L^{-1} d^{-1})	Removal efficiency (%)	
		$N\text{-}NO_3^-$	$P\text{-}PO_4^{3-}$
NRRE-1	8.23 ± 1.17	18.33 ± 0.56	65.56 ± 2.17
NSRE-1	8.21 ± 1.19	17.77 ± 1.68	64.38 ± 2.42
NSRE-2	9.36 ± 1.39	17.49 ± 0.69	64.67 ± 1.64

approximately 90% of the N was removed. Microalgae were also cultivated in the modified Guillard f/2 medium using a similar strategy, adding limited amounts of P to conserve a 14 N/P ratio applied to the Guillard f/2 medium. For the three strains studied, the results were similar to those found using the aquaculture wastewater-based medium, which indicates the viability of aquaculture wastewater-based mediums for marine microalgae cultivation and marine microalgae viability for aquaculture wastewater treatment.

NRRE-1 maximum biomass concentration and biomass productivity in the aquaculture wastewater-based medium supplemented with P were significantly higher at the 14 N/P ratio than at the 9 and 12 N/P ratios (P < 0.05). At the 14 N/P ratio, maximum biomass concentration and biomass productivity increased from 158.8 ± 0.26 to 720 ± 25.14 mg L^{-1} and from 6.8 to 17.04 mg L^{-1} d^{-1}, in comparison with the aquaculture wastewater-based medium non-supplemented with P. Besides, the $N\text{-}NO_3^-$ removal efficiency rose from 25 ± 0.73 to 90.38 ± 0.19%, while $P\text{-}PO_4^{3-}$ removal efficiency was maintained (~95%; Fig. 5, Table 5). NSRE-1 showed similar results, maximum biomass concentration and biomass productivity were significantly higher at 14 N/P ratio, in which the maximum biomass concentration increased from 175.37 ± 1.91 to 826.66 ± 12.57 mg L^{-1}, and biomass productivity from 8.35 to 24.37 mg L^{-1} d^{-1} in comparison with the aquaculture wastewater-based medium non-supplemented with P (P < 0.05). $N\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$ removal efficiency raised from 23.9 ± 3.27 to 94.46 ± 0.24% and from 34.65 ± 2.6 to 96.74 ± 0.39%, respectively (Fig. 5). For NSRE-2 maximum biomass concentration and biomass productivity were significantly higher at 9 and 12 N/P ratios, however, the maximum biomass concentration and productivity were at 9 N/P ratio (P < 0.05). Maximum biomass concentration at 9 N/P ratio increased from 189.6 ± 0.31 to 680 ± 12.6 mg L^{-1} while biomass productivity raised from 8.2 to 18.18 mg L^{-1} d^{-1} in comparison with the aquaculture wastewater-based medium not supplemented with P. $N\text{-}NO_3^-$ removal efficiency also improved since removal efficiency when the aquaculture wastewater-based medium was supplemented with P was 92.54 ± 0.94% while using the aquaculture wastewater-based medium non-supplemented with P was 22.54 ± 1.78%. Finally, for this strain, $P\text{-}PO_4^{3-}$ removal efficiency was maintained at ~99% (Fig. 5, Table 5). Lipid content in the microalgae biomass for all the N/P ratios studied was between 8 and 10%. Therefore, the N/P ratios studied did not significantly affect the microalgae lipid content (P < 0.05).

DISCUSSION

N/P ratio effect on growth profiles of microalgae cultivated on aquaculture wastewater-based medium

The results of this study align with previous findings in the literature (Rasdi & Qin 2015). Cellular death at low N/P ratios (2, 4, and 6) can be attributed to N limitation in the medium due to excess P, which may inhibit photosynthetic activity. A lack of N is known to reduce microalgal protein synthesis rates (Berdalet et al. 1994) and hinder the formation of essential structural and functional components such as chlorophyll, proteins, ribosomal RNA, and ribosomal proteins (Turpin 1991); ultimately, leads to a decline in the protein pool (Falkowski et al. 1989) and a subsequent decrease in the net photosynthetic rate.

Conversely, P becomes increasingly limited at higher N/P ratios (18 and 20), leading to microalgae death, particularly in the NSRE-1 strain. The results suggest that N limitation at low N/P ratios does not have the same impact as P limitation at high N/P ratios, which is evident from the significantly higher maximum biomass concentration of NRRE-1 at 18 and 20 N/P ratios (~100 mg L^{-1}) compared to 2, 4, and 6 N/P ratios (~60 mg L^{-1}; P < 0.05). Similarly, for NSRE-1 and NSRE-2, maximum biomass concentration at an 18 N/P ratio (~115 mg L^{-1}) was significantly higher than at 4 and 6 N/P ratios (~80 mg L^{-1}; P < 0.05).

