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## Microalgae-bacteria consortia in high-rate ponds for treating urban wastewater: Elucidating the key state indicators under dynamic conditions

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Abstract: This work evaluated the performance of a microalgae-bacteria consortium during the start-up period of a pilot-scale high-rate pond for urban wastewater treatment. The key state indicators of the process were screened via multivariate projection analysis. The system was started-up without seed of either bacterial or microalgal biomass. It took around 19 days to fully develop a microalgal community assimilating nutrients significantly. Slight increases in the biomass productivities in days 26-30 suggest that the minimum time for establishing a performant bacteria-microalgae consortium could be of around one month for non-inoculated systems. At this point the process was fully functional, meeting the European discharge limits for protected areas. The results of the statistical analyses show that both the pH and the dissolved oxygen concentration represent accurately the biochemical processes taking place. Both pH and DO represented accurately also the HRAP performance, being affordable, easily-implemented, options for monitoring the start-up of industrial-scale processes.

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Dear Editor,

Attached you will find the manuscript entitled “**Microalgae-bacteria consortia in high-rate ponds for treating urban wastewater: elucidating the key state indicators during the start-up period**” submitted for possible publication as an original research article in *Bioresource Technology*.

This work aims to evaluate the performance of a microalgae-bacteria consortium during the start-up period of a pilot-scale high-rate pond for urban wastewater treatment. Moreover, the key state indicators of the process were screened via multivariate projection analysis. The results of the statistical analyses show that both pH and DO represented accurately also the HRAP performance, being affordable, easily-implemented, options for monitoring the start-up of industrial-scale processes.

Yours sincerely,  
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**Microalgae-bacteria consortia in high-rate ponds for treating urban wastewater:  
elucidating the key state indicators during the start-up period**

Ángel Robles <sup>a,\*</sup>, Gabriel Capson-Tojo <sup>b</sup>, Amandine Galès <sup>c</sup>, María Victoria Ruano <sup>a</sup>, Bruno Sialve <sup>c</sup>, José Ferrer <sup>d</sup>, Jean-Philippe Steyer <sup>c</sup>

**Graphical abstract**



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**Highlights**

- A stable functional bacterial community was established in less than 10 days
- It took 19 days to develop a microalgal community assimilating nutrients
- Fully functional process after 26-30 days, meeting the EU discharge limits
- The pH and DO were accurate parameter for process monitoring

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## Graphical abstract



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## 1. Introduction

Although it is generally accepted that the existing wastewater treatment (WWT) technology is a remarkable human achievement, modern times call for the development of novel processes,



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able to cope with the necessities of a modern society. The development of the so-called water resource recovery facilities (WRRFs) is moving in this direction, aiming at recovering all the valuables that are contained within wastewaters (*i.e.* reclaimed water, carbon and nutrients).

Autotrophic microalgae can play a major role in WRRFs. Microalgae are photosynthetic microorganisms able to grow using inorganic carbon (*i.e.* CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup>) as carbon source, gathering the required energy for growth and metabolism from sunlight. In addition, microalgae need macronutrients (nitrogen and phosphorous), which are consumed in their soluble inorganic forms, namely ammonium (NH<sub>4</sub><sup>+</sup>) and phosphate (PO<sub>4</sub><sup>3-</sup>). In this process, microalgal biomass is produced, together with a variety of organic compounds that are precursors of different forms of bio-energy (*e.g.* biomass itself, biodiesel, bio-ethanol and bio-butanol) and other value-added products (*e.g.* proteins) (Wang et al., 2016).

The application of microalgae-based systems in WRRFs for nutrient uptake has attracted the interest of the scientific community in the last years and several different applications can be found in the literature, such as industrial WWT (Mohd Udaiyappan et al., 2017), treatment of anaerobic digestion effluents (González-Camejo et al., 2017; Uggetti et al., 2014; Viruela et al., 2018) or integration with membrane units (Luo et al., 2017). This increased attention has occurred, not only due to the development of more sustainable WWT processes, but also due to the possibility of reducing the cost of microalgae production, which has been reported to be around \$20–\$200 per kg (Wang et al., 2016). The reduced carbon footprint of the WWT process thanks to carbon dioxide biofixation by microalgae is another major advantages of microalgae-based processes (Lardon et al., 2009; Mata et al., 2010).

High rate algal ponds (HRAPs) and closed photobioreactors (PBRs) are the most commonly used technologies for microalgae cultivation (Vasumathi et al., 2012). Although higher biomass productivities have been reported using PBRs when compared with HRAPs (Ugwu et al., 2008), the latter present different advantages when dealing with wastewater-based

1 microalgae cultivation: (i) smaller investment and operational costs, (ii) easier operation and  
2 maintenance, (iii) lower specific energy demand, (iv) natural selection of the most productive  
3 colonial algae and (iv) lower carbon and environmental footprint. Because of these reasons,  
4 HRAPs have been widely implemented for large-scale microalgae cultivation worldwide  
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10 (Craggs et al., 2011; Kumar et al., 2015). Moreover, commercial production rates in HRAPs  
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12 up to  $40 \text{ g dry weight} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$  have been reported, which represent acceptable values for an  
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14 industrial process for microalgae cultivation (Dalrymple et al., 2013).

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16 However, although microalgae cultivated in wastewater have been reported to reduce the  
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18 concentrations of nutrients to very low values (*e.g.*  $2.20 \text{ mg} \cdot \text{L}^{-1}$  and  $0.15 \text{ mg} \cdot \text{L}^{-1}$  for  $\text{NH}_4\text{-N}$   
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20 and  $\text{PO}_4\text{-P}$  respectively), these photosynthetic organisms are not able to assimilate the high  
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22 organic matter contents present in many waste streams, such as urban wastewater (UWW)  
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24 (Boelee et al., 2011). Therefore, the treatment of wastewaters with high chemical oxygen  
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26 demand (COD) concentrations via microalgae-based systems is frequently combined with  
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28 anaerobic pretreatments or with wastewater dilution (Wang et al., 2015). One solution to this  
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30 issue is the application of microalgae-bacteria consortia for WWT. In these systems, a  
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32 synergy occurs that favors the growth of both algae and bacteria (Galès et al., 2019; Wang et  
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34 al., 2016): while bacteria remove the input COD via heterotrophic growth (producing carbon  
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36 dioxide), microalgae assimilate the nutrients and the carbon dioxide generated by the bacteria,  
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38 supplying at the same time the oxygen that bacteria need. Furthermore, other advantages have  
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40 been postulated when compared to traditional microalgal cultures: (i) both algae and bacteria  
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42 can supply vitamins and other compounds beneficial for the respective partner, (ii) the  
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44 extracellular matrix generated by some microalgae can provide attachment sites for bacteria  
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46 and act as organic carbon source, (iii) bacteria are known to favor the flocculation of algae,  
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48 increasing the floc size and favoring biomass harvesting and (iv) a clear decrease in the spatial  
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50 distance for  $\text{O}_2$  and  $\text{CO}_2$  exchange exists (Wang et al., 2016).  
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Recent studies have been carried out to demonstrate the feasibility of applying algae-bacteria consortia for WWT. Novoveská et al. (2016) carried out a long-term study using offshore PBRs for UWW treatment. They efficiently removed the nutrients via microalgal uptake, achieving removals of 75 % of total nitrogen ( $N_T$ ) and 93 % of total phosphorus ( $P_T$ ). At the same time, the aeration of the reactors by the photosynthetically produced oxygen supported bacterial growth, removing 92 % of the biological chemical demand (BOD) present in the influent. They also reached biomass productivities of  $3.5\text{-}23\text{ g}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$  during continuous operation, with the productivity being mainly driven by the temperature (T) and the harvesting frequency. More recently, Foladori et al. (2018) used photo-sequencing batch reactors to treat UWW, reaching removal efficiencies of 87 % of the chemical oxygen demand (COD) and 98 % of the total Kjeldahl nitrogen (TKN) using again only the oxygen from photosynthesis to support bacterial growth and thus avoiding the need of external aeration.

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The performance of HRAPs for WWT depends on several factors, such as light intensity, light mitigation (related mainly to the pond depth), mixing intensity, pH (usually around 7-9), temperature (T), dissolved oxygen (DO), salinity and carbon dioxide and nutrient concentrations (Faried et al., 2017; Kumar et al., 2015; Larsdotter, 2006; Park et al., 2011).

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Therefore, if productivities are to be improved and stable processes are to be achieved, the aforementioned parameters must be monitored and controlled (if possible), together with the most relevant process outputs, such as biomass productivity, nutrient removal rate, photosynthetic efficiency or solar-to-biomass conversion efficiency (Havlik et al., 2013). In large-scale processes, this monitoring requires reliable sensors for on-line, in situ measurement of both physicochemical and biological process variables. Because of this, long-term studies of HRAPs for nutrient recovery from wastewater are generally fully monitored, measuring on-line the influent flow-rate, solar radiation, T, DO and pH. Among the different

1 off-line measurements that are generally applied, the most relevant are the concentrations of  
2 suspended solids (SS), COD,  $N_T$  and  $P_T$  (Arbib et al., 2013; Fernández et al., 2016; Solimeno  
3 et al., 2017a; Tran et al., 2014). Real-time monitoring also serves for optimization and control  
4 of WWT processes. By coupling monitoring to different modelling and control approaches,  
5 several studies have shown improved performances. Relevant examples are: the optimization  
6 of the dilution rate to maximize the microalgal biomass production in PBRs (De Andrade et  
7 al., 2016); advanced control strategies for pH control to reduce  $CO_2$  losses in tubular PBRs  
8 (Berenguel et al., 2004); model-driven optimization of the biomass production in HRAPs via  
9 closed-loop control of the operational variables (*e.g.* T, pH, or nutrient feeding rate) (Muñoz-  
10 Tamayo et al., 2013); mathematical modelling of sunlight incidence and light distribution in  
11 PBRs for optimization of the biomass production (De Andrade et al., 2016); or application of  
12 Internet of Things for monitoring and control of a microalgae cultivation coupled with a  
13 decision support system (Esposito et al., 2017). Together with an efficient monitoring of the  
14 process variables, novel models have the potential of allowing the prediction of the  
15 microalgae production rates, serving also for optimizing the operation in microalgae-bacteria  
16 consortia cultivated in HRAPs (Solimeno et al., 2017b).

17 The first step to achieve a proper monitoring of HRAPs treating wastewater using microalga-  
18 bacteria consortia is the selection of the most relevant variables affecting the process, which  
19 will serve as key state indicators. In addition, as the long and failure-prone start-up period in  
20 HRAPs is one of main challenges in microalgae-bacteria consortia for WWT, a proper  
21 monitoring of this period is crucial to achieve an efficient and stable process (Liu et al., 2017).

