Manuscript Details

Manuscript number	ALGAL_2019_264_R4
Title	Assessing the influence of the hydraulic retention time and carbon/nitrogen ratio on urban wastewater treatment in a new anoxic-aerobic algal-bacterial photobioreactor configuration.
Article type	Full Length Article

Abstract

The influence of the hydraulic retention time (HRT) (2 and 4 days) and the carbon/nitrogen ratio (C/N) (7, 8 and 9) of the wastewater on the treatment of synthetic domestic wastewater was evaluated in a new anoxic-aerobic algal-bacterial photobioreactor configuration operated at solids retention time of 10 d by biomass recycling and withdrawal. The removal of chemical oxygen demand remained between 84% and 89% regardless of the operational conditions. However, the decrease in the HRT from 4 to 2 d entailed reductions in the removal of total nitrogen (TN) and P-PO43- from 87±2% to 62±2% and from 22±5% to 11±1%, respectively. On the other hand, the decrease in the C/N ratio of the wastewater from 9 to 8 and 7 at a HRT of 2 d induced TN removals of 62±4% and 48±4%, respectively. In contrast, P-PO43- removals unexpectedly increased from 11±1% at a C/N ratio of 9 to 53±3% and 47±5% at C/N ratios of 8 and 7, respectively. Finally, biomass settling and recycling supported the enrichment of an algal-bacterial population with good settleability characteristics (suspended solids removals in the settler ~98%), being Chlorella vulgaris the dominant microalga specie at a C/N ratio of 9 which was gradually replaced by Phormidium sp., as a result of the reduction in the C/N ratio of the wastewater.

Keywords	Algal-bacterial processes; anoxic-aerobic photobioreactor; C/N ratio; nitrification-denitrification; photosynthetic oxygenation.
Taxonomy	Biological Nutrient Removal, Environmental Biotechnology, Algal Photobioreactor Performance, Algae Based Waste Treatment
Manuscript category	Algal Biotechnology

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Assessing the influence of the hydraulic retention time and carbon/nitrogen ratio on urban wastewater treatment in a new anoxic-aerobic algal-bacterial photobioreactor configuration.

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Abstract

The influence of the hydraulic retention time (HRT) (2 and 4 days) and the carbon/nitrogen ratio (C/N) (7, 8 and 9) of the wastewater on the treatment of synthetic domestic wastewater was evaluated in a new anoxic-aerobic algal-bacterial photobioreactor configuration operated at solids retention time of 10 d by biomass recycling and withdrawal. The removal of chemical oxygen demand remained between 84% and 89% regardless of the operational conditions. However, the decrease in the HRT from 4 to 2 d entailed reductions in the removal of total nitrogen (TN) and $P\text{-}PO_4^{3-}$ from 87±2% to 62±2% and from 22±5% to 11±1%, respectively. On the other hand, the decrease in the C/N ratio of the wastewater from 9 to 8 and 7 at a HRT of 2 d induced TN removals of 62±4% and 48±4%, respectively. In contrast, $P\text{-}PO_4^{3-}$ removals unexpectedly increased from 11±1% at a C/N ratio of 9 to 53±3% and 47±5% at C/N ratios of 8 and 7, respectively. Finally, biomass settling and recycling supported the enrichment of an algal-bacterial population with good settleability characteristics (suspended solids removals in the settler ~98%), being *Chlorella vulgaris* the dominant microalga specie at a C/N ratio of 9 which was gradually replaced by *Phormidium* sp., as a result of the reduction in the C/N ratio of the wastewater.

Keywords:

Algal-bacterial processes; anoxic-aerobic photobioreactor; C/N ratio; nitrification-denitrification; photosynthetic oxygenation.

Abbreviations

C: Carbon

C_{Bio}: Carbon content in biomass

C/N: Carbon/Nitrogen ratio

C/N/P: Carbon/Nitrogen/Phosphorous ratio

COD: Chemical oxygen demand

DO: Dissolved oxygen concentration

HRAP: High rate algal pond

HRT: Hydraulic retention time

IC: Inorganic carbon concentration

N: Nitrogen

NH_4^+: Ammonium

NO_2^-: Nitrite

NO_3^-: Nitrate

P: Phosphorous

PAR: Photosynthetically active radiation

P_b: Biomass productivity

Q_{Eff}: Effluent flowrate

Q_{SWW}: Influent flowrate

REs: Removal efficiencies

SRT: Solids retention time

SWW: Synthetic domestic wastewater

TN: Total nitrogen concentration

TOC: Total organic carbon concentration

TSS: Suspended solids concentration

WWTPs: Wastewater treatment plants

$X_{i\ Eff}$: Concentrations of either COD, TOC, IC, TN, NH_4^+ or $P\text{-}PO_4^{3-}$ in the effluent

$X_{i\ sww}$: Concentrations of either COD, TOC, IC, TN, NH_4^+ or $P\text{-}PO_4^{3-}$ in the influent

1. Introduction

Algal-bacterial processes have emerged in the past decades as a cost-effective and environmentally friendly platform technology to remove carbon (C), nitrogen (N) and phosphorus (P) from wastewaters [1]. The synergistic interactions between microalgae and bacteria are based on the *in-situ* supply of O_2 (produced by photosynthesis) for aerobic heterotrophic bacteria and autotrophic nitrifiers, and the subsequent assimilation of C, N, and P in the form of valuable algal-bacterial biomass [2,3]. This green biotechnology significantly reduces oxygenation costs when compared to activated sludge systems and enhances nutrient recovery compared to anaerobic digestion technologies in conventional wastewater treatment plants (WWTPs) [2,4].

Despite the above-mentioned advantages, the efficiency of microalgae-based wastewater treatment processes is often limited by the C/N/P ratio of the secondary wastewater (100/25/12) or centrate (100/207/5), which hinders a complete nutrient assimilation due to carbon limitation [5,6] In this sense, only wastewaters with balanced C/N/P ratios (e.g. 100/14/2 on mass basis) are favorable for microalgae growth and can therefore support N and P removals by assimilation, which opens the investigation niche for innovative photobioreactor configurations capable of supporting an effective N and P removals at low C/N ratios [3,7]. In this context, the complete nitrification-denitrification process represents a key metabolic pathway to remove C and N in wastewaters with low C/N ratios in algal-bacterial photobioreactors [3,8]. Nevertheless, the relatively low hydraulic retention times (HRTs =2-6 days) applied in high rate algal ponds (HRAPs) devoted to wastewater treatment limit the occurrence of nitrifying bacterial communities in the cultivation broth. Typically, the oxidation of NH_4^+ requires HRT >8 d for complete nitrification [4,9]. Therefore, a new generation of anoxic-aerobic algal-bacterial photobioreactors based on decoupling the HRT from the solids retention time (SRT) were

developed and successfully tested at laboratory scale [3,9]. In these systems, photosynthesis supports the oxidation of NH_4^+ to NO_2^- or NO_3^- required for carbon oxidation in the anoxic bioreactor through an internal recirculation. For instance, De Godos *et al*. [8] reported C (95%) and N (98%) removals from synthetic wastewater in a 1 L anoxic bioreactor coupled to a 3.5 L closed photobioreactor operated at HRTs of 2-4.5 d and SRTs of 9-31 d. These authors also reported that the recirculation of the harvested biomass from a 1 L settler to the anoxic bioreactor (external recirculation) avoided the washout of nitrifying bacteria and supported process operation at biomass concentrations of 1.0-1.5 g volatile suspended solids (VSS) L^{-1}. Alcántara *et al*. [3] observed the absence of nitrification at high dissolved oxygen concentrations (DO), 21 ± 4 mgO_2 L^{-1}, during synthetic wastewater treatment in a similar anoxic-aerobic algal-bacterial closed photobioreactor configuration; Additionally, light-dark cycles combined with process aeration during dark periods were tested in order to elucidate a light-mediated inhibition on nitrifying activity. These authors recorded total organic carbon (TOC), inorganic carbon (IC) and total nitrogen (TN) removal efficiencies (REs) of ~ 80% at 2 d of HRT and SRTs of 20 d, the N removal mechanisms being governed by the light intensity and DO. This particular configuration has also been tested for the treatment of domestic wastewater [9] and synthetic textile wastewater [10] coupled to biogas upgrading and flue gas scrubbing, respectively. CO_2 supply overcame the IC limitation, enhanced N and P removal by assimilation and supported an efficient nitrification-denitrification process. However, the local availability of an external CO_2 source is not always technical or economically feasible.

On the other hand, despite the high biomass productivities reached in closed photobioreactors, the high construction and operating costs limit their scalability [11]. Thus, HRAPs are typically the preferred photobioreactor configuration for microalgae-

based wastewater treatment [5]. However, the use of a HRAP as process oxygenation unit for this novel anoxic-aerobic configuration has not been evaluated yet. Furthermore, there is a lack of studies assessing the influence of the C/N ratio of the wastewater on the performance of wastewater treatment in anoxic-aerobic algal-bacterial systems. Therefore, this work aim at evaluating the influence of the HRT and the C/N ratio of the wastewater on the C, N and P removal in an anoxic-aerobic algal-bacterial photobioreactor with a HRAP as process oxygenation unit. Mass balance calculations were conducted to elucidate the global carbon and nutrient removal mechanisms. Finally, a characterization of the biomass harvesting efficiency and the microalgae population structure was carried out during the different operational conditions assessed.

2. Materials and methods

2.1 Algal-bacterial inoculum

The inoculum consisted in a mixture of secondary activated sludge from the Valladolid WWTP (which operates with a nitrification-denitrification configuration) and a microalgae consortium collected from an outdoors pilot HRAP treating digestate located at the Department of Chemical Engineering and Environmental Technology of the University of Valladolid, Spain.

2.2 Synthetic domestic wastewater

The synthetic domestic wastewater (SWW) was prepared according to Frutos *et al*. [12] with the follow composition in g L^{-1}: 0.16 of casein peptone, 0.11 of meat extract, 0.03 of NH_2COH_2, 0.007 of NaCl, 0.004 of $CaCl_2 \cdot 2H_2O$, 0.002 of $MgSO_4 \cdot 7H_2O$, $5 \cdot x10^{-6}$ of $CuCl_2 \cdot 2H_2O$, 0.112 of $K_2HPO_4 \cdot 3H_2O$, 0.25 of glucose, and 1.1 of $NaHCO_3$. The main characteristics of the SWW were chemical oxygen demand (COD) concentration of

632±45 mg L^{-1}, TOC of 196±9 mg L^{-1}, IC of 195±12 mg L^{-1}, TN of 43±3 mg L^{-1}, N-NH_4^+ of 24±3 mg L^{-1}, P-PO_4^{3-} of 13.1±0.8 mg L^{-1} and pH of 7.7±0.2.

2.3 Experimental set-up

The experimental set-up consisted of a 3.75 L enclosed anoxic bioreactor (15 cm long, 15 cm wide, 17 cm deep), an 11.25 L open photobioreactor (HRAP) (30 cm long, 15 cm wide, 25 cm deep) and a 1 L conical settler (Fig. 1). The agitation of the cultivation broth in the anoxic bioreactor and in the HRAP was provided by Eheim compact 300 immersion pumps (Spain) (one pump in the anoxic bioreactor and two pumps in the HRAP). The HRAP was illuminated at an average photosynthetically active radiation (PAR) of 1314±12 μmol m^{-2} s^{-1} (light:dark cycles of 12:12 h) by high-intensity LED PCBs (Phillips SA, Spain). The internal liquid recirculation from the HRAP to the anoxic bioreactor supported the denitrification process, while the external liquid recirculation from the bottom of the settler to the anoxic bioreactor mediated controlling the SRT according to table 1.

The bioreactors were initially filled with SWW and inoculated to have an initial total suspended solids concentrations (TSS) of 0.2 g TSS L^{-1} of microalgae and 0.6 g TSS L^{-1} of activated sludge. The SWW was fed to the anoxic bioreactor at 4 and 2 d of total HRT (HRT of anoxic bioreactor + HRT of HRAP) (Table 1). These operational conditions promote the nitrification-denitrification process [13]. The flow rates of the internal and external recirculation (Watson Marlow 120 S pump, UK, and Masterflex 7021-24, USA, respectively) corresponded to 200% and 50% of the SWW flow rate, respectively, and were adjusted depending on the HRT tested. The SRT of the system was fixed at 10 d regardless of the operational stage by means of harvesting a volume of the external recirculation (wasted biomass ~1.5 g TSS d^{-1} under a steady biomass concentration in the

reactor). This volume was adjusted in accordance with the TSS concentration recorded in the wastage stream.