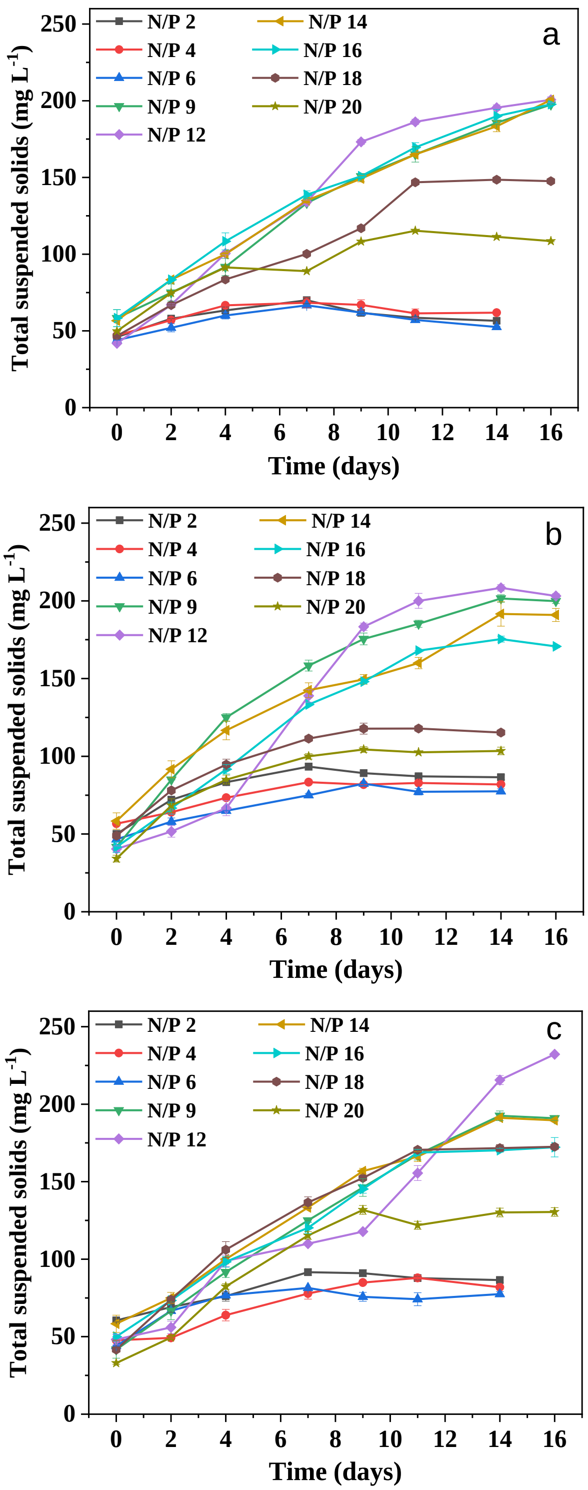

Figure 2. Microalgae growth curves for a) *Nannochloris* (NRRE-1), b) *Nannochloropsis* 1 (NSRE-1), and c) *Nannochloropsis* 2 (NSRE-2) cultivated in modified Guillard f/2 medium.

The distinct cellular responses to N and P limitations can explain this difference. Under N limitation at low N/P ratios, microalgae cells cease division, decreasing protein and chlorophyll production. In contrast, cell division may continue under P limitation, but growth is impaired, often resulting in altered cell morphology and reduced biomass production (Danesh et al. 2017). A key indicator of P limitation in microalgae is reduced RNA content (Berdalet et al. 1994). Therefore, when P concentrations become too low, as seen at a 20 N/P ratio, cell division may eventually halt, leading to cellular death.

These results align with findings from Rasdi & Qin (2015), who investigated the effects of six N/P ratios (5, 10, 20, 30, 60, and 120) on the growth of *Tisochrysis lutea* and *Nannochloropsis oculata* using F/2 medium. Their study showed that as the N/P ratio increased from 5 to 20, the specific growth rate of *T. lutea* and *N. oculata* peaked at 1.42 and 1.49 d^{-1}, respectively. Cell densities at an N/P ratio of 20 at the end of the stationary phase were significantly higher than those at other tested ratios ($P < 0.05$).

Similarly, Magyar et al. (2024) reported that a nutrient solution with an N/P ratio of 14 enhanced microalgae activity rates by 71.2% compared to the control sample (BG-11, N/P ratio = 35) in a *Chlorella vulgaris* culture. Mayers et al. (2014) found that the maximum biomass productivity for *Nannochloropsis* sp. (56.6 ± 2.21 mg L^{-1} d^{-1}) occurred at an N/P ratio of 16. In another study, Huo et al. (2020) identified an optimal N/P ratio of 30 for the growth of *Tribonema* sp. in swine anaerobic effluent, achieving a significant biomass accumulation of 2.04 g L^{-1} after 14 days of cultivation.