22 It must be mentioned that, although this has been widely researched in microalgae cultures,  
23 the results cannot be directly extrapolated to microalga-bacteria mixed systems, mainly  
24 because in the latter no external carbon dioxide is generally supplied. Addition of carbon  
25 dioxide into algae-based HRAPs for WWT has been used, not only for improving the algal

1 growth (Craggs et al., 2011), but also for preventing free ammonia inhibition (Park et al.,  
2 2011) and for pH control in long-term studies (González-Camejo et al., 2019; Novoveská et  
3 al., 2016; Tran et al., 2014). Therefore, not adding carbon dioxide into a HRAP implies that  
4 the sole mechanisms controlling the pH of the system are the biochemical processes  
5 performed by the microorganisms.  
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10 The main objective of this work was to evaluate the start-up period of a HRAP treating UWW  
11 using a microalgae-bacteria consortium, screening at same time the key state indicators that  
12 would allow to monitor the performance of a potential industrial process (in terms of biomass  
13 productivity, nutrient removal rate, photosynthetic efficiency and carbon dioxide biofixation).  
14 For this purpose, experiments were carried out using a 56 m<sup>2</sup> pilot-scale HRAP (working  
15 volume of 22 m<sup>3</sup>), enabling the extrapolation of the observed results to industrial-scale  
16 processes. A statistical multivariate projection approach was followed for screening of the  
17 monitoring variables.  
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## 31 **2. Materials and methods**

### 32 *2.1. Start-up of the HRAP and influent wastewater*

33 No active inoculation of the HRAP was performed, implying that an initial natural selection  
34 of the predominant microorganisms occurred. This approach facilitates a potential industrial  
35 application of this technology.  
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45 Table 1 shows the main characteristics of the wastewater fed into the system. The synthetic  
46 UWW was weekly prepared according to Nopens et al. (2001) and it was continually fed to  
47 the HRAP from a continuously-stirred tank with a volume of 500 L and refrigerated at 4 °C.  
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**Table 1.** Characteristics of the synthetic UWW

Parameter	Units	Mean $\pm$ SD
NH <sub>4</sub> -N	mg N·L <sup>-1</sup>	17.3 $\pm$ 8.1
N <sub>T</sub>	mg N·L <sup>-1</sup>	45.5 $\pm$ 24.2
PO <sub>4</sub> -P	mg P·L <sup>-1</sup>	3.9 $\pm$ 1.6
P <sub>T</sub>	mg P·L <sup>-1</sup>	6.1 $\pm$ 2.2
COD <sub>T</sub>	mg·L <sup>-1</sup>	332 $\pm$ 55
VSS	mg·L <sup>-1</sup>	89 $\pm$ 24

SD stands for standard deviation, NH<sub>4</sub>-N for ammonium-N, N<sub>T</sub> for total nitrogen, PO<sub>4</sub>-P for phosphate-P, P<sub>T</sub> for total phosphorous, COD<sub>T</sub> for total chemical oxygen demand and VSS for volatile suspended solids

## 2.2. Description and operation of the pilot plant

This study was performed using a continuous HRAP with a working volume of 22 m<sup>3</sup>. It had a liquid depth of 0.3 m and a solar irradiance area of approximately 56 m<sup>2</sup>. The HRAP (located in the south of France, Lat. 43.156711, Long. 2.995075) was operated outdoors (*i.e.* under variable solar irradiance and T) at a hydraulic retention time (HRT) of 6 days. The reactor was continuously stirred by a paddlewheel. During the period of study, the daily average photosynthetic active radiation (PAR) and the culture T were 433 $\pm$ 113  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  and 22 $\pm$ 3 °C, respectively. The pH varied freely according to variations in the concentration of carbon dioxide occurring due to the activity of microorganisms.

## 2.3. On-line and off-line monitoring of the pilot plant

Different on-line sensors were placed in the HRAP, which allowed continuous data acquisition. The on-line sensors used in this study were: (i) a pH-T transmitter (METTLER TOLEDO InPro<sup>®</sup> 4260 SG), (ii) a DO probe (METTLER TOLEDO InPro<sup>®</sup> 6800 G Amperometric Oxygen Sensor) and (iii) an irradiation sensor (Skye PAR Quantum Sensor) for measuring the PAR.

Besides the on-line process monitoring, samples were taken from the influent and the effluent streams to assess the performance of the biological processes. The concentrations of the

1 following parameters were determined: total and soluble COD (COD<sub>T</sub> and COD<sub>S</sub>,  
2 respectively), N<sub>T</sub>, P<sub>T</sub>, inorganic nutrients (NH<sub>4</sub><sup>+</sup>, NO<sub>2</sub><sup>-</sup>, NO<sub>3</sub><sup>-</sup> and PO<sub>4</sub><sup>3-</sup>), total suspended solids  
3 (TSS), and volatile suspended solids (VSS). Additionally, optical density at 680 nm (OD<sub>680</sub>)  
4 was used for VSS estimation (VSS<sub>680</sub>). Regarding the biomass composition, the copies per  
5 liter of 18S rDNA from chlorophyte and bacterial 16S rDNA were also determined.  
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#### 10 11 *2.4. Analytical methods and microbial analyses*

12 The concentrations of COD<sub>T</sub>, COD<sub>S</sub>, N<sub>T</sub>, P<sub>T</sub>, TSS and VSS were analyzed according to  
13 Standard Methods (APHA, 2005). The concentrations of inorganic nutrients (NH<sub>4</sub><sup>+</sup>, NO<sub>2</sub><sup>-</sup>,  
14 NO<sub>3</sub><sup>-</sup> and PO<sub>4</sub><sup>3-</sup>) were measured by ion chromatography, as described in Capson-Tojo et al.  
15 (2017). The eukaryotic and prokaryotic cell numbers were estimated by quantitative  
16 polymerase chain reaction (qPCR). The presence of microalgal biomass was estimated  
17 targeting a partial sequence of 18S rDNA from chlorophyte or bacillariophyte, whilst the total  
18 bacterial content was estimated using universal primers and probes for the 16S rDNA. A more  
19 extended description can be found in Turon et al. (2015). For direct identification of the  
20 microalgae species, samples from the HRAP were fixed with 4 % formaldehyde (final  
21 concentration) and microscopic observations were conducted with an Olympus upright  
22 fluorescence microscope (BX53).  
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#### 41 *2.5. HRAP performance monitoring*

42 The process indicators used to monitor the performance of the microalgae-bacteria-based  
43 HRAP were the nitrogen removal rate (NRR) (g N·m<sup>-3</sup>·d<sup>-1</sup>), the biomass productivity per  
44 working volume (BP<sub>V</sub>), the daily photosynthetic efficiency (PE), and the carbon dioxide  
45 biofixation (CO<sub>2BF</sub>) (kg CO<sub>2</sub> per m<sup>3</sup> of treated water), which were calculated according to Eq.  
46 1, Eq. 2, Eq. 3, and Eq. 4, respectively.  
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$$55 \text{NRR} = \frac{Q \cdot (N_i - N_e)}{V} \quad (\text{Eq. 1})$$

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$$BP_V = \frac{Q \cdot X_{VSS}}{V} \quad (\text{Eq. 2})$$

Where  $Q$  is the treatment flow rate ( $\text{m}^3 \cdot \text{d}^{-1}$ ),  $N_i$  is the influent nitrogen concentration ( $\text{g N} \cdot \text{m}^{-3}$ ),  $N_e$  is the effluent nitrogen concentration ( $\text{g N} \cdot \text{m}^{-3}$ ),  $V$  is the reaction volume ( $\text{m}^3$ ), and  $X_{VSS}$  is the biomass concentration ( $\text{g VSS} \cdot \text{L}^{-1}$ ).

$$PE (\%) = \frac{r_G \cdot H_B}{I \cdot S \cdot f} \cdot 100 \quad (\text{Eq. 3})$$

$$CO_{2BF} = \frac{r_G}{Y_{CO_2} \cdot Q} \quad (\text{Eq. 4})$$

Where  $r_G$  is the daily microalgae growth ( $\text{kg VSS} \cdot \text{d}^{-1}$ ),  $H_B$  is the lower heating value of dry biomass ( $22,900 \text{ kJ} \cdot \text{kg VSS}^{-1}$ ),  $I$  is the photosynthetic active radiation ( $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ ),  $f$  is a conversion factor ( $18.78 \text{ kJ} \cdot \text{s} \cdot \mu\text{mol photons}^{-1} \cdot \text{d}^{-1}$ ),  $S$  is the surface of the open pond ( $\text{m}^2$ ) and  $Y_{CO_2}$  is the stoichiometric  $\text{CO}_2$  capture for microalgae growth ( $0.52 \text{ kg VSS} \cdot \text{kg CO}_2^{-1}$ ).

For stoichiometric calculations of microalgae biomass composition, the chemical formula used in Viruela et al. (2018) was applied (*i.e.*  $\text{C}_{106}\text{H}_{181}\text{O}_{45}\text{N}_{16}\text{P}$ ).

In order to assess the best key state indicators related to the performance of the microalgae-bacteria consortium (*i.e.* NRR,  $BP_V$ , PE, and  $CO_{2BF}$ ), different variables were calculated from on-line DO and pH measurements, using a microalgae kinetic model (Fernández et al., 2016). This allowed to confirm that the measured variables represented properly the physicochemical and biological processes occurring in the HRAP. At the same time, this process allowed obtaining variables that could lead to more precise predictions of the outputs/indicators (*i.e.* NRR,  $BP_V$ , PE, and  $CO_{2BF}$ ) when compared to the raw DO or pH values.

Assuming non-inhibitory DO and pH conditions, Eq. 5 (see Fernández et al. (2016)) can be used to determine the photosynthesis rate (oxygen production rate;  $\mu_{O_2}$ ) as a function of  $I_i$  (a function of the average light irradiance ( $I_{av}$ )) and a net, constant respiration/consumption rate ( $r_{O_2}$ ).

$$\mu_{O_2} = \mu_{O_2max} \cdot I_i - \alpha_s \cdot r_{O_2} \quad (\text{Eq. 5})$$



Where  $\mu_{O_2_{max}}$  is the maximum oxygen production rate, and  $\alpha_S$  is a distributed factor to account the shadow influences on the photosynthesis rate.

