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Assessing the effect of Fe_2O_3 nanoparticle addition on microalgae wastewater treatment and biomass composition

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ABSTRACT

The addition of Fe_2O_3 nanoparticles to microalgae cultures has gained popularity since it has been demonstrated to enhance microalgae growth and metabolite accumulation. However, most of the literature has been focused on small batch laboratory-scale studies under controlled conditions and the need of continuous studies under real environmental conditions is needed to demonstrate the feasibility of this process. In this study, the effect of Fe_2O_3 nanoparticles on the metabolism and nutrient uptake of a microalgae-cyanobacteria consortium cultivated in wastewater was elucidated. Different concentrations of Fe_2O_3 nanoparticles (10, 20, 30 and 70 mg L^{-1}) were assessed at least for 21 days in outdoor cylindrical PBRs at 7 days of hydraulic retention time. No significant difference in microalgae growth, microalgae biomass composition and nutrient uptake was observed at concentrations <30 mg L^{-1}. Moreover, when 30 mg L^{-1} were added to the culture, the carbohydrate content increased up to 38 % but a decrement in biomass concentration of 18 % was observed by the deposition of nanoparticles on the cyanobacteria cell wall. The addition of 70 mg Fe_2O_3 L^{-1} reduced the content of carbohydrate and biomass concentration but did not influence the nutrient uptake from wastewater. In brief, Fe_2O_3 NP supplementation at 30 mg L^{-1} can be added as a strategy to stimulate carbohydrate content during microalgae-based wastewater treatment.

1. Introduction

Microalgae are considered a promising platform for wastewater treatment, CO_2 capture and the production of biofuel [1]. Wastewater cultivation became attractive since it contains the main nutrients required by microalgae *i.e.* nitrogen and phosphorous [2]. Microalgae have demonstrated to have the ability to remediate wastewater with high nitrogen and phosphorous removals [3], however, their biomass productivities and biofuel production yields (*i.e.* biodiesel) remain low. For instance, Arias et al. [4] studied the growth of microalgae in digestate diluted with secondary effluent and a biomass concentration of 1 gTSS L^{-1} was recorded though the study. The authors attributed this low biomass concentration to the low nutrient content, which were 9, 16 and 2 mg L^{-1} of N-NH_4^+, N-NO_3^- and P-PO_4^{3-}, respectively. Similarly, Morais et al. [5], studied the all-year-growth of a microalgal consortia in secondary effluent with an average concentration of N-NH_4^+; N-NO_3^- and P-PO_4^- of 0.5–30; 2.8–90; and 5–11 mg L^{-1}, respectively. The authors reported biomass concentration of 50 mg L^{-1} mainly due to the low nutrient concentration in the wastewater. In this context, even if the growth of wastewater cultivated microalgae is governated by the nutrient content, several techniques have been implemented to improve and stimulate microalgae growth [6,7].

Recently, the use of nanotechnology in microalgae cultures is gaining attention since it has been demonstrated that the addition of some metallic nanoparticles (NPs) can enhance biomass productivity, lipid and carbohydrate accumulation; CO_2 sequestration; and light absorption [8]. Notwithstanding, most of the literature is focused on the toxic effect of NPs on microalgae cultures since NPs of Ag [9], TiO_2 [10], Co

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[11] and ZnO [12] have demonstrated harmful effects on microalgae metabolism leading to cell death. On the other hand, NPs composed of SiO_2, Fe_2O_3 and mesoporous carbons have been recently reported to exhibit beneficial effects on microalgae metabolism, improving both biomass productivities and metabolite accumulation as a result of an enhanced CO_2 availability to the cultures [13–17]. In this regard, if research is focused on the beneficial NPs, a promising technology to improve microalgae cultivation and valorization processes could be developed.

In this sense, Jeon et al. [13] observed that the addition of SiO_2 NPs increased *Chlorella vulgaris* production by 177 % and its lipid content by 340 % [13]. Similarly, Fe_2O_3 NPs also demonstrated beneficial effects on some microalgae species. For instance, Rana et al. [14] reported an increment in *Chlorella pyrenoidosa* biomass concentration of 33.75 % when 20 mg L^{-1} of Fe_2O_3 NPs were added to the culture. Additionally, *Chlorella pyrenoidosa* lipid concentration increased up to 16.89 % (dw) when 30 mg L^{-1} were added [14]. Similarly, Bibi et al. [15] revealed that the addition of 50 and 100 mg L^{-1} of Fe_2O_3 NPs stimulated the lipid accumulation of *C. vulgaris* by 39.7 % and 25.5 %, respectively [15]. However, even if NPs have demonstrated to have a positive influence in some species of microalgae, most of the reported studies are limited to batch conditions, synthetic mineral media, controlled conditions and time exposures up to 35 days. Recently, Xia et al. [18] evaluated the effect of Fe_2O_3 NPs addition to synthetic wastewater cultivated *C. vulgaris* cultures for 100 days under indoor conditions. The authors reported that the addition of 50 mg L^{-1} of Fe_2O_3 NPs supported biomass yields of 2.02 g L^{-1} and lipid contents up to 12.3 % [18]. Unfortunately, studies on the long-term exposure of microalgae to NPs under real wastewater and environmental conditions are scarce, which is preferable at industrial scale.

Hence, this study aimed to elucidate the long-term exposure (over 150 days) of a mixed microalgae-cyanobacterial consortium cultivated in real wastewater under outdoor conditions to Fe_2O_3 NPs. The effect of NPs on biomass production, nutrient uptake and biomass composition was investigated.

2. Materials and methods

2.1. Nanoparticles

Fe_2O_3 NPs were synthetized according to Norouzi and Nezamzadeh-Ejhieh [19]. Briefly, 6 g of $Fe(NO_3)_3$ were dissolved in 150 mL of deionized water, afterwards 12 mL of ethanol were added (1 mL per minute) under continuous stirring. Then, the pH was adjusted to 11 with 8 M NaOH and the solution was left to settle at room temperature for 1 h. After, several washes were carried out with deionized water and the precipitate was dried at 80 °C for 2 h. Finally, the Fe_2O_3 precipitate was calcined at 500 °C for 4 h. A nitrogen physisorption analysis was carried out in an ASAP 2050 (Micromeritics, USA) at 77 K to obtain the average pore diameter, pore volume and surface area of the NPs. The BET method and BJH equation were used to determine the pore characteristics and specific surface area. Scanning electron microscopy (SEM) (JEOL JSM-6490LV) and energy-dispersive spectroscopy (EDS) (EDX-700/800, Hitachi, Japan) were conducted to obtain the elemental composition and surface morphology of the target NPs.

