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Impact of inoculum on domestic wastewater treatment in high-rate ponds in pilot-scale: Assessment of organic matter and nutrients removal, biomass growth, and content

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ABSTRACT

Discharging untreated wastewater deteriorates water bodies, but biological treatment methods can mitigate this by reducing organic matter, nutrients, and phosphorus. Systems using microalgae-bacteria consortia are promising nature-based solutions (NbS) that require no artificial aeration and can produce valuable products. This study, conducted on a pilot scale with domestic wastewater, involved two high-rate ponds (HRP1 and HRP2) with a depth of 0.30 m and lengths of 6.0 m. While HRP1 was not inoculated, HRP2 received activated sludge, and both were operated in two sequential batches followed by continuous operation with a four-day hydraulic detention time. After 20 days, biomass and system stabilization were primarily observed in HRP2, which achieved total nitrogen removal of 84.2 %, phosphorus removal of 63.7 %, and organic matter removal of 74.7 %. The inoculated pond showed increased stability and sedimentation capacity, with average volatile suspended solids of 152.75 ± 120.53 mg/L, a flocculation efficiency exceeding 90 %, and a sludge volume index of 19.83 ± 28.54 mL/g. Chlorophyll-a concentrations were higher in HRP2 (0.84 ± 0.48 mg/L), indicating higher algal biomass. Protein concentrations were 98.86 ± 47.78 mg/gVSS in HRP2, while carbohydrate concentrations averaged 67.87 ± 135.07 mg/gVSS, slightly superior for HRP2. Additionally, HRP2 demonstrated a lipid content of 23.03 %, attributed to a dominant *Chlorella* sp. presence. These findings underscore the need to optimize operational parameters and microbial consortia for effective biomass production and resource recovery in HRPs. Such low-cost NbS contribute to pollution control and align with the UN's sustainability objectives (SDGs) 3, 6, 11, 13, and 14.

1. Introduction

High-rate lagoons (HRL) or high-rate ponds (HRP), both used in wastewater treatment and post-treatment processes, have demonstrated significant potential for resource recovery. Concentrating high-value products within the biomass of microalgae-bacteria consortia (MABA) presents an efficient approach to promoting a circular economy. Notably, separating the biomass from the liquid phase is a critical step in this process [1–3]. Enhanced commensal interactions between microalgae and bacteria may accelerate the process of extracting the MABA from treated wastewater [4] and improve their retention within the system, optimizing overall performance [5,6].

According to Coggins et al. [7] and Satiro et al. [8], effective HRP treatment relies on a mutualistic and symbiotic relationship between the organisms. Oxygen generated through algal photosynthesis is utilized by bacteria to oxidize organic matter, while carbon dioxide required by algae is supplied by bacterial metabolism in the pond. Additionally, Rezvani et al. [9] reported that the assimilation and dissimilation of nitrogen forms can be enhanced by the application of the consortia. Moreover, several external and internal factors, including dissolved

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oxygen, pH, temperature, and radiation, can influence the performance of these systems [1,10].

Microalgae perform optimally at temperatures between 25 °C and 30 °C, although this range can vary depending on the species. At lower temperatures, energy absorption and consumption become unbalanced. It is important to note that light is absorbed regardless of temperature; however, enzymatic processes function less efficiently under suboptimal conditions. As photosynthetic and metabolic enzymes lose structure and catalytic activity, they can be gradually suppressed [11–13]. Conversely, elevated temperatures directly affect all enzymes within microalgal cells, disrupting their balance within the system. When the temperature exceeds the optimal range, growth rates decline significantly. Thermal stress mostly affects proteins and membrane structures and can increase the number of reactive oxygen species in cells [11,14].

Carbon, nitrogen, and phosphorus are the most important limiting elements for microalgal growth in ponds [3,15]. The most common nitrogen removal methods in HRPs include ammonia volatilization, ammonia and nitrate assimilation by microalgae, nitrification, and denitrification by aerobic bacteria, as well as particulate organic nitrogen sedimentation. Depending on the pond's characteristics and local climate conditions, these mechanisms may operate simultaneously or even dominate the process [16–18].

According to the literature, the presence of specific types of microorganisms is required for the total removal of organic matter and nitrogen [19,20]. Autotrophic bacteria use an aerobic process to nitrify the system, producing nitrite and nitrate, while ammonia-oxidizing bacteria (AOB) are responsible for converting ammonia to nitrite [21,22]. The generated nitrite can be removed from the system through denitrification, which converts NO_2^- to atmospheric N_2 [23,24]. There are also two known NH_4^+ oxidation processes: anammox, where nitrite acts as an electron acceptor under anoxic conditions and a low C/N ratio (0.2–2.5) [25,26], and comammox, where ammonia is completely oxidized to nitrate [27].

To enhance the sedimentation of HRP biomass, some studies have utilized activated sludge as an inoculum [28,29]. Inoculating activated sludge during reactor startup is a common strategy to expedite the formation of active biomass and stabilize biological processes, particularly in systems with microalgae-bacteria consortia [8,30]. This approach offers several advantages, including reducing the time needed to achieve stable operational conditions, optimizing nutrient and organic matter removal through symbiotic interactions between microalgae and bacteria [24,31–33], and improving system resilience to influent variability due to the microbial diversity present in activated sludge. However, there are notable drawbacks, such as competition for nutrients between heterotrophic bacteria and microalgae, the potential for microbial predators to reduce algal biomass, and the increased complexity of maintaining symbiotic balance under variable operational conditions [4,34]. Thus, the use of activated sludge as an inoculum must be carefully evaluated, considering its effects on microalgal performance and overall system stability.

In recent years, wastewater treatment systems coupled with algae biomass production have gained attention for their ability to leverage the synergistic relationship between microalgae and bacteria to remove organic matter and nutrients [19,29,35]. These systems offer a cost-effective alternative to conventional treatments by minimizing energy consumption and improving biomass harvesting efficiency [3,36,37]. Biomass recovery, a critical step for producing high-value products, accounts for 20 % to 30 % of total production costs [38]. The bioflocculation process presents a sustainable solution [39], enabling the production of sedimentable biomass without chemical additives or resource-intensive technologies, such as mechanical and electrical treatments, which can significantly increase operation costs [40].

The by-products derived from the MABA play a pivotal role in advancing sustainable industries. These consortia enable the recovery of valuable resources, including biofuels (biodiesel, bioethanol, and biohydrogen) [24], biopolymers [41], phosphorus [42], biochar [43], and animal feed [44]. As such, systems utilizing MABA not only provide an eco-friendly wastewater treatment approach but also contribute to the circular economy by transforming waste into valuable products.

Microalgae in high-rate pond systems represent a promising sustainable NbS for resource recovery (water circularity and nutrient circularity), pollution control, and public health protection, aligning with UN sustainability goals 3 (good health and well-being), 6 (clean water and sanitation), 11 (sustainable cities and communities), 13 (climate action) and 14 (life belongs water) [45]. Compared to continuous and sequential batch reactors, HRPs are more cost-effective and environmentally sustainable [4,8,46]. This study aims to evaluate the biological removal process of organic matter, nitrogen, and phosphorus in HRPs under conventional operation and systems inoculated with activated sludge. Special emphasis is placed on the formation of photogranules and biomass growth in the microalgae-bacteria consortium. Additionally, the work investigates biomass sedimentation capacity and composition, contributing to a deeper understanding of HRP system optimization.

2. Materials and methods

2.1. System configuration

The high-rate ponds used in this experiment were located at the Mangueira Wastewater Treatment Station in Pernambuco, Brazil, situated at a latitude of 8°04′40.22″ south and a longitude of 34°55′29.39″ west. The station is designed for the treatment of domestic wastewater and includes preliminary treatment followed by secondary treatment with UASB reactors (each with a capacity of 810 m^3 and a hydraulic retention time (HRT) of 7 h) before the effluent is discharged at a high rate. Three HRPs were installed at the station (Fig. 1), but data from two ponds (HRP1 and HRP2) were evaluated for this investigation. These fiberglass HRPs have a length of 6 m and a surface area of 8 m^2 and were operated with a water depth of 0.3 m. A motor-reducer system operating at 0.5 m/s was used to circulate the liquid mass. Finally, the ponds were operated outside, for this reason there was no control of lighting and aeration. Also, there was no inoculation of microalgae in the HRPs, the

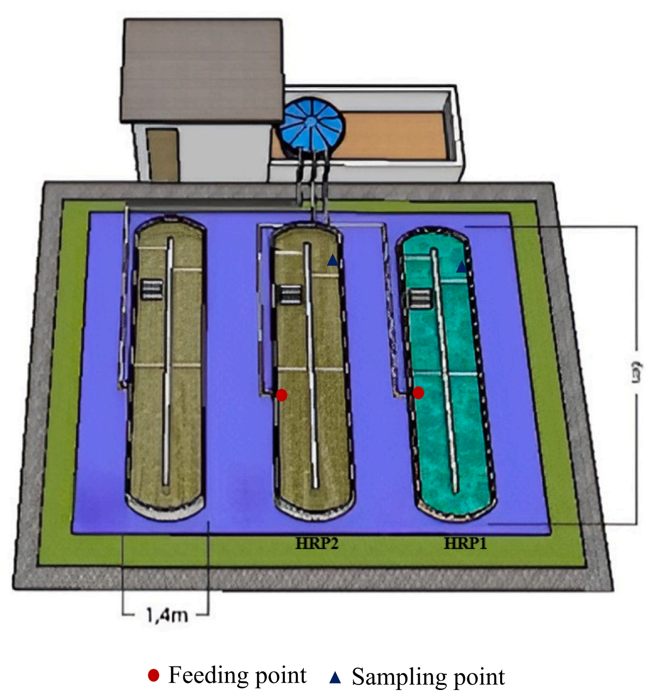

Fig. 1. Pilot-scale experimental apparatus (high-rate ponds).

microalgae species developed throughout the operating time.