Stage I lasted 47 days in which the system was operated at an HRT of 4 d by feeding the SWW with a C/N ratio of 9 (COD concentration of 669±6 mg L^{-1}). Afterwards, the HRT was decreased to 2 d during stages II, III and IV, while the C/N ratio of the wastewater was step-wise decreased from 9 to 8 and 7, respectively, by means of decreasing the glucose concentration (corresponding to COD concentrations of 669±6 mg L^{-1}, 493±11 mg L^{-1} and 434± 11 mg L^{-1}, respectively). Stages II, III and IV were maintained for 40 d (~4 times the SRT) to achieve consistent steady states values.

2.5 Analytical methods

Samples (50 mL) from the SWW, anoxic bioreactor, HRAP, settler and effluent were drawn twice per week in order to monitor the concentrations of dissolved TOC, IC, and TN in a Shimadzu TOC-VCSH analyzer with TNM-1 module (Japan). The NH_4^+ concentration of samples was determined by using an Electrode Orion Dual Star (Thermo Scientific, The Netherlands), while the NO_2^-, NO_3^- and PO_4^{3-} concentrations by HPLC-IC according to Posadas *et al*. [14]. The pH (Eutech Cyberscan pH 510, The Netherlands), dissolved oxygen concentration (DO) (OXI 330i oximeter, WTW, Germany) and temperature were daily monitored in situ (anoxic bioreactor and HRAP). Furthermore, the TSS concentrations in the anoxic bioreactor, HRAP, settler, and effluent were monitor twice per week according to standard methods [15]. The concentration of COD in the SWW and treated effluent was only assessed under steady state (last three days from each operational stage) by the closed reflux method [15]. The influent and effluent flowrates were daily recorded in order to determine the water evaporation rate, while the PAR was weekly monitored (LI-250A, LI-COR Biosciences, Germany). The algal-bacterial

biomass harvested from the bottom of the settler under steady state was washed three times with distilled water and dried for 24 hours at 105 ºC prior determination of its elemental composition C, N, and P (LECO CHNS-932 analyzer). Finally, the morphological identification of the microalgae population in the HRAP was carried out at steady state. Two biomass samples were preserved with lugol acid at 5% and formaldehyde at 10%, respectively, and stored at 4 ºC prior analysis. The quantification and morphological identification of photosynthetic microorganisms were carried out according to Sournia [16] in an inverted microscope (OLYMPUS IX70, USA).

2.6 Mass balance calculation

The global mass balance calculation for C, N, and P were conducted based on the average concentrations of all their chemical species at the inlet (SWW) and outlet (effluent).

Carbon mass balance:

$$(TOC_{SWW} + IC_{SWW})\, Q_{SWW} = (TOC_{Eff} + IC_{Eff})\, Q_{Eff} + C_{Bio}\, P_b + C\text{-}CO_{2\text{-stripping}} \quad \text{Eq. 1}$$

Nitrogen mass balance:

$$TN_{SWW}\, Q_{SWW} = TN_{Eff}\, Q_{Eff} + N_{Bio}\, P_b + (N\text{-}NH_4^{+}{}_{volatilization} + N_2) \quad \text{Eq. 2}$$

Phosphorous mass balance:

$$P\text{-}PO_4^{3-}{}_{SWW}\, Q_{SWW} = P\text{-}PO_4^{3-}{}_{Eff}\, Q_{Eff} + P_{Bio}\, P_b \quad \text{Eq. 3}$$

The carbon and nutrients recovery as algal-bacterial biomass and their removal efficiencies (REs) were calculated as follows:

Carbon recovery= C_{Bio} P_b / ((TOC_{SWW} + IC_{SWW}) Q_{SWW}) × 100 Eq. 4

Nitrogen recovery= N_{Bio} P_b / (TN_{SWW} Q_{SWW}) × 100 Eq. 5

Phosphorous recovery= P_{Bio} P_b / ($P\text{-}PO_4^{3-}{}_{SWW}$ Q_{SWW}) × 100 Eq. 6

$$RE_i = \frac{(X_{i,SWW} \times Q_{SWW}) - (X_{i,Eff} \times Q_{Eff})}{X_{i,STWW} \times Q_{SWW}} \times 100 \quad \text{Eq. 7}$$

where X_i accounts for the corresponding COD, TOC, IC, TN, NH_4^+ or $P\text{-}PO_4^{3-}$ concentrations (g L^{-1}) in the influent (SWW) and effluent (Eff). Q_{SWW} stands for the influent SWW flowrate (L d^{-1}) and Q_{Eff} for the effluent flowrate (L d^{-1}). P_b stands for the biomass productivity (g d^{-1}), C_{Bio} for the carbon content in biomass (g g^{-1}), N_{Bio} for the nitrogen content in biomass (g g^{-1}) and P_{Bio} for the phosphorous content in biomass (g g^{-1}). *)*. Finally, $C\text{-}CO_{2\text{-stripping}}$ contribution was calculated as the difference between the total carbon input and the sum of the total carbon in the effluent and biomass wastage. Similarly, the contribution of $N\text{-}NH_4^+{}_{volatilization}$ + N_2 from denitrification was calculated as the difference between the total nitrogen input and the sum of total nitrogen in the effluent and biomass wastage. All data are reported as means ± SD, n = 4 (in steady-state).

3. Results and discussion

3.1 Environmental parameters

The DO concentration in the anoxic bioreactor remained lower than 0.3±0.2 mgO_2 L^{-1} during all operational stages which is suitable to support an effective denitrification process (it typically requires DO concentrations <1 mgO_2 L^{-1}) [4]. During stage I, the low oxygen demand induced by the HRT tested (lowest organic matter load) promoted the highest DO concentration in the HRAP (7.8±3.6 mgO_2 L^{-1}). Afterward, the decrease in

the HRT (2 d) applied during stage II resulted in a severe decrease in the DO concentration to 0.4±0.1 mgO_2 L^{-1}. In contrast, the decrease in the C/N ratio promoted oxygen concentrations of 3.4±2.6 and 4.7±3.6 mgO_2 L^{-1} during stages III and IV, respectively, due to the lower oxygen demand required for oxidizing the organic matter of the SWW with lower C/N ratios. Furthermore, the algal photosynthetic activity in the HRAP supported higher pHs compared to those recorded in the anoxic bioreactor (Table 1). However, the decreasing C/N ratios of the SWW fed during stages III and IV entailed a reduction of the pH in the HRAP, which equaled the pH in the anoxic bioreactor during stage IV. Nonetheless, the pHs were optimum to support a successful SWW treatment [17].

The seasonal increase of temperature slightly increased the temperature in the anoxic bioreactor and HRAP from stage I to IV, being the HRAP temperature higher than the anoxic bioreactor due to the heating associated with LED lighting (Table 1). Temperatures were always suitable to support effective nitrification, denitrification, photosynthesis and aerobic organic matter biodegradation [17]. Nonetheless, the evaporation rates ranged from 13 to 17 L m^{-2} d^{-1}, mainly due to the temperature of the cultivation broth (Table 1). These values were significantly higher than those typically observed at industrial scale (~3-8 L m^{-2} d^{-1}) as a result of the high turbulence prevailing in this lab-scale HRAP [18].

3.2 Carbon removal

COD removals of 87±0%, 84±0%, 89±1%, and 86±1% were recorded during stages I, II, III and IV, respectively, while TOC-REs accounted for 93±3%, 88±2%, 87±8%, and 82±5%, respectively (Fig. 2a). These removal efficiencies allowed average COD effluent concentrations of 89±4 mg L^{-1}, 116±4 mg L^{-1}, 61±5 mg L^{-1}, and 67±7 mg L^{-1},

respectively, and TOC effluent concentrations of 15±5 mg L^{-1}, 24±3 mg L^{-1}, 13±2 mg L^{-1}, and 19±3 mg L^{-1}, respectively (Fig. 2a). As expected, the decrease in the C/N ratio applied during stages III and IV mediated the lowest COD concentrations in the effluent, while the highest COD concentration was achieved during stage II when the highest organic loading rate (HRT of 2 d and C/N ratio of 9) and the lowest DO concentrations in the HRAP occurred. Furthermore, the lower C/N ratio applied in stage IV caused an organic carbon limitation that likely affected the algal-bacterial metabolism, which ultimately affected COD and TN removal (Section 3.3). However, the organic matter removal in this novel anoxic-aerobic algal-bacterial photobioreactor complied with the limits for COD concentration (≤125 mg L^{-1}) of the wastewater discharged into the environment regardless of the operational conditions [19]. Furthermore, the recorded TOC-REs were similar to those reported by Alcántara *et al*. [3] (88±2%) in an anoxic bioreactor of 1 L interconnected to an enclosed photobioreactor of 3.5 L operated at 2 d of HRT (SWW with C/N ratio of ~2) and 20 d of SRT. In this sense, a higher SRT typically entails higher oxidations rates of C and NH_4^+, although no significant increase in TOC, NH_4^+ or TN removal is expected when increasing the SRT from 10 to 20 days since no washout of key microbial communities occurs in this SRT range.

IC-REs of 18±5%, 0±1%, 15±4%, and 9±1% were recorded during stages I, II, III and IV, respectively, which corresponded to IC effluent concentrations of 191±6 mg L^{-1}, 197±10 mg L^{-1}, 190±5 mg L^{-1}, and 187±3 mg L^{-1}, respectively (Fig. 2b). The REs here observed were lower than the 30-40% IC-REs reported by De Godos *et al*. [8] during the operation of a 1 L anoxic bioreactor coupled to a 3.5 L enclosed photobioreactor, in which the IC consumption by nitrifying bacteria at DO concentrations ranging from 12 to 20 mg L^{-1} likely enhanced the IC-REs. Nonetheless, according to the heterotrophic TOC

removal above reported, among 580 to 1250 mg C-CO_2 d^{-1} were produced which supported the imposed biomass productivity (SRT=10 d) of ~1.5 g TSS d^{-1} (section 3.4). Therefore, CO_2 stripping and phototrophic microalgae production can explain the removal of inorganic carbon, which was not significantly impacted by the low nitrifying activity recorded in our system (Section 3.3). Finally, the carbon recoveries in the harvested biomass accounted for 56±8%, 43±7%, 36±2% and 73±9% of the total (TOC+IC) carbon removal during stages I, II, III and IV, respectively (carbon content in the biomass was 38.8±0.6% during the four operational stages). Thus, the increase in the total carbon-loading rate mediated by the decrease in HRT from 4 to 2 days slightly affected the C recovery. However, decreasing the C/N ratio (stages III and IV) clearly induced the assimilatory carbon removal (higher carbon recovery) with the associated CO_2 stripping reduction (lower IC-RE observed in stage IV). Figure 3 shows a schematic representation of mass balance performed during stage IV:

3.3 Nitrogen and phosphorous removal

TN-REs of 87±2%, 62±2%, 62±4%, and 48±4% were recorded during stages I, II, III and IV, respectively, which resulted in TN effluent concentrations of 7±1 mg L^{-1}, 18±2 mg L^{-1}, 17±1 mg L^{-1}, and 23±2 mg L^{-1}, respectively (Fig. 4a). Hence, TN effluent concentrations only complied with the EU Water Framework Directive during stage I, since requires TN concentrations lower than 15 mg L^{-1} [19]. The decrease in the HRT applied during stage II mediated lower TN-REs likely due to low photosynthetic activity (DO concentration of 0.4±0.1 mgO_2 L^{-1}) that prevented the complete nitrification-denitrification process. Photosynthetic activity is typically correlated with the dissolved oxygen concentration in the cultivation broth. Therefore, oxygen limitation or availability may inhibit or boost nitrifying activity, respectively, which ultimately impacts on the

performance of the denitrification process. In fact, during stage II neither NO_2^- nor NO_3^- were returned from the photobioreactor via the internal and external recirculations to the anoxic bioreactor (Fig. 4b), and therefore, no significant denitrification occurred. Furthermore, the impact of the C/N ratio on TN removal was significant at a ratio of 7, where a decrease in TN-RE caused by a severe organic carbon limitation was observed [9,10].

$N\text{-}NH_4^+$-REs of 86±11%, 45±4%, 50±3%, and 43±4% were recorded during stages I, II, III and IV, respectively, which resulted in $N\text{-}NH_4^+$ effluent concentrations of 4±3 mg L^{-1}, 13±2 mg L^{-1}, 12±1 mg L^{-1}, and 18±2 mg L^{-1}, respectively (Fig. 4a). The low DO concentrations prevailing in the cultivation broth of the HRAP during stages II, III and IV limited $N\text{-}NH_4^+$ oxidation. Indeed, $N\text{-}NO_3^-$ was only detected in the HRAP cultivation broth during stage I at a maximum concentration of 2.4 mg L^{-1} (Fig. 4b). Average effluent $N\text{-}NO_2^-$ concentrations of 1.4±1.1 mg L^{-1}, 0.0±0.0 mg L^{-1}, 0.9±0.9 mg L^{-1}, and 1.4±0.8 mg L^{-1} were recorded during stage I, II, III and IV respectively. Despite the fact that the DO concentration remained >2 mgO_2 L^{-1}, the fluctuations in DO concentration during the illuminated period along with the high temperature in the HRAP (>28 ºC) likely favored the accumulation of NO_2^-.