2.2. Microalgae and culture media

The microalgae used for inoculation of the photobioreactors (PBRs) was *Chlorella vulgaris*, which was kindly donated by the Metropolitan Autonomous University (UAM), Mexico City, Mexico. An inoculum was grown in SK medium enriched with peptone, glucose and yeast extract according to Vargas-Estrada et al. [17]. The PBRs were inoculated with 200 mg L^{-1} of *C. vulgaris* in wastewater obtained from the wastewater treatment plant of the Renewable Energy Institute (IER-UNAM), Temixco, Mexico, which treats water from the bathrooms, cafeteria and the laboratories. The PBRs were allowed to stabilize for 30 days before the beginning of the experiment, until reaching a steady state of species dominance, biomass concentration and nutrient recovery to ensure the reproducibility of the study. After the stabilization period, a change in the composition of the biomass was observed. The microorganisms dominating the culture were the cyanobacterium *Leptolyngbya foveolarum,* followed by the microalgae *Scenedesmus obtusus* and *Desmodesmus abundans*.

2.3. Experimental set-up

The experiments were conducted under environmental outdoor conditions at the Hydrogen Laboratory, IER-UNAM, Temixco, Mexico, using two closed cylindrical photobioreactors (PBR) of 15 cm of diameter, 50 cm of height and a working volume of 7 L (Fig. 1). The PBRs were daily fed with 1 L of wastewater using peristaltic pumps to maintain a hydraulic retention time of 7 days. Additionally, air was pumped to prevent culture sedimentation and to provide CO_2. The output liquor was collected in a settler and weekly harvested. The harvested liquor was centrifuged at 5200 rpm for 10 min, freeze-dried and kept at −20 °C until its characterization. One PBR was used as a control (PBR-Control) and was only fed with wastewater, while the other PBR was used to assess the influence of the Fe_2O_3 NPs (PBR-NPs) on the microalgae culture. The nanoparticles were added by preparing a stock solution of 200 mg L^{-1} of Fe_2O_3 NPs in wastewater. Every two days, wastewater was prepared at a different concentration tested to feed the PBR-NPs. Four different concentrations of Fe_2O_3 NPs were assessed: 10 mg L^{-1}, 20 mg L^{-1}, 30 mg L^{-1} and 70 mg L^{-1}, each condition lasted at least 21 days.

2.4. Operational conditions and sampling procedures

The PBRs were initially operated for 30 days to acclimatize and stabilize the microalgae-cyanobacteria culture. Subsequently, stage I (days 0–10) was intended as a control stage to assess the reproducibility in biomass growth and wastewater treatment performance of both PBRs. Then, Fe_2O_3 NPs were added to the PBR-NPs in stage II (days 11–50) to reach a final concentration of 10 mg L^{-1}, and were also daily fed in the inlet wastewater. In stage III (days 51–81) the concentration of NPs was increased to 20 mg L^{-1} in both the PBR-NPs and the inlet wastewater. In stage IV (days 82–120), the concentration in the PBR-NPs and in the fed wastewater was increased up to 30 mg L^{-1}, while in stage V (days 121–150) the concentration was increased to 70 mg L^{-1}.

The pH of the PBRs was measured daily in the morning, while the temperature of the PBRs was measured daily twice a day (morning and afternoon). Direct solar irradiance and environmental temperature were measured daily at 9:00 am and 18:00 pm and were kindly provided by the solar energy group (ESOLMET) of the IER-UNAM. Twice a week, samples of 100 mL of the PBRs broth and wastewater were taken to obtain the concentration of N-NH_4^+, N-NO_2^-, N-NO_3^-, P-PO_4^{3-}, total suspended solids (TSS) and volatile suspended solids (VSS).

The collected biomass in the settler was harvested, frozen at −30 °C (Equitec) and finally freeze-dried (−110 °C, 0.049 hPa) (Labconco, USA) for subsequent characterization in terms of carbohydrate and lipid content.

2.5. Analytical procedures

2.5.1. Wastewater characterization

The pH was measured with an Orion Star A211 sensor (ThermoFisher Scientific, US). A submersible thermometer (4339, Control Company) was used to measure the temperature. N-NH_4^+ was measured according to Solórzano [20], briefly 2 mL of sodium nitroprusside 0.5 %, 2 mL of phenol 10 % solution and 5 mL of oxidizing solution were added to 50 mL of filtered sample, after 90 min the absorbance was read at 640 nm. N-NO_3^- concentration was obtained by the cadmium reduction method

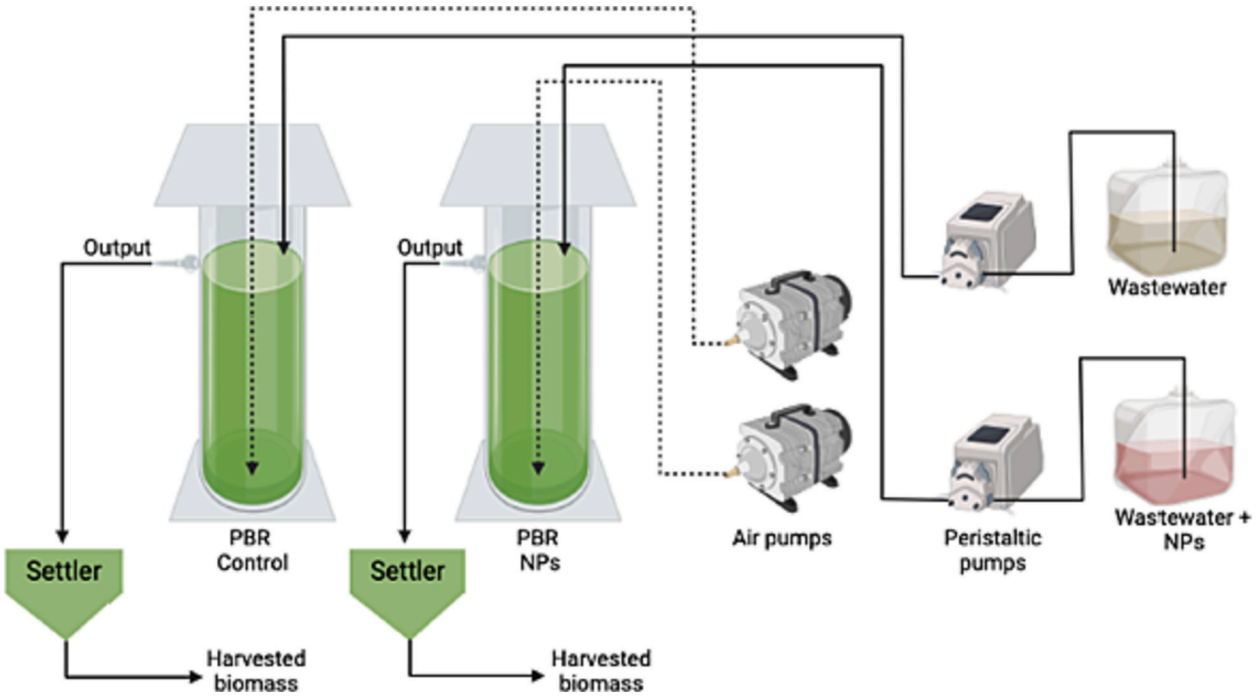

Fig. 1. The experimental set up. Continuous lines represent liquid flow, dotted lines represent air flow.