Four normalizing factors related to  $I_{av}$  ( $I_i$ ) were considered in this work for representing different behaviors of microalgae in the HRAP.  $I_1$  is a modified Monod-type factor reported by Fernández et al. (2016) (Eq. 6).  $I_2$  is analogous to the duty cycle, which is the proportion of time at which microalgae are exposed to light (Fernández-Sevilla et al., 2018) (Eq. 7).  $I_3$  is a Monod-type factor where  $I_{av}$  acts as substrate that is proposed based on  $I_1$  (Eq. 8). Lastly,  $I_4$  is proposed based on  $I_3$  where instead of  $k_i$ , PAR serves as semisaturation “constant” (Eq. 9).

$$I_1 = \frac{I_{av}^n}{k_i \cdot e^{m \cdot I_{av}} + I_{av}^n} \quad (\text{Eq. 6})$$

$$I_2 = \frac{I_{av}}{PAR} \quad (\text{Eq. 7})$$

$$I_3 = \frac{I_{av}}{I_{av} + k_i} \quad (\text{Eq. 8})$$

$$I_4 = \frac{I_{av}}{I_{av} + PAR} \quad (\text{Eq. 9})$$

Where  $n$  is a form exponent (1.045),  $k_i$  is a form parameter representing the optimum light intensity ( $174 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ ),  $m$  is a form parameter (0.0021), and PAR is the solar photosynthetically active radiation received by the HRAP ( $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ ).

The  $I_{av}$ , which integrates the light availability over the culture volume (Acién Fernández et al., 1997) can be calculated using Eq. 10.

$$I_{av} = \frac{I_0}{K_a \cdot C_b \cdot h} \cdot (1 - e^{-K_a \cdot C_b \cdot h}) \quad (\text{Eq. 10})$$

Where  $I_0$  is the solar irradiance on the pond surface ( $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ ),  $K_a$  is an extinction coefficient ( $80 \text{ m}^2 \cdot \text{kg SSV}^{-1}$ ),  $C_b$  is the biomass concentration ( $\text{kg} \cdot \text{m}^{-3}$ ), and  $h$  is the liquid height (m).

In addition, the carbon dioxide kinetics ( $\mu_{CO_2}$ ) can be calculated from Eq. 11 considering a one-to-one molar ratio between oxygen and carbon dioxide (Fernández et al., 2016).

$$\mu_{CO_2} = -\mu_{O_2} \quad (\text{Eq. 11})$$

Hence, Eq. 5 can be used for modelling the oxygen kinetics of the microalgae-bacteria consortia during daylight and night-time hours. Microalgae growth is represented by the first term of the right side of Eq. 5 if oxygen consumption is assumed to be negligible during daylight hours compared to oxygen production. On the other hand, microalgae respiration and bacteria growth are represented by the second term of the right side of Eq. 5 during night-time hours. Similarly, Eq. 11 can be used for modelling the carbon dioxide kinetics (pH dynamics). Table 2 summarizes the different variables calculated in this study based on DO and pH measurements. The variables related to microalgae growth were also normalized to account for the effect of  $I_{av}$  using Eq. 6 to Eq. 9. Specifically, the variables related to the first term of the right side of Eq. 5 were divided by the  $I_i$  function (considering the four  $I_{av}$  factors,  $I_1$ ,  $I_2$ ,  $I_3$ , and  $I_4$ ) to approximate to the maximum growth rate of microalgae in the system. The resulting variables were noted as variable:  $I_i$  (e.g.  $DO_{AVE}:I_i$ ). This normalization was not performed for those variables related to microalgae respiration and bacterial growth, since they represented the second term of the right side of Eq. 5. The process indicators NRR,  $BP_V$ , PE and  $CO_{2BF}$  were also normalized by the  $I_i$  function.

**Table 2.** Variables based on DO and pH measurements

Variable acronym	Description <sup>1</sup>
DO <sub>AVE</sub>	Dissolved oxygen average
DO <sub>MEDIAN</sub>	Dissolved oxygen median
DO <sub>SD</sub>	Dissolved oxygen standard deviation
DO <sub>MIN</sub>	Minimum dissolved oxygen
DO <sub>MAX</sub>	Maximum dissolved oxygen
DO <sub>RANGE</sub>	Dissolved oxygen range
DO <sub>MIN SLOPE1h</sub>	Minimum value of dissolved oxygen one-hour slope <sup>2</sup>
DO <sub>MAX SLOPE1h</sub>	Maximum value of dissolved oxygen one-hour slope <sup>3</sup>
DO <sub>AVE SLOPE1h</sub>	Absolute average of DO <sub>MIN SLOPE1h</sub> and DO <sub>MAX SLOPE1h</sub>
DO <sub>MIN SLOPE2h</sub>	Minimum value of dissolved oxygen two-hour slope <sup>2</sup>
DO <sub>MAX SLOPE2h</sub>	Maximum value of dissolved oxygen two-hour slope <sup>3</sup>
DO <sub>AVE SLOPE2h</sub>	Absolute average of DO <sub>MIN SLOPE2h</sub> and DO <sub>MAX SLOPE2h</sub>
DO <sub>INTEGRAL</sub>	Dissolved oxygen integral
DO <sub>MAX:MIN ABS SLOPE1</sub>	Absolute value of DO <sub>MAX SLOPE1h</sub> to DO <sub>MIN SLOPE1h</sub> ratio
DO <sub>MAX:MIN ABS SLOPE2h</sub>	Absolute value of DO <sub>MAX SLOPE2h</sub> to DO <sub>MIN SLOPE2h</sub> ratio
DO <sub>SLOPE1h RANGE</sub>	Range of dissolved oxygen one-hour slope
DO <sub>SLOPE2h RANGE</sub>	Range of dissolved oxygen two-hour slope
pH <sub>AVE</sub>	pH average
pH <sub>MEDIAN</sub>	pH median
pH <sub>MIN</sub>	Minimum pH
pH <sub>MAX</sub>	Maximum pH
pH <sub>MAX:MIN</sub>	Maximum pH to minimum pH ratio
pH <sub>RANGE</sub>	pH range
pH <sub>MIN SLOPE1h</sub>	Minimum value of pH one-hour slope <sup>2</sup>
pH <sub>MAX SLOPE1h</sub>	Maximum value of pH one-hour slope <sup>3</sup>
pH <sub>AVE SLOPE1h</sub>	Absolute average of pH <sub>MIN SLOPE1h</sub> and pH <sub>MAX SLOPE1h</sub>
pH <sub>MIN SLOPE2h</sub>	Minimum value of pH two-hour slope <sup>2</sup>
pH <sub>MAX SLOPE2h</sub>	Maximum value of pH two-hour slope <sup>3</sup>
pH <sub>AVE SLOPE2h</sub>	Absolute average of pH <sub>MIN SLOPE2h</sub> and pH <sub>MAX SLOPE2h</sub>
pH <sub>INTEGRAL</sub>	pH integral
pH <sub>MAX:MIN ABS SLOPE1</sub>	Absolute value of pH <sub>MAX SLOPE1h</sub> to pH <sub>MIN SLOPE1h</sub> ratio
pH <sub>MAX:MIN ABS SLOPE2h</sub>	Absolute value of pH <sub>MAX SLOPE2h</sub> to pH <sub>MIN SLOPE2h</sub> ratio
pH <sub>SLOPE1h RANGE</sub>	Range of pH one-hour slope
pH <sub>SLOPE2h RANGE</sub>	Range of pH two-hour slope

1. Variables calculated within a time interval of one day

2. Minimum slopes were determined during night periods

3. Maximum slopes were determined during daylight periods

## 2.6. Multivariate projection analysis

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2 Statistical analyses based on multivariate projection were performed to assess potential key  
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4 state indicators based on pH and DO measurements affecting the HRAP performance. The  
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6 mixOmics package (<http://www.mixOmics.org>) implemented in the R statistical software  
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8 version 3.2.3 (<http://www.R-project.org>) was used for this purpose. Firstly, a set of calculated  
9  
10 variables was statistically analyzed through principal component analysis (PCA) for screening  
11  
12 and validation of potential key state indicators for real-time HRAP monitoring. On the other  
13  
14 hand, the relations between inputs (pH and DO variables) and the output process indicators  
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16 (NRR, BP<sub>V</sub>, PE and CO<sub>2BF</sub>) were analyzed through partial least squares regression (PLSR).  
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### 2.6.1. Principal component analysis (PCA)

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24 PCA is a type of multivariate analysis that enables the identification of trends or patterns on a  
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26 single dataset. In addition, it also gives information regarding the major sources of variation  
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28 (Wold et al., 1987). This method reduces the dimensionality of the data by creating  
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30 uncorrelated artificial variables called principal components (PCs) that combine linearly the  
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32 original variables, retaining as much information as possible. The data is projected into the  
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34 space given by the PCs.  
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### 2.6.2. Partial least squares regression (PLSR)