Microalgal growth responses to N/P ratios vary among species and depend on their physiological nutrient requirements (Lagus et al. 2004). Therefore, understanding the role of N/P ratios in the metabolic processes of indigenous microalgal strains is crucial for optimizing biomass productivity, particularly when using wastewater as a culture medium. The findings of this study suggest that for NRRE-1, NSRE-1, and NSRE-2, an optimal N/P ratio for cultivation in aquaculture wastewater ranges between 9 and 14, as these ratios significantly enhanced biomass productivity ($P < 0.05$).

The maximum biomass concentrations of NRRE-1, NSRE-1, and NSRE-2 were not significantly different when cultivated in aquaculture wastewater-based medium with N/P ratios of 9, 12, and 14 compared to the modified Guillard F/2 medium ($P < 0.05$). These results suggest that aquaculture wastewater is a suitable medium for marine microalgae cultivation. However, the achieved biomass concentrations were lower than

Table 2. Biomass productivity and N-NO_3^- and P-PO_4^{3-} removal efficiency of *Nannochloris* (NRRE-1) cultivated in an aquaculture wastewater-based medium under different N/P ratios. Data are presented as mean ± standard deviation (n = 3). Statistical differences among N/P ratios were assessed using one-way ANOVA ($P < 0.05$). [a]Bold text indicates statistically significant differences.

N/P ratio	Biomass productivity (mg L^{-1} d^{-1})	Removal efficiency (%)	
		N-NO_3^-	P-PO_4^{3-}
2	3.87	13.84 ± 0.19	30.34 ± 0.11
4	4.03	14.93 ± 0.38	50.08 ± 0.16
6	3.5	21.38 ± 0.21	62.83 ± 1.44
9	**6.61**[a]	18.86 ± 0.21	75.50 ± 0.29
12	**10.05**[a]	25.83 ± 0.39	64.54 ± 0.01
14	**9.92**[a]	24.11 ± 0.43	97.1 ± 0.75
16	8.7	18.62 ± 0.67	42.35 ± 0.99
18	5.06	17.53 ± 0.12	72.82 ± 1.8
20	3.66	12.93 ± 0.20	77.07 ± 0.69

Table 3. Biomass productivity, N-NO_3^- and P-PO_4^{3-} removal efficiency of *Nannochloropsis* 1 (NSRE-1) cultivated in an aquaculture wastewater-based medium under different N/P ratios. Data are presented as mean ± standard deviation (n = 3). Statistical differences among N/P ratios were assessed using one-way ANOVA ($P < 0.05$). [a]Bold text indicates statistically significant differences.

N/P ratio	Biomass productivity (mg L^{-1} d^{-1})	Removal efficiency (%)	
		N-NO_3^-	P-PO_4^{3-}
2	3.37	17.20 ± 1.2	30.52 ± 0.95
4	2.29	14.07 ± 1.4	40.37 ± 0.95
6	2.8	17.51 ± 0.87	40.27 ± 1.69
9	**9.98**[a]	23.9 ± 1.28	46.27 ± 1.16
12	**10.14**[a]	22.09 ± 0.64	44.66 ± 0.23
14	**8.35**[a]	21.0 ± 0.97	21.89 ± 1.08
16	**8.32**[a]	18.13 ± 1.13	36.29 ± 0.96
18	6.27	13.21 ± 0.97	43.21 ± 0.73
20	6.38	7.5 ± 1.05	56.29 ± 0.92

Table 4. Biomass productivity, N-NO_3^- and P-PO_4^{3-} removal efficiency of *Nannochloropsis* 2 (NSRE-2) cultivated in an aquaculture wastewater-based medium under different N/P ratios. Data are presented as mean ± standard deviation (n = 3). Statistical differences among N/P ratios were assessed using one-way ANOVA ($P < 0.05$). [a]Bold text indicates statistically significant differences.

N/P ratio	Biomass productivity (mg L^{-1} d^{-1})	Removal efficiency (%)	
		N-NO_3^-	P-PO_4^{3-}
2	2.47	14.28 ± 0.92	26.69 ± 0.59
4	2.78	11.53 ± 1.57	19.77 ± 1.08
6	3.38	14.24 ± 1.1	9.8 ± 1.52
9	**10.68**[a]	22.54 ± 1.78	99.43 ± 0.2
12	**10.38**[a]	19.09 ± 0.93	99 ± 0.91
14	**8.20**[a]	21.9 ± 0.8	62.01 ± 1.88
16	**9.6**[a]	16.9 ± 0.94	47.09 ± 1.66
18	**8.18**[a]	13.59 ± 0.87	67.78 ± 1.03
20	6.09	13.31 ± 0.89	59.14 ± 0.92