2.2. Domestic wastewater and sludge characteristics

The high-rate ponds were fed with domestic wastewater that had undergone full-scale treatment by an up-flow anaerobic reactor with a sludge blanket (UASB reactor); the effluent parameters are detailed in Table 1. Additionally, HRP2 had a total volume of 2400 L and was inoculated with 240 L of activated sludge, approximately 10 % of the total volume, sourced to facilitate the formation of microalgae-bacteria photogranules. The recovered aerobic inoculum contains flocculent biomass with a high concentration of organic materials (Table 1).

2.3. Operation of high-rate ponds

The experiment consisted of three stages spanning a total of 94 days. The mechanisms for batch1 and batch2 were selected based on the work of Satiro et al. [3], which outlined strategies for startup reactors with microalgae-bacteria consortium:

i) *First batch phase* (*Batch1*): both ponds were filled with UASB effluent, and the liquid was circulated in the HRP1 and HRP2 for 11 days. Since no photogranules were observed, the rotor was turned off, and approximately 80 % of the volume was purged to reduce turbidity in the water column;
ii) *Second batch phase* (*Batch2*): this phase began immediately after the first batch phase and lasted 13 days to allow the consolidation of the MABA. Once photogranules were observed in the system, especially in HRP2, semi-continuous feeding was initiated, as described in (iii).
iii) *Semi-continuous fed phase* (*SC*): both HRPs were fed with UASB's effluent for 70 days with an HRT of 4 days. Feeding was semi-continuous, carried out for 16 h daily (predominantly during daylight), following the approach described by Sutherland et al. [47] and Arbib et al. [48].

2.4. Evaluation of organic matter and nutrient removal

The removal of organic matter, nitrogen, and phosphorus was evaluated in the soluble phase of water samples collected at the inlet and outlet of HRP after filtration with 0.45 mm pore size filters and based on removal efficiencies and variations in mass loads. Organic matter was quantified through the determination of chemical oxygen demand (COD), nitrogen as ammonia nitrogen (NH_4-N), nitrite nitrogen (NO_2-N) and nitrate nitrogen (NO_3-N), and phosphorus as phosphate phosphorus (PO_4-P), according to standard methods [49]. Dissolved oxygen (DO) and pH were also measured through HACH CO HQ40d (Germany) multiparameter equipment.

Table 1
Characterization in triplicates of domestic wastewater and aerobic sludge inoculated in HRP.

Parameter	Unit	Domestic wastewater	Activated sludge
COD	mg·L^{-1}	160.51 ± 96.11	12,470.40 ± 439.20
BOD	mg·L^{-1}	80.00 ± 42.41	1200.00 ± 70.70
TN	mg N·L^{-1}	32.25 ± 6.58	315.30 ± 17.90
TP	mg P·L^{-1}	5.40 ± 1.33	118.40 ± 3.90
VSS	mg VSS·L^{-1}	60.33 ± 54.14	9960.00 ± 410.10

COD: chemical oxygen demand; BOD: biochemical oxygen demand; TN: total nitrogen; TP: total phosphorous; VSS: volatile suspended solids.

2.5. Profile of in-situ monitoring of high-rate ponds

During continuous operation, after establishing a stable state of organic matter and nutrient removal, temporal profiles of the HRPs were conducted on days 67 and 81 of the operating period. Monitoring was carried out every hour, starting at 8:00 a.m. and concluding at 7:00 p.m. pH and dissolved oxygen were used as key parameters for analysis, measured using a HACH HQ40d multiparameter probe. This technique was employed to study HRP's behavior regarding photosynthetic activity and the consumption of dissolved oxygen in the medium.

2.6. Identification and classification of microalgae species

To perform both quantitative and qualitative analyses of microorganisms, 300 mL samples of mixed liquor from HRP1 and HRP2 were collected during each sampling event and preserved in a 5 % acid Lugol solution. The microalgae populations were then identified and counted using an inverted microscope (Leica-DME) equipped with a Bel View 7.1 photonics camera, following the procedures outlined in the phytoplankton manual [50].

2.7. Analysis of biomass formation, stabilization, and content

Volatile suspended solids (VSS) were analyzed using the standard methods [49]. Chlorophyll-a (CHLA) was measured using the methodology developed by Leong et al. [32]. Volatile suspended solids (VSS) were analyzed using standard methods [49]. Chlorophyll-a (CHLA) was measured following the methodology developed by Leong et al. [32]. For this, 40 mL of the raw sample (mixed liquor) from each LAT was centrifuged for 10 min at 4000 rpm. The supernatant was discarded, and 20 mL of 90 % acetone along with 0.05 g of $CaCO_3$ were added to each sample. The samples were then homogenized using a vortex for 1 min. Afterward, they were stored in the dark by wrapping the falcon tubes in aluminum foil and refrigerated at 4 °C for 24 h. Finally, the samples were centrifuged again for 10 min at 4000 rpm, and the resulting supernatant was analyzed for optical density (OD) at 750 nm, 663 nm, 645 nm, and 630 nm, as described in Eq. 1.

$$\mathrm{CHLA}\left(\frac{\mathrm{mg}}{\mathrm{L}}\right)=\frac{[11.64\ (\mathrm{OD663}-\mathrm{OD750})-2.16\ (\mathrm{OD645}-\mathrm{OD750})+0.10\ (\mathrm{OD630}-\mathrm{OD70})\,]\times\mathrm{V}}{\mathrm{VT}} \tag{1}$$

where V represents the acetone volume; VT total volume of the sample.

The sludge volume index (SVI) was determined according to the method proposed by Schwarzenbeck et al. [51]. To perform the flocculation efficiency (FE), the methodology described by Satiro et al. [3] was followed. A 50 mL aliquot of the mixed liquor sample was extracted and gently homogenized by stirring. The OD was measured at a wavelength of 650 nm. The sample was then allowed to settle for 20 min, and the OD was measured again at the same wavelength, with the new aliquot extracted up to a maximum depth of 2.5 cm from the surface. The biomass flocculation efficiency (%) was calculated using Eq. 2.

$$\mathrm{FE}\ (\%)=\left(1-\frac{\mathrm{OD650i}}{\mathrm{OD650f}}\right)\times 100 \tag{2}$$

where OD650i represents the non-homogenized sample, and OD650f represents the sample after 20 min.

To assess the biomass content, chloroform acid was used to extract the extracellular polymeric substances (EPS) for 1 h at 90 °C, following the method described by Arcila and Buitrón [28]. After EPS extraction, the samples were analyzed for carbohydrates (PS) according to Dubois et al. [52] and proteins (PN) following the method of Lowry et al. [53]. For oil extraction and lipid quantification, the biomass was dried and placed in a Soxhlet apparatus for 8 h, using a continuous wash of 2:1 methanol, as described by Bligh et al. [54]. Finally, the contents were evaporated at 40 °C in a water bath until a dry mass was obtained. The residual content in the tubes was determined using gravimetry, following the method described by Satiro et al. [55] and presented in Eq. 3.

$$\text{Lipid content } (\%) = \left(\frac{\text{L1}}{\text{L2}}\right) \times 100 \tag{3}$$

where L1 is the weight of lipid content in the tubes; and L2 is the weight of dry biomass.

2.8. Statistical analysis

A statistical evaluation of the process performance indicators was conducted using ANOVA at a 95 % confidence level, along with Tukey's significance test, to compare the performance of the high-rate ponds under both batch and semi-continuous regimes. Samples were taken in triplicate for all experiments.