(method 8171MR, HACH), briefly 1 powder pillow of NitraVer 5 was added to 10 mL of filtered sample, after 5 min the absorbance was read with a portable spectrophotometer HACH. N-NO_2^- concentration was obtained by the diazotization method (method 8507, HACH), briefly 1 powder pillow of NitriVer 3 was added to 10 mL of filtered sample, after 15 min the absorbance was read with a portable spectrophotometer HACH. P-PO_4^{3-} concentration was obtained by the phospho-vanadium-molybdate method according to standard methods [21], briefly 10 mL of vanadium molybdate reagent were added to 20 mL of filtered and previously hydrolyzed sample. The solution was made up to 50 mL and within 8 min the absorbance was read at 420 nm.

2.5.2. Microalgae biomass concentration and characterization

TSS and VSS were determined according to standard methods [21], briefly 10 mL of culture sample was filtered through glass-fiber filter 934AH, 47 mm, Whatman. Afterwards, the filters were dried overnight at 105 °C and then calcined at 550 °C for 20 min. The harvested biomass was characterized in terms of carbohydrates and lipid content according to Vargas-Estrada et al. [22], briefly, for carbohydrates determination 2 mg of biomass (freeze-dried) was hydrolyzed with 2 mL of HCl 1 N for 2 h. Afterwards, the sample was centrifuged (10,000 rpm, 5 min) and 100 μL of the supernatant was carefully placed in a glass vial where 100 μL of 5 % phenol solution and 2.5 mL of concentrated H_2SO_4 were added to finally read the absorbance at 490 nm. On the other hand, lipids were determined by the chloroform:methanol method, briefly 100 mg of biomass (freeze-dried) was placed in glass tubes and 10 mL of chloroform:methanol solution was added. Then, the glass tubes were sonicated for 1 h and left over night at 37 °C. The next day, 1.25 mL of chloroform was added and the glass tubes were sonicated for 30 min. Afterwards, the samples were filtered and the filtrate was carefully recovered, and placed in new tubes containing 1.25 mL of distilled water. Further, the solution was centrifugated and the chloroform phase was carefully recovered, placed in a clean glass vial and left to dry, lipid content was determined gravimetrically. Finally, microalgae population structure identification was carried out by microscopic examination in a Nikon E600 microscope (Japan), with interdifferential contrast and photographs were taken with a Nikon DXM1200 digital camera. The basic bibliography for species identification was Komárek and Anagnostidis [23,24] and Komárek and Fott [25]. All the species were corroborated with specialized bibliography.

2.6. Statistical analysis

The results were analyzed to determine the effect of the NPs on microalgae growth and biomass composition considering $\alpha = 0.05$ by ANOVA followed by Tuckey's test. Additional *t*-test ($\alpha = 0.05$) was carried out to confirm significant differences in microalgae concentration (VSS) and carbohydrate and lipid content during stage III and IV.

3. Results and discussions

3.1. Nanoparticle characterization

The Fe_2O_3 NPs exhibited a nanorod morphology with particle size of 25 nm (Fig. 2a, b), this particular morphology supports better magnetic and electrochemical properties and high specific surface area compared to other Fe_2O_3 morphologies [26–28]. The chemical composition of these particular NPs was: O = 59 %, Fe = 37.40 % and Na = 3.44 % (in atomic percentage) (Fig. 2c, d). The presence of Na in the NPs is attributed to trace levels of the catalyst used for their synthesis. Finally, the BET surface area, pore volume and average pore diameter were determined as 32.10 $m^2\ g^{-1}$, 0.38 $cm^3\ g^{-1}$ and 47.50 nm as previously described by Vargas-Estrada et al. [16].

3.2. Environmental parameters

The environmental parameters recorded in both PBRs are presented in Table 1. The pH of both PBRs did not present a significant change during the experiment and remained above 8.5 for 150 days. This particular pH is correlated to the lack of CO_2 injection to the cultures throughout the study. The temperature in the PBRs decreased concomitantly with the decrease in ambient temperature. The ambient temperature was in average of 35.4 ± 0.9 °C in stage I and decreased to 27.3 ± 0.9 °C in stage V. The same behavior was observed in the direct solar irradiance. In this regard, the decrease in both temperature and direct solar irradiance in stage V likely caused reduction in biomass