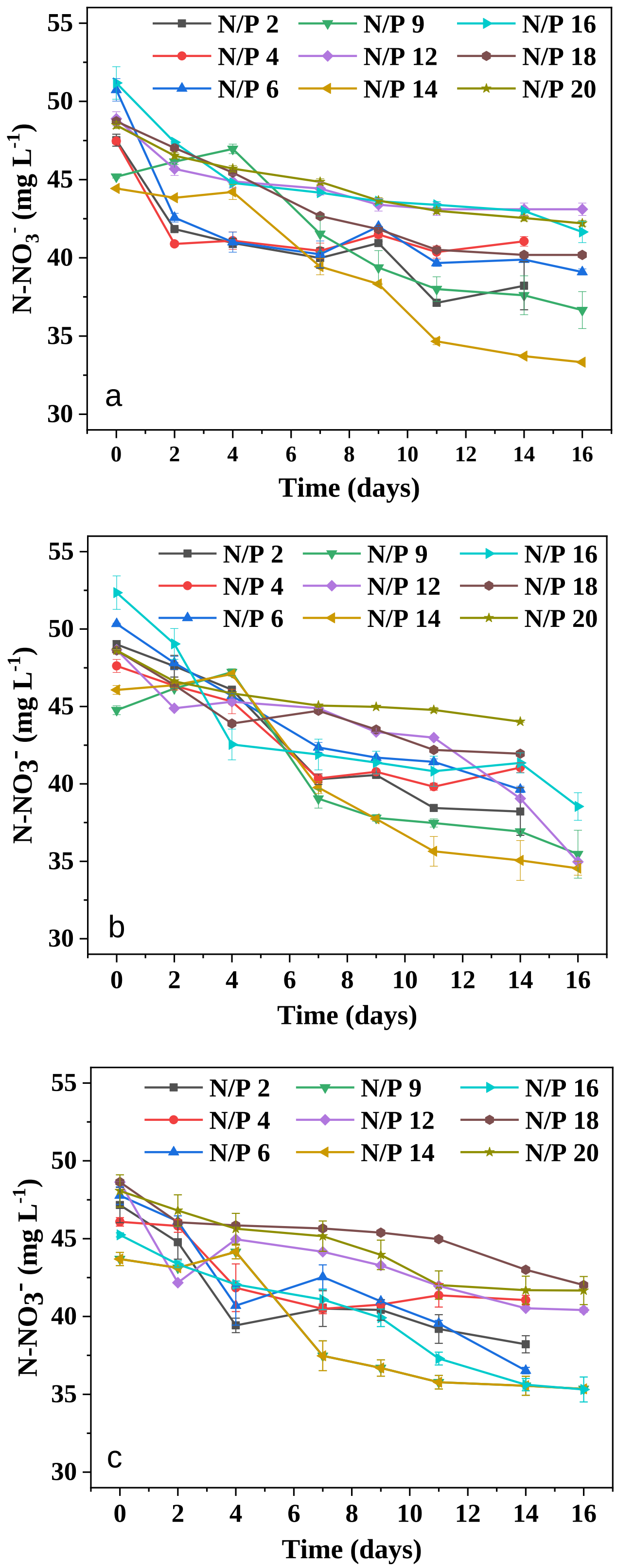

Figure 3. Effect of the N/P ratio on the N-NO_3^- removal rate by a) *Nannochloris* (NRRE-1), b) *Nannochloropsis* 1 (NSRE-1), and c) *Nannochloropsis* 2 (NSRE-2) cultivated in an aquaculture wastewater-based medium.

those reported in the literature. Additionally, nutrient removal -particularly nitrogen- was relatively low, limiting the effectiveness of microalgae as a wastewater treatment method.

The low nitrogen removal efficiency can be attributed to P limitation at N/P ratios of 9, 12, and 14, which may have restricted N uptake (Yu et al. 2017). Further optimization strategies should be explored to enhance biomass productivity and nutrient removal efficiency. Regarding lipid content, microalgae biomass across all studied N/P ratios contained between 8% and 10% lipids, indicating that N/P variations did not significantly affect lipid accumulation ($P < 0.05$).

Aquaculture wastewater treatment optimization by P supplementation

In the first stage of the study, the three strains exhibited significantly higher biomass productivity in the aquaculture wastewater-based medium at N/P ratios of 9, 12, and 14. Therefore, these ratios were selected for further optimization of wastewater treatment through P supplementation. As previously discussed, nutrient removal in the aquaculture wastewater-based medium was low across all evaluated N/P ratios, particularly for N (~20%). This low removal efficiency can be attributed to P limitation after six days of cultivation (Fig. 6), during which 30-40% of the available P was depleted, while only ~10% of N was removed. This imbalance led to a significant increase in the N/P ratio.