3. Results and discussion

3.1. System startup — single batches

Table 2 shows the process parameter results from the initial stages of pond operation, spanning the first batch (11 days), the second batch (13 days), and the transition to semi-continuous operation. The impact of activated sludge inoculation on organic matter and nutrient concentrations (ammoniacal nitrogen, nitrite, nitrate, and phosphorus) is emphasized. Ammoniacal nitrogen, nitrite, and nitrate concentrations were measured at the start of the first batch with values close to zero, indicating that the nitrification process had not yet begun following the initial mixing of the ponds. This inactivity could be attributed to the high concentration of solids, which may have impeded the growth of autotrophic microorganisms. Additionally, most particles in this batch were smaller than 0.250 mm, suggesting limited synergistic interactions between microalgae and bacteria.

Low dissolved oxygen concentrations were observed during the first batch, likely due to the effluent's origin from anaerobic treatment, which may have contributed to a low population of autotrophic microorganisms. The ammoniacal nitrogen concentrations measured in the first and second batches, approximately 18.40 mg·L^{-1} and 31.80 mg·L^{-1}, respectively, can be attributed to the anaerobic conversion of organic nitrogen. Ammonia is released in this pathway as amino acids degrade.

The initial pH values in the first batch were 7.61 and 7.50 for the two ponds, both close to neutrality, which received anaerobic influent feeding and limited photosynthetic activity at the start of the system. However, these pH values rapidly increased during the subsequent batches, reaching approximately 10.80 and 9.66 for HRP1 and HRP2, respectively. This increase was attributed to CO_2 consumption by microalgae during photoautotrophic growth, as Su et al. [56] and Satiro et al. [3] reported.

An increase in dissolved oxygen (DO) levels in the medium was observed at the end of each batch. Initially, these values were 0.17 mg·L^{-1} and 0.10 mg·L^{-1}, increasing to 8.68 mg·L^{-1} and 10.40 mg·L^{-1} by the end of the second batch for HRP1 and HRP2, respectively. The increase in DO content in the ponds can be attributed to the enhanced photosynthetic activity of microalgae in the HRPs. Similar findings were reported in high-rate pond systems by Rodrigues et al. [44], where initial DO levels of 1.00 mg·L^{-1} increased to 8.00 mg·L^{-1} following microalgae adaptation and biomass growth.

Soluble COD removal, excluding biomass, reached 89 % for both ponds at the end of the second batch. These results for organic matter removal align with findings observed by Arcila and Buitrón [28] in similar systems; although their experiments were conducted over a 10-day batch period. After the significant degradation of organic matter and the increase in biomass within ponds, the second batch concluded after 24 days of operation in single-batch mode, transitioning the systems to semi-continuous feeding.

Fig. 2 illustrates the conditions observed in both ponds at the end of each single-batch mode. Water samples were collected from HRP1 and HRP2 at the end of Batch1 and Batch2 and placed in beakers for 10 min. Fig. 2a, corresponding to batch 1, shows a higher concentration of suspended particles, which increased medium turbidity and hindered the growth of the microalgae-bacteria consortium in HRP2. Elevated turbidity reduced light penetration into the water column, limiting the growth and formation of photogranules. Fig. 2b shows that the medium's turbidity decreased after the initial days of the second batch's operation, facilitating the development of the microalgae-bacteria photogranules.

In addition to the factors mentioned above, the solids in HRPs may have been produced not only by the growth of microalgae but also by the proliferation of other microorganisms such as bacteria and zooplankton. After 11 days of the first single batch, a rapid degradation of organic debris and nutrients was observed. The biomass increase in HRP2 may have been limited due to the lower abundance of microalgae, as the CO_2 produced by the microalgae was facultative, which is critical for bacterial growth. Zhu et al. [56] reported that when organic carbon is depleted in a single batch mode, bacteria are unable to develop in the medium. Consequently, microalgae became predominant in the ecosystem, functioning as photoautotrophic organisms that capture atmospheric CO_2 to continue multiplying.

3.2. Semi-continuous fed operation

3.2.1. Assessment of pH and DO variation

Dissolved oxygen in the HRP1 medium was approximately 15.79 ± 3.81 mg·L^{-1}, indicating an elevated level of photosynthetic activity and, consequently, higher growth of microalgae. In contrast, the average DO level in HRP2 was reported 12.8 ± 2.89 mg·L^{-1}. The lower DO levels in HRP2 may be attributed to oxygen consumption by aerobic bacteria within photogranules, considering that this pond exhibited better

Table 2
Characterization of the UASB effluent and HRP outputs in the batches.

Parameter	Unit	AF 1ª batch	Start 1ª batch		AF 2ª batch	Start 2ª batch		Batch end	
			HRP1	HRP2		HRP1	HRP2	HRP1	HRP1
pH	–	7.37	7.61	7.50	7.30	8.18	7.52	10.08	9.66
DO	mg·L^{-1}	1.19	0.17	0.10	0.28	8.66	0.27	8.68	10.40
COD	mg·L^{-1}	160.51	149.51	633.63	255.15	94.21	272.88	255.86	240.97
$NO_4^+ - N$	mg·L^{-1}	18.40	18.40	17.40	31.80	12.98	16.66	0.34	0.48
$NO_2^- - N$	mg·L^{-1}	0.008	0.008	0	0.005	0.004	0.122	0	0
$NO_3^- - N$	mg·L^{-1}	0.520	0.52	0	0	0	0.005	0	0
$PO_4^{3-} - P$	mg·L^{-1}	3.32	3.32	8.12	5.75	5.75	6.76	4.90	7.62

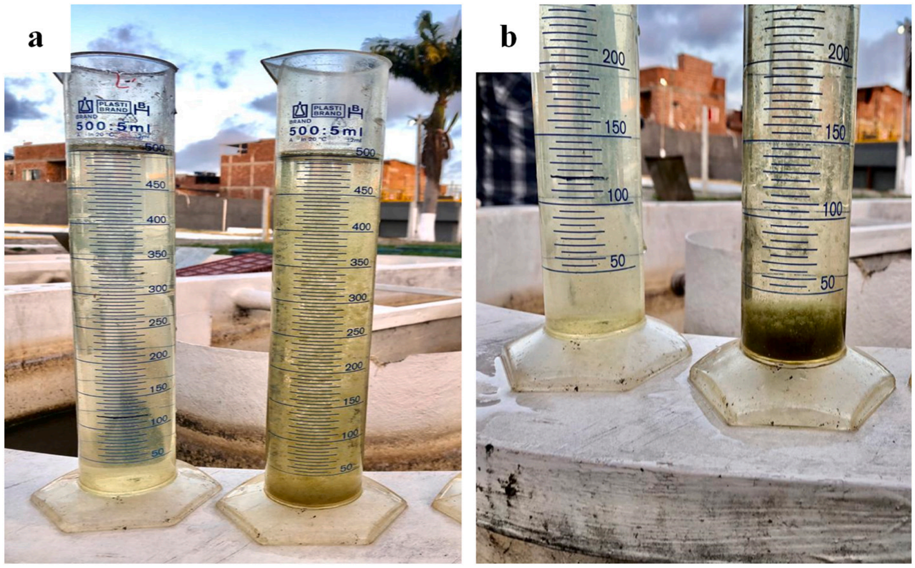

Fig. 2. Samples collected at the end of each batch experiment: a) Batch1 (from HRP1 to the left, from HRP2 to the right); b) Batch2 (from HRP1 to the left, from HRP2 to the right).

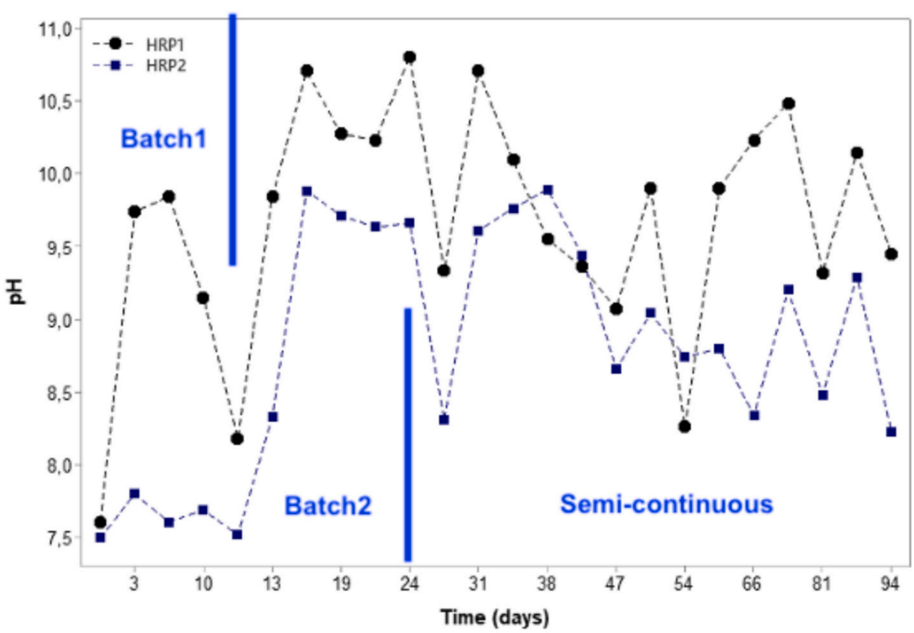

Fig. 3. pH profiles in both HRP for all operation periods.

formation of the microalgae-bacteria consortium, as discussed in the previous section on single batch mode. This observation is supported by Coggins et al. [7], who noted that heterotrophic organisms consume CO_2 generated by microalgae in mutualistic and symbiotic relationships within utilizing microalgae-bacteria consortium.