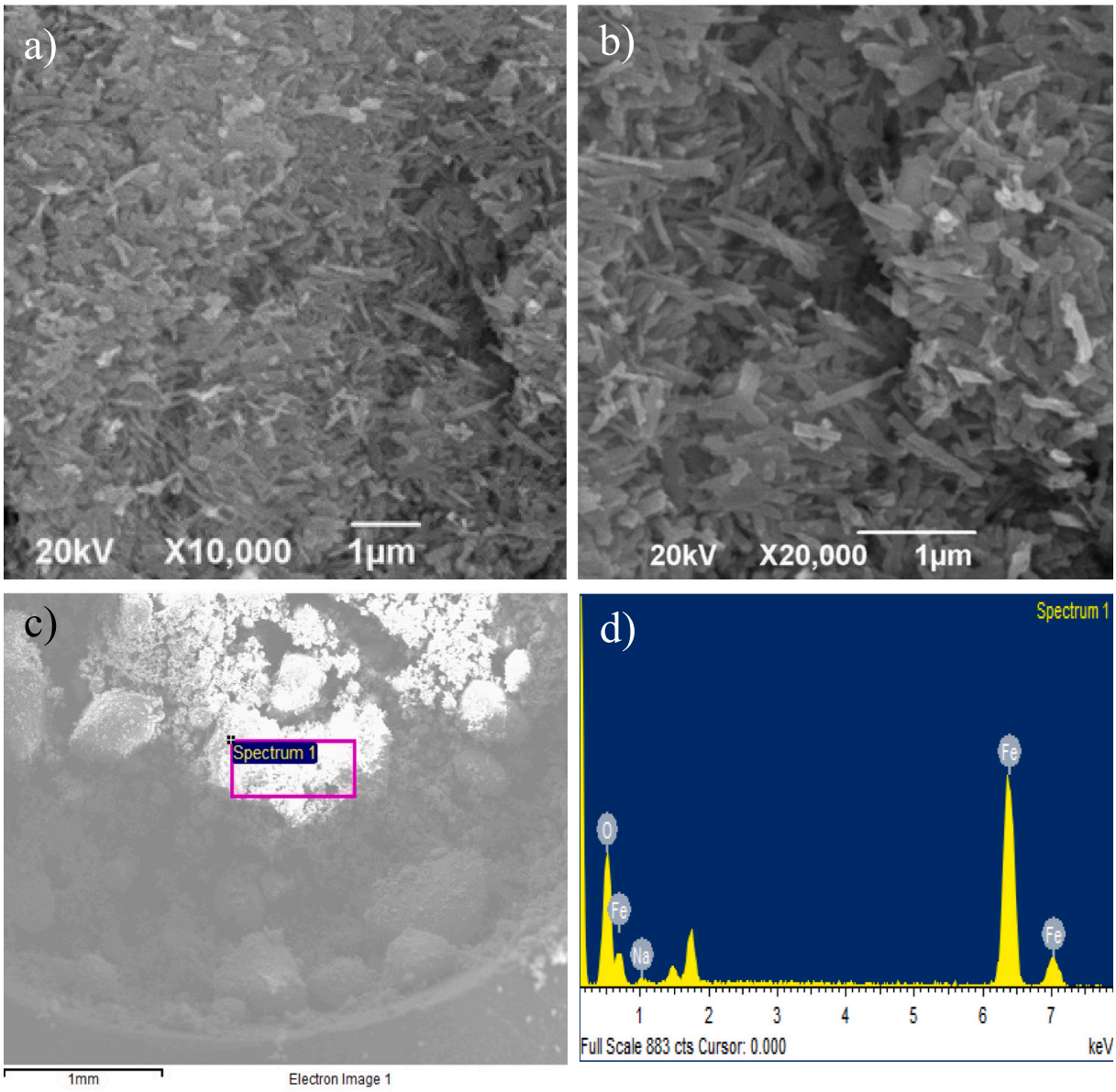

Fig. 2. a) SEM micrograph of the Fe_2O_3 nanoparticles 10,000 X; b) SEM micrograph of the Fe_2O_3 nanoparticles 20,000 X; c) and d) Elemental analysis (EDS) results of the Fe_2O_3 nanoparticles.

concentration in both PBRs.

3.3. Microalgae biomass growth in the PBRs

The microalgae population structure changed in each PBR after 150 days of cultivation. At the beginning of the study, an active growth of *Leptolyngbya foveolarum* that formed clusters on which other cyanoprokaryota (such as *Gloeocapsa atrata*) and isolated coenobium of *Scenedesmus obtusus* and *Desmodesmus abundans* incorporated, was observed. Additionally, a slight growth of *Stigeoclonium tenue* was observed in PBR-NPs. At the end of the study, the culture in PBR-Control was composed of *Scenedesmus obtusus*, *Merismopedia punctata*, *Desmodesmus abundans*, *Closteriopsis acicularis*, *Phormidium aerugineo-coeruleum*, *L. foveolarum*, *Choricysits parasítica*, *Pectinodesmus javanensis* and ciliates. Similarly, the species observed in PBR-NPs at the end of the study were *Scenedesmus obtusus*, *Desmodesmus abundans*, *Merismopedia punctata*, *Closteriopsis acicularis* and ciliates. The species identified are shown in the Supplementary material (Fig. 1S–13S). Interestingly, the presence of *L. foveolarum* in PBR-NPs was not observed. The absence of *L. foveolarum* was mainly ascribed to the fact that the Fe_2O_3 NPs formed agglomerates that were deposited through the filamentous species (Fig. 3). The agglomeration of NPs has been previously stablished as one of the major inhibiting mechanisms of microalgae growth [8]. However, in the present study the optical images show that Fe_2O_3 NPs were easily deposited on the filamentous species. To the best of the authors' knowledge, the literature on the effect of Fe_2O_3 NPs on cyanobacteria is scarce and no specific mechanisms of interaction between NPs and cyanobacteria have been proposed. However, by the observations recorded in this particular study, the Fe_2O_3 NPs formed agglomerates that were preferably deposited on filamentous cyanobacteria, probably due to their higher surface area or since the Fe_2O_3 agglomerates were trapped in the filamentous cells, additional images can be found in the Supplementary material (Fig. 14S-16S). Moreover, the agglomeration of NPs comes along with the shading effect, which also governs microalgae growth [29]. Demir et al. [30] reported that Fe_2O_3 NPs caused the flocculation and growth inhibition of *Nannochloropsis sp.* Similarly, Chen et al. [11] observed a similar behavior with Co-NPs and *Skeletonema costatum*. In this regard, the absence of *L. foveolarum* is in agreement with the previously reported

Table 1
Environmental parameters of the PBRs during the experiment.

Stage	PBR	Temperature* (°C)		Direct Irradiance (W m^{-2})		pH
		Morning	*Afternoon*	*Morning*	*Afternoon*	
I	*Control*	24.2 ± 2.3	35.1 ± 0.3	668.7 ± 73.4	226.8 ± 89.4	8.9 ± 0.4
	NPs	24.4 ± 2.1	35.1 ± 0.3			9.0 ± 0.5
II	*Control*	27.7 ± 2.1	32.1 ± 2.3	439.4 ± 271.7	62.3 ± 102.3	8.9 ± 0.4
	NPs	22.7 ± 2.1	32.4 ± 2.6			8.8 ± 0.4
III	*Control*	21.7 ± 1.7	28.9 ± 1.3	482.1 ± 311.7	199.3 ± 207.3	8.8 ± 0.4
	NPs	21.7 ± 1.5	28.9 ± 1.3			8.5 ± 0.3
IV	*Control*	21.1 ± 1.3	28.0 ± 1.6	460.5 ± 280.1	128.2 ± 188.5	8.7 ± 0.5
	NPs	21.1 ± 1.4	29.1 ± 1.2			8.8 ± 0.6
V	*Control*	21.2 ± 1.0	27.5 ± 1.5	472.1 ± 296.5	28.4 ± 39.9	9.3 ± 0.9
	NPs	21.2 ± 1.0	27.5 ± 1.4			9.2 ± 0.7

Note: *temperature of the PBRs, **Direct irradiance at Temixco, Mexico.

studies suggesting that the interaction of Fe_2O_3 NPs and the algal culture was a physical effect rather than a cell or DNA damage.