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41 PLSR is a type of multivariate analysis (two-block predictive PLS) for relating two data  
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43 matrices, predictors (X) and responses (Y), by a linear multivariate model (Wold et al., 2001).  
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46 In contrast to the traditional multiple linear regression approach, this method is capable of  
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48 modelling the structure of X and Y, allowing the analysis of data sets with many X-variables,  
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50 with strong collinearity and outliers, being noisy, and even with incomplete objects in both X  
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52 and Y. Hence, PLSR allows modelling one or several Y-variables from a set of X-variables,  
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54 while reducing the dimensionality of the explanatory variables. Moreover, this method  
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57 identifies the predictors that better explain the information (explained variability) between the  
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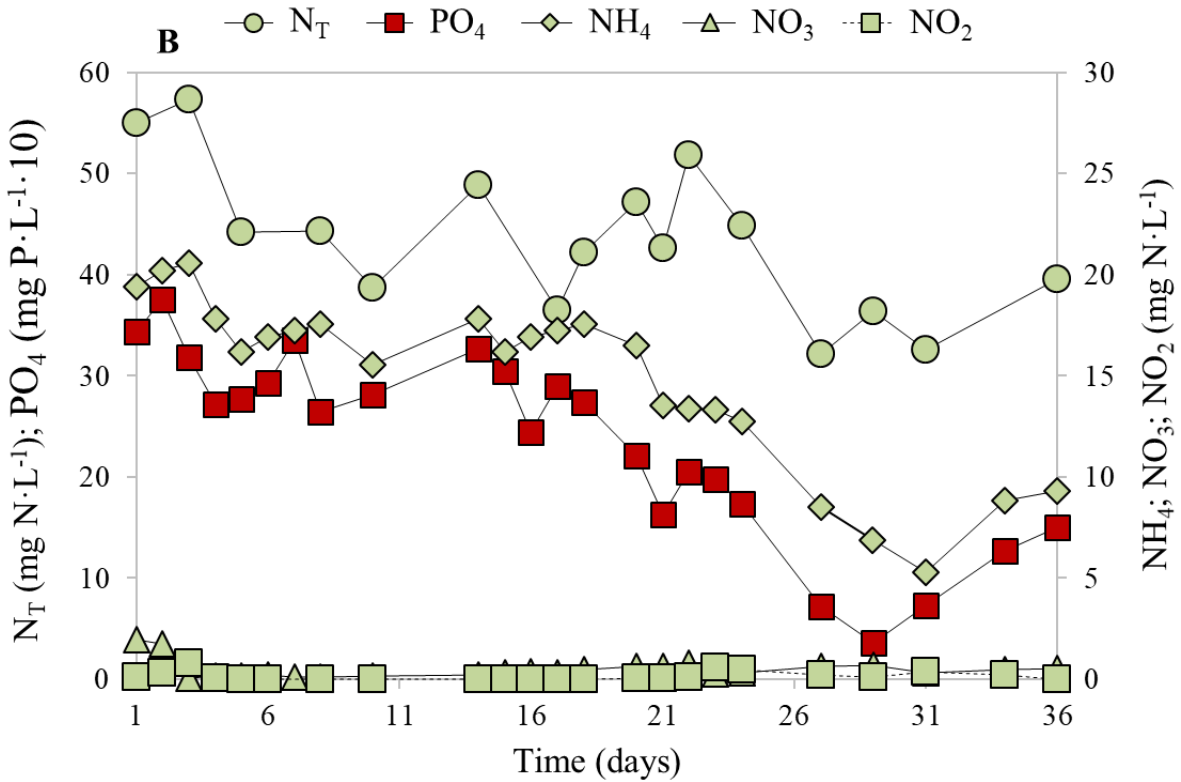
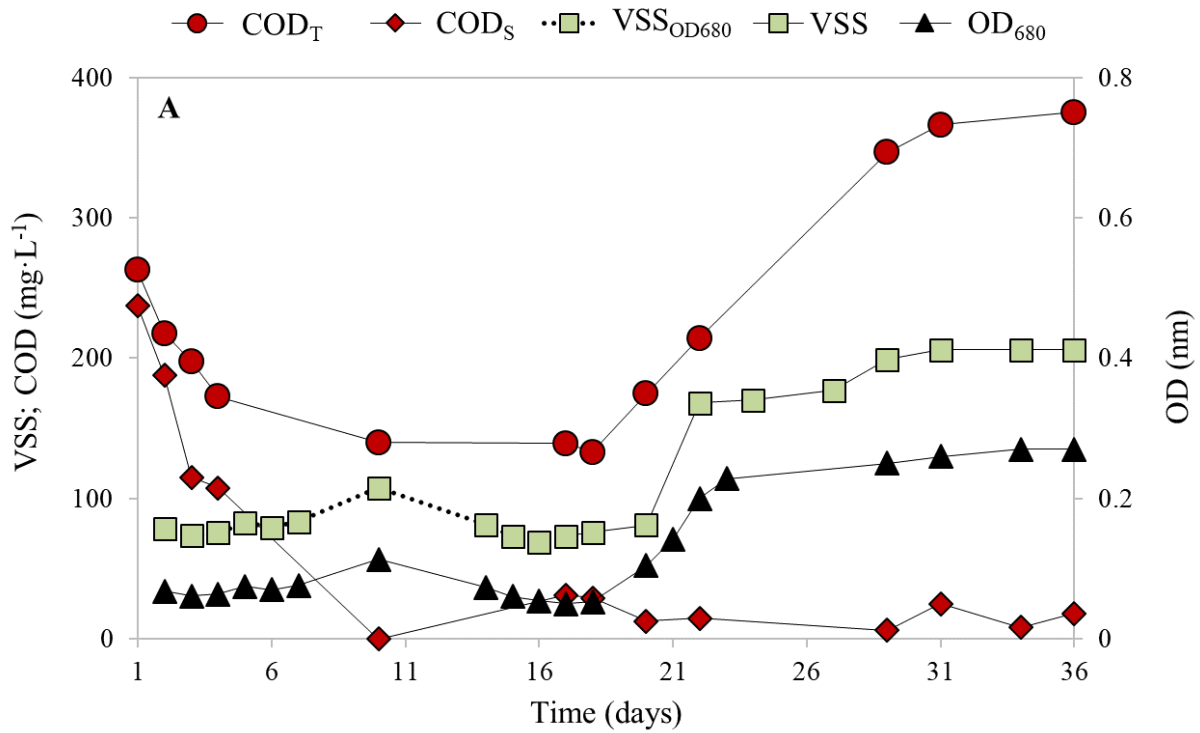
1 X and Y datasets. By handling numerous and collinear predictors (X) and responses (Y),  
2 PLSR allows investigating complex problems whilst analyzing data in a fairly realistic way.  
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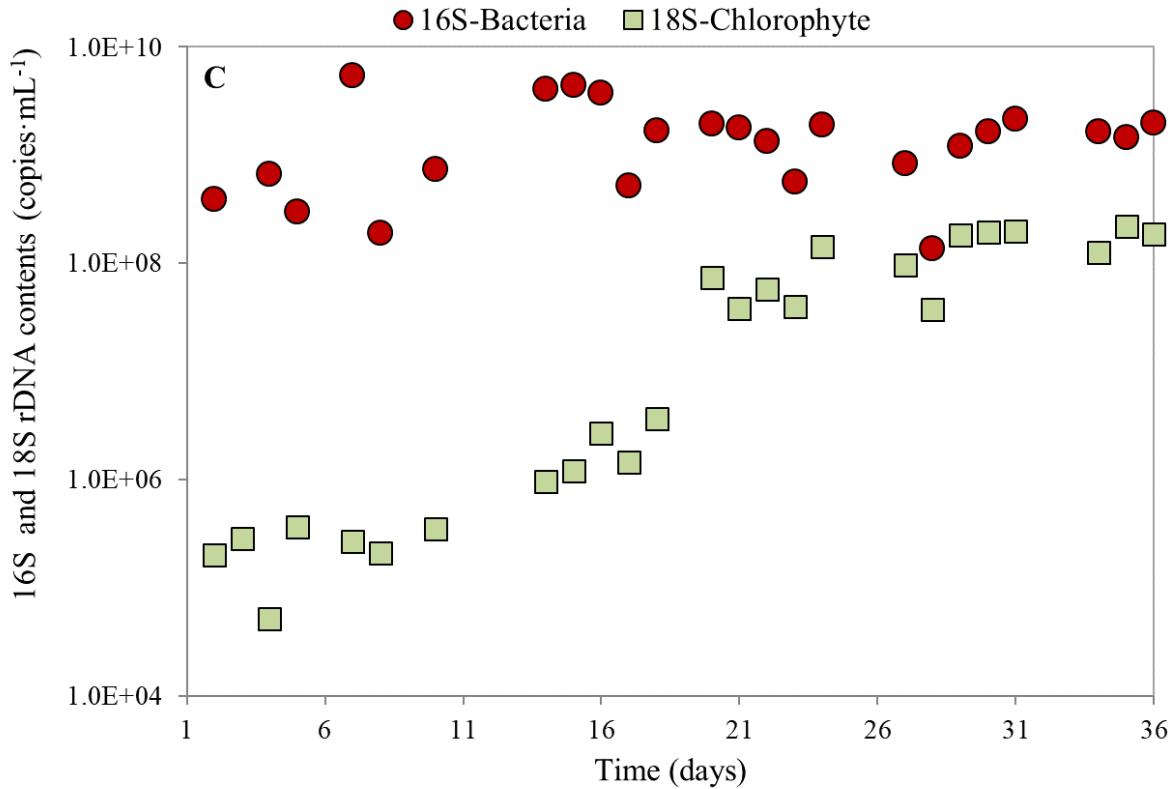
### 6 **3. Results and discussion**

#### 7 *3.1. Performance of the HRAP*

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10 Figure 1A shows the evolution of the COD, VSS and OD concentrations in the mixed liquor  
11 during the experimental period. As it can be observed, the COD<sub>S</sub> was rapidly consumed  
12 initially (days 1 to 5), which indicated a quick appearance and adaptation of heterotrophic  
13 bacteria in the system. Afterwards, the COD<sub>S</sub> was kept at 15±11 mg·L<sup>-1</sup> from day 10 until the  
14 end of the experimental period. This suggests that a stable community of heterotrophic  
15 bacteria was established in the system only after 10 days of operation. The stable values of the  
16 COD<sub>T</sub> from day 10 to 18 corroborated this statement. During this period, no significant  
17 photosynthetic activity was observed, with the number of 18S rDNA copies from chlorophyte  
18 remaining at low values (below 10<sup>6</sup> copies·mL<sup>-1</sup>; see Figure 1C), which implies that relatively  
19 stable concentrations of both heterotrophic bacteria (10<sup>8</sup>-10<sup>10</sup> copies 16S rDNA·mL<sup>-1</sup>) and  
20 chlorophyte had been reached at that time (no significant algae growth occurred initially). The  
21 values of the VSS, of 64±17 mg·L<sup>-1</sup> until day 18 (Figure 1A), also indicate that no significant  
22 variations in the biomass concentration existed during this initial operating stage. After day  
23 18, a significant increase in the COD<sub>T</sub> and VSS concentrations occurred, indicating the  
24 development of a microalgal community in the HRAP. This is in agreement with the increase  
25 in the 18S rDNA copies (chlorophyte) from day 11 until days 18-23, confirming microalgae  
26 growth. Therefore, a period of 18 days was needed in this study for the natural selection,  
27 development and adaptation of the most suitable phototrophic species within the cultivation  
28 medium and under the environmental and operating conditions applied. It is important to  
29 highlight that the system was started-up with nor seed of bacterial biomass neither microalgae  
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biomass, implying that the more suitable species were naturally selected.