Fig. 3 illustrates the pH behavior in the ponds during the semi-continuous regime. The average pH values for HRP1 and HRP2 were 9.9 ± 0.67 and 9.04 ± 0.57, respectively, highlighting that pH control agents were applied [3]. A decrease in pH was observed primarily on days of heavy rainfall, such as days 24 and 54. As documented by Sutherland et al. [47], precipitation events dilute the system, contributing to these pH drops. Additionally, pH levels above 9.5 may promote the release of chemicals into the atmosphere, such as ammonia. However, all ponds recovered their pH, suggesting that the system's startup and operation strategies were adequate.

In general, the typical pH value in HRP1 was approximately 10.0, which could be attributed to the medium's high photosynthetic activity and elevated solar radiation levels. Variations in solar radiation influence pH levels by affecting the consumption of inorganic carbon. In HRP2, the mean pH values were below 9.0, possibly due to the self-regulating effect induced by the microalgae-bacteria consortium. Notably, the optimal pH range for microalgae growth is between 7.00 and 9.00 [57].

3.2.2. *Monitoring profile*

The monitoring profile was designed to assess the behavior of the ponds, specifically focusing on photosynthetic activity and oxygen consumption. This evaluation was conducted in the HRPs on two different days (days 67 and 81 of operation) when a steady state of organic matter and nutrient removal was reached. This assessment

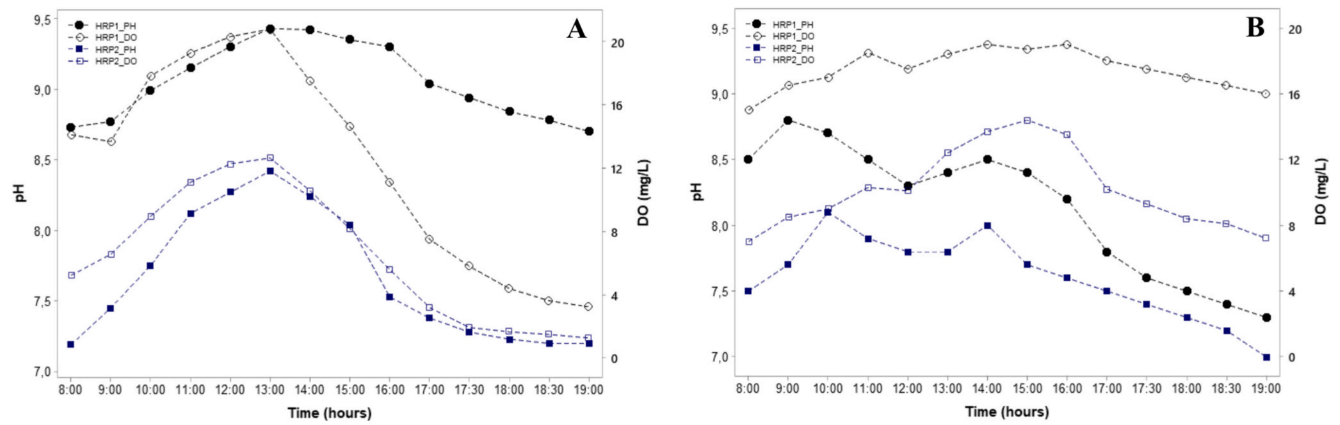

Fig. 4. Temporal profile of pH and DO evaluation: a) day 67; and b) day 81.

aimed to corroborate the findings by Park and Craggs [58], which indicated that a decrease in photosynthetic activity directly influences the respiration of the biomass within the system.

On the first evaluated day (Fig. 4a), higher DO and pH values were observed in HRP1, which had not been inoculated with activated sludge. At 8:00 a.m., the initial pH and DO were 8.73 and 14.11 mg·L^{-1}, respectively. As photosynthetic activity increased, serving as the primary mechanism for dissolved oxygen production, between 1:00 p.m. and 2:00 p.m., the time of highest solar radiation, pH and DO values reached 9.43 and 20.77 mg·L^{-1}, respectively. These values gradually decreased with increased respiration during the dark phase and the assimilation of ammoniacal nitrogen by microorganisms.

The second profile (Fig. 4b) showed lower photosynthetic activity values. On this day, HRP1's initial pH and DO were 8.91 and 13.81 mg·L^{-1}, respectively. No significant changes in pH and DO were observed until 2:00 p.m., which can be attributed to the poor weather conditions, particularly low temperatures, and rain during the monitoring period. The highest average values for HRP2 in the two pH and DO profiles were 8.46 and 11.67 mg·L^{-1}, respectively. The lower pH and DO levels in HRP2 may be due to the higher concentration of suspended solids in this pond. This observation aligns with the findings of Nijboer et al. [59], who reported that the reduced efficacy of solar radiation in high-rate ponds results in lower effluent temperatures and, consequently, reduced biomass respiration rates.

This study of pH and DO changes discovered that during the light phase, when there was more solar radiation, photosynthetic activity increased. There is a decrease in these variables during the dark phase, from 17:00 h onwards, when the radiation is substantially reduced. This decrease can be explained by the phase in which microorganisms' respiration predominates.

3.2.3. *Assessment of organic matter removal*

As previously mentioned, the mean influent COD values during the continuous operation regime of the high-rate ponds, receiving domestic wastewater from a UASB reactor, were 160.51 ± 96.11 mg·L^{-1}. Fig. 5 illustrates the concentrations of soluble COD in the effluent and the

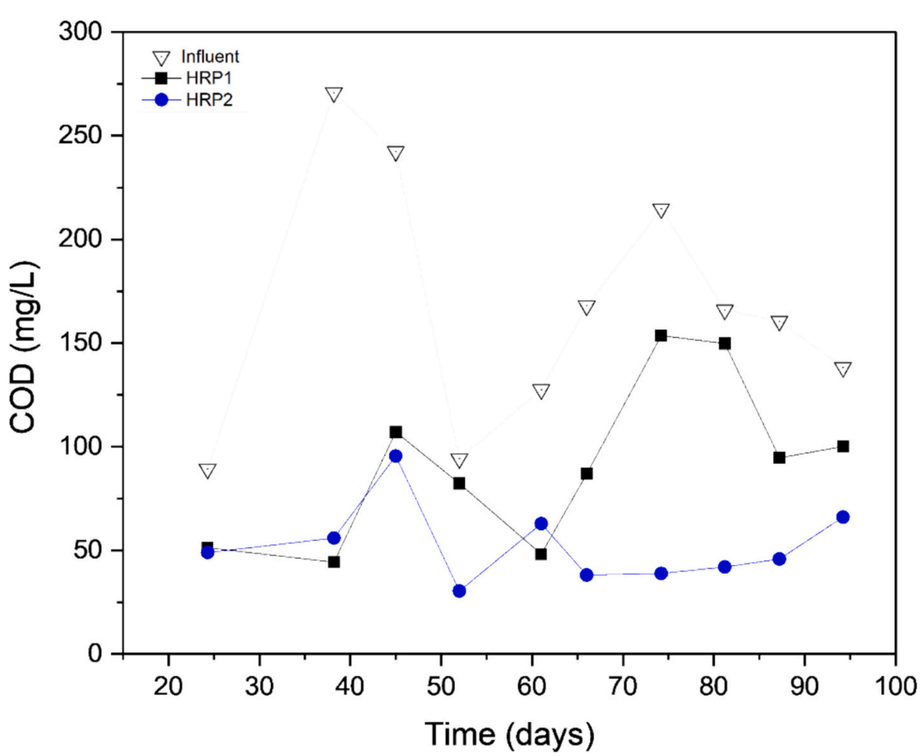

Fig. 5. The COD concentration of the influent and soluble effluent of the HRPs.

removal efficiencies achieved throughout the system's semi-continuous flow.

HRP1 and HRP2 achieved average COD removal rates of 51.62 % and 74.67 %, respectively. HRP2, which was inoculated with activated sludge to enhance the production of the microalgae-bacteria consortium, outperformed HRP1, which was not inoculated. Additionally, HRP1 showed higher variability in total COD removal during the operational period, possibly due to less aggregation and consequently higher susceptibility to variations such as feed composition, precipitation, temperature, radiation, and other variables.