Figure 6 illustrates P removal for NRRE-1 across various N/P ratios (2, 4, 6, 9, 12, 14, 16, 18, and 20). The threshold at which P limitation impacts algal growth varies by species. However, under severe P limitation, microalgal cells cannot produce nucleic acids, which inhibits cell division and reduces protein synthesis, often leading to C accumulation (Åkerström et al. 2014). Microalgae can simultaneously absorb and utilize N and P. Still, the assimilation rate for each nutrient differs, leading to an imbalance in the culture medium, especially concerning P levels. This nutrient imbalance creates non-ideal conditions for the microalgae, potentially affecting growth and metabolic efficiency. Notably, the simultaneous uptake of N and P means that low P concentrations can impair N removal efficiency, ultimately reducing the overall effectiveness of nutrient removal (Qian et al. 2024).

While microalgae can over-absorb P when exposed to excess P, this behavior is not observed for N. Furthermore, both high and low N/P ratios in the culture can lead to the generation of reactive oxygen species (ROS). These highly reactive molecules can

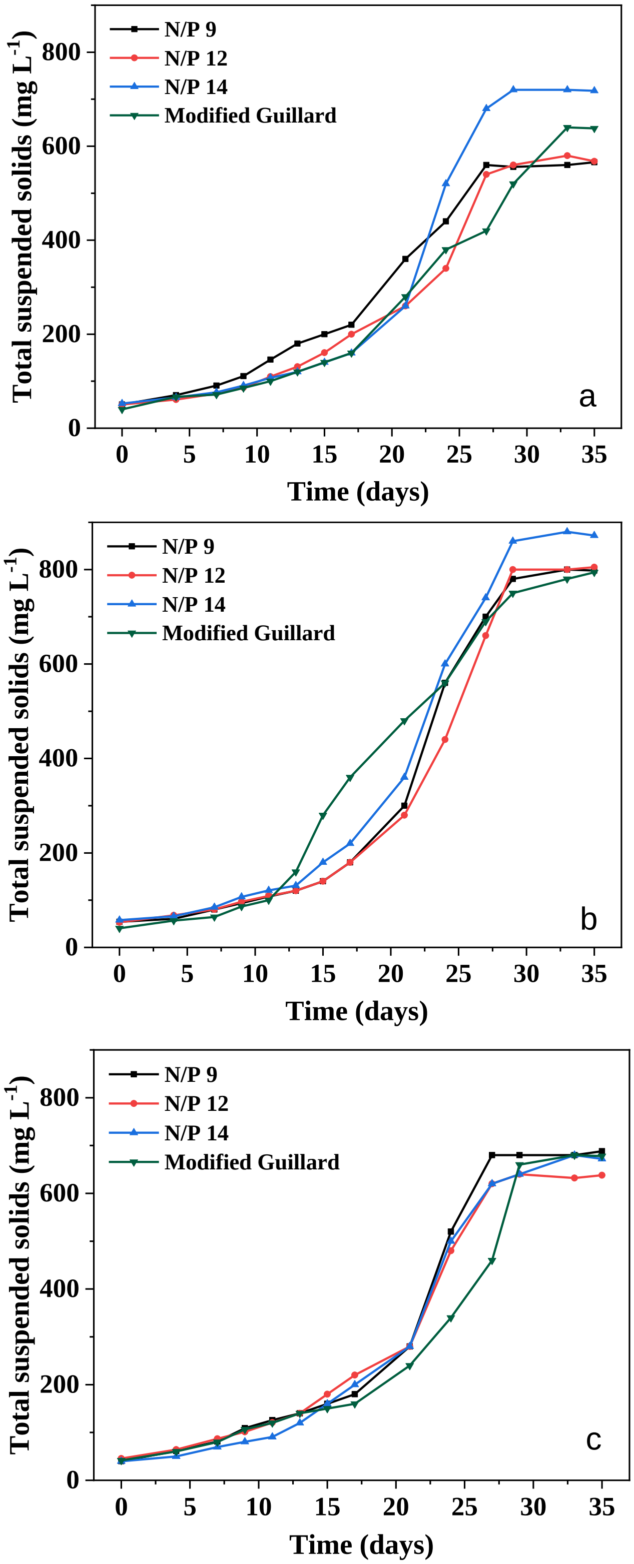

Figure 4. Growth curves of microalgae a) *Nannochloris* (NRRE-1), b) *Nannochloropsis* 1 (NSRE-1), and c) *Nannochloropsis* 2 (NSRE-2) cultivated in an aquaculture wastewater-based medium and modified Guillard medium supplemented with P under N/P ratios of 9, 12, or 14.

induce oxidative stress, damaging crucial biomolecules such as lipids, carbohydrates, proteins, and DNA, impairing cellular function (Gill & Tuteja 2010).