Overall, the decrease in the HRT from 4 to 2 d reduced the rate of nitrification due to the low DO concentrations prevailing in the cultivation broth, while the decrease in the organic carbon load ultimately limited the denitrification process and TN removal. Indeed, this limited denitrification resulted in lower TN-RE compared to Alcántara *et al*. [3]) and De Godos *et al*. [8], who reported TN-REs of 68-79% and 90%, respectively, in a similar experimental set-up. The use of a HRAP as oxygenation unit showed low activity of nitrifying bacteria, compared to similar anoxic-aerobic configurations engineered in enclosed photobioreactors, due to the lower DO concentrations in the

cultivation broth mediated by the O_2 exchange with the open atmosphere and the lower illuminated area/volume ratio [3,8].

Neither the HRT nor the C/N ratio influenced the N biomass content, which averaged 7.4±0.3% along the four operational stages. The nitrogen mass balance showed average N recoveries in the harvested biomass of 56±5%, 52±3%, 37±3% and 73±2% during stages I, II, III and IV, respectively. Despite the high pHs (8.4 to 9.1) prevailing in the HRAP, the open nature of the system and the low rates of nitrification recorded, likely induced $N\text{-}NH_4^+$ losses by volatilization. The nitrogen mass balance also confirmed the limited denitrification activity occurring in the anoxic bioreactor during stage IV, as previously hypothesized.

The REs of $P\text{-}PO_4^{3-}$ accounted for 22±5%, 11±1%, 53±3% and 47±5% during stages I, II, III and IV, respectively, which corresponded to $P\text{-}PO_4^{3-}$ effluent concentrations of 11±1 mg L^{-1}, 13±1 mg L^{-1}, 6±1 mg L^{-1} and 7±1 mg L^{-1}, respectively (Fig. 5). $P\text{-}PO_4^{3-}$ effluent concentrations did not comply with the EU Water Framework Directive, which requires $P\text{-}PO_4^{3-}$ concentrations lower than 2 mg L^{-1} prior to wastewater discharge [19]. The decrease in $P\text{-}PO_4^{3-}$ REs when decreasing the HRT from 4 to 2 d was likely mediated by the overload of the assimilation capacity of algal-bacterial consortium present in the anoxic-aerobic photobioreactor. This finding agreed with the results obtained by Posadas *et al*. [14], who reported a $P\text{-}PO_4^{3-}$ REs decreasing from 57±17% to 36±22% when the HRT was reduced from 5.2 d to 3.1 d during secondary domestic wastewater treatment in a 31 L open algal-bacterial biofilm photobioreactor. The highest P removals observed at C/N ratios of 8 and 7 compared to that recorded at a C/N ratio of 9 were likely mediated by a luxury phosphorus uptake at the lowest C/P ratios in the SWW [20,21]. In fact,

microalgae can store acid-insoluble polyphosphate when phosphorous concentration in the media becomes limiting [22].

3.4 Biomass concentration and settling efficiency

The average TSS concentrations in the anoxic bioreactor during stages I, II, III, and IV were 0.5±0.1 g L^{-1}, 0.7±0.1 g L^{-1}, 0.5±0.1 g L^{-1}, and 0.6±0.1 g L^{-1}, respectively; while the TSS concentrations in the HRAP averaged 0.9±0.1 g L^{-1}, 1.0±0.1 g L^{-1}, 0.7±0.1 g L^{-1}, and 1.0±0.1 g L^{-1}, respectively. The decreasing organic loads during process operation at C/N ratios of 8 and 7 did not imply lower TSS concentrations in the system likely due to the decreasing carbon losses by stripping. The slightly higher TSS concentrations in the HRAP than in the anoxic bioreactor were caused by the higher retention time and superior C and N assimilation mediated by algal activity in the photobioreactor [14]. This difference in TSS concentration was also in agreement with De Godos *et al*. [8] who reported VSS concentrations of 0.57-0.94 g L^{-1} in the anoxic bioreactor and of 0.69-1.4 g L^{-1} in the enclosed photobioreactor.

Process operation at 10 d of SRT supported biomass productivities of 1.4-1.6 g TSS d^{-1} regardless of the HRT and C/N ratio, which resulted in the low variations in the biomass concentrations of the cultivation broth of the anoxic bioreactor and HRAP above mentioned. In this sense, decoupling the SRT from the HRT by means of recycling the settled biomass allowed washing out from the system the poorly settleable species while keeping a constant the biomass productivity. This operating strategy represents a cost-effective method for algal biomass production/harvesting in spite of the variation of the operating parameters during the wastewater treatment [23]. Furthermore, the wastage stream TSS concentration averaged 6.7±0.6 g L^{-1}, 5.9±1.0 g L^{-1}, 5.8±1.5 g L^{-1}, and 6.8±1.5 g L^{-1} in stage I, II, III and IV, respectively; while the TSS removal efficiency in

the settler averaged 98±1%, 92±1%, 97±1% and 98±5%, respectively. This resulted in TSS concentrations in the effluent along stages I to IV of 0.02±0.01 g L^{-1}, 0.08±0.01 g L^{-1}, 0.02±0.01 g L^{-1}, and 0.03±0.01 g L^{-1}, respectively. Therefore, effluent TSS concentrations complied with the EU Water Framework Directive, which requires TSS concentrations ≤35 mg TSS L^{-1} prior to discharge [19].

3.5 Microalgae population dynamics

The microalgae inoculum was composed of (percentage of cells) 49% of *Chlorella vulgaris*, 20% of *Chlorella kessieri*, 20% of *Chlamydomonas altera*, 7% of *Chlorella minutissima*, 3% of *Scenedesmus obliquus* and 2% of *Chlamydomonas* sp. (Fig. 6). During steady state I, *Chlorella vulgaris* became the dominant microalga accounting for 54% of the total number of cells. Species such as *S. obliquus* increased up to 22% while *Chlamydomonas altera* disappeared and others such as *Chloroccum* sp. (11%) and *Synechococcus* sp. (9%) appeared during stage I. These variations in microalgae population were caused by the imposed biomass productivity (throughout controlling the biomass withdrawal) and the acclimation to anoxic-oxic cycles, irradiation, and the wastewater characteristics. The decrease in the HRT from 4 to 2 d mediated a slight increase in the dominance of *C. vulgaris* (accounting for 61% of the total number of cells), while the abundance of *S. obliquus* decreased to 14% in stage II. Other species of *Chlorella* such as *Chlorella kessieri* and *Chlorella minutissima* were identified with abundances of 16% and 6%, respectively. Overall, the results revealed that the decrease in HRT did not change the most abundant microalga species. In contrast, the decrease in the C/N ratio to 8 applied during stage III resulted in a reduction in the number of cells of *C. vulgaris* (38%), while *Phormidium* sp. showed up with an abundance of 21% and *S. obliquus* population increased to 31%. The subsequent reduction in the C/N ratio to 7

induced a further decrease in the number of *C. vulgaris* to 32%, while the population of *Phormidium* sp. increased to 44% (Fig. 6). Thus, the C/N ratio of the wastewater played a key role in the structure of the microalgae population. Nonetheless, it is worth noticing that the morphology of microalgae could depend on these factors and therefore might bias microalgae identification at the species level. On the other hand, there is still an ongoing active discussion about the appropriate DNA fragment to be sequenced during molecular identification of microalgae for a clear species identification. ITS-2 is often used for phylogenetic studies of microalgae at species level, but shows difficulties with the alignment of sequences and the prediction of the secondary structure. In our particular study, the high microalgae diversity recorded in this research was in agreement with the observations of Alcántara *et al*. [3] in an anoxic-aerobic algal-bacterial photobioreactor treating domestic wastewater. Furthermore, the genera *Chlorella* and *Phormidium* have typically ranked among the 12 microalgae genera most tolerant to organic pollution in HRAPs [24].

4. Conclusions

This work represents, to the best of our knowledge, the first systematic evaluation of the influence of the HRT and C/N ratio on the wastewater treatment performance of an anoxic-aerobic algal-bacterial photobioreactor using a HRAP as oxygenation unit. The effluent COD concentrations complied with the EU Water Framework Directive regardless of the HRT and C/N ratio. However, the low DO concentrations in the cultivation broth of the HRAP during process operation at 2 d of HRT limited the nitrification-denitrification process. Therefore, the effluent TN concentrations only complied with the EU Directive at 4 d of HRT. Similarly, the effluent $P\text{-}PO_4^{3-}$ concentrations were over the discharge limits at all operating conditions tested. Biomass

settling and recycling mediated the enrichment of algal-bacterial biomass with good settleability properties, which resulted in TSS discharge levels complying with EU Directive. Finally, *C. vulgaris* was the dominant species at a C/N ratio of 9 regardless of the HRT and was gradually replaced by *Phormidium* sp. when decreasing this ratio to 7.

Acknowledgments

This research was supported by Elecnor S.A. Araceli Crespo and Jonatan Prieto are also gratefully acknowledged for their technical support. The financial support from the Regional Government of Castilla y Leon and the EU-FEDER programme is also acknowledged (CLU-2017-09 and UIC 71). Finally, to Fondo Sectorial CONACyT-SENER, CEMIE-Bio project No. 247006.

Declaration of competing interest: None.

Statement of informed consent, human/animal rights: No conflicts, informed consent, or human or animal rights are applicable to this study.

Declaration of contributions: Dr. Toledo-Cervantes participated actively in the discussion of the results, critically reviewed the article and corrected the manuscript. Dr. Posadas conducted the experimentation and drafted the manuscript. Isabel Berton participated in the analysis and interpretation of the data. Sara Turiel participated in the interpretation of data and the discussion of results. Ana Alcoceba participated actively in the discussion of the results. Dr. Muñoz obtained the financial support for conducting the experimentation, designed and supervised the experimentation and reviewed the manuscript for its final approval.

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Figure captions

Figure 1. Schematic diagram of the anoxic-aerobic algal-bacterial photobioreactor configuration.

Figure 2. Time course of the concentration of (**a**) total organic carbon and (**b**) total inorganic carbon in the influent (▲), the output of the anoxic bioreactor (x) and effluent (○). TOC and IC removal efficiencies (■) are displayed in the secondary Y-axis.

Figure 3. Schematic representation of mass balance for total carbon (TC), total nitrogen (TN) and phosphorous ($P\text{-}PO_4^{3-}$) during stage IV.

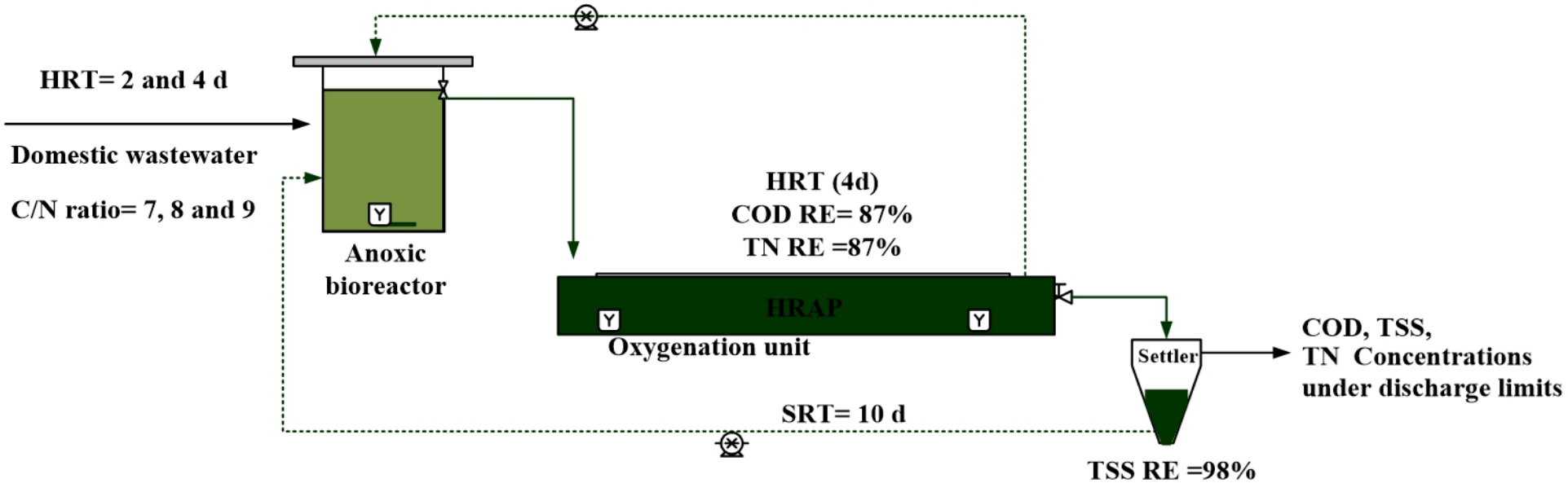
Figure 4a. Time course of the concentration of $N\text{-}NH_4^+$ in the influent (▲) and effluent (○) and the TN removal efficiencies (■, displayed in the secondary Y-axis); **4b**. Time course of the $N\text{-}NO_2^-$ (♦) and $N\text{-}NO_3^-$ (◊) concentrations in the effluent.

