Wastewater Nutrient Removal in a Mixed Microalgae-bacteria Culture: Effect of Light and Temperature on the Microalgae-bacteria Competition.

J. González-Camejo[b], R. Barat[b*], M. Pachés[b], M. Murgui [a], A. Seco [a], J. Ferrer [b]

[a]*Departamento de Ingeniería Química, Universitat de València, Avinguda de la Universitat, s/n., 46100 Burjassot, Valencia, Spain.E-mail addresses: monica.murgui@uv.es; aurora.seco@uv.es. Tel.: +34 963 543 927; fax: +34 963 544 898.*

[b]*Instituto de Ingeniería del Agua y Medio Ambiente, IIAMA, Universitat Politècnica de València, Camí de Vera, s/n., 46022 Valencia, Spain. E-mail addresses: jogonca4@upv.es;rababa@dihma.upv.es;mapacgi@upvnet.upv.es;jferrer@hma.upv.es. Tel.: +34 963 877 007.*

* Corresponding author

Abstract

The aim of this study was to evaluate the effect of light intensity and temperature on nutrient removal and biomass productivity in a microalgae-bacteria culture and their effects on the microalgae-bacteria competition. Three experiments were carried out at constant temperature and various light intensities: 40, 85 and 125 $\mu E \cdot m^{-2} \cdot s^{-1}$. Other two experiments were carried out at variable temperatures: 23 ± 2 and 28 ± 2 ºC at light intensity of 85 and 125 $\mu E \cdot m^{-2} \cdot s^{-1}$, respectively. The photobioreactor was fed by the effluent from an anaerobic membrane bioreactor. High nitrogen and phosphorus removal efficiencies (about 99%) were achieved under the following operating

conditions: 85 - 125 $\mu E \cdot m^{-2} \cdot s^{-1}$ and 22 ± 1 ºC. In the microalgae-bacteria culture studied, increasing light intensity favoured microalgae growth and limited the nitrification process. However, a non-graduated temperature increase (up to 32ºC) under the light intensities studied caused the proliferation of nitrifying bacteria and the nitrite and nitrate accumulation. Hence, light intensity and temperature are key parameters in the control of the microalgae-bacteria competition.

Biomass productivity significantly increased with light intensity, reaching 50.5 ± 9.6, 80.3 ± 6.5 and 94.3 ± 7.9 $mgVSS \cdot L^{-1} \cdot d^{-1}$ for a light intensity of 40, 85 and 125 $\mu E \cdot m^{-2} \cdot s^{-1}$, respectively.

1. Introduction

In recent decades wastewater treatment plants (WWTPs) are evolving to more efficient technology in order to reduce the total carbon footprint of the process. In this regard, anaerobic treatments have several advantages compared to aerobic processes: no aeration is needed, slow sludge production because of the low biomass yield of anaerobic organisms [1] and biogas production, which improves the energetic balance [2].

Anaerobic Membrane Bioreactors (AnMBR) technology appears as a useful application for anaerobic wastewater treatment [3], since it allows decoupling hydraulic retention time (HRT) and biomass retention time (BRT). The AnMBR system achieves a high quality effluent in terms of total suspended solids (TSS) and chemical oxygen demand

(COD) [2]. Nevertheless, this system is not able to efficiently remove inorganic nutrients (mainly nitrogen and phosphorus) which cannot be directly discharged into the aquatic environment as they could cause serious eutrophication problems, affecting water quality. In fact, the European Union Council Directive 91/271/EEC [4] indicates that the discharge limits of nitrogen and phosphorus for a WWTP (> 100,000 p.e.) are 10 $mgN \cdot L^{-1}$ and 1 $mgP \cdot L^{-1}$, respectively, considerably lower than the typical values obtained for these nutrients in the effluent of an AnMBR process [5].

Microalgae offer a win-win alternative for tertiary wastewater treatment due to their ability to simultaneously remove inorganic nitrogen and phosphorus to meet the effluent standards and at the same time produce valuable biomass which may be used to produce biofuels [6, 7], biogas [8] and other valuable products [6,9]. In addition, as photosynthetic organisms, they are able to absorb carbon dioxide, thus contributing to reducing greenhouse gas emissions. It is remarkable that microalgae have been already presented as a potential technology able to reduce the nutrient concentration in the effluent from an AnMBR [5].

Nevertheless, in mixed microalgae cultures, the other microorganisms compete with algae for nutrients. For instance, the coexistence of microalgae and nitrifying bacteria (both ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB)) has been already reported in the treatment of wastewater [10]. AOB transform ammonium into nitrite, while NOB carry out the second nitrification step by oxidizing nitrite into nitrate. The nitrification process therefore implies two negative effects in the use of microalgae for wastewater treatment: (i) the reduction of ammonium by AOB does not involve a net nitrogen reduction as it is oxidized to nitrate, which is not as easily assimilated by microalgae as ammonium [11]; (ii) the limitation of microalgae growth

when the concentration of ammonium in the culture is low. In fact, Meseck *et al.* [12] concluded that the presence of nitrifying bacteria in a microalgae culture of *Tetraselmis chui* reduced the specific growth rate due to their competition for nutrients. However, Risgaard-Petersen *et al.* [13] observed that microalgae became the predominant group under non-limiting ammonium due to their higher growth rate. Thus, if maximum biomass productivity of microalgae is desired, the optimal conditions for microalgae growth must be applied for them to dominate the competition.

Two key factors that affect the microalgae activity are light and temperature [14]. Microalgae growth is a direct function of the light intensity supplied as long as it remains under the optimum light intensity. Over this value, the photosystem II reactor centres of the microalgae can be damaged, which implies a reduction of the carbon needed to carry out the photosynthesis process, causing photoinhibition [15]. On the other hand, if the supplied light intensity is low, the availability of light is restricted to the surface area of the photobioreactor [16], therefore photolimitation occurs.