The observed removal of organic matter in HRP2 indicates that bacteria consume organic matter at a substantially faster rate than microalgae. Tang et al. [60] highlighted the characteristic interaction of MABA, which contributes to evaluating HRP2 with activated sludge inoculation. In these systems, the release of CO_2 by microalgae promotes biomass growth, resulting in lower soluble COD concentrations. However, the COD removal values obtained in this study were lower than similar high-rate pond systems with MABA [61,62], which achieved removal efficiencies of around 90 % of COD. These systems operated the HRPs for longer durations and recirculated biomass, potentially enhancing the incorporation and removal of carbonaceous material.

When the microalgae-bacteria consortium system reaches symbiosis, there is mutualistic transfer of CO_2 and DO, with bacteria oxidizing carbonaceous material concurrently assimilated by microalgae [7]. The lower average removal values in HRP1 may be attributed to reduced aggregation, leading to fewer interactions between bacteria and microalgae. This could result in an increase in pH above 9.5 due to microalgae activity, a condition that limits organic matter degradation in the pond. In studies treating domestic wastewater, organic matter removals exceeded 95 % after 30 days of semi-continuous operation [63–65]. These high removal rates were observed after achieving higher stability of photogranules, with a higher content of extracellular polymeric substances.

3.2.4. Assessment of nitrogen removal

Fig. 6 illustrates the nitrogen removal performance during a semi-

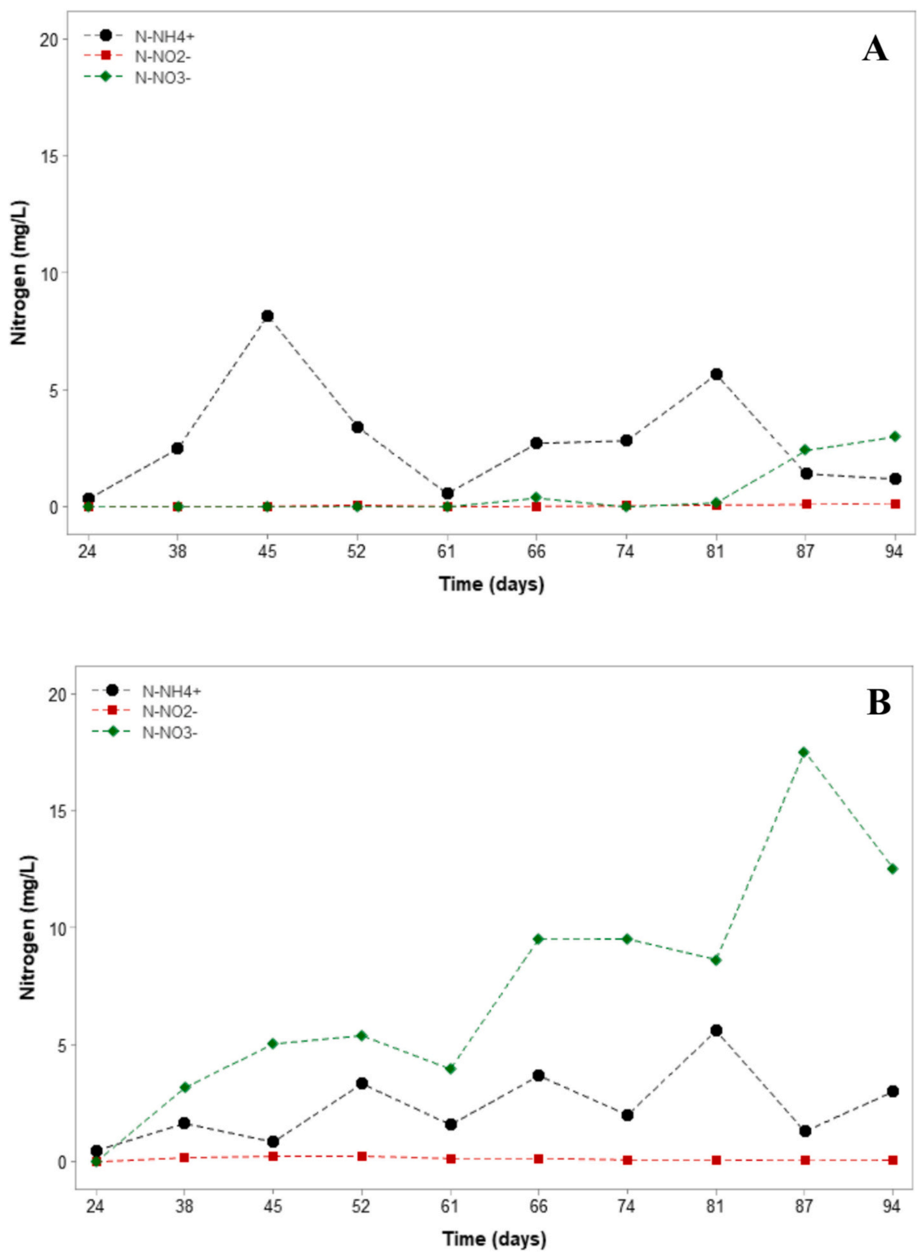

Fig. 6. Nitrogen balance in a) HRP1 and b) HRP2.

continuous regime. HRP1 and HRP2 showed similar average total nitrogen removal (83.50 % ± 6.30 and 84.15 % ± 6.75, respectively). Ammoniacal nitrogen predominated in HRP1 due to the higher concentration of microalgae in the system, whereas nitrate predominated in HRP2, attributed to nitrification by autotrophic bacteria present in the MABA. According to Satiro et al. [24], microalgae preferentially absorb nitrogen in the ammoniacal form (NH_4^+-N). Nitrogen removal in high-rate ponds typically occurs through three pathways: first, nitrification by autotrophic bacteria; second, assimilation, involving the conversion of ammoniacal nitrogen to organic nitrogen; and finally, through a physical-chemical process when pH exceeds 9.0.

The first hypothesis is that the variation in biomass concentration in the medium, influenced by climatic variations, may be related to ammonia volatilization due to pH values exceeding 9.0. Over time, Garcia et al. [66] and Robles et al. [33] have demonstrated that ammonia volatilization is the primary pathway for removing ammoniacal nitrogen in HRPs, particularly during clear phases characterized by high pH values.

Nitrogen loss through volatilization in the MABA system, such as in HRP2, is expected to be negligible due to natural pH control below 9.0, as discussed earlier. In similar studies with HRPs, Arcila and Buitrón [28] achieved NH_4^+-N removal rates of 99.0 % and 86.0 %, respectively, operating with high-rate ponds with HRT of 10 and 6 days. This success is attributed to biomass recirculation within the system, facilitating higher retention and incorporation of nitrifying bacteria into the biomass, which have slow growth rates. HRP2 demonstrated early and sustained nitrate formation during semi-continuous operation, which can be attributed to its operational characteristics and the inoculation of activated sludge, enhancing cell retention and subsequent nitrifying bacteria activity [28,62].

3.2.5. Assessment of phosphorus removal

The performance of phosphorus removal in the HRPs is as follows: The influent phosphorus levels directed to the HRPs are within the range of 4.39 ± 1.50 mg·L^{-1}. Both HRP1 and HRP2 exhibit similar average removal rates. Fig. 7 illustrates the overall phosphorus concentrations observed in the ponds.

HRP1 achieved a phosphorus removal rate of 71.40 %, while HRP2 achieved 62.69 %. Both ponds were operated at a design depth of 0.30 m, which allows for higher penetration of solar radiation into the water column, facilitating the assimilation of phosphorus by microalgae. Among the monitored high-rate ponds, HRP1 demonstrated superior performance in phosphorus removal. This outcome may be attributed to the following factors: (i) solar radiation — the design depth of 0.30 m likely allowed for sufficient solar radiation to penetrate the water column, enhancing the photosynthetic activity of microalgae and promoting phosphorus assimilation; (ii) pH conditions — HRP1 maintained a pH above 9.5, which is conducive to the chemical precipitation of phosphates. According to Lei et al. [67], phosphorus tends to precipitate as hydroxyapatite or struvite under alkaline conditions, forming small crystals that settle out of the system; and (iii) microbial process — microalgae in HRP1 likely enhanced phosphorus removal through biological uptake, with subsequent incorporation into biomass, followed by sedimentation. These combined factors contributed to the higher phosphorus removal efficiency observed in HRP1 compared to HRP2, despite both ponds operating under similar design conditions.