The steady state biomass concentration during stage I averaged 0.42 ± 0.14 and 0.46 ± 0.15 g L^{-1} for PBR-Control and PBR-NPs, respectively (Fig. 4). Similarly, no significant difference in steady state biomass concentration was observed in stage II, where biomass concentration averaged 0.56 ± 0.28 and 0.51 ± 0.25 g L^{-1} for PBR-Control and PBR-NPs, respectively. Interestingly, biomass concentration in PBR-NPs was significantly reduced compared to PBR-Control by 36 and 18 % during stage III and IV ($p < 0.05$), which corresponds to Fe_2O_3 NPs concentrations of 20 and 30 mg L^{-1}, respectively. Even if biomass concentration during these two stages was significantly lower than that recorded in the control PBR, the biomass concentration in PBR-NPs in stage IV exhibited the maximum value recorded in this study (1.21 g L^{-1}). The latter could be attributed to the fact that Fe_2O_3 NPs were deposited onto *L. foveolarum*, causing biomass sedimentation and cell death of this particular species. However, since most NPs were attached to *L. foveolarum*, the culture media could have presented a lower concentration of suspended Fe_2O_3 NPs, which did not inhibit the growth of the other microalgae species. In this context, biomass concentration in PBR-NPs in stage IV recovered either by the adaptation of the microalgae to the NPs concentration; by sedimentation of the *L. foveolarum* + Fe_2O_3 NPs flocs, which led to a lower NPs concentration in the culture broth; or by the enhanced environmental conditions, as a result of the NPs addition, that could have stimulated the growth of microalgae. Interestingly, PBR-Control presented the same growth behavior regardless of the addition of the NPs. Finally, biomass concentration in stage V decreased in both PBRs, suggesting that the biomass decrement in the PBRs was more likely by the influence of environmental conditions or by nutrient limitation.

Hence, the addition of NPs to the mixed microalgae-cyanobacteria culture resulted in the inhibition of *L. foveolarum* likely due to the attachment of the NPs to its cell wall. Interestingly, Fe_2O_3 NPs added at a concentration of 70 mg L^{-1} did not inhibit microalgae growth, which is contrary to the results reported by Bibi et al. [15], who reported that concentrations >50 mg L^{-1} of α-Fe_2O_3 inhibited the growth of *C. vulgaris* but enhanced its lipid production, which confirms that the effect of Fe_2O_3 NPs is species specific. In this context, the low biomass concentrations recorded during stage V was more likely due to a change in environmental conditions such as ambient temperature and solar irradiance, rather than an effect of the NPs. Additionally, it is important to highlight that the biomass concentration recorded throughout the study is in accordance with the results previously reported for wastewater cultivated microalgae, where biomass concentration typically ranges between 0.5 and 1 g VSS L^{-1} [4,5], mainly due to the low nutrient content in the wastewater.

3.4. Nutrient uptake

P-PO_4^{3-} concentration in the influent wastewater decreased during stage II and remained stable until the beginning of stage V (Fig. 5a). This can be explained by the constant changes in the population and activities at the IER-UNAM. It is important to address that P-PO_4^{3-} concentration in the wastewater remained above 10 mg L^{-1} during the study. Even if concentrations of 10 mg L^{-1} P-PO_4^{3-} have been reported as the optimum for microalgae growth [31], this particular nutrient has also been reported as the limiting factor for microalgae growth [22,32]. Thus, the low P-PO_4^{3-} concentration in the wastewater could have limited microalgae growth in this study during stage III, IV and V. On the other hand, the P-PO_4^{3-} in the PBRs gradually decreased during stage II and remained ≤2 mg L^{-1}, and no significant differences in the P-PO_4^{3-} concentrations in both PBRs were observed during the study. The decrease in the P-PO_4^{3-} concentration in the PBRs during stage II was mainly attributed to microalgae consumption for their growth. Indeed, P-PO_4^{3-} concentration was correlated to microalgae growth, since microalgae concentration was <0.60 g L^{-1} during stage I and II, and P-PO_4^{3-} removals averaged up to 60 and 44 % in PBR-Control; and 68 and 36 % for PBR-NPs during stage I and II, respectively. An intense P-PO_4^{3-} consumption (above 70 %) was observed in both PBRs in stage III, which was correlated with biomass growth. As biomass concentration in both PBRs increased, P-PO_4^{3-} consumption decreased as well. However, P-PO_4^{3-} concentration in both PBRs slightly increased by the end of stage IV, which is in accordance with the biomass decay observed by the end of this stage. This behavior suggests that biomass concentration was related to the P-PO_4^{3-} concentration, and indeed this nutrient was a limiting factor for microalgae growth.

N-NH_4^+ concentration in the influent wastewater ranged from 10 to 50 mg L^{-1} (Fig. 5b). Even if the concentration of N-NH_4^+ in the wastewater remained low compared to those previously reported by Vargas-Estrada et al. [22], these values are in the range of N-NH_4^+ required to support the growth of most microalgae species (42–49 mg L^{-1}) [33]. The lowest N-NH_4^+ removals were recorded during stage I, and up to 92 % of the N-NH_4^+ was consumed by microalgae despite the conditions tested. Moreover, N-NH_4^+ removals reached up to 99 % in stage II regardless of the addition of Fe_2O_3 NPs. This behavior indicates that the lower N-NH_4^+ removals recorded during stage I and the first part of stage II can be mainly attributed to the acclimation of the microalgae to the media, which is correlated with the biomass concentration recorded in these stages. The N-NH_4^+ concentration in the PBRs remained almost negligible from the middle of stage II onwards. The intense N-NH_4^+ consumption is attributed to microalgae growth since this particular nitrogen specie is the one preferred by microalgae due to its easy assimilation [34]. The latter suggests that the addition of Fe_2O_3 NPs did not influence the nutrient uptake of PBR-NPs, contrary to the findings previously reported by Xia et al. [18] who observed that Fe_2O_3 NPs enhanced the N-NH_4^+ uptake by *Chlorella vulgaris* [18].