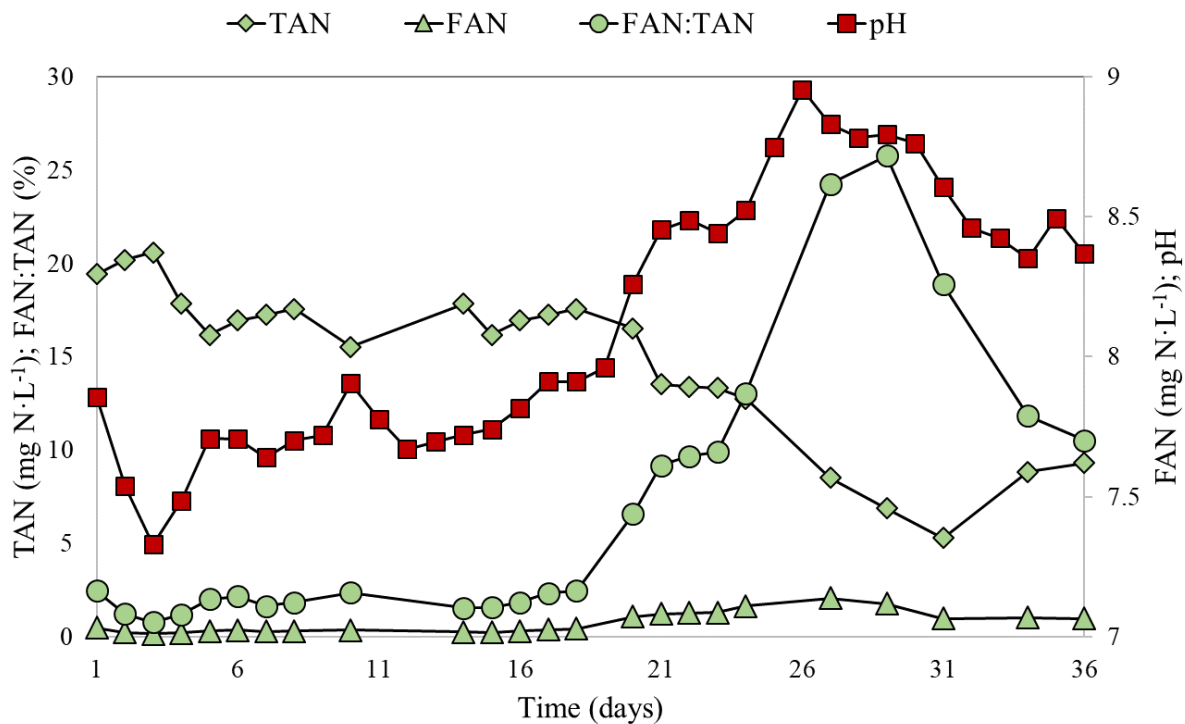
**Figure 1.** Evolution of (A) the total and soluble chemical oxygen demand ( $COD_T$  and  $COD_S$ ), the measured and estimated volatile suspended solids ( $VSS$  and  $VSS_{OD680}$ ) and the optical density at 680 nm ( $OD_{680}$ ), (B) the total nitrogen concentration ( $N_T$ ) and the concentrations of inorganic nutrients ( $PO_4^{3-}$ ,  $NH_4^+$ ,  $NO_3^-$  and  $NO_2^-$ ) and (C) the 16S rDNA and chlorophyte contents ( $copies \cdot mL^{-1}$ )

Regarding the concentrations of different inorganic nutrients in the mixed liquor (*i.e.*  $PO_4^{3-}$ ,  $NH_4^+$ ,  $NO_3^-$  and  $NO_2^-$ ), the results are shown in Figure 1B, together with the evolution of the concentration of  $N_T$ . As it could be expected from the previous results, the concentrations of  $NH_4$ -N and  $PO_4$ -P remained unchanged until day 18 due to the lack of algal growth, with values of  $17.7 \pm 1.5 \text{ mg } NH_4\text{-N} \cdot L^{-1}$  and  $3.0 \pm 0.4 \text{ mg } PO_4\text{-P} \cdot L^{-1}$ , respectively. Nevertheless, when microalgae started to grow (after day 18), the concentrations of both nutrients started to decrease due to uptake by microalgae, reaching values of  $7.8 \pm 1.7 \text{ mg } NH_4\text{-N} \cdot L^{-1}$  and  $0.9 \pm 0.5$

1 mg  $\text{PO}_4\text{-P}\cdot\text{L}^{-1}$  when the process was fully functional (days 26-36). Considering that the  
2 European discharge limits in protected areas of treatment flows below 100,000 PE are 15 mg  
3  
4  $\text{NH}_4\text{-N}\cdot\text{L}^{-1}$  and 2 mg  $\text{PO}_4\text{-P}\cdot\text{L}^{-1}$  (European Council Directive 91/271/CEE), it can be  
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6 concluded that, once a working microalgae population was developed, the proposed system  
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8 was able to treat the UWW efficiently in terms of nutrient content. Moving forwards, the  $\text{N}_\text{T}$   
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10 concentration showed a slight decrease from day 24 until the end of the experimental period  
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12 (*i.e.*  $\text{N}_\text{T}$  decreased from an average of 44.8 mg  $\text{N}\cdot\text{L}^{-1}$  between days 5-24 to 35.1 mg  $\text{N}\cdot\text{L}^{-1}$   
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14 between days 25-36). This can be attributed to different factors: i) variations in the nitrogen  
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16 loading rate to the system (influent  $\text{N}_\text{T}$  was  $45.5\pm 24.2$  mg  $\text{N}\cdot\text{L}^{-1}$ ), ii) increased nitrification-  
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18 denitrification rates, or iii) stripping of  $\text{NH}_3$  due to the relatively high pH values reached in  
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20 the media during this period (above 8). The occurrence of nitrification-denitrification could  
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22 not be verified by the nitrate and nitrite concentrations, which remained close to zero  
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24 throughout the whole experimental period due to the switch between aerobic-anoxic  
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26 conditions in the daylight and night-time hours (a maximum nitrate concentration of around 1  
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28 mg  $\text{NO}_3\text{-N}\cdot\text{L}^{-1}$  was reached on day 21). Regarding  $\text{NH}_3$  stripping, Figure 2 shows the  
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30 evolution of the total ammonia nitrogen concentration (sum of  $\text{NH}_4\text{-N}$  and free ammonia  
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32 nitrogen ( $\text{NH}_3\text{-N}$ ; FAN); TAN) during the experimental period. These results were obtained  
33  
34 using the Davies equation included in the geochemical equilibrium speciation model  
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36 MINTEQA2 (Allison et al., 1991) via Visual MINTEQ (Gustafsson, 2012). As it can be  
37  
38 observed, the FAN to TAN ratio significantly increase as the pH of the culture media  
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40 increased due to the phototrophic consumption of  $\text{CO}_2$  by microalgae. Indeed, the FAN:TAN  
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42 ratio increased with the pH. When the pH was above 8, the amount of FAN increased up to  
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44 25.8% of the TAN, suggesting a possible stripping of  $\text{NH}_3$  to the atmosphere. In absolute  
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46 terms, nevertheless, FAN concentration reached maximum values of around 2 mg  $\text{N}\cdot\text{L}^{-1}$ .  
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58 Thus,  $\text{NH}_3$  stripping cannot explain by itself the decrease in  $\text{N}_\text{T}$  observed in the system at the  
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end of the experimental period. This suggests that other processes, such as variations in influent nitrogen and nitrification-denitrification, occurred.

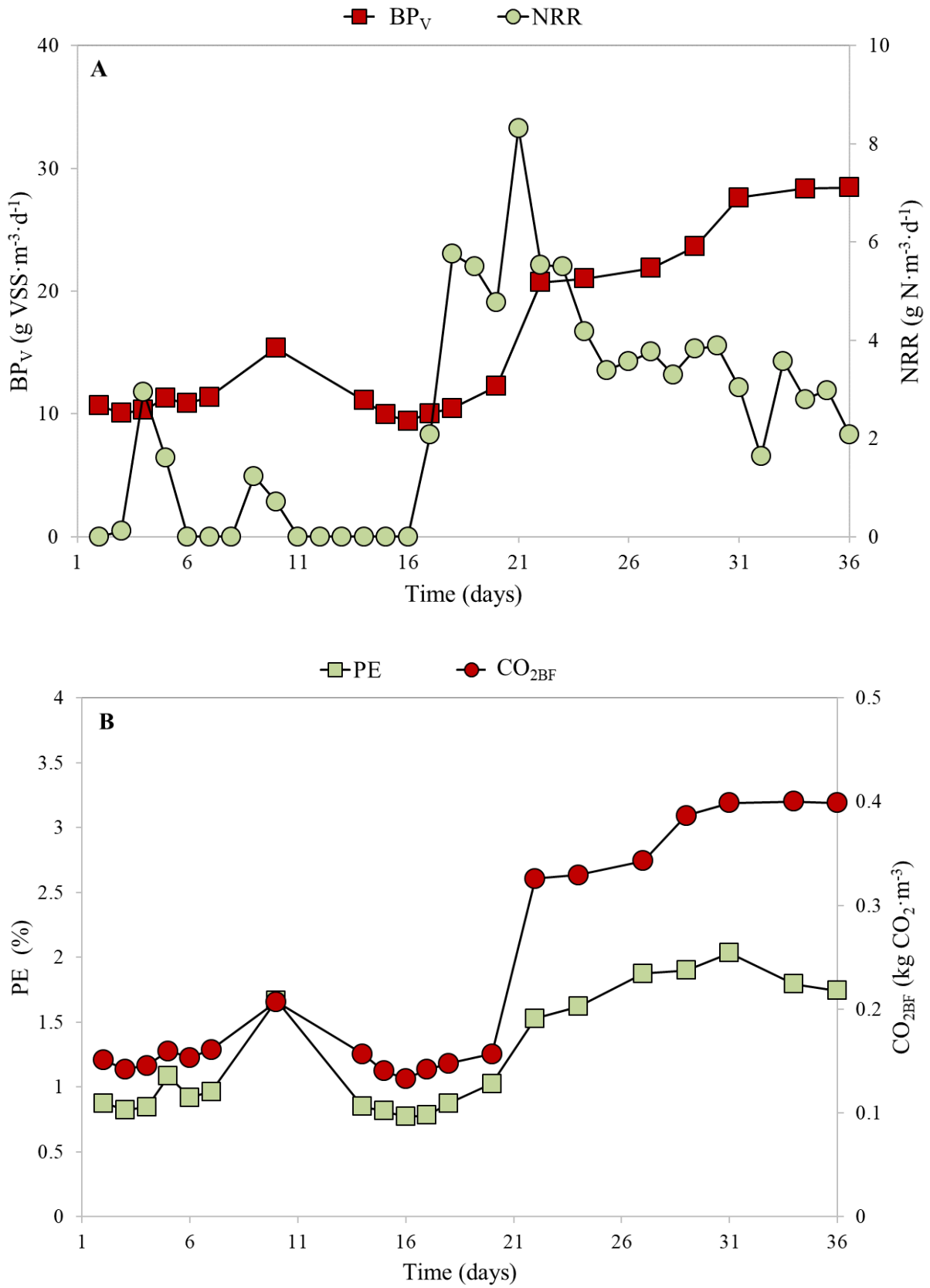


**Figure 2.** Evolution of the total ammonia nitrogen (TAN), the free ammonia nitrogen (FAN), the FAN to TAN ratio (FAN:TAN), and the pH of the media. All values represent daily averages

As key state indicators of the microbial performance of the HRAP, Figure 3A shows the evolution of the  $BP_V$  and NRR in the HRAP. As expected, both variables showed a significant increase around day 18, confirming the development of the microalgal community mentioned previously. After this point, the NRR experimented a sharp increase, reaching values up to  $9 \text{ g N}\cdot\text{m}^{-3}\cdot\text{d}^{-1}$  and progressively decreasing from day 21 to 26 until reaching nearly stable values around  $4 \text{ g N}\cdot\text{m}^{-3}\cdot\text{d}^{-1}$ . This behavior is consistent with the variables described previously (*i.e.* inorganic nutrient concentration and 18S rDNA copies content), confirming that the

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autotrophic growth of algae was directly responsible for the reduction in the concentrations of the inorganic compounds measured. The higher NRR observed from days 18 to 23 can be attributed to a more efficient usage of the sunlight due to the lower initial biomass concentrations (*i.e.* lower biomass shading effect, allowing higher microalgal growth rates). In addition, the sharp decrease of the inorganic nutrient concentrations from day 21 to day 26 reduced their availability, which might have significantly reduced the microalgal growth rates. As previously, these results suggest that conditions of equilibrium were achieved after day 26, indicating a full start-up of the system. However, it must be mentioned that the biomass productivity experimented a slight increase around day 30, which could indicate that the minimum time for establishing adequate consortia between bacteria and microalgae could be of around one month of continuous operation.  $BP_V$  values during the steady-state period at the end of the experiment were around  $30 \text{ g VSS} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$ , which are similar to those reported by Blanco et al. (2007) ( $40 \text{ g VSS} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$ ).