Notably, the two HRPs used in this study outperformed those used in a comparable high-rate pond system with biomass recirculation, which achieved phosphorus removal of around 30 % under an HRT of 6 and 10 days. In that system, the authors operated with an initial concentration of PO_4^{3-}-P of 14.3 mg·L^{-1} and a pH range of around 8.2. Moreover, when operating with HRPs at a depth of 0.30 m, phosphorus removal rates ranged from 41.4 % to 53.2 %, yielding overall removal efficiencies lower than those observed in this present study [68].

Throughout the continuous regimen, the mean N/P ratios of HRP1 and HRP2 were 4.39 and 7.53, respectively. Studies indicate that the optimal ratio for achieving good phosphorus removal in systems with microalgae-bacteria consortium is between 6 and 10. This supported

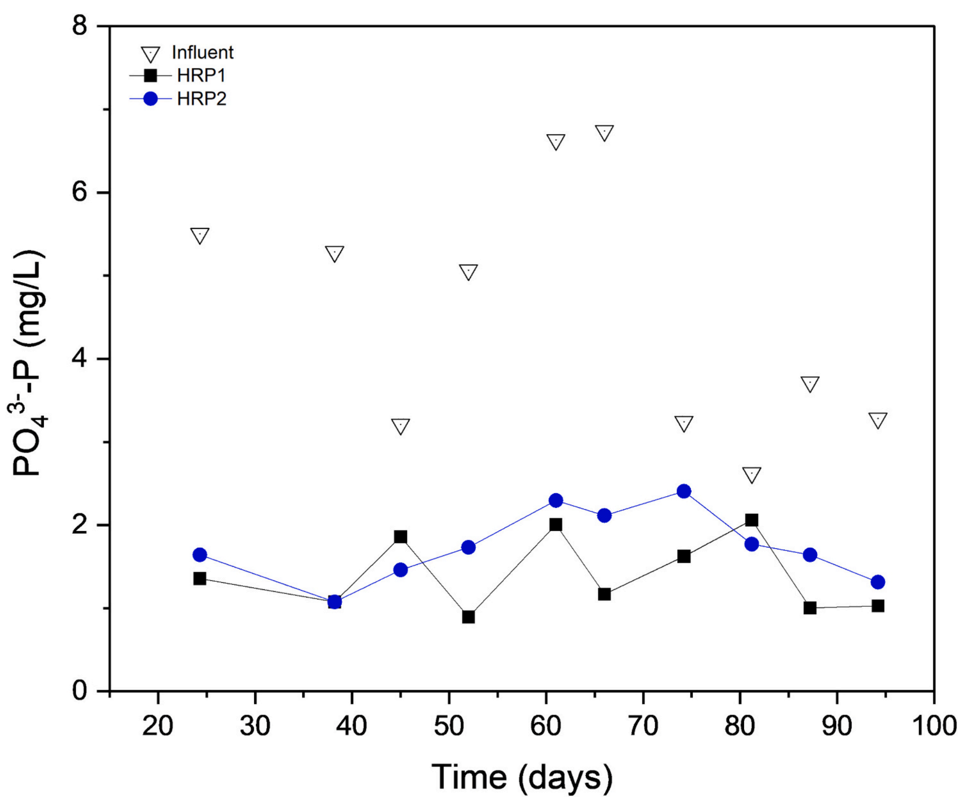

Fig. 7. Average soluble phosphorus concentration in HRP effluent.

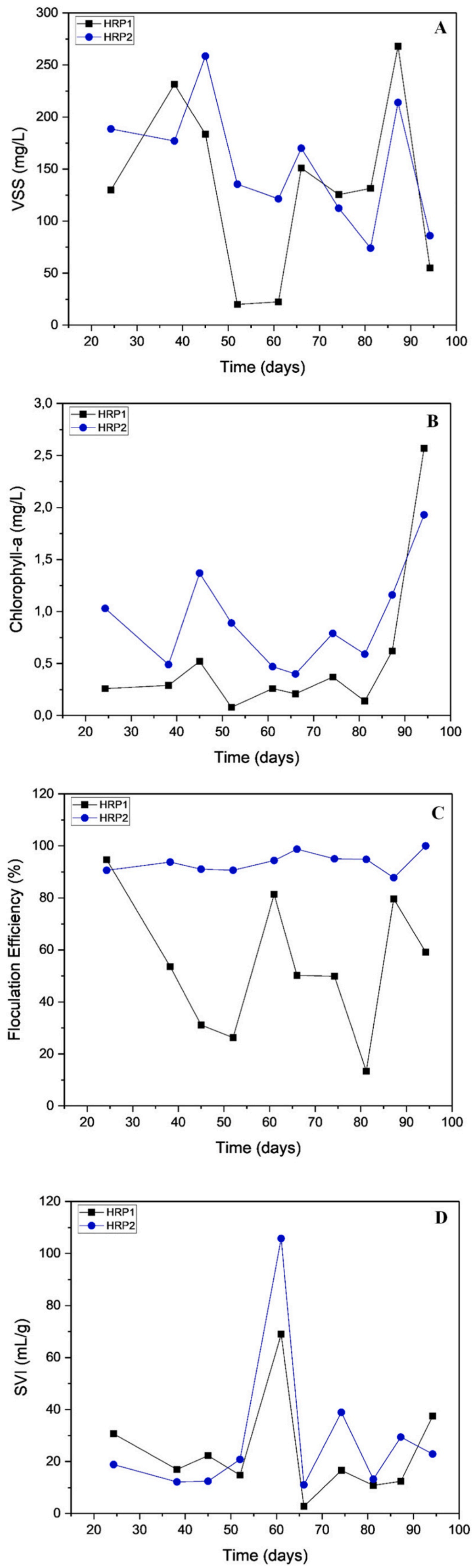

Fig. 8. Biomass growth, stabilization, and sedimentation parameters measured during the continuous operation of the HRPs: (a) volatile suspended solids (VSS); (b) chlorophyll-a (CHLA); (c) flocculation efficiency (FE); and (d) sludge volume index (SVI).

HRP2's ability to sustain strong phosphorus removals via biomass assimilation, potentially reducing the need for chemical removal [28,69].

3.3. Assessment of biomass growth and stabilization

This section examines the parameters that allow the assessment of microalgae-bacteria consortium formation. Various factors can influence this process, including operational parameters such as hydraulic retention time, the concentration of organic load, and the nutrient content in the effluent to be treated. Fig. 8 illustrates the behavior of solids, chlorophyll, sludge volume index, and flocculation efficiency in a continuous operating regime.

To begin this discussion, it is essential to highlight that the biomass produced in high-rate ponds comprises a mixture of microalgae, bacteria, zooplankton, and detritus [3]. As shown in Fig. 8a, which presents the solids data from the biomass after the initial two batches used to start up the system, the average concentrations during the semi-continuous phase were 131.25 ± 72.03 mg/L and 152.75 ± 120.53 mg/L for HRP1 and HRP2, respectively. These values suggest similar performance between the two ponds, with the slightly higher concentration in HRP2 likely due to the bioflocculation process between microalgae and bacteria. In related studies, Garcia et al. [70] reported VSS values of 300 ± 100 mg/L and 260 ± 93 mg/L for HRPs with hydraulic retention times of 4 and 7 days, respectively, highlighting that optimizing the HRT is a critical parameter to prevent biomass washout from the system.

In HRP1, the VSS concentration values are not directly linked to the stabilization behavior of the microalgae-bacteria aggregates, as this pond did not employ the activated sludge inoculation mechanism to stratify the system. The variation in behavior in this HRP is primarily due to the abundance of microalgae species that dominate the environment. It is important to note that on rainy days, certain species tend to predominate, while at higher temperatures, others adapt better to the environment [71,72]. This fluctuation in microalgae abundance within the HRP limits the production of CO_2, which hinders the growth of heterotrophic bacteria, ultimately reducing the VSS concentration in the pond [62].

On the other hand, when evaluating the time required for microalgae and bacteria to achieve stability, structure, and a more consistent concentration in the medium, it was observed that after 24 days of continuous operation in HRP2, the VSS values became more linear and stable [8]. This suggests that the initial batches were sufficient to establish a stabilized consortium of microalgae and bacteria after initiating the system. In similar work, Robles et al. [62] reported that approximately 30 days of continuous operation were necessary to stabilize MABAs in high-rate ponds.

The average chlorophyll-a concentrations in the semi-continuous operation are presented in Fig. 8b, showing values of 0.28 ± 0.64 mg/L for HRP1 and 0.84 ± 0.48 mg/L for HRP2. According to Couto et al. [73], the chlorophyll concentration values in high-rate ponds indicate the growth of the algal biomass produced. However, dos Santos Neto et al. [29] caution that in open systems, such as in this study, environmental, climatic, and operational conditions can directly influence the amount of pigments produced in the microalgal cells.