N-NO_2^- concentration remained almost negligible (Fig. 5d), but in stage IV and V a maximum concentration was recorded in the wastewater and in the PBRs which can be explained by the variations in the wastewater and the nitrification process. The latter is explained by the fact that N-NO_3^- (Fig. 5c) was present in the wastewater, and microalgae in the PBRs could have used it as a source of nitrogen since the N-NH_4^+ was rapidly consumed as a result of biomass growth. Interestingly, the N-NO_3^- concentration in PBR-Control was lower than in PBR-NPs, which is attributed to the consumption of microalgae and is correlated to the higher microalgae concentration in the PBR-Control. Additionally, the N-NO_2^- concentration in PBR-Control reached maximum values during

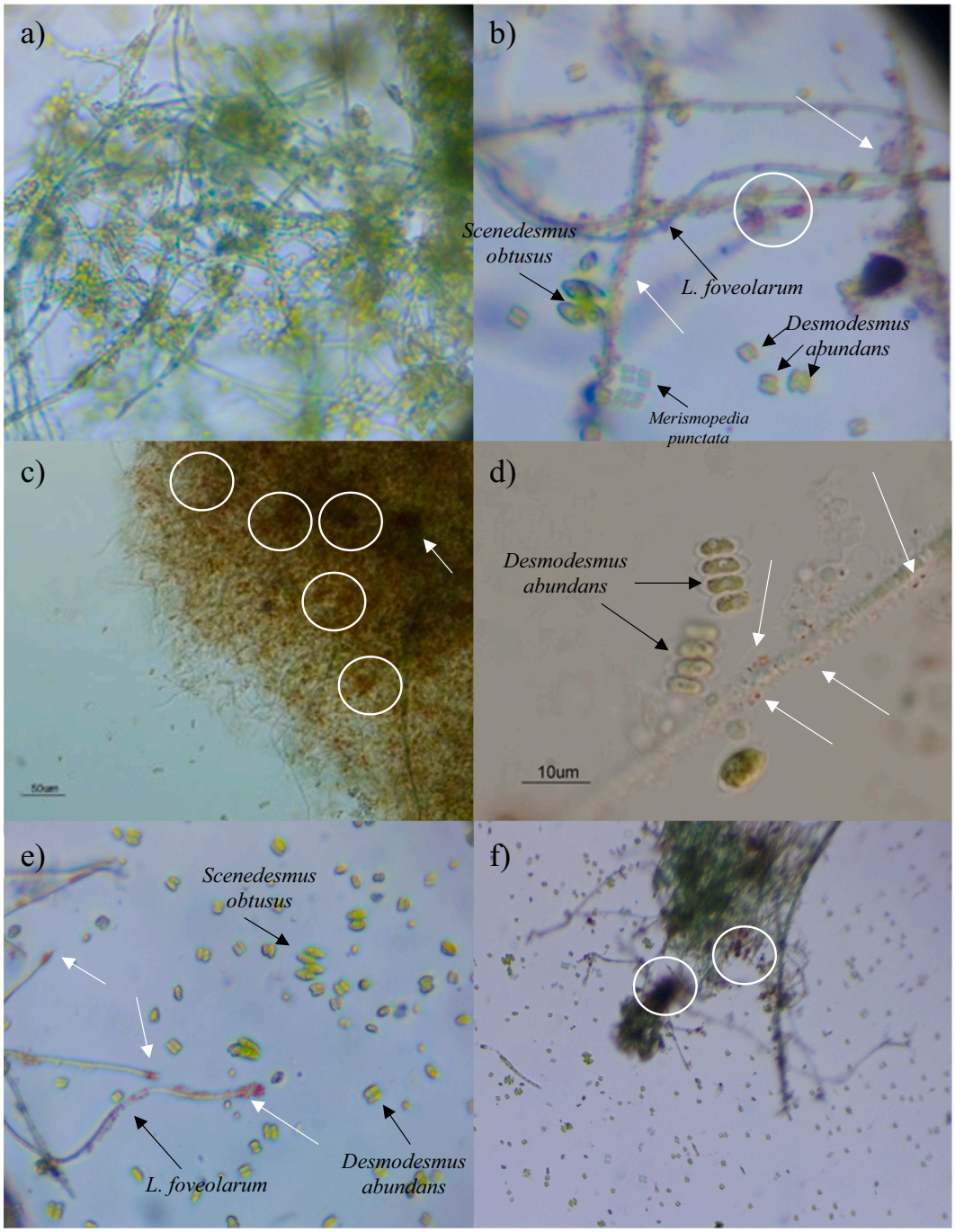

Fig. 3. Optical microscope images of the effect of Fe_2O_3 nanoparticles on microalgae-cyanobacteria consortium. a) control PBR 100×, b) Fe_2O_3 nanoparticles attached to *Leptolyngbya foveolarum* 100×; c) Fe_2O_3 nanoparticles deposited on the filamentous species contained in PBR-NPs 20×; d) Fe_2O_3 nanoparticles attached to *L. foveolarum*; e) and f) show the preference of Fe_2O_3 for filamentous cyanobacteria. The white circles and arrows indicate the presence of Fe_2O_3 nanoparticles.

stage IV and V, which was likely mediated by the partial denitrification of $N\text{-}NO_3^-$. It is important to addressed that the higher $N\text{-}NO_3^-$ concentration in the PBR-NPs is due to the lower nitrogen consumption as a result of the lower biomass concentration and not an interaction of Fe_2O_3 with the nitrogen sources, since no accumulation of $N\text{-}NO_3^-$ and $N\text{-}NO_2^-$ was observed in the PBR-NPs during the study.

In this regard, the Fe_2O_3 NPs neither influenced negatively the nutrient uptake nor acted as nutrient competitors for microalgae. Thus, it can be hypothesized that the slight decrease in the biomass concentration was related to the deposition of the NPs onto the filamentous microorganisms and not by an interaction of the Fe_2O_3 NPs.

3.5. Biomass composition

The changes in the biomass composition of each PBR are shown in Fig. 6. The lipid content in both PBRs remained under 12 % (dw) throughout the entire study. Even if it has been widely established that Fe_2O_3 NPs can enhance the lipid content in microalgae, mainly by the formation of reactive oxygen species (ROS) [8], this theory has contradictory results in literature. Bibi et al. [15] previously reported that *C. vulgaris* experienced a lipid enhancement of 39.7 % and 25.5 % when 50 and 100 mg L^{-1} of Fe_2O_3 NPs respectively were added to the culture [15]. On the other hand, Vargas-Estrada et al. [16] and Vargas-Estrada et al. [17] recently demonstrated that the addition of Fe_2O_3 to a mixed