Figure 5. Time course of the concentration of $P\text{-}PO_4^{3-}$ in the influent (▲), the output of the anoxic bioreactor (x) and effluent (○). $P\text{-}PO_4^{3-}$ removal efficiency (■) is displayed in the secondary Y-axis.

Figure 6. Time course of the structure of the microalgae population in the HRAP: ■ *Chlamydomonas* sp., ■ *Chlamydomonas altera*, □ *Chlorella Kessieri,* ■ *Chlorella minutissima*, ▰ *Chlorella vulgaris*, ▰ *Chlorococcum* sp., ▌▌ *Phormidium* sp., ▩ *Scenedesmus obliquus* and ▤ *Synechococcus* sp.

Highlights

- A new anoxic-aerobic algal-bacterial photobioreactor configuration was assessed
- High COD removals regardless of the HRT and C/N ratio applied were obtained
- Low DO concentrations at 2 d HRT limited the nitrification-denitrification process
- TP remained over regulation discharge limits at the HRT and C/N ratios tested
- Biomass settling and recycling allowed effluent TSS under discharge limits


HRT= 2 and 4 d
Domestic wastewater
C/N ratio= 7, 8 and 9
Anoxic
bioreactor
HRT (4d)
COD RE= 87%
TN RE =87%
HRAP
Oxygenation unit
Settler
COD, TSS,
TN Concentrations
under discharge limits
SRT= 10 d
TSS RE =98%

Assessing the influence of the hydraulic retention time and carbon/nitrogen ratio on urban wastewater treatment in a new anoxic-aerobic algal-bacterial photobioreactor configuration.

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Abstract

The influence of the hydraulic retention time (HRT) (2 and 4 days) and the carbon/nitrogen ratio (C/N) (7, 8 and 9) of the wastewater on the treatment of synthetic domestic wastewater was evaluated in a new anoxic-aerobic algal-bacterial photobioreactor configuration operated at solids retention time of 10 d by biomass recycling and withdrawal. The removal of chemical oxygen demand remained between 84% and 89% regardless of the operational conditions. However, the decrease in the HRT from 4 to 2 d entailed reductions in the removal of total nitrogen (TN) and $P\text{-}PO_4^{3-}$ from 87±2% to 62±2% and from 22±5% to 11±1%, respectively. On the other hand, the decrease in the C/N ratio of the wastewater from 9 to 8 and 7 at a HRT of 2 d induced TN removals of 62±4% and 48±4%, respectively. In contrast, $P\text{-}PO_4^{3-}$ removals unexpectedly increased from 11±1% at a C/N ratio of 9 to 53±3% and 47±5% at C/N ratios of 8 and 7, respectively. Finally, biomass settling and recycling supported the enrichment of an algal-bacterial population with good settleability characteristics (suspended solids removals in the settler ~98%), being *Chlorella vulgaris* the dominant microalga specie at a C/N ratio of 9 which was gradually replaced by *Phormidium* sp., as a result of the reduction in the C/N ratio of the wastewater.

Keywords:

Algal-bacterial processes; anoxic-aerobic photobioreactor; C/N ratio; nitrification-denitrification; photosynthetic oxygenation.

Abbreviations

C: Carbon

C_{Bio}: Carbon content in biomass

C/N: Carbon/Nitrogen ratio

C/N/P: Carbon/Nitrogen/Phosphorous ratio

COD: Chemical oxygen demand

DO: Dissolved oxygen concentration

HRAP: High rate algal pond

HRT: Hydraulic retention time

IC: Inorganic carbon concentration

N: Nitrogen

NH_4^+: Ammonium

NO_2^-: Nitrite

NO_3^-: Nitrate

P: Phosphorous

PAR: Photosynthetically active radiation

P_b: Biomass productivity

Q_{Eff}: Effluent flowrate

Q_{SWW}: Influent flowrate

REs: Removal efficiencies

SRT: Solids retention time

SWW: Synthetic domestic wastewater

TN: Total nitrogen concentration

TOC: Total organic carbon concentration

TSS: Suspended solids concentration

WWTPs: Wastewater treatment plants

$X_{i\ Eff}$: Concentrations of either COD, TOC, IC, TN, NH_4^+ or $P\text{-}PO_4^{3-}$ in the effluent

$X_{i\ sww}$: Concentrations of either COD, TOC, IC, TN, NH_4^+ or $P\text{-}PO_4^{3-}$ in the influent

1. Introduction

Algal-bacterial processes have emerged in the past decades as a cost-effective and environmentally friendly platform technology to remove carbon (C), nitrogen (N) and phosphorus (P) from wastewaters [1]. The synergistic interactions between microalgae and bacteria are based on the *in-situ* supply of O_2 (produced by photosynthesis) for aerobic heterotrophic bacteria and autotrophic nitrifiers, and the subsequent assimilation of C, N, and P in the form of valuable algal-bacterial biomass [2,3]. This green biotechnology significantly reduces oxygenation costs when compared to activated sludge systems and enhances nutrient recovery compared to anaerobic digestion technologies in conventional wastewater treatment plants (WWTPs) [2,4].

Despite the above-mentioned advantages, the efficiency of microalgae-based wastewater treatment processes is often limited by the C/N/P ratio of the secondary wastewater (100/25/12) or centrate (100/207/5), which hinders a complete nutrient assimilation due to carbon limitation [5,6] In this sense, only wastewaters with balanced C/N/P ratios (e.g. 100/14/2 on mass basis) are favorable for microalgae growth and can therefore support N and P removals by assimilation, which opens the investigation niche for innovative photobioreactor configurations capable of supporting an effective N and P removals at low C/N ratios [3,7]. In this context, the complete nitrification-denitrification process represents a key metabolic pathway to remove C and N in wastewaters with low C/N ratios in algal-bacterial photobioreactors [3,8]. Nevertheless, the relatively low hydraulic retention times (HRTs =2-6 days) applied in high rate algal ponds (HRAPs) devoted to wastewater treatment limit the occurrence of nitrifying bacterial communities in the cultivation broth. Typically, the oxidation of NH_4^+ requires HRT >8 d for complete nitrification [4,9]. Therefore, a new generation of anoxic-aerobic algal-bacterial photobioreactors based on decoupling the HRT from the solids retention time (SRT) were

developed and successfully tested at laboratory scale [3,9]. In these systems, photosynthesis supports the oxidation of NH_4^+ to NO_2^- or NO_3^- required for carbon oxidation in the anoxic bioreactor through an internal recirculation. For instance, De Godos *et al*. [8] reported C (95%) and N (98%) removals from synthetic wastewater in a 1 L anoxic bioreactor coupled to a 3.5 L closed photobioreactor operated at HRTs of 2-4.5 d and SRTs of 9-31 d. These authors also reported that the recirculation of the harvested biomass from a 1 L settler to the anoxic bioreactor (external recirculation) avoided the washout of nitrifying bacteria and supported process operation at biomass concentrations of 1.0-1.5 g volatile suspended solids (VSS) L^{-1}. Alcántara *et al*. [3] observed the absence of nitrification at high dissolved oxygen concentrations (DO), 21 ± 4 mgO_2 L^{-1}, during synthetic wastewater treatment in a similar anoxic-aerobic algal-bacterial closed photobioreactor configuration; Additionally, light-dark cycles combined with process aeration during dark periods were tested in order to elucidate a light-mediated inhibition on nitrifying activity. These authors recorded total organic carbon (TOC), inorganic carbon (IC) and total nitrogen (TN) removal efficiencies (REs) of ~ 80% at 2 d of HRT and SRTs of 20 d, the N removal mechanisms being governed by the light intensity and DO. This particular configuration has also been tested for the treatment of domestic wastewater [9] and synthetic textile wastewater [10] coupled to biogas upgrading and flue gas scrubbing, respectively. CO_2 supply overcame the IC limitation, enhanced N and P removal by assimilation and supported an efficient nitrification-denitrification process. However, the local availability of an external CO_2 source is not always technical or economically feasible.

On the other hand, despite the high biomass productivities reached in closed photobioreactors, the high construction and operating costs limit their scalability [11]. Thus, HRAPs are typically the preferred photobioreactor configuration for microalgae-

based wastewater treatment [5]. However, the use of a HRAP as process oxygenation unit for this novel anoxic-aerobic configuration has not been evaluated yet. Furthermore, there is a lack of studies assessing the influence of the C/N ratio of the wastewater on the performance of wastewater treatment in anoxic-aerobic algal-bacterial systems. Therefore, this work aim at evaluating the influence of the HRT and the C/N ratio of the wastewater on the C, N and P removal in an anoxic-aerobic algal-bacterial photobioreactor with a HRAP as process oxygenation unit. Mass balance calculations were conducted to elucidate the global carbon and nutrient removal mechanisms. Finally, a characterization of the biomass harvesting efficiency and the microalgae population structure was carried out during the different operational conditions assessed.

2. Materials and methods

2.1 Algal-bacterial inoculum

The inoculum consisted in a mixture of secondary activated sludge from the Valladolid WWTP (which operates with a nitrification-denitrification configuration) and a microalgae consortium collected from an outdoors pilot HRAP treating digestate located at the Department of Chemical Engineering and Environmental Technology of the University of Valladolid, Spain.

2.2 Synthetic domestic wastewater

The synthetic domestic wastewater (SWW) was prepared according to Frutos *et al*. [12] with the follow composition in g L^{-1}: 0.16 of casein peptone, 0.11 of meat extract, 0.03 of NH_2COH_2, 0.007 of NaCl, 0.004 of $CaCl_2 \cdot 2H_2O$, 0.002 of $MgSO_4 \cdot 7H_2O$, $5 \cdot x10^{-6}$ of $CuCl_2 \cdot 2H_2O$, 0.112 of $K_2HPO_4 \cdot 3H_2O$, 0.25 of glucose, and 1.1 of $NaHCO_3$. The main characteristics of the SWW were chemical oxygen demand (COD) concentration of

632±45 mg L^{-1}, TOC of 196±9 mg L^{-1}, IC of 195±12 mg L^{-1}, TN of 43±3 mg L^{-1}, N-NH_4^+ of 24±3 mg L^{-1}, P-PO_4^{3-} of 13.1±0.8 mg L^{-1} and pH of 7.7±0.2.

2.3 Experimental set-up

The experimental set-up consisted of a 3.75 L enclosed anoxic bioreactor (15 cm long, 15 cm wide, 17 cm deep), an 11.25 L open photobioreactor (HRAP) (30 cm long, 15 cm wide, 25 cm deep) and a 1 L conical settler (Fig. 1). The agitation of the cultivation broth in the anoxic bioreactor and in the HRAP was provided by Eheim compact 300 immersion pumps (Spain) (one pump in the anoxic bioreactor and two pumps in the HRAP). The HRAP was illuminated at an average photosynthetically active radiation (PAR) of 1314±12 μmol m^{-2} s^{-1} (light:dark cycles of 12:12 h) by high-intensity LED PCBs (Phillips SA, Spain). The internal liquid recirculation from the HRAP to the anoxic bioreactor supported the denitrification process, while the external liquid recirculation from the bottom of the settler to the anoxic bioreactor mediated controlling the SRT according to table 1.

The bioreactors were initially filled with SWW and inoculated to have an initial total suspended solids concentrations (TSS) of 0.2 g TSS L^{-1} of microalgae and 0.6 g TSS L^{-1} of activated sludge. The SWW was fed to the anoxic bioreactor at 4 and 2 d of total HRT (HRT of anoxic bioreactor + HRT of HRAP) (Table 1). These operational conditions promote the nitrification-denitrification process [13]. The flow rates of the internal and external recirculation (Watson Marlow 120 S pump, UK, and Masterflex 7021-24, USA, respectively) corresponded to 200% and 50% of the SWW flow rate, respectively, and were adjusted depending on the HRT tested. The SRT of the system was fixed at 10 d regardless of the operational stage by means of harvesting a volume of the external recirculation (wasted biomass ~1.5 g TSS d^{-1} under a steady biomass concentration in the

reactor). This volume was adjusted in accordance with the TSS concentration recorded in the wastage stream.