Although high N/P ratios did not significantly impact microalgae biomass productivity, they could induce oxidative stress, which eventually affected the growth state of the microalgae (Rhee et al. 2018). Therefore, maintaining an optimal N/P ratio is essential for ensuring both effective nitrogen removal and biomass production. To achieve this, a limited amount of P was supplemented to the aquaculture wastewater-based medium, using a 1,000 mg P-PO_4^{3-} L^{-1} solution, to maintain N/P ratios of 9, 12, or 14 over 33 days of cultivation. The N/P ratio adjustments (9, 12, or 14) were based on the analysis of N-NO_3^- and P-PO_4^{3-} concentrations every 72 h. KH_2PO_4 was used to add P-PO_4^{3-} to the aquaculture wastewater-based medium, and no significant changes in pH were observed following the addition of P-PO_4^{3-}.

P supplementation in the aquaculture wastewater-based medium was critical in optimizing wastewater treatment by maintaining N/P ratios of 9, 12, or 14. P supplementation significantly increased biomass productivity by 350 to 470% and improved N and P removal efficiency to approximately 90% across the three strains. This success can be attributed to the consistent P analysis and supplementation, which ensured nutrient balance in the culture medium, thereby maintaining the microalgae in an optimal growth state.

These results are consistent with those found by Wang & Li (2024), who also used a nutrient supplementation strategy for *Chlorella pyrenoidosa* in BG-11 medium. In their study, nutrient supplementation began after 72 h of cultivation and was repeated every 3 days with varying amounts of BG-11 medium. They observed a 42.9% increase in microalgae biomass concentration compared to traditional methods, with the highest biomass concentration reaching approximately 1.2 g L^{-1} after 10 days of cultivation (Wang & Li 2024).

Aquaculture circular bioeconomy based on microalgae and future perspectives

The circular bioeconomy aims to achieve sustainable production by utilizing biological resources as feedstock while optimizing resource use in a closed loop to minimize the depletion of virgin resources (Leong et al. 2021). This strategy mitigates climate change by recycling renewable C sources and nutrients (Banu et al. 2020) and addresses critical global sustainability challenges such as climate change, biodiversity loss, and resource depletion. Furthermore,