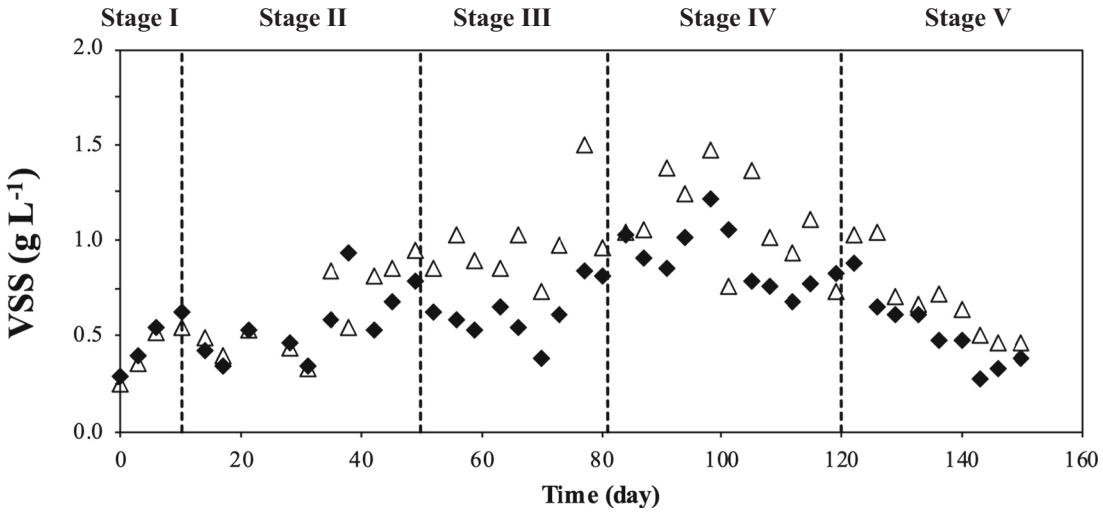

Fig. 4. Time course of biomass concentration in PBR-control (empty triangles) and PBR-NPs (dark diamonds).

culture of microalgae and bacteria, and to *Chlorella sorokiniana* culture did not stimulate the lipid production. Thus, the effect of Fe_2O_3 NPs on microalgae species is strongly related to NP concentration, its crystal phase, the time of exposure and the microalgae strain. It is important to address that the dominant species in both PBRs at the beginning of stage I was the cyanobacteria *L. foveolarum*. Cyanobacteria are known for their high protein or glycogen and polyhydroxybutyrate content and usually nitrogen starvation leads to high concentration of polyhydroxybutyrate [35]. However, nitrogen sources such as $N\text{-}NO_3^-$ and $N\text{-}NO_2^-$ were always present in the PBRs, and as it has been stated elsewhere [22], even when NH_4^+ concentration is negligible in the culture media, the presence of other nitrogen species stimulates the nitrification/denitrification process, resulting in low lipid content. Additionally, the dominant species changed throughout the study, which could have directly influenced macromolecule accumulation. Thereby, the low lipid content herein observed can be attributed to the fact that i) the concentration of Fe_2O_3 NPs tested was low and did not induce an oxidative environment and ii) the species used did not exhibit a high lipid accumulation capacity.

The carbohydrate content averaged 7.0 and 6.3 % during stage I in the PBR-Control and PBR-NPs, respectively, where no significant difference was observed. During stage II the content of carbohydrate in the biomass present in both PBRs increased up to 12.3 and 15.6 % in PBR-Control and PBR-NPs, respectively, but no significant difference was observed either. During stage III, the same behavior was observed and no significant difference was observed regardless of the addition of the NPs ($p = 0.237$). At stage IV, the content of carbohydrate in the biomass of PBR-Control remained as 18.3 %, whilst the carbohydrate content of PBR-NPs biomass increased from 15.45 to 27.52 %, which was significantly higher than the PBR-Control ($p < 0.05$). At the middle of stage IV, the carbohydrate content in PBR-NPs experienced a maximum value of 38 %. Similar results were recently reported by Vargas-Estrada et al. [16], who assessed the influence of Fe_2O_3 NPs in a mixed microalgal-bacterial consortium and observed that the addition of the NPs under UV–visible light stimulated the carbohydrate content of a consortium, mainly attributed to a protective response. In the present study, the increased carbohydrate content can be ascribed to the fact that the addition of Fe_2O_3 NPs stimulated the metabolism of microalgae, and carbohydrates were subsequently accumulated. It is important to address that stage IV could have been prolonged to confirm this tendency. However, the shown data suggests that when 30 mg L^{-1} of Fe_2O_3 NPs were added, the content of carbohydrate in the culture constantly increased until a maximum value of 38 % (dw). In this sense, 30 mg L^{-1} of Fe_2O_3 could be considered as an optimal concentration to stimulate carbohydrate accumulation in wastewater cultivated mixed cultures. Moreover, when the concentration of the Fe_2O_3 NPs in stage V increased to 70 mg L^{-1} the carbohydrate content of the PBR-NPs was significantly reduced from 27.52 to 11.29 %, suggesting that concentrations higher than 30 mg L^{-1} could have a negative impact on microalgae carbohydrate accumulation. The latter was confirmed by the fact that during stage V the carbohydrate content in PBR-NPs continued to decrease gradually mainly due to the presence of the NPs. Therefore, it can be concluded that even if the macromolecule accumulation in the biomass was species dependent in this study, the carbohydrate production in PBR-NPs was stimulated when 30 mg L^{-1} Fe_2O_3 NPs was added. On the other hand, the addition of 70 mg L^{-1} Fe_2O_3 NPs resulted in a carbohydrate content reduction likely due to the oxidation of intracellular storage compounds.

4. Conclusions

The long-term exposure of microalgae-cyanobacteria to Fe_2O_3 nanoparticles resulted in cyanobacterial death mediated by the attachment of Fe_2O_3 nanoparticles to their cell wall when concentrations $\geq$30 mg L^{-1} were added to the culture. The environmental conditions and nutrient concentration in the culture medium played a key role in microalgae growth. $P\text{-}PO_4^{3-}$ concentration was the limiting nutrient regardless of the addition of the nanoparticles, since biomass growth and $P\text{-}PO_4^{3-}$ concentration were strongly related. On the other hand, the Fe_2O_3 nanoparticles did not interfere with the nutrient uptake, suggesting that their interaction was limited to the activation of microalgal metabolism for carbohydrate accumulation. However, the carbohydrate content significantly decreased when 70 mg L^{-1} Fe_2O_3 nanoparticles were added to the culture, suggesting that this order of concentration can induce an oxidative damage to microalgae.

CRediT authorship contribution statement

Laura Vargas-Estrada: Methodology, Writing – original draft. **P.U. Okoye:** Writing – review & editing. **Raúl Muñoz:** Conceptualization, Writing – review & editing. **Eberto Novelo Maldonado:** Methodology. **Armando González Sánchez:** Writing – review & editing. **P.J. Sebastian:** Supervision, Writing – review & editing.

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.