Stage I lasted 47 days in which the system was operated at an HRT of 4 d by feeding the SWW with a C/N ratio of 9 (COD concentration of 669±6 mg L^{-1}). Afterwards, the HRT was decreased to 2 d during stages II, III and IV, while the C/N ratio of the wastewater was step-wise decreased from 9 to 8 and 7, respectively, by means of decreasing the glucose concentration (corresponding to COD concentrations of 669±6 mg L^{-1}, 493±11 mg L^{-1} and 434± 11 mg L^{-1}, respectively). Stages II, III and IV were maintained for 40 d (~4 times the SRT) to achieve consistent steady states values.

2.5 Analytical methods

Samples (50 mL) from the SWW, anoxic bioreactor, HRAP, settler and effluent were drawn twice per week in order to monitor the concentrations of dissolved TOC, IC, and TN in a Shimadzu TOC-VCSH analyzer with TNM-1 module (Japan). The NH_4^+ concentration of samples was determined by using an Electrode Orion Dual Star (Thermo Scientific, The Netherlands), while the NO_2^-, NO_3^- and PO_4^{3-} concentrations by HPLC-IC according to Posadas *et al*. [14]. The pH (Eutech Cyberscan pH 510, The Netherlands), dissolved oxygen concentration (DO) (OXI 330i oximeter, WTW, Germany) and temperature were daily monitored in situ (anoxic bioreactor and HRAP). Furthermore, the TSS concentrations in the anoxic bioreactor, HRAP, settler, and effluent were monitor twice per week according to standard methods [15]. The concentration of COD in the SWW and treated effluent was only assessed under steady state (last three days from each operational stage) by the closed reflux method [15]. The influent and effluent flowrates were daily recorded in order to determine the water evaporation rate, while the PAR was weekly monitored (LI-250A, LI-COR Biosciences, Germany). The algal-bacterial

biomass harvested from the bottom of the settler under steady state was washed three times with distilled water and dried for 24 hours at 105 ºC prior determination of its elemental composition C, N, and P (LECO CHNS-932 analyzer). Finally, the morphological identification of the microalgae population in the HRAP was carried out at steady state. Two biomass samples were preserved with lugol acid at 5% and formaldehyde at 10%, respectively, and stored at 4 ºC prior analysis. The quantification and morphological identification of photosynthetic microorganisms were carried out according to Sournia [16] in an inverted microscope (OLYMPUS IX70, USA).

2.6 Mass balance calculation

The global mass balance calculation for C, N, and P were conducted based on the average concentrations of all their chemical species at the inlet (SWW) and outlet (effluent).

Carbon mass balance:

$$(TOC_{SWW} + IC_{SWW})\, Q_{SWW} = (TOC_{Eff} + IC_{Eff})\, Q_{Eff} + C_{Bio}\, P_b + C\text{-}CO_{2\text{-stripping}} \quad \text{Eq. 1}$$

Nitrogen mass balance:

$$TN_{SWW}\, Q_{SWW} = TN_{Eff}\, Q_{Eff} + N_{Bio}\, P_b + (N\text{-}NH_4^{+}{}_{volatilization} + N_2) \quad \text{Eq. 2}$$

Phosphorous mass balance:

$$P\text{-}PO_4^{3-}{}_{SWW}\, Q_{SWW} = P\text{-}PO_4^{3-}{}_{Eff}\, Q_{Eff} + P_{Bio}\, P_b \quad \text{Eq. 3}$$

The carbon and nutrients recovery as algal-bacterial biomass and their removal efficiencies (REs) were calculated as follows:

Carbon recovery= $C_{Bio}\ P_b$ / ((TOC_{SWW} + IC_{SWW}) Q_{SWW}) × 100 Eq. 4

Nitrogen recovery= $N_{Bio}\ P_b$ / ($TN_{SWW}\ Q_{SWW}$) × 100 Eq. 5

Phosphorous recovery= $P_{Bio}\ P_b$ / ($P\text{-}PO_4^{3-}{}_{SWW}\ Q_{SWW}$) × 100 Eq. 6

$$RE_i = \frac{(X_{i,SWW} \times Q_{SWW}) - (X_{i,Eff} \times Q_{Eff})}{X_{i,STWW} \times Q_{SWW}} \times 100 \quad \text{Eq. 7}$$

where X_i accounts for the corresponding COD, TOC, IC, TN, NH_4^+ or $P\text{-}PO_4^{3-}$ concentrations (g L^{-1}) in the influent (SWW) and effluent (Eff). Q_{SWW} stands for the influent SWW flowrate (L d^{-1}) and Q_{Eff} for the effluent flowrate (L d^{-1}). P_b stands for the biomass productivity (g d^{-1}), C_{Bio} for the carbon content in biomass (g g^{-1}), N_{Bio} for the nitrogen content in biomass (g g^{-1}) and P_{Bio} for the phosphorous content in biomass (g g^{-1}). *)*. Finally, $C\text{-}CO_{2\text{-stripping}}$ contribution was calculated as the difference between the total carbon input and the sum of the total carbon in the effluent and biomass wastage. Similarly, the contribution of $N\text{-}NH_4^+{}_{volatilization}$ + N_2 from denitrification was calculated as the difference between the total nitrogen input and the sum of total nitrogen in the effluent and biomass wastage. All data are reported as means ± SD, n = 4 (in steady-state).

3. Results and discussion

3.1 Environmental parameters

The DO concentration in the anoxic bioreactor remained lower than 0.3±0.2 $mgO_2\ L^{-1}$ during all operational stages which is suitable to support an effective denitrification process (it typically requires DO concentrations <1 $mgO_2\ L^{-1}$) [4]. During stage I, the low oxygen demand induced by the HRT tested (lowest organic matter load) promoted the highest DO concentration in the HRAP (7.8±3.6 $mgO_2\ L^{-1}$). Afterward, the decrease in

the HRT (2 d) applied during stage II resulted in a severe decrease in the DO concentration to 0.4±0.1 mgO_2 L^{-1}. In contrast, the decrease in the C/N ratio promoted oxygen concentrations of 3.4±2.6 and 4.7±3.6 mgO_2 L^{-1} during stages III and IV, respectively, due to the lower oxygen demand required for oxidizing the organic matter of the SWW with lower C/N ratios. Furthermore, the algal photosynthetic activity in the HRAP supported higher pHs compared to those recorded in the anoxic bioreactor (Table 1). However, the decreasing C/N ratios of the SWW fed during stages III and IV entailed a reduction of the pH in the HRAP, which equaled the pH in the anoxic bioreactor during stage IV. Nonetheless, the pHs were optimum to support a successful SWW treatment [17].

The seasonal increase of temperature slightly increased the temperature in the anoxic bioreactor and HRAP from stage I to IV, being the HRAP temperature higher than the anoxic bioreactor due to the heating associated with LED lighting (Table 1). Temperatures were always suitable to support effective nitrification, denitrification, photosynthesis and aerobic organic matter biodegradation [17]. Nonetheless, the evaporation rates ranged from 13 to 17 L m^{-2} d^{-1}, mainly due to the temperature of the cultivation broth (Table 1). These values were significantly higher than those typically observed at industrial scale (~3-8 L m^{-2} d^{-1}) as a result of the high turbulence prevailing in this lab-scale HRAP [18].

3.2 Carbon removal

COD removals of 87±0%, 84±0%, 89±1%, and 86±1% were recorded during stages I, II, III and IV, respectively, while TOC-REs accounted for 93±3%, 88±2%, 87±8%, and 82±5%, respectively (Fig. 2a). These removal efficiencies allowed average COD effluent concentrations of 89±4 mg L^{-1}, 116±4 mg L^{-1}, 61±5 mg L^{-1}, and 67±7 mg L^{-1},

respectively, and TOC effluent concentrations of 15±5 mg L^{-1}, 24±3 mg L^{-1}, 13±2 mg L^{-1}, and 19±3 mg L^{-1}, respectively (Fig. 2a). As expected, the decrease in the C/N ratio applied during stages III and IV mediated the lowest COD concentrations in the effluent, while the highest COD concentration was achieved during stage II when the highest organic loading rate (HRT of 2 d and C/N ratio of 9) and the lowest DO concentrations in the HRAP occurred. Furthermore, the lower C/N ratio applied in stage IV caused an organic carbon limitation that likely affected the algal-bacterial metabolism, which ultimately affected COD and TN removal (Section 3.3). However, the organic matter removal in this novel anoxic-aerobic algal-bacterial photobioreactor complied with the limits for COD concentration (≤125 mg L^{-1}) of the wastewater discharged into the environment regardless of the operational conditions [19]. Furthermore, the recorded TOC-REs were similar to those reported by Alcántara *et al*. [3] (88±2%) in an anoxic bioreactor of 1 L interconnected to an enclosed photobioreactor of 3.5 L operated at 2 d of HRT (SWW with C/N ratio of ~2) and 20 d of SRT. In this sense, a higher SRT typically entails higher oxidations rates of C and NH_4^+, although no significant increase in TOC, NH_4^+ or TN removal is expected when increasing the SRT from 10 to 20 days since no washout of key microbial communities occurs in this SRT range.

IC-REs of 18±5%, 0±1%, 15±4%, and 9±1% were recorded during stages I, II, III and IV, respectively, which corresponded to IC effluent concentrations of 191±6 mg L^{-1}, 197±10 mg L^{-1}, 190±5 mg L^{-1}, and 187±3 mg L^{-1}, respectively (Fig. 2b). The REs here observed were lower than the 30-40% IC-REs reported by De Godos *et al*. [8] during the operation of a 1 L anoxic bioreactor coupled to a 3.5 L enclosed photobioreactor, in which the IC consumption by nitrifying bacteria at DO concentrations ranging from 12 to 20 mg L^{-1} likely enhanced the IC-REs. Nonetheless, according to the heterotrophic TOC

removal above reported, among 580 to 1250 mg C-CO_2 d^{-1} were produced which supported the imposed biomass productivity (SRT=10 d) of ~1.5 g TSS d^{-1} (section 3.4). Therefore, CO_2 stripping and phototrophic microalgae production can explain the removal of inorganic carbon, which was not significantly impacted by the low nitrifying activity recorded in our system (Section 3.3). Finally, the carbon recoveries in the harvested biomass accounted for 56±8%, 43±7%, 36±2% and 73±9% of the total (TOC+IC) carbon removal during stages I, II, III and IV, respectively (carbon content in the biomass was 38.8±0.6% during the four operational stages). Thus, the increase in the total carbon-loading rate mediated by the decrease in HRT from 4 to 2 days slightly affected the C recovery. However, decreasing the C/N ratio (stages III and IV) clearly induced the assimilatory carbon removal (higher carbon recovery) with the associated CO_2 stripping reduction (lower IC-RE observed in stage IV). Figure 3 shows a schematic representation of mass balance performed during stage IV:

3.3 Nitrogen and phosphorous removal

TN-REs of 87±2%, 62±2%, 62±4%, and 48±4% were recorded during stages I, II, III and IV, respectively, which resulted in TN effluent concentrations of 7±1 mg L^{-1}, 18±2 mg L^{-1}, 17±1 mg L^{-1}, and 23±2 mg L^{-1}, respectively (Fig. 4a). Hence, TN effluent concentrations only complied with the EU Water Framework Directive during stage I, since requires TN concentrations lower than 15 mg L^{-1} [19]. The decrease in the HRT applied during stage II mediated lower TN-REs likely due to low photosynthetic activity (DO concentration of 0.4±0.1 mgO_2 L^{-1}) that prevented the complete nitrification-denitrification process. Photosynthetic activity is typically correlated with the dissolved oxygen concentration in the cultivation broth. Therefore, oxygen limitation or availability may inhibit or boost nitrifying activity, respectively, which ultimately impacts on the

performance of the denitrification process. In fact, during stage II neither NO_2^- nor NO_3^- were returned from the photobioreactor via the internal and external recirculations to the anoxic bioreactor (Fig. 4b), and therefore, no significant denitrification occurred. Furthermore, the impact of the C/N ratio on TN removal was significant at a ratio of 7, where a decrease in TN-RE caused by a severe organic carbon limitation was observed [9,10].