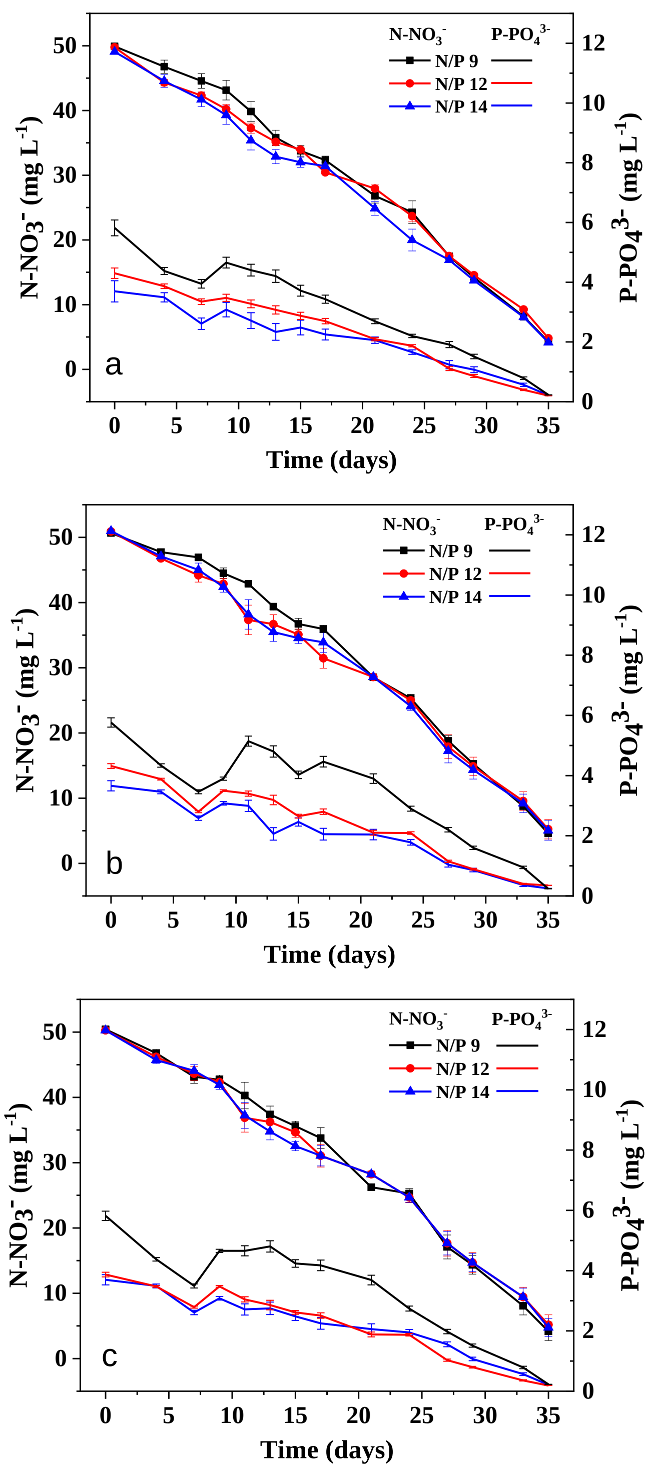

Figure 5. N-NO$_3^-$ and P-PO$_4^{3-}$ removal rates by a) *Nannochloris* (NRRE-1), b) *Nannochloropsis* 1 (NSRE-1), and c) *Nannochloropsis* 2 (NSRE-2) cultivated in an aquaculture wastewater-based medium and modified Guillard medium supplemented with P under N/P ratios of 9, 12, or 14.

it drives economic growth and enhances societal well-being, making it a collective mission (Sharma & Malaviya 2023).

A promising circular bioeconomy strategy is aquaculture wastewater treatment using microalgae. As a primary food source for many aquatic organisms, microalgae play a crucial role in the aquaculture food chain. Studies have shown that incorporating microalgae into aquaculture enhances productivity by improving survival rates, boosting immune responses, maintaining water quality, and meeting consumer demand for antibiotic-free aquaculture products.

Villar-Navarro et al. (2022) cultivated *Tetraselmis chui* in recirculating aquaculture system (RAS) wastewater, achieving a productivity of 36 mg L^{-1} d^{-1} while keeping pollutant levels -including suspended solids and total dissolved N, P, and C- within discharge limits. Biomass quality analysis showed that the microalgae cultivated in RAS wastewater was highly comparable to commercial *T. chui*. The experimental biomass contained 41.6 g 100^{-1} g of total protein, 10.1 g 100^{-1} g of total lipids, and 48.3 g 100^{-1} g of total carbohydrates, compared to 55.2, 10.1, and 34.7 g 100^{-1} g, respectively, in commercial *T. chui* biomass.

Similarly, Jakhwal et al. (2024) evaluated the feasibility of using *Nannochloropsis oculata*, *Pavlova gyrans*, *Tetraselmis suecica*, and *Phaeodactylum tricornutum* for biomass production and nutrient removal from RAS wastewater. Their findings showed that *N. oculata* exhibited the highest biomass production (0.4 g L^{-1}). At the same time, *T. suecica* demonstrated the highest nitrate (N-NO$_3^-$) removal efficiency (80.88 ± 2.08%) and optimal fatty acid composition, making it a promising candidate for fish feed in a circular bioeconomy.

Despite its potential, a microalgae-based circular bioeconomy's feasibility and long-term viability in aquaculture must be ensured before large-scale implementation. Techno-economic analyses suggest that optimizing cultivation conditions could reduce production costs by up to 92%. One of the most significant expenses-accounting for approximately 50% of total costs-is the procurement of live microalgal biomass for hatchery applications. This cost can be mitigated by enhancing photosynthetic efficiency. Achieving a photosynthetic efficiency of 7% could significantly lower microalgal production costs, making it competitive with fishmeal (Vázquez-Romero et al. 2022). Additionally, using concentrated microalgal biomass presents a viable strategy to improve economic feasibility further.

Table 5. Biomass productivity, N-NO_3^- and P-PO_4^{3-} removal efficiency of *Nannochloris* (NRRE-1), *Nannochloropsis* 1 (NSRE-1), and *Nannochloropsis* 2 (NSRE-2) cultivated in an aquaculture wastewater-based medium under 9, 12, and 14 N/P ratios. Data are presented as mean ± standard deviation (n = 3). *The N/P ratio was adjusted through P supplementation based on nutrient analysis results in all experiments. Statistical differences among N/P ratios were assessed using one-way ANOVA, ($P < 0.05$). [a]Bold text indicates statistically significant differences.

N/P ratio*	Biomass productivity (mg L^{-1} d^{-1})	Removal efficiency (%)	
		N-NO_3^-	P-PO_4^{3-}
NRRE-1			
9	15.44	83.79 ± 0.56	93.93 ± 1.06
12	15.36	89.71 ± 0.21	94.58 ± 0.89
14	**17.04**[a]	90.38 ± 0.19	95 ± 0.76
NSRE-1			
9	22.59	88.80 ± 1.09	89.16 ± 1.7
12	22.62	89.29 ± 1.15	94.76 ± 1.21
14	**24.37**[a]	94.46 ± 0.24	96.74 ± 0.39
NSRE-2			
9	**18.18**[a]	92.54 ± 0.94	99 ± 0.95
12	**17.39**[a]	91.07 ± 1.51	94.81 ± 0.78
14	16.79	89.60 ± 1.03	94.46 ± 1.14