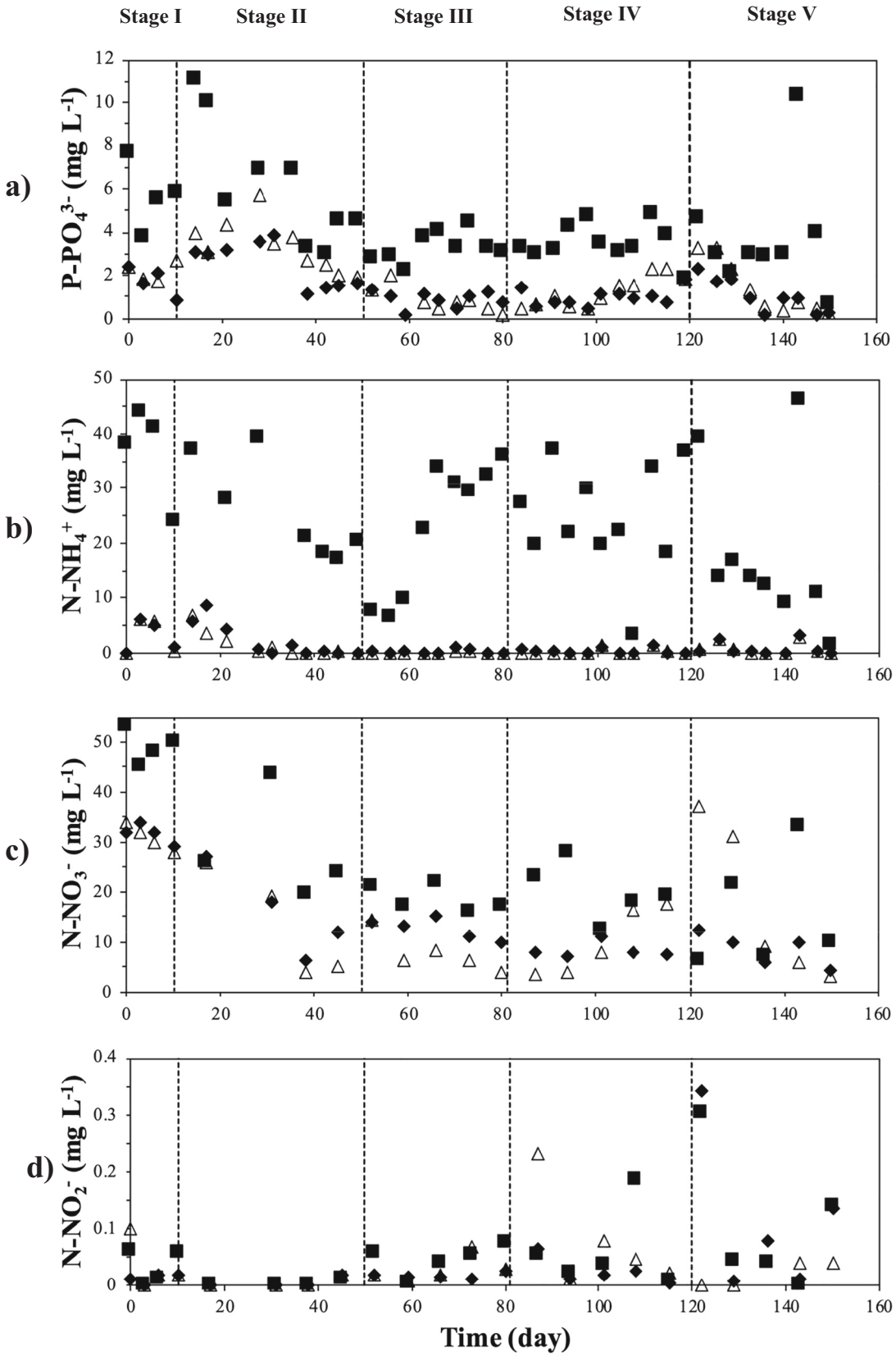

Fig. 5. Time course of a) $P\text{-}PO_4^{3-}$; b) $N\text{-}NH_4^+$; c) $N\text{-}NO_3^-$; d) $N\text{-}NO_2^-$ concentrations in the PBR-control (empty triangles), PBR-NPs (dark diamonds) and influent wastewater (dark squares).

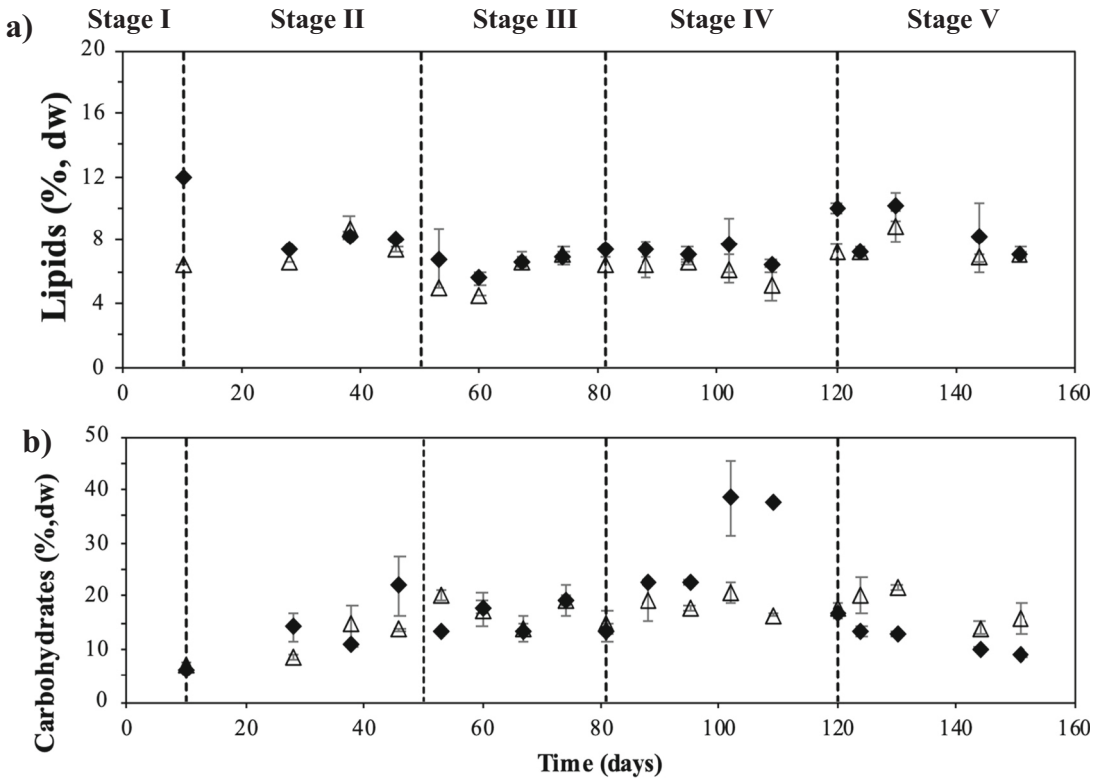

Fig. 6. Time course of a) lipids and b) carbohydrate content in the microalgal-cyanobacterial consortium present in PBR-control (empty triangles) and PBR-NPs (dark diamonds).

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2024.103399.

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