$N\text{-}NH_4^+$-REs of 86±11%, 45±4%, 50±3%, and 43±4% were recorded during stages I, II, III and IV, respectively, which resulted in $N\text{-}NH_4^+$ effluent concentrations of 4±3 mg L^{-1}, 13±2 mg L^{-1}, 12±1 mg L^{-1}, and 18±2 mg L^{-1}, respectively (Fig. 4a). The low DO concentrations prevailing in the cultivation broth of the HRAP during stages II, III and IV limited $N\text{-}NH_4^+$ oxidation. Indeed, $N\text{-}NO_3^-$ was only detected in the HRAP cultivation broth during stage I at a maximum concentration of 2.4 mg L^{-1} (Fig. 4b). Average effluent $N\text{-}NO_2^-$ concentrations of 1.4±1.1 mg L^{-1}, 0.0±0.0 mg L^{-1}, 0.9±0.9 mg L^{-1}, and 1.4±0.8 mg L^{-1} were recorded during stage I, II, III and IV respectively. Despite the fact that the DO concentration remained >2 mgO_2 L^{-1}, the fluctuations in DO concentration during the illuminated period along with the high temperature in the HRAP (>28 ºC) likely favored the accumulation of NO_2^-.

Overall, the decrease in the HRT from 4 to 2 d reduced the rate of nitrification due to the low DO concentrations prevailing in the cultivation broth, while the decrease in the organic carbon load ultimately limited the denitrification process and TN removal. Indeed, this limited denitrification resulted in lower TN-RE compared to Alcántara *et al*. [3]) and De Godos *et al*. [8], who reported TN-REs of 68-79% and 90%, respectively, in a similar experimental set-up. The use of a HRAP as oxygenation unit showed low activity of nitrifying bacteria, compared to similar anoxic-aerobic configurations engineered in enclosed photobioreactors, due to the lower DO concentrations in the

cultivation broth mediated by the O_2 exchange with the open atmosphere and the lower illuminated area/volume ratio [3,8].

Neither the HRT nor the C/N ratio influenced the N biomass content, which averaged 7.4±0.3% along the four operational stages. The nitrogen mass balance showed average N recoveries in the harvested biomass of 56±5%, 52±3%, 37±3% and 73±2% during stages I, II, III and IV, respectively. Despite the high pHs (8.4 to 9.1) prevailing in the HRAP, the open nature of the system and the low rates of nitrification recorded, likely induced $N\text{-}NH_4^+$ losses by volatilization. The nitrogen mass balance also confirmed the limited denitrification activity occurring in the anoxic bioreactor during stage IV, as previously hypothesized.

The REs of $P\text{-}PO_4^{3-}$ accounted for 22±5%, 11±1%, 53±3% and 47±5% during stages I, II, III and IV, respectively, which corresponded to $P\text{-}PO_4^{3-}$ effluent concentrations of 11±1 mg L^{-1}, 13±1 mg L^{-1}, 6±1 mg L^{-1} and 7±1 mg L^{-1}, respectively (Fig. 5). $P\text{-}PO_4^{3-}$ effluent concentrations did not comply with the EU Water Framework Directive, which requires $P\text{-}PO_4^{3-}$ concentrations lower than 2 mg L^{-1} prior to wastewater discharge [19]. The decrease in $P\text{-}PO_4^{3-}$ REs when decreasing the HRT from 4 to 2 d was likely mediated by the overload of the assimilation capacity of algal-bacterial consortium present in the anoxic-aerobic photobioreactor. This finding agreed with the results obtained by Posadas *et al*. [14], who reported a $P\text{-}PO_4^{3-}$ REs decreasing from 57±17% to 36±22% when the HRT was reduced from 5.2 d to 3.1 d during secondary domestic wastewater treatment in a 31 L open algal-bacterial biofilm photobioreactor. The highest P removals observed at C/N ratios of 8 and 7 compared to that recorded at a C/N ratio of 9 were likely mediated by a luxury phosphorus uptake at the lowest C/P ratios in the SWW [20,21]. In fact,

microalgae can store acid-insoluble polyphosphate when phosphorous concentration in the media becomes limiting [22].

3.4 Biomass concentration and settling efficiency

The average TSS concentrations in the anoxic bioreactor during stages I, II, III, and IV were 0.5±0.1 g L^{-1}, 0.7±0.1 g L^{-1}, 0.5±0.1 g L^{-1}, and 0.6±0.1 g L^{-1}, respectively; while the TSS concentrations in the HRAP averaged 0.9±0.1 g L^{-1}, 1.0±0.1 g L^{-1}, 0.7±0.1 g L^{-1}, and 1.0±0.1 g L^{-1}, respectively. The decreasing organic loads during process operation at C/N ratios of 8 and 7 did not imply lower TSS concentrations in the system likely due to the decreasing carbon losses by stripping. The slightly higher TSS concentrations in the HRAP than in the anoxic bioreactor were caused by the higher retention time and superior C and N assimilation mediated by algal activity in the photobioreactor [14]. This difference in TSS concentration was also in agreement with De Godos *et al*. [8] who reported VSS concentrations of 0.57-0.94 g L^{-1} in the anoxic bioreactor and of 0.69-1.4 g L^{-1} in the enclosed photobioreactor.

Process operation at 10 d of SRT supported biomass productivities of 1.4-1.6 g TSS d^{-1} regardless of the HRT and C/N ratio, which resulted in the low variations in the biomass concentrations of the cultivation broth of the anoxic bioreactor and HRAP above mentioned. In this sense, decoupling the SRT from the HRT by means of recycling the settled biomass allowed washing out from the system the poorly settleable species while keeping a constant the biomass productivity. This operating strategy represents a cost-effective method for algal biomass production/harvesting in spite of the variation of the operating parameters during the wastewater treatment [23]. Furthermore, the wastage stream TSS concentration averaged 6.7±0.6 g L^{-1}, 5.9±1.0 g L^{-1}, 5.8±1.5 g L^{-1}, and 6.8±1.5 g L^{-1} in stage I, II, III and IV, respectively; while the TSS removal efficiency in

the settler averaged 98±1%, 92±1%, 97±1% and 98±5%, respectively. This resulted in TSS concentrations in the effluent along stages I to IV of 0.02±0.01 g L^{-1}, 0.08±0.01 g L^{-1}, 0.02±0.01 g L^{-1}, and 0.03±0.01 g L^{-1}, respectively. Therefore, effluent TSS concentrations complied with the EU Water Framework Directive, which requires TSS concentrations ≤35 mg TSS L^{-1} prior to discharge [19].

3.5 Microalgae population dynamics

The microalgae inoculum was composed of (percentage of cells) 49% of *Chlorella vulgaris*, 20% of *Chlorella kessieri*, 20% of *Chlamydomonas altera*, 7% of *Chlorella minutissima*, 3% of *Scenedesmus obliquus* and 2% of *Chlamydomonas* sp. (Fig. 6). During steady state I, *Chlorella vulgaris* became the dominant microalga accounting for 54% of the total number of cells. Species such as *S. obliquus* increased up to 22% while *Chlamydomonas altera* disappeared and others such as *Chloroccum* sp. (11%) and *Synechococcus* sp. (9%) appeared during stage I. These variations in microalgae population were caused by the imposed biomass productivity (throughout controlling the biomass withdrawal) and the acclimation to anoxic-oxic cycles, irradiation, and the wastewater characteristics. The decrease in the HRT from 4 to 2 d mediated a slight increase in the dominance of *C. vulgaris* (accounting for 61% of the total number of cells), while the abundance of *S. obliquus* decreased to 14% in stage II. Other species of *Chlorella* such as *Chlorella kessieri* and *Chlorella minutissima* were identified with abundances of 16% and 6%, respectively. Overall, the results revealed that the decrease in HRT did not change the most abundant microalga species. In contrast, the decrease in the C/N ratio to 8 applied during stage III resulted in a reduction in the number of cells of *C. vulgaris* (38%), while *Phormidium* sp. showed up with an abundance of 21% and *S. obliquus* population increased to 31%. The subsequent reduction in the C/N ratio to 7

induced a further decrease in the number of *C. vulgaris* to 32%, while the population of *Phormidium* sp. increased to 44% (Fig. 6). Thus, the C/N ratio of the wastewater played a key role in the structure of the microalgae population. Nonetheless, it is worth noticing that the morphology of microalgae could depend on these factors and therefore might bias microalgae identification at the species level. On the other hand, there is still an ongoing active discussion about the appropriate DNA fragment to be sequenced during molecular identification of microalgae for a clear species identification. ITS-2 is often used for phylogenetic studies of microalgae at species level, but shows difficulties with the alignment of sequences and the prediction of the secondary structure. In our particular study, the high microalgae diversity recorded in this research was in agreement with the observations of Alcántara *et al*. [3] in an anoxic-aerobic algal-bacterial photobioreactor treating domestic wastewater. Furthermore, the genera *Chlorella* and *Phormidium* have typically ranked among the 12 microalgae genera most tolerant to organic pollution in HRAPs [24].

4. Conclusions

This work represents, to the best of our knowledge, the first systematic evaluation of the influence of the HRT and C/N ratio on the wastewater treatment performance of an anoxic-aerobic algal-bacterial photobioreactor using a HRAP as oxygenation unit. The effluent COD concentrations complied with the EU Water Framework Directive regardless of the HRT and C/N ratio. However, the low DO concentrations in the cultivation broth of the HRAP during process operation at 2 d of HRT limited the nitrification-denitrification process. Therefore, the effluent TN concentrations only complied with the EU Directive at 4 d of HRT. Similarly, the effluent $P\text{-}PO_4^{3-}$ concentrations were over the discharge limits at all operating conditions tested. Biomass

settling and recycling mediated the enrichment of algal-bacterial biomass with good settleability properties, which resulted in TSS discharge levels complying with EU Directive. Finally, *C. vulgaris* was the dominant species at a C/N ratio of 9 regardless of the HRT and was gradually replaced by *Phormidium* sp. when decreasing this ratio to 7.

Acknowledgments

This research was supported by Elecnor S.A. Araceli Crespo and Jonatan Prieto are also gratefully acknowledged for their technical support. The financial support from the Regional Government of Castilla y Leon and the EU-FEDER programme is also acknowledged (CLU-2017-09 and UIC 71). Finally, to Fondo Sectorial CONACyT-SENER, CEMIE-Bio project No. 247006.

Declaration of competing interest: None.

Statement of informed consent, human/animal rights: No conflicts, informed consent, or human or animal rights are applicable to this study.

Declaration of contributions: Dr. Toledo-Cervantes participated actively in the discussion of the results, critically reviewed the article and corrected the manuscript. Dr. Posadas conducted the experimentation and drafted the manuscript. Isabel Berton participated in the analysis and interpretation of the data. Sara Turiel participated in the interpretation of data and the discussion of results. Ana Alcoceba participated actively in the discussion of the results. Dr. Muñoz obtained the financial support for conducting the experimentation, designed and supervised the experimentation and reviewed the manuscript for its final approval.

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Figure captions

Figure 1. Schematic diagram of the anoxic-aerobic algal-bacterial photobioreactor configuration.

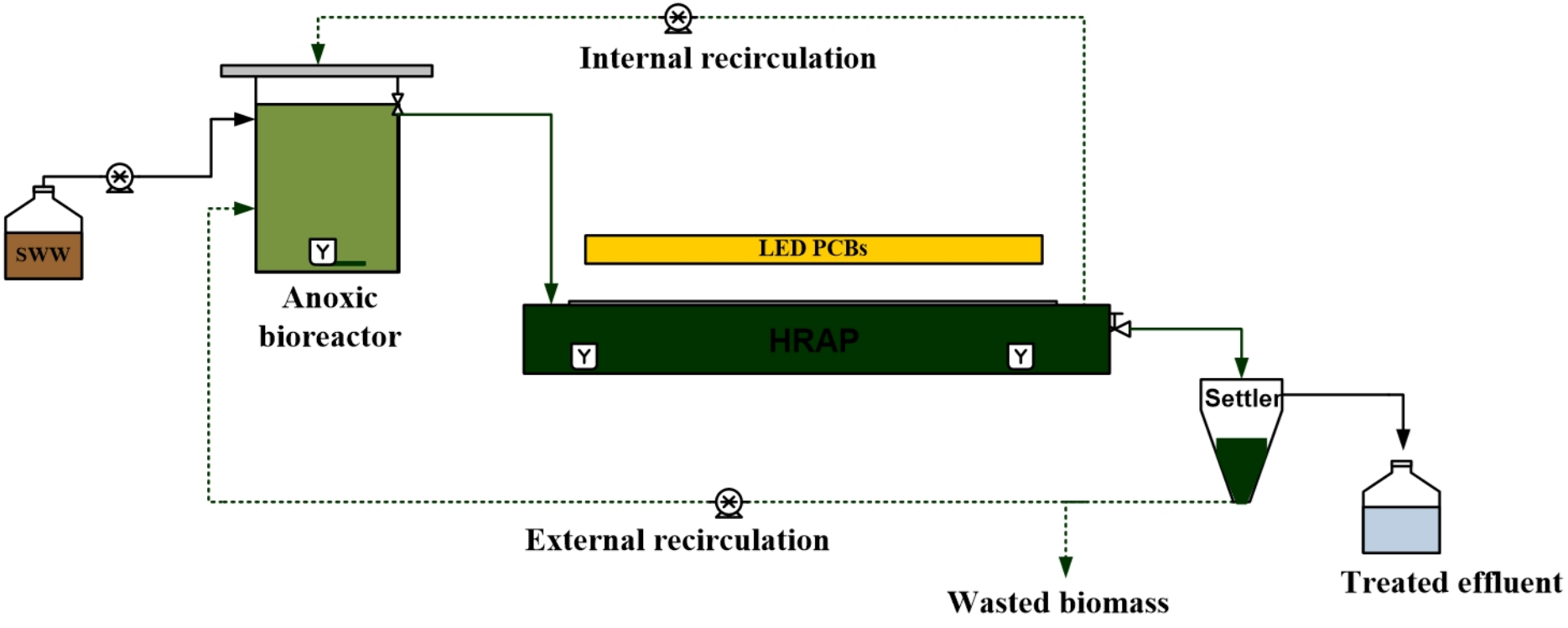
Figure 2. Time course of the concentration of (**a**) total organic carbon and (**b**) total inorganic carbon in the influent (▲), the output of the anoxic bioreactor (x) and effluent (○). TOC and IC removal efficiencies (■) are displayed in the secondary Y-axis.