It was observed that in HRP2, chlorophyll levels remained more stable, despite the climatic variations that occurred during the operational period. Zhao et al. [65] attributed this stability to the effective formation of photogranules, which increased in size and became denser and more compact after the batch regime period. As a result, HRP2 exhibited lower biomass loss and higher pigment concentrations, regardless of the seasonal fluctuations affecting the systems. Furthermore, in studies involving HRPs with water column depth of 0.45 m and 0.30 m, Craggs et al. [6] reported higher VSS concentrations in the HRP with a 0.45 m water column; however, they noted that chlorophyll concentrations in both ponds were similar.

Investigating the flocculation efficiency values (Fig. 8c), which reflect biomass sedimentation capacity, HRP1 and HRP2 exhibited average efficiencies of 51.89 ± 25.94 % and 94.08 ± 3.80 %, respectively, during the semi-continuous operation. In this context, HRP2, which operated with a microalgae-bacteria consortium, developed more stable photogranules, achieving flocculation values exceeding 90 % from the early stage of semi-continuous operation. Conversely, HRP1 displayed a more erratic behavior, indicating a deficit in the linearity of biomass sedimentation. These results suggest that HRP2, utilizing aerobic sludge systems for start-up, produced biomass with higher potential and effectiveness for resource recovery [55]. In a similar study, Arcila and Buitrón [28], evaluated sedimentation percentages in high-rate ponds with hydraulic retention times of 10, 6, and 2 days, reporting values of 98.3 %, 92.7 %, and 0.0 %, respectively. Thus, the satisfactory flocculation and sedimentation of biomass using microalgae-bacteria aggregates can be attributed to the system's start-up with two sequential batches, as well as the presence of diatoms, which are commonly found in aggregates. The silica-based cell walls of these organisms aid in the sedimentation of biomass [8].

Fig. 8d presents data on biomass sedimentation capacity. Although this parameter is not commonly used in studies on biomass production in high-rate ponds, it is frequently applied in systems operating with activated sludge and aerobic granular sludge to evaluate the sedimentation characteristics of the produced biomass. Supporting the previously discussed flocculation efficiency values, the average SVI_{30} values for HRP1 and HRP2 were 16.8 ± 18.83 mL/g and 19.83 ± 28.54 mL/g, respectively. These results indicate good sedimentation of the biomass, suggesting that the strategy of utilizing two batches for the formation of photogranules is a viable and effective process for the bioagglutination of microalgae with bacteria.

Good bioagglutination enhances wastewater treatment and biomass recovery, reducing the need for decanters and facilitating the effective harvesting of biomass for the production of value-added products [55]. Ahmad et al. [74] working with synthetic wastewater in continuous-flow photobioreactors, reported sedimentation values (SVI_{30}) of approximately 44 mL/g for biomass with microalgae-bacteria consortium. In contrast, Arcila and Buitrón [28] achieved SVI_{30} values of 40 mL/g using high-rate ponds with microalgae-bacteria aggregation, although their systems operated with a hydraulic retention time of 10 days. To conclude this discussion on the formation and sedimentation of biomass in HRP1 and HRP2, Fig. 9 presents microscopic images illustrating the interaction between bacteria and microalgae during semi-continuous operation.

3.4. Identification of microalgae species

The relative abundances of the species present in the ponds are shown in Fig. 10, along with the taxonomic identification of each species observed throughout the experimental period. In HRP1, Chlorophyceae predominated, having adapted to the environmental conditions provided. In this HRP, with lower turbidity, it was likely easier for sunlight

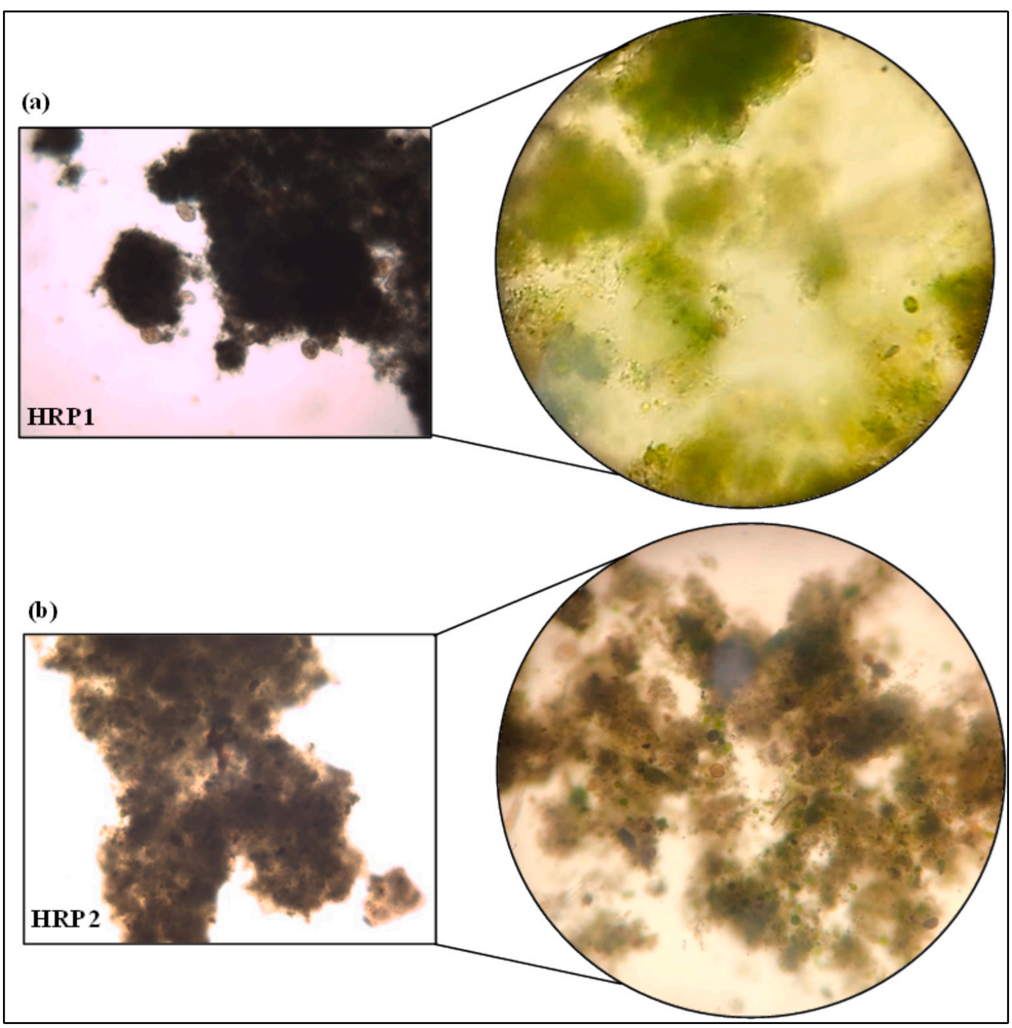

Fig. 9. Optical microscope images illustrating the granular and symbiotic differences between microalgae and bacteria in the two high-rate ponds (HRPs): (a) HRP1 at 10× magnification with subsequent 40× magnification; (b) HRP2 at 10× magnification with subsequent 40× magnification.

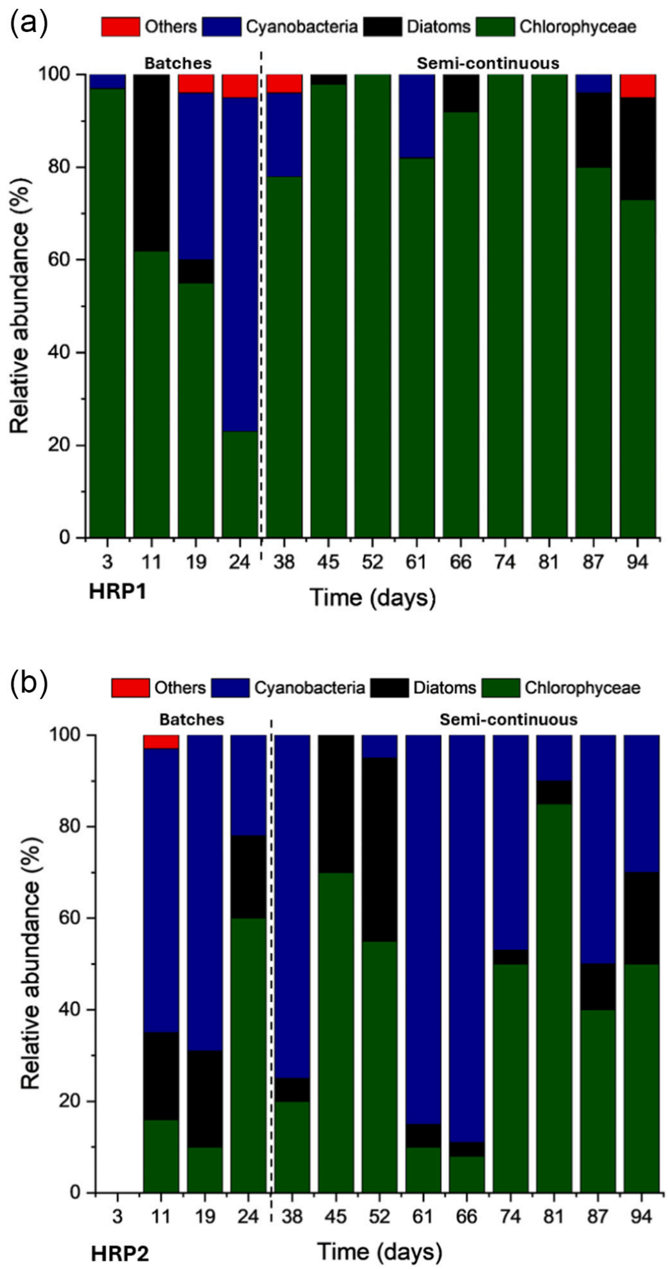

Fig. 10. Relative abundance of microalgae species in batch regime and semi-continuous regime: (a) HRP1; and (b) HRP2.