**Figure 3.** Evolution of (A) the biomass productivity per working volume (BP<sub>V</sub>) and the

1 nitrogen removal rate (NRR) and (B) the photosynthetic efficiency (PE) and the carbon  
2 dioxide biofixation ( $\text{CO}_{2\text{BF}}$ )  
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7 Figure 3B shows the evolution of the PE and the  $\text{CO}_{2\text{BF}}$  in the HRAP. As expected, both PE  
8 and  $\text{CO}_{2\text{BF}}$ , which depend on biomass productivity, showed a similar trend when compared to  
9  $\text{BP}_V$ . PE yielded values of 2 % on day 30, which are below the theoretical maximum of 8-12  
10 % (Romero Villegas et al., 2017). This suggests that further optimization of the process is  
11 possible. However, microalgae cultivation at industrial-scale, even at optimum conditions,  
12 rarely exceeded 1.5 to 2.0 % (Nwoba et al., 2019). In this respect, the light path and the light  
13 limitation of the outdoor configurations play a key role in light use efficiency. Several studies  
14 have assessed the effect of the culture depth (Arbib et al., 2017; Fernández et al., 2016) and  
15 how the light regime at which the microalgae are exposed to are far from the optimal values in  
16 outdoor raceway ponds (Barceló-Villalobos et al., 2019). Optimization of light use efficiency  
17 in system such as the one proposed in this study clearly deserve further research efforts.  
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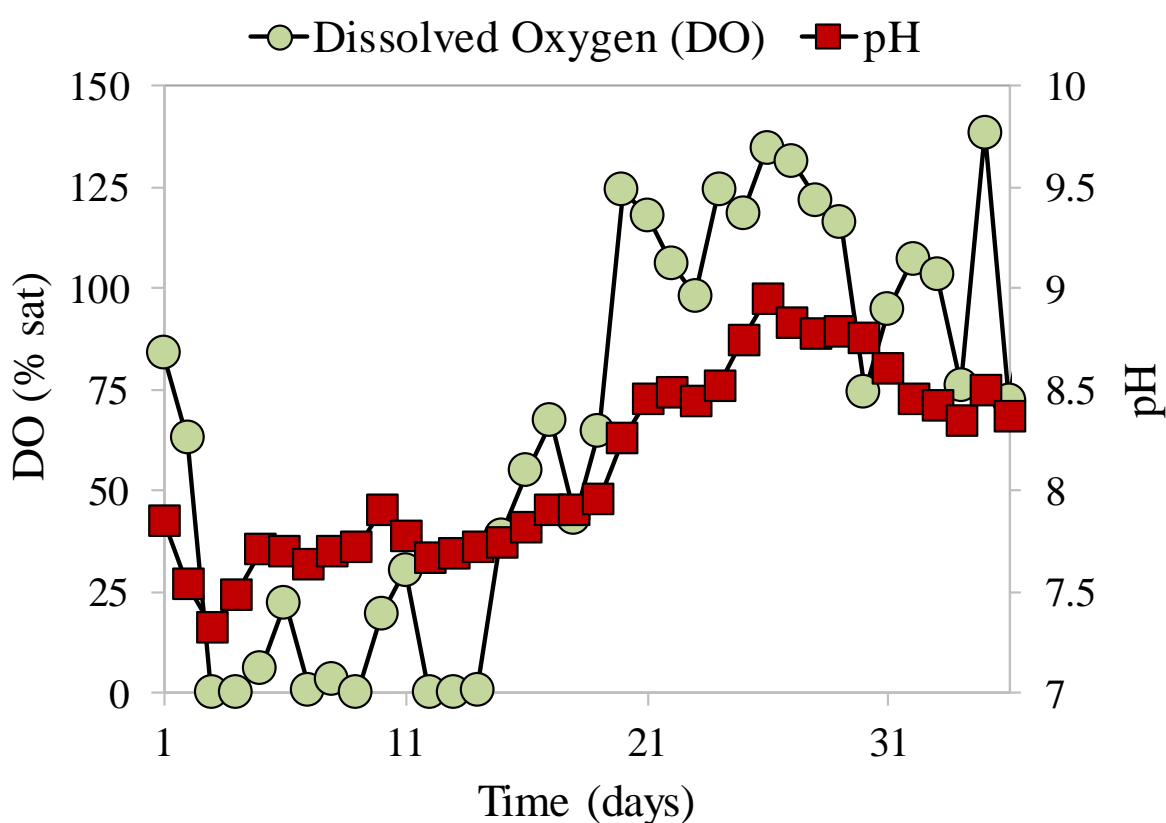
32 Regarding the  $\text{CO}_{2\text{BF}}$ , at the end of the experiment its value was around 0.4 kg  $\text{CO}_2$  per  $\text{m}^3$  of  
33 treated water (assuming  $\text{C}_{106}\text{H}_{181}\text{O}_{45}\text{N}_{16}\text{P}$  as typical composition of algal biomass (Green et  
34 al., 1996)). Viruela et al. (2018) reported maximum  $\text{CO}_{2\text{BF}}$  of 0.51 kg  $\text{CO}_2$  per  $\text{m}^3$  of treated  
35 water in a membrane photobioreactor (MPBR), similar to the results presented in this study,  
36 even if a less efficient microalgae cultivation systems was used (HRAP). This reinforces the  
37 economic and environmental feasibility of this technology for UWW treatment.  
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### 48 *3.2. Navigating on-line key state indicators*

#### 49 *3.2.1. Results of the on-line monitoring of the process*

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51 As described previously, the DO and the pH were the variables measured on-line in this  
52 experiment. Figure 4 shows the evolution of the daily average DO and pH values in the mixed  
53 liquor throughout the experimental period. The DO decreased sharply at the very beginning of  
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the start-up period. This was caused by the initial activity of heterotrophic bacteria, corresponding to the initial COD consumption shown in Figure 1A. However, in accordance to the results presented in Figures 1-3, a sharp increase in the DO was observed between days 14 and 23, reaching values about 125 % of the DO saturation in water. During this period, the inorganic nutrient concentrations decreased and the chlorophyte concentrations increased. It was also during this period when the maximum NRR values were achieved. Therefore, it is clear that the DO increase was caused by the photosynthetic activity of the microalgae. Regarding the pH, its value remained fairly constant until day 18 (around 7.7), when it increased up to almost 9 due to the consumption of CO<sub>2</sub> related again to the photosynthetic activity of the microalgae. Relatively stable values of around 8.4 were maintained after day 26, suggesting that an equilibrium of the carbon dioxide production-consumption had been reached.



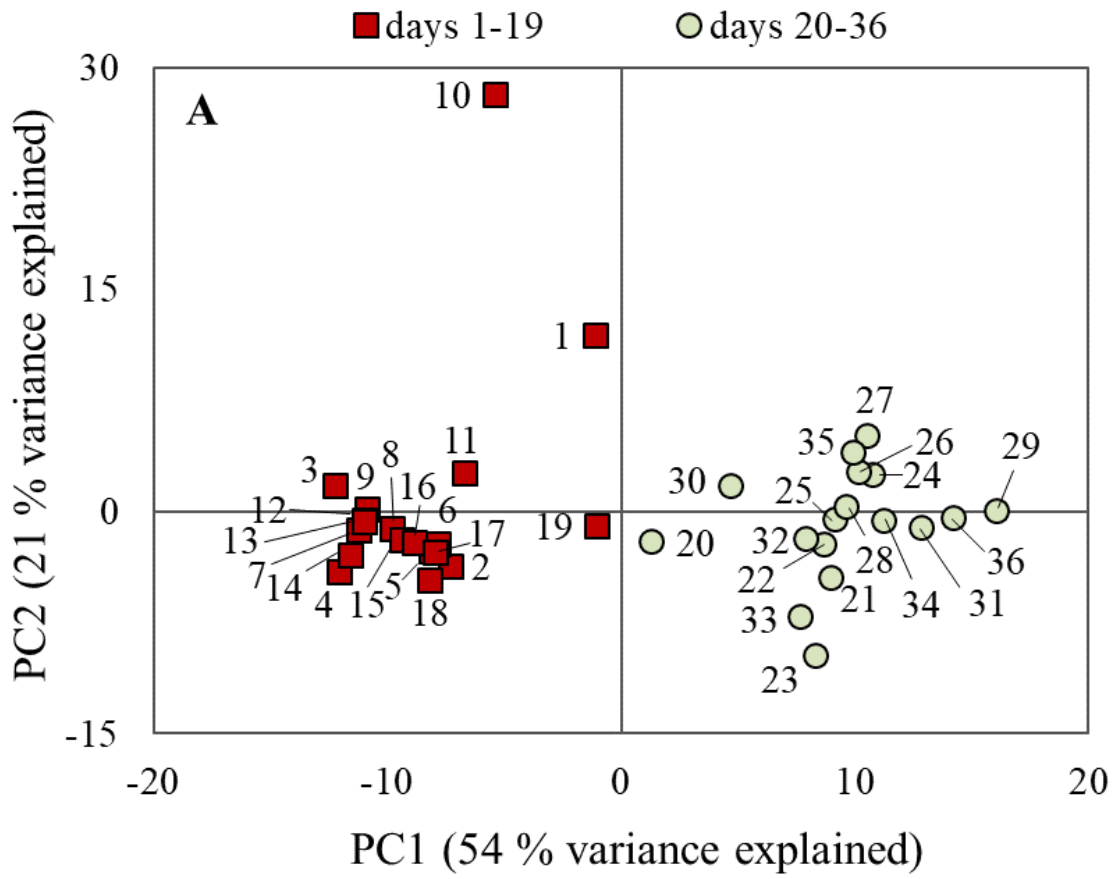
**Figure 4.** Evolution of the dissolved oxygen concentration (DO) and the pH in the mixed