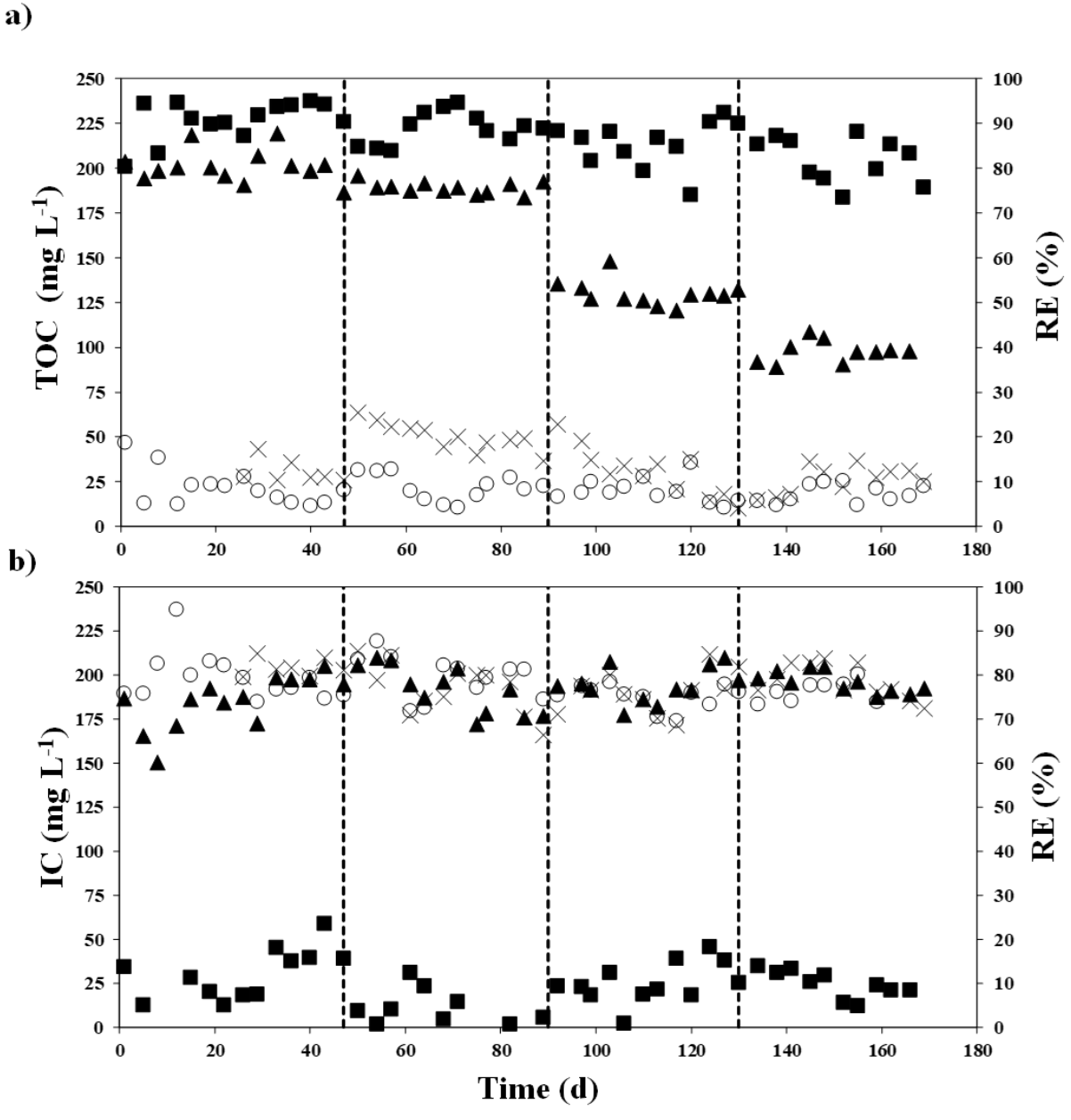
Figure 3. Schematic representation of mass balance for total carbon (TC), total nitrogen (TN) and phosphorous ($P\text{-}PO_4^{3-}$) during stage IV.

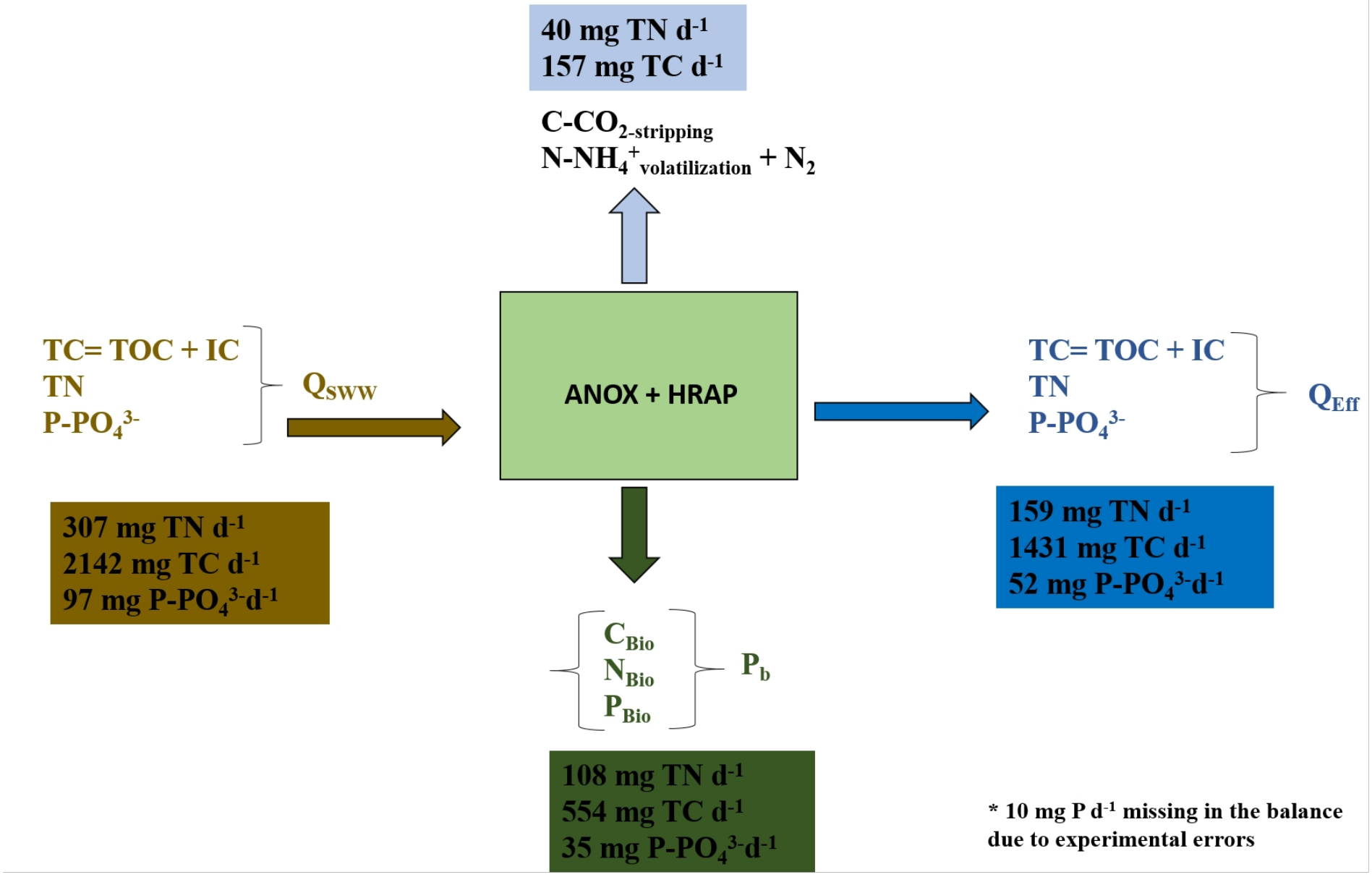
Figure 4a. Time course of the concentration of $N\text{-}NH_4^+$ in the influent (▲) and effluent (○) and the TN removal efficiencies (■, displayed in the secondary Y-axis); **4b**. Time course of the $N\text{-}NO_2^-$ (♦) and $N\text{-}NO_3^-$ (◊) concentrations in the effluent.

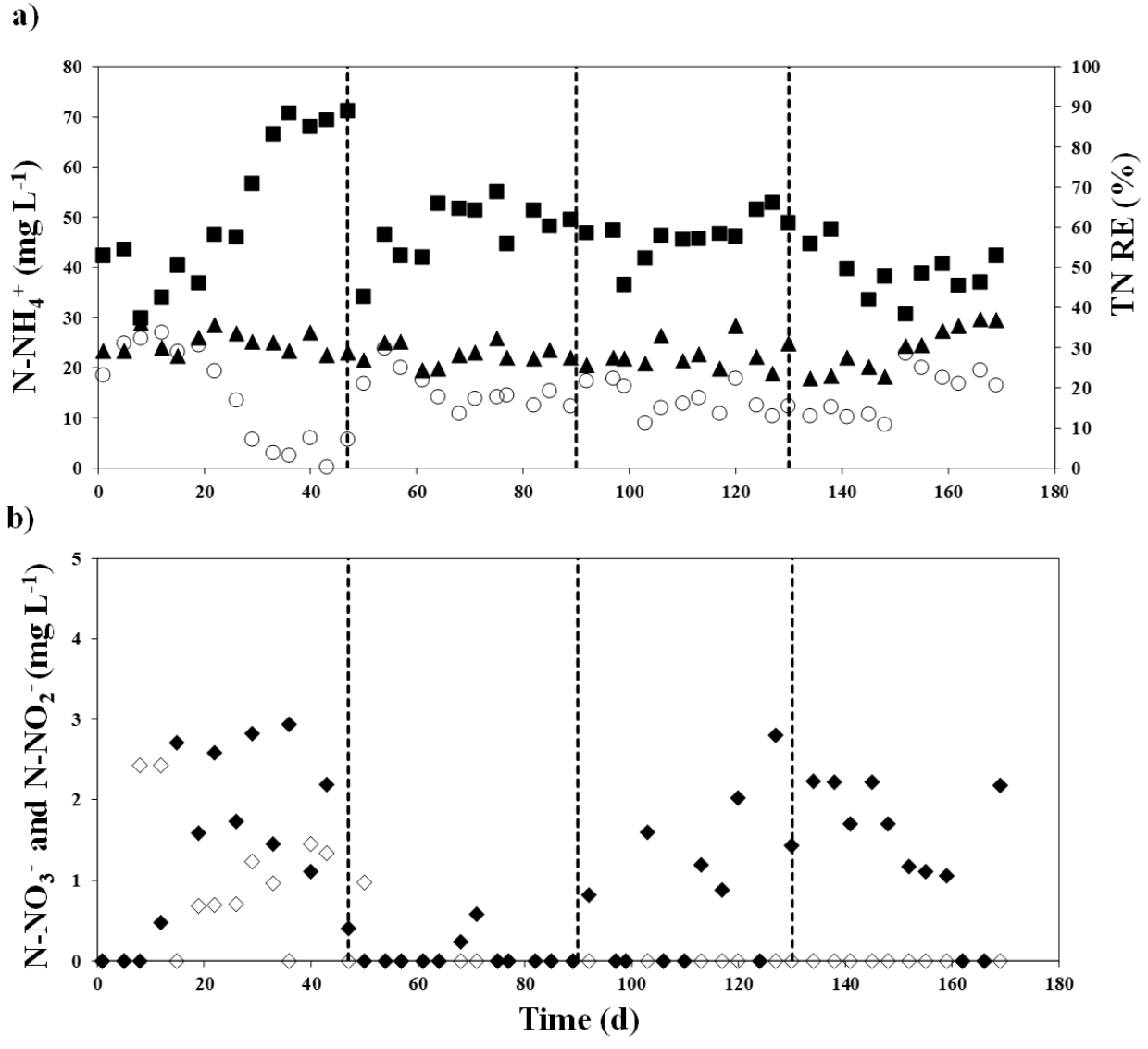
Figure 5. Time course of the concentration of $P\text{-}PO_4^{3-}$ in the influent (▲), the output of the anoxic bioreactor (x) and effluent (○). $P\text{-}PO_4^{3-}$ removal efficiency (■) is displayed in the secondary Y-axis.