to penetrate the water column, as this pond was not inoculated at startup. In contrast, HRP2 exhibited a higher distribution in the relative abundance of species. It is important to note that diatoms are essential organisms in the formation of MABA, as they contribute to the stability of the consortium, and their silica-based cell walls enhance the sedimentation of particles. Additionally, in HRP2, the presence of filamentous cyanobacteria was observed throughout the experimental period, which may have facilitated the symbiotic process between microalgae and bacteria.

In HRP1, Chlorophyceae predominated. Initially, during the batch regime, the most abundant species was *Chlorella* sp., with an average abundance of 44 %. From the semi-continuous regime onwards, the average abundance of the most frequent species shifted to *Oocystis* sp. and *Chlamydomonas* sp., with average abundances of 85 % and 82 %, respectively. These species are characteristic of periods with lower temperatures [73,75], which can be attributed to the colder days and high rainfall during the experimental period. Additionally, in HRP1, a low abundance of diatoms was observed; these species began to appear more frequently in the last 10 days of operation in this pond.

On the other hand, for HRP2, it was not possible to visualize the morphological structures of the species on operational day 3, as shown in Fig. 10b. In this pond, the environmental diversity was higher during the operational period, with the presence of Chlorophyceae, cyanobacteria, and diatoms. The species that predominated in this pond was *Chlorella* sp., with approximately 60 % relative abundance. This is a promising indication for future studies on biomass recovery for biofuel generation, considering that this genus is capable of accumulating between 20 % and 60 % lipids by dry mass [55,76].

The authors Santiago et al. [77] studied HRPs with a depth of 0.30 m and observed the presence of *Chlorella* sp. and *Desmodesmus* sp., highlighting the importance of these species due to their ability to withstand high organic loads, as well as their high growth and respiration rates. Additionally, it is essential to emphasize the abundant presence of *Planktothrix* sp. (55 ± 20 %), which played a fundamental role in the formation of the MABA in HRP2. These filamentous organisms facilitate the adhesion of microalgae and bacteria to one another. In the study conducted by Arcila and Buitrón [28], the most abundant genus of filamentous organisms was *Stigeoclonium*, which, due to its flagellated structure, contributed to an increased contact area with other species of microalgae and bacteria. Furthermore, Satiro et al. [8] indicated that cyanobacteria can adapt quickly in systems with limited light penetration, a characteristic feature of HRP2.

3.5. Assessment of biomass content

Throughout the semi-continuous operating period, the biomass content was evaluated in HRP1 and HRP2 (Table 3). During this period, the average protein concentration values were 61.29 ± 65.23 mg/gVSS for HRP1 and 98.86 ± 47.78 mg/gVSS for HRP2. The protein content (EPS-PN) in HRP2 was higher than in HRP1, which supports the better performance of this pond in terms of sedimentability and stability of the consortium. According to Zhao et al. [65], a higher EPS-PN content in photogranules is associated with higher stabilization capacity and serves as an indicator of the maturation process of the granules. Regarding the potential by-products generated from protein concentration, Barreiro-Vescovo et al. [78] suggest that proteins extracted from biomass produced in high-rate ponds can be utilized to create animal feed and food supplements.

The carbohydrate concentrations (EPS-PS) were similar between the two ponds, with HRP2 exhibiting a slight advantage. The concentrations measured for the dry mass of suspended solids were 56.46 ± 29.26 mg/gVSS for HRP1 and 67.87 ± 135.07 mg/gVSS for HRP2. According to Biller and Ross [79], carbohydrate concentrations can vary based on operating conditions and the species of microalgae present in the medium. For instance, Valdez et al. [80] found that algal biomass composed of the microalga *Scenedesmus* sp. contained 31 % carbohydrates, while Biller and Ross [79] reported a carbohydrate content of 9 % in their biomass with *Chlorella vulgaris*.

Regarding oil extraction and the lipid content of the biomass, higher oil content was observed at the end of the batches and the beginning of the semi-continuous regime, with values of 15.86 % for HRP1 and 21.82 % for HRP2. This increased oil content at the end of the batches may be related to the nutritional stress experienced by the species during this feeding regime, as no organic load was introduced into the system [55].

Table 3
Average chemical composition of protein, carbohydrate, and lipid content found in the biomass of high-rate ponds during the continuous operating regime.

HRP	Proteins (%)	Carbohydrates (%)	Lipids (%)
HRP1	46.72	42.75	10.53
HRP2	44.47	32.50	23.03

This observation aligns with the findings of Chen et al. [81], who noted that the growth rate of organisms increases alongside cell density until nutrient depletion occurs. Subsequently, when the growth rate declines, there is an increase in the storage of high-energy compounds, such as lipids, due to alterations in metabolic processes [3].

The average lipid content values during the semi-continuous regime were 10.53 % for HRP1 and 23.03 % for HRP2. In a similar study of high-rate ponds with hydraulic retention times of 10 days, 6 days, and 2 days, Arcila and Buitrón [28] reported an average lipid content of 9 % across the three ponds. This variation may be attributed to the microalgae species that predominated in these HRPs, as certain species have a higher capacity to store lipids in their cells. Notably, in this study, the highest concentrations of lipids were recorded on operational day 87, when the microalga *Chlorella* sp. was particularly abundant, accounting for 71.4 % of the biomass in HRP2, while in HRP1, its relative abundance was 37.9 %. Supporting this discussion, Sajjadi et al. [76] indicated that lipid production from *Chlorella* sp. can reach up to 58 % oil content. Therefore, in conjunction with efficient biomass harvesting, these lipid yield results are advantageous to produce value-added products.

4. Conclusion

According to the results of this study, HRP1 and HRP2, with a water column depth of 0.30 m, performed well in terms of nutrient and organic matter removal. This performance could be attributed to the higher levels of solar light reaching the HRPs, increasing the system's photosynthetic activity. It was also shown that at HRP2, which used activated sludge inoculation to start the system and produce the photogranules, there was a higher concentration of nitrates due to the nitrification process. Consequently, it was found that bacteria and microalgae in the medium were unable to break down this type of nitrogen. On the day with the most solar radiation, the pH and DO profiles exhibited higher values, indicating an increase in photosynthetic activity. Lower DO and pH values were found in the second profile, with lower radiation values and gloomy skies, due to reduced photosynthetic activity by the microalgae.

The results indicate that the biomass and the system stabilized after 20 days of semi-continuous operation, particularly in HRP2. This system achieved a total nitrogen removal of 84.2 %, while HRP1 reached 83.5 %. Regarding organic matter removal, measured in COD, HRP2 demonstrated an efficiency of 74.7 %, compared to 51.6 % for HRP1. Based on the observations, it is determined that HRP2 has a larger capacity for full-scale application, showing satisfactory results in sanitary wastewater treatment, associated with the development and stability of the microalgae-bacteria consortium. The higher protein and lipid content in HRP2 indicates its potential for producing value-added products like animal feed. Optimizing these systems enhances biomass production and aligns with sustainable development goals, promoting public health and restoring aquatic ecosystems.

CRediT authorship contribution statement

Josivaldo Sátiro: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Antônio dos Santos Neto:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Jucélia Tavares:** Writing – original draft, Visualization, Investigation, Formal analysis. **Idayana Marinho:** Visualization, Investigation. **Bruna Magnus:** Writing – original draft, Visualization. **Mario Kato:** Writing – review & editing, Writing – original draft, Visualization, Resources, Funding acquisition. **António Albuquerque:** Writing – review & editing, Writing – original draft, Visualization. **Lourdinha Florencio:** Writing – review & editing, Writing – original draft, Visualization, Resources, Project administration, Funding acquisition.

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Declaration of competing 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.

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Data availability

Data will be made available on request.

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