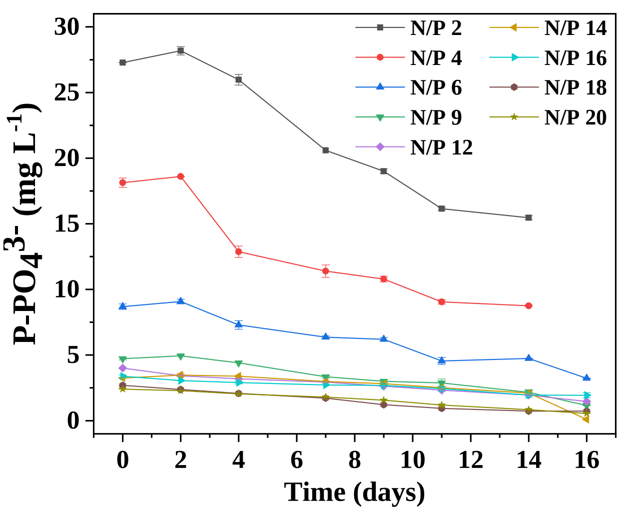

Figure 6. P-PO_4^{3-} removal rate for *Nannochloris* (NRRE-1) under N/P ratios of 2, 4, 6, 9, 12, 14, 16, 18, and 20.

While microalgae hold great promise for aquaculture, further research is needed to unlock their full potential. Future efforts should prioritize the development of cost-effective, easy-to-operate microalgae culture reactors suitable for small- to medium-scale hatcheries, as these could significantly reduce aquaculture feed production costs (Erbland et al. 2020). Commercial-scale microalgal feed production has also outperformed terrestrial feed sources in terms of environmental sustainability, particularly by minimizing land use change and reducing global warming potential (Taelman et al. 2013).

Scaling up microalgae cultivation can significantly reduce production costs, ranging from 108.26 to 44 € kg DW^{-1} (~20-60%). Vázquez-Romero et al. (2022) emphasize that these costs are heavily influenced by scale and climatic conditions. Therefore, once cultivation conditions are optimized, cost-reduction strategies should focus on using low-cost materials, improving light distribution, enhancing photosynthetic efficiency, and cultivating indigenous microalgae species adapted to controlled environments. Implementing these measures could bring microalgal biomass costs in line with the aquafeed market, making it a competitive alternative.

However, P supplementation in large-scale microalgae-based wastewater treatment presents economic and regulatory challenges, particularly in the European Union. P is a non-renewable resource and is classified as a Critical Raw Material. The cost of P supplementation must also be factored in, as phosphate rock was priced at approximately US$0.076 kg^{-1} (~€0.064 kg^{-1}) in 2020 (World Bank). Addressing phosphorus dependency through alternative nutrient sources is crucial for the long-term viability of this approach.

Large-scale wastewater treatment must balance efficiency, environmental impact, and economic feasibility. Reducing aquaculture's environmental footprint while improving wastewater treatment efficiency is key to advancing a sustainable and economically viable microalgae-based circular bio-economy in aquaculture (Pradel & Aissani 2019).

CONCLUSIONS

The cultivation optimization strategy applied in this study is an efficient technology that may significantly increase the sustainability of aquaculture wastewater treatment by microalgae, due to the increased microalgal cell density and nutrient (N and P) removal efficiency requirements. Microalgae cultivation on the wastewater-based medium supplemented with P led to a substantial increase in cell density by 453, 472, and 358.71% for NRRE-1, NSRE-1, and NSRE-2, respectively. Similarly, N removal significantly increased by 74.88, 66.44, and 68.55% for NRRE-1, NSRE-1, and NSRE-2, respectively. Also, the results suggested that studying an adequate N/P ratio for cultivating a specific microalgae strain in conjunction with the aquaculture wastewater-based medium enriched with P allows for optimizing the aquaculture wastewater treatment and biomass production. These findings provide valuable insights into the practical implications of sustainable microalgae cultivation and more efficient and economical processes. However, it is crucial to consider the cost of P in this context.

Credit author contribution

R.M. Gonzalez-Balderas: conceptualization, methodology, investigation, writing; T. Toledano-Thompson: supervision, writing-review & editing; C. Rosas-Vásquez: writing-review & editing; I.G. Albarrán: writing-review & editing; R. Valdez-Ojeda: supervision, project administration, writing-review & editing.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ACKNOWLEDGMENTS

This research work was carried out at the Renewable Energy Unit of the Yucatán Scientific Research Center (CICY). To the Consejo Nacional de Humanidades, Ciencia y Tecnología (CONAHCYT) for the scholarship awarded for the project entitled "Estudio nutrimental en microalgas marinas para potenciar su capacidad lipídica" (2364085) within the framework of the Estancias Posdoctorales por México program.

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Received: October 17, 2024; Accepted: May 10, 2025