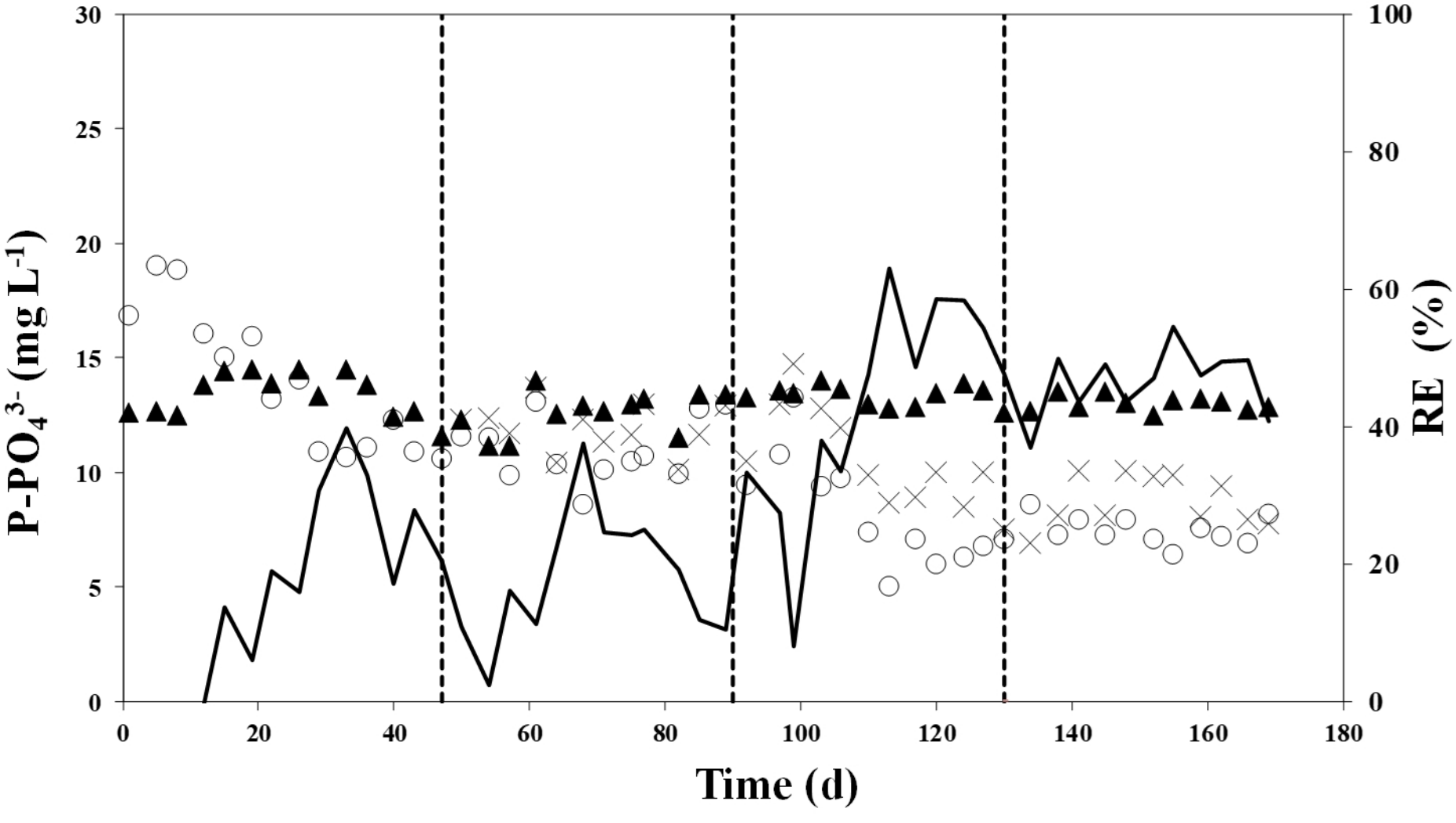
Figure 6. Time course of the structure of the microalgae population in the HRAP: ■ *Chlamydomonas* sp., ■ *Chlamydomonas altera*, □ *Chlorella Kessieri,* ■ *Chlorella minutissima*, ■ *Chlorella vulgaris*, ■ *Chlorococcum* sp., ▌▌ *Phormidium* sp., ■ *Scenedesmus obliquus* and ■ *Synechococcus* sp.


Internal recirculation
SWW
Y
Anoxic
bioreactor
LED PCBs
HRAP
Y
Y
Settler
External recirculation
Wasted biomass
Treated effluent


a)
b)
TOC (mg L-1)
IC (mg L-1)
RE (%)
Time (d)
0
20
40
60
80
100
120
140
160
180
0
25
50
75
100
125
150
175
200
225
250
0
10
20
30
40
50
60
70
80
90
100


40 mg TN d-1
157 mg TC d-1
C-CO2-stripping
N-NH4+volatilization + N2
TC= TOC + IC
TN
P-PO43-
Qsww
ANOX + HRAP
TC= TOC + IC
TN
P-PO43-
QEff
307 mg TN d-1
2142 mg TC d-1
97 mg P-PO43-d-1
159 mg TN d-1
1431 mg TC d-1
52 mg P-PO43-d-1
CBio
NBio
PBio
Pb
108 mg TN d-1
554 mg TC d-1
35 mg P-PO43-d-1
* 10 mg P d-1 missing in the balance due to experimental errors


a)
N-NH4+ (mg L-1)
TN RE (%)
b)
N-NO3- and N-NO2- (mg L-1)
Time (d)


P-PO4 3- (mg L-1)
RE (%)
Time (d)
0
5
10
15
20
25
30
0
20
40
60
80
100
120
140
160
180
0
20
40
60
80
100

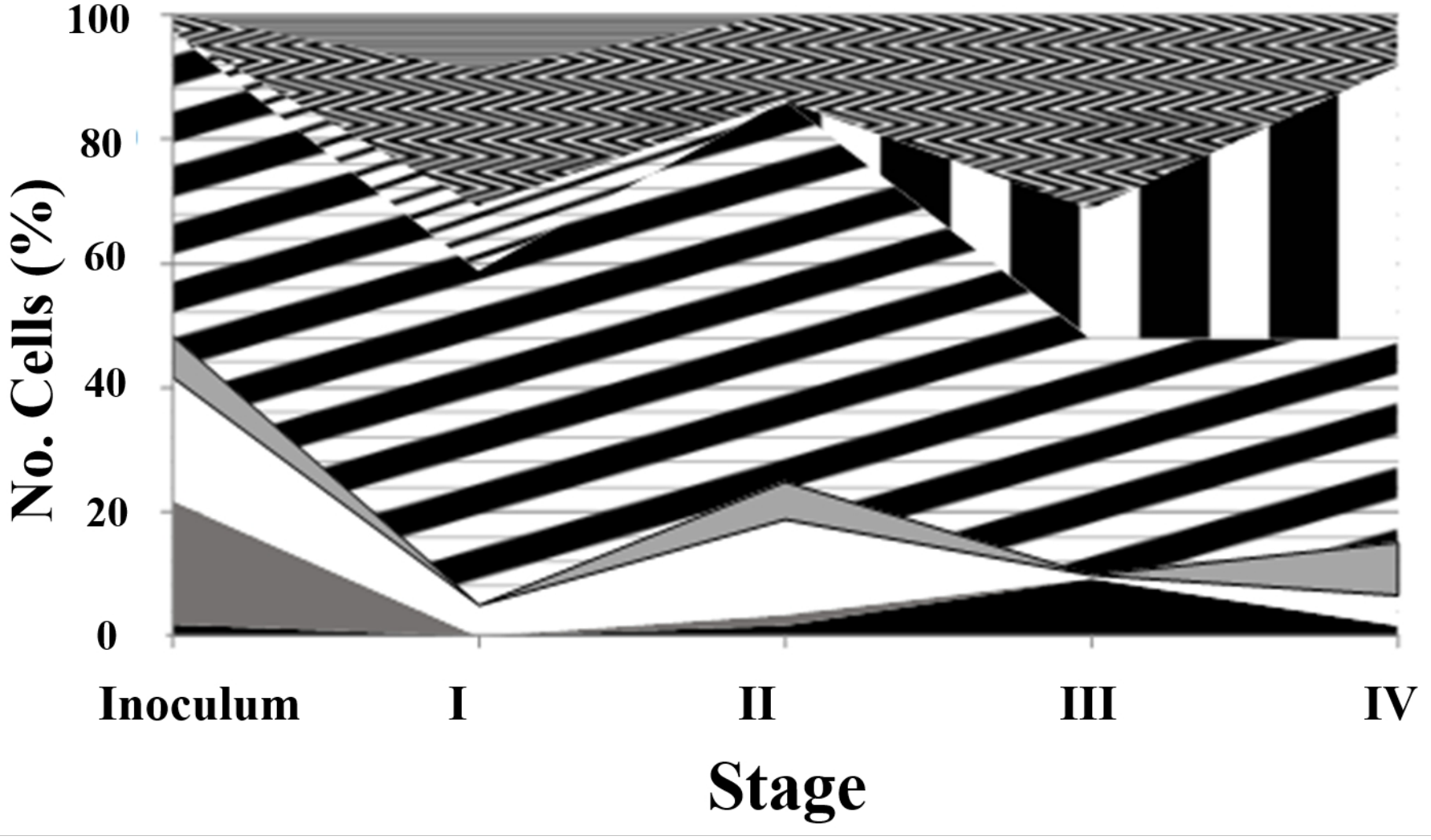

100
80
60
40
20
0
No. Cells (%)
Inoculum
I
II
III
IV
Stage

Table 1. Operational parameters and steady-state values recorded in the anoxic bioreactor (AX) and HRAP during the four operational stages.

	Stage I	**Stage II**	**Stage III**	**Stage IV**
HRT (d)	4	2	2	2
C/N_{ratio} (g/g)	9	9	8	7
COD (mg O_2 L^{-1})	595±27	669±6	493±11	434±11
Evaporation rate (L m^{-2} d^{-1})	13±0	14±1	15±2	15±2
pH_{AX}	8.2±0.1	8.2±0.2	8.1±0.1	8.2±0.2
pH_{HRAP}	9.1±0.1	9.0±0.1	8.7±0.1	8.4±0.3
DO_{AX} (mg O_2 L^{-1})*	0.2±0.1	0.1±0.1	0.2±0.1	0.3±0.2
DO_{HRAP} (mg O_2 L^{-1})*	7.8±3.6	0.4±0.1	3.4±2.6	4.7±3.6
T_{AX} (ºC)	22±0	24±1	26±2	27±1
T_{HRAP} (ºC)	24±0	26±0	28±2	29±1

*-DO measured during the illuminated period. Data shown are the mean ± SD, n = 4.

Declarations of interest: Authors declare no conflict of interest

Contributions declaration

Alma Toledo-Cervantes

Dr. Toledo-Cervantes participated actively in the discussion of the results, critically reviewed the article and corrected the manuscript.

Esther Posadas:

Dr. Posadas conducted the experimentation and drafted the manuscript

Isabel Bertol

Isabel Berton participated in the analysis and interpretation of the data

Sara Turiel

Sara Turiel participated in the interpretation of data and the discussion of results.

Ana Alcoceba

Ana Alcoceba participated actively in the discussion of the results.

Raul Muñoz

Dr. Muñoz obtained the financial support for conducting the experimentation, designed and supervised the experimentation and reviewed the manuscript for its final approval.

Dr. Alma Toledo-Cervantes (almatolecerv@gmail.com) and Dr. Raul Muñoz (mutora@iq.uva.es) take responsibility for the integrity of this work.