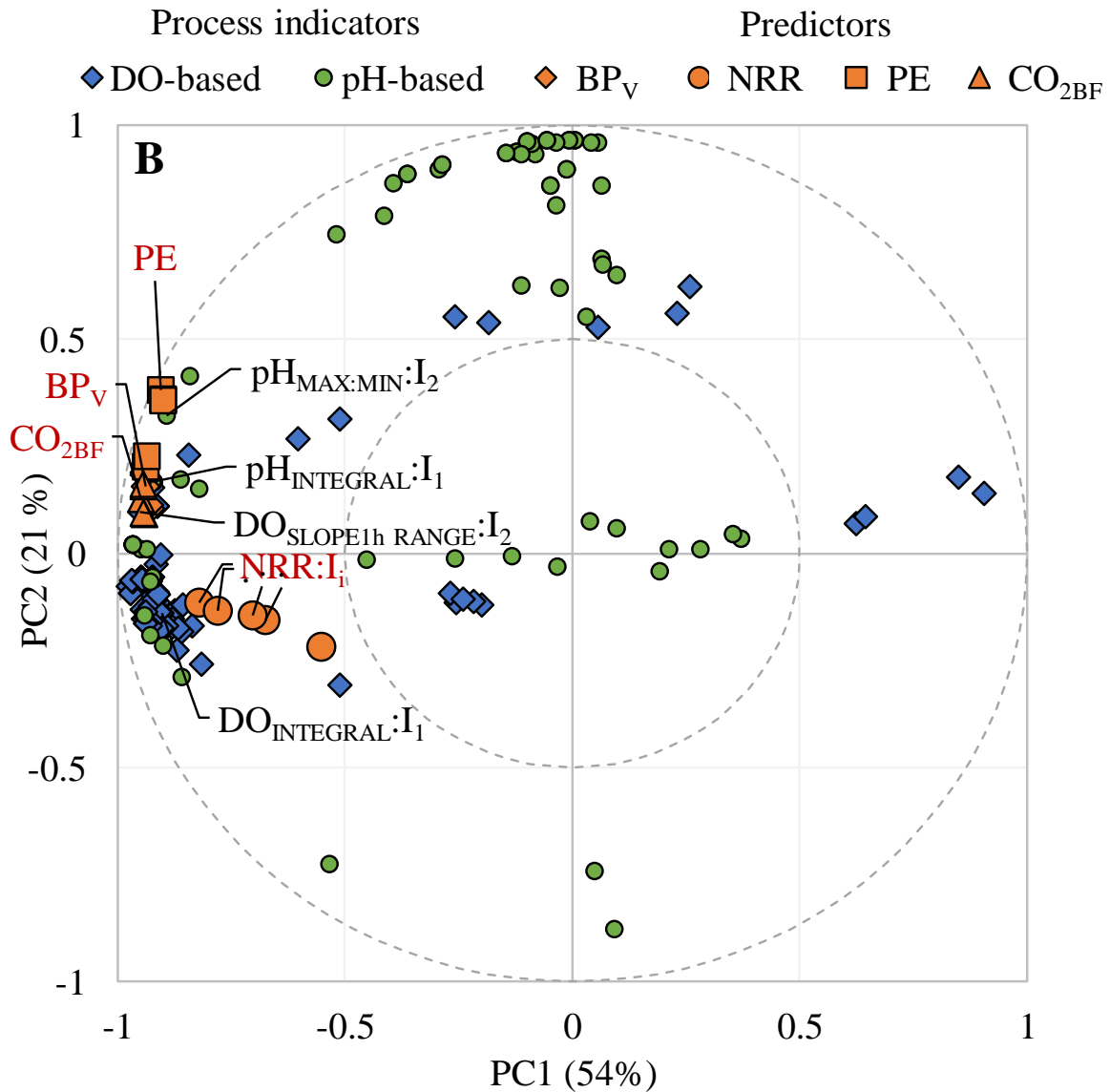
liquor throughout the experimental period

As expected, the evolution of the monitored variables (*i.e.* pH and DO) could easily explain the results presented above (Figure 1 to Figure 3). To obtain straight-forward results, statistical analyses were carried out to determine if these variables could be accurately used as predictors for key state indicators of the performance of microalgae-bacteria consortia.

### *3.2.2. Statistical identification of the key state indicators*

Initially, PCA and PLSR analyses were carried out using all the available data (with data from 36 days). The obtained results are presented in Figure 5a and Figure 5b. Two PCs accounted for a cumulative explained variance of 75 %, indicating that the given results represent most of the contained information. The results of Figure 5a further confirm the conclusions from the previous section, since two clusters were observed, which corresponded to data from day 1 to day 19 (bacteria community was predominant), and data from day 20 to day 36 (microalgae-bacteria community). These results confirm also the different behaviour of the HRAP plant with respect to the microalgal activity. Days 1 and 10 were considered outliers of the PCA, since day 1 corresponded to the start-up of the plant and day 10 was attributed to the activity of different microalgae species that were not successfully adapted to the operating and environmental conditions applied.





**Figure 5.** (A) Score plot for the first two components of the PCA model and (B) weight plot of the first two components of the PLSR model

Continuing with Figure 5B, the PLSR results show that the output process indicators BP<sub>V</sub>, PE, and CO<sub>2BF</sub> (and to a lesser extent NRR) are close to each other since they are all directly related to the biomass activity. The clusters obtained between some parameters indicate that they represented virtually the same information. For instance, the output process indicators (NRR:I<sub>i</sub>, BP<sub>V</sub>:I<sub>i</sub>, PE:I<sub>i</sub>, and CO<sub>2BF</sub>:I<sub>i</sub>), represent the same information with model I<sub>1</sub> and I<sub>3</sub> or



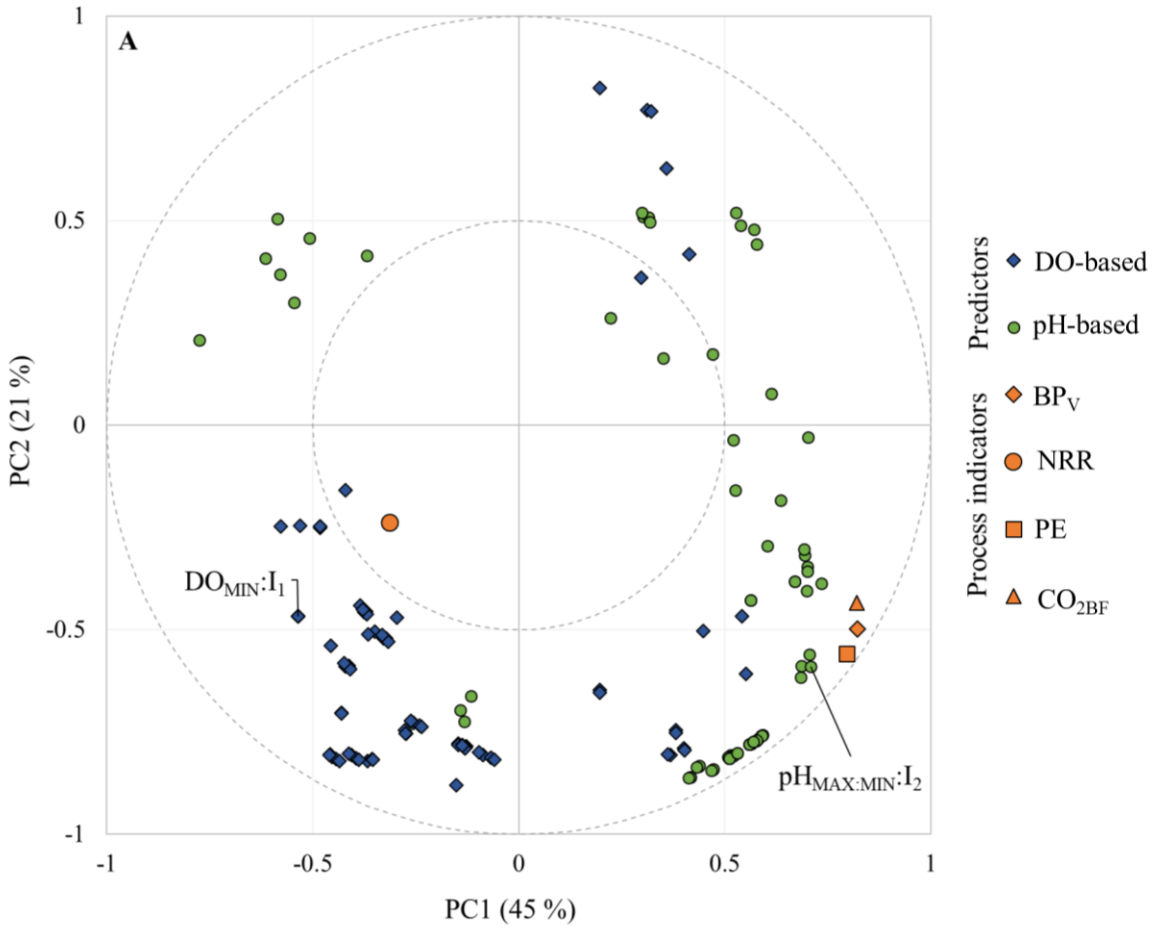
1 the model  $I_2$  and  $I_4$ . This implies that redundant values existed and therefore only one  
2 representative parameter of each cluster should be considered. In addition, non-standardized  
3 output process indicators ( $BP_V$ , PE, and  $CO_{2BF}$ ; marked in red in Figure 5B) indicate similar  
4 information to the standardized ones except for NRR. In contrast, the best fitting of these  
5 output process indicators with the predictors was achieved when using  $I_i$ -normalized  
6 parameters based on pH and DO measurements ( $NRR:I_1$  marked in red in Figure 5B).  
7 Concerning the correlations between the predictors and the responses, the results of Figure 5B  
8 support the findings from the previous section due to the good correlations obtained.  
9 Specifically, stronger direct correlations were observed for the following pairs of parameters:  
10  $pH_{INTEGRAL}:I_1$  and  $BP_V$ ,  $DO_{SLOPE1h\ RANGE}:I_2$  and  $CO_{2BF}$ ,  $pH_{MAX:MIN}:I_2$  and PE, and  
11  $DO_{INTEGRAL}:I_1$  and  $NRR:I_1$ .

12 The  $pH_{INTEGRAL}:I_1$  parameter represents the  $CO_2$  availability in the media within the day  
13 derived from the microalgae-bacteria consortia performance. It is important to highlight that  
14 the biomass productivity was mainly related to microalgae growth, confirmed by particle size  
15 distribution analysis (data not shown). Thus, at higher concentrations of  $CO_2$  available, higher  
16 microalgae activities are expected (with concomitant higher biomass productivities). The  
17  $DO_{SLOPE1h\ RANGE}:I_2$  parameter represents the  $O_2$  availability in the media within the day. The  
18 oxygen production indeed derives from the photosynthetic activity, which is directly  
19 correlated to  $CO_2$  biofixation. The  $pH_{MAX:MIN}:I_2$  parameter represents microalgae growth rate  
20 versus the respiration rate of the microalgae. This respiration rate can be regarded as an  
21 indirect indicator of the maximum capacity of the system, since this process was not limited  
22 by the operating conditions. Hence, higher microalgae growth efficiencies (represented by  
23  $pH_{MAX:MIN}:I_2$ ) correspond to higher photosynthetic efficiencies. The  $DO_{INTEGRAL}:I_1$  parameter  
24 also represents the  $O_2$  availability in the media within a day. At higher DO production rates,  
25 higher nitrogen uptake rates by microalgae and higher nitrification capacities of the system

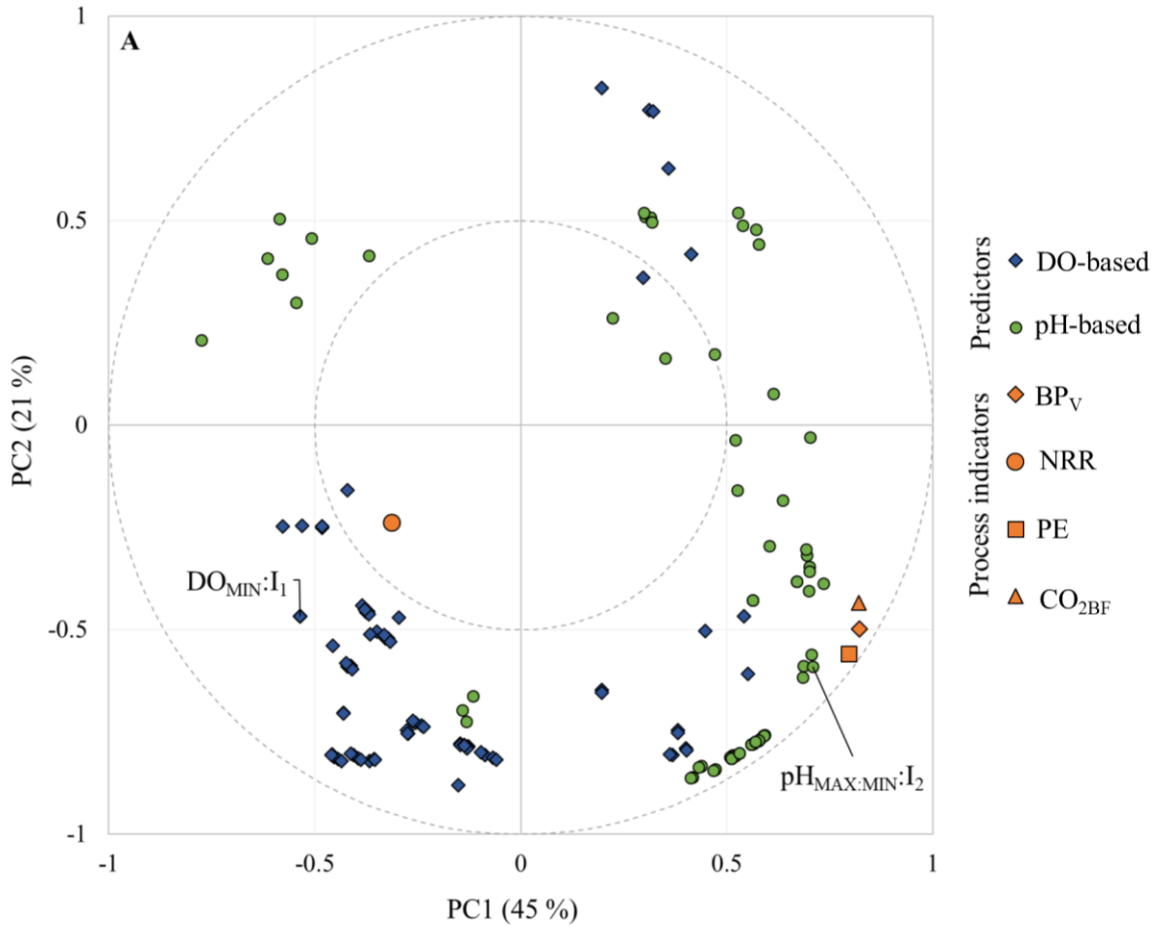
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can be expected. These results prove that pH and DO-derived variables can accurately be used to predict the general performance of the HRAP.

Nevertheless, since two representative sets of parameters were observed in Figure 5a (days 1-19 and days 20-36), two PLSR models (one per data set) were conducted aiming at determining which variables predicted more accurately the process outputs depending on the behavior of the system (Figure 6). Similar to Figure 5B, Figure 6A (showing the data set corresponding to days 1-19) shows that process indicators  $BP_V$ , PE, and  $CO_{2BF}$  are nearby in the PLS plot. NRR was not significantly correlated to any other variable since neither significant microalgal activity nor nitrification were observed until day 14. In this case, the PLSR results show a strong correlation between the outputs and the predictor  $pH_{MAX:MIN:I_2}$ . As commented before, this parameter is related to the microalgae activity. Thus, this variable would be useful for assessing the initial dynamics of microalgae growth (days 14-19; see Figure 3), representing precisely  $BP_V$ ,  $CO_{2BF}$  and PE during this period. NRR could be predicted by different DO-based variables, such as  $DO_{MIN:I_1}$ , suggesting that this parameter can be used for monitoring the initial performance of the system. This is a logical output, as NRR was mainly determined at this point by bacterial growth, which were responsible for oxygen consumption.



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**Figure 6.** Weight plot (correlation circles) of the first two components of the PLSR model for (A) days 1-19, and (B) days 20-36

Figure 6B shows the results from the PLSR model conducted with data during the period of stable operation of plant (days 20-36). Indeed, all the responses evaluated in the PLSR model resulted in the same location in the PLS plot, indicating a fully developed consortium. The direct correlation of NRR and the other process indicators further confirms the dominant microalgal activity. In this case, the predictor that better correlates with the responses is the variable  $DO_{MIN\ SLOPE2h}$ . This variable represents the consumption of oxygen by respiration of microalgae, by growth of heterotopic microorganisms and by nitrification, which are the main processes occurring in a fully developed microalgae-bacteria consortia during the night (when

the minimum slopes were determined).

1  
2 These results suggest that both pH and DO can be accurately used for a proper monitoring of  
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4 the microbial processes occurring in HRAPs treating UWW by microalgae-bacteria consortia.  
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6 They could also be applied potentially for the prediction of the performances that can be  
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8 achieved, applying simple regression analysis. Other than being in agreement with different  
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10 studies performed with microalgae in HRAPs (Galès et al., 2019; Havlik et al., 2013), the  
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12 conclusions from this work also agree with the results presented in Foladori et al. (2018),  
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14 another study dealing with monitoring of microalgae-bacteria consortia for WWT. In their  
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16 research, the DO, the pH and the ORP were monitored in real-time using on-line probes  
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18 installed in a sequential PBR, revealing that the evolution of the WWT process could be  
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20 followed by measuring the aforementioned parameters. The real-time on-line monitoring of  
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22 both the pH and the DO offers an easily-applicable option for the follow up of the start-up  
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24 period of industrial-scale HRAPs, using affordable probes already available in the market.  
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26 The normalized variables can also provide accurate indications of the performance of the  
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28 HRAP when compared to an optimal behavior, serving as markers for disturbances in the  
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30 system. This approach has a great potential, not only to monitor and control the proper  
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32 operation of the proposed WWT system, but also to optimize the achieved performances and  
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34 to increase the understanding of the underlying processes when coupling monitoring with  
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36 novel mathematical models, such as the one presented in Solimeno et al. (2017b).  
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#### 48 **4. Conclusions**

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50 A stable bacterial community existed after 10 days. It took around 19 days to develop a  
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52 microalgal community able to uptake nutrients significantly. After 26 days, the HRAPs was  
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54 fully functional, meeting the European discharge limits. Variations in the biomass  
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56 productivities in days 26-30 suggest that the minimum time required for establishing a  
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1 performant microbial population could be one month. The statistical analyses show that pH-  
2 based and DO-based variables can represent accurately the biochemical processes taking  
3 place. Both the pH and the DO could be used to accurately describe the HRAP performance.  
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5 This represents an affordable, easily-implemented option for monitoring the start-up of  
6  
7 industrial-scale processes.  
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## Figure and table captions

**Figure 1.** Evolution of (A) the total and soluble chemical oxygen demand ( $\text{COD}_T$  and  $\text{COD}_S$ ), the measured and estimated volatile suspended solids (VSS and  $\text{VSS}_{\text{OD680}}$ ) and the optical density at 680 nm ( $\text{OD}_{680}$ ), (B) the total nitrogen concentration ( $\text{N}_T$ ) and the concentrations of inorganic nutrients ( $\text{PO}_4^{3-}$ ,  $\text{NH}_4^+$ ,  $\text{NO}_3^-$  and  $\text{NO}_2^-$ ) and (C) the 16S rDNA and chlorophyte contents ( $\text{copies}\cdot\text{mL}^{-1}$ )

**Figure 2.** Evolution of the total ammonia nitrogen (TAN), the free ammonia nitrogen (FAN), the FAN to TAN ratio (FAN:TAN), and the pH of the media. All values represent daily averages

**Figure 3.** Evolution of (A) the biomass productivity per working volume ( $\text{BP}_V$ ) and the nitrogen removal rate (NRR) and (B) the photosynthetic efficiency (PE) and the carbon dioxide biofixation ( $\text{CO}_{2\text{BF}}$ )

**Figure 4.** Evolution of the dissolved oxygen concentration (DO) and the pH in the mixed liquor throughout the experimental period

**Figure 5.** (A) Score plot for the first two components of the PCA model and (B) weight plot of the first two components of the PLSR model

**Figure 6.** Weight plot (correlation circles) of the first two components of the PLSR model for (A) days 1-19, and (B) days 20-36

**Table 1.** Characteristics of the synthetic UWW

**Table 2.** Variables based on DO and pH measurements