Temperature is another parameter which must be taken into account in microalgal growth. Many species of microalgae are able to grow in a wide range of temperature, but all of them show an optimum value in which they are inhibited if they pass it [17], and may even lead to cell death [18]. In fact, Martínez *et al.* [19] observed a considerable reduction in the specific growth rate and biomass productivity of *Scenedesmus obliquus* when the temperature was over 30 ºC. Li *et al.* [20] reported that *Scenedesmus* sp. LX1 could grow within a wide temperature range: 10-30ºC; reaching the maximum specific growth rate at 25ºC.

It must be highlighted that most of previous studies have been focused on the effect of light intensity and temperature over pure cultures and many of them have used an

artificial medium as nutrients source, but scarce studies have shown the effect of key parameters of microalgae growth, such as light and temperature, over the microalgae-bacteria competition for nutrients in real wastewater.

The aim of the present study is to analyse the effect of light intensity and temperature on nutrient removal and biomass productivity in an indigenous microalgae-bacteria culture dominated by *Scenedemus* spp. and their effect over the competition microalgae-bacteria. The system was fed by nutrient-loaded (ammonium and phosphate) effluent from an AnMBR which treated urban wastewater.

2. Material and Methods

2.1 Influent wastewater composition

The effluent of an anaerobic membrane bioreactor pilot plant was used as microalgae PBR influent. Further details of the characteristics of the AnMBR may be found in [2, 21].

The AnMBR effluent was collected every day and taken to the laboratory to feed the lab-scale photobioreactor (PBR). The main characteristics of the AnMBR effluent during the whole experimental period were: ammonium of 57.4 ± 2.2 mgN-NH$_4$·L^{-1}, phosphate of 7.0 ± 0.9 mgP-PO$_4$·L^{-1}, N:P molar ratio of 17.6 ± 1.4 molN·molP^{-1}, total COD of 51 ± 8 mgCOD·L^{-1}, alkalinity of 737 ± 38 mgCaCO3·L^{-1}, volatile fatty acids of 1.5 ± 0.5 mgHAc·L^{-1}, and sulphide of 100.3 ± 12.4 mgS·L^{-1}. Nitrite and nitrate were negligible in the AnMBR effluent.

It is relevant that the N:P molar ratio in the effluent during this time remained at similar values to the one reported by Rawat *et al.* [7] and Reynolds [22]: 16. Hence, the

AnMBR effluent allowed microalgae to remove nitrogen and phosphorus efficiently and simultaneously.

Regarding organic matter loading, soluble COD in this AnMBR effluent is inert, thus it enhanced photoautotrophic metabolism typical of microalgae and limited heterotrophic activity. Due to the high sulfide concentration, the AnMBR effluent was previously aerated to oxidize the sulphide to sulphate since sulphide acts as a specific inhibitor of microalgae [23].

2.2 Inoculum and pre-culture conditions

The microalgae used as inoculum in the present study were isolated from the walls of the secondary clarifier in the Carraixet WWTP (Alboraya, Spain), which consisted of a complex ecosystem containing microalgae, algae and bacteria (including *Cyanobacteria*). Prior to the inoculation of the lab-scale PBR, the sample from the secondary clarifier was passed through a laboratory paper filter to remove filamentous bacteria and zooplankton from the reactor. The culture was adapted to the growth medium with continuous illumination (85 $\mu E \cdot m^{-2} \cdot s^{-1}$). The medium consisted of a nutrient-loaded effluent from the aforementioned AnMBR pilot plant (see Section 2.1). Temperature and pH were maintained at 22 ± 1 ºC and 7.4 ± 0.3, respectively. Ten days later, under the cited cultivation conditions, microalgae reached a concentration of 447 $mgVSS \cdot L^{-1}$, which was considered appropriate for the PBR to be fed in semicontinuous mode (see Section 2.3). *Scenedesmus* spp. became the main microalgae genus present in the PBR. *Scenedesmus* have been consistently reported as an adaptable microalgae genus able to grow in several types of wastewater [24, 25].

2.3 Photobioreactor: description, control and operation

The PBR consisted of a cylindrical transparent tank (20 cm internal diameter) with a working volume of 8L (see Figure 1a). To achieve suitable culture homogenisation and avoid biofilms on the walls, the culture was stirred with air at a flow rate of 1.0–1.2 L·min^{-1} through four fine bubble diffusers placed crosswise on the bottom. In order to maintain fixed pH in the PBR, pure CO_2 (99.9%) was injected into the gas flow from a pressurised cylinder at 1–1.5 bar pressure when the pH value rose above 7.5. Controlling pH in the reactor helped to prevent undesirable phenomena such as phosphate precipitation and the ammonia stripping losses. This stripping can be considered negligible when pH values are under 9.0 and ammonia concentration is under 65 mgN·L^{-1} [26].

The physical-chemical parameters of the algae culture such as temperature, pH and dissolved oxygen were monitored online in the PBR and logged in a PC using data acquisition software. For pH, the signal from the corresponding electrodes was processed by a multiparametric analyser (CONSORT C832, Belgium), while temperature and dissolved oxygen were measured by a 4-Star multiparametric analyser (Thermo Scientific) connected to a 087003RDO probe.

The PBR was operated in a semi-continuous mode without biomass retention; therefore BRT and HRT had the same value. The feed was the nutrient-loaded effluent from the AnMBR system used in the pre-culture (see section 2.1). To keep BRT and HRT constant at 8 days, every six hours (4 feed cycles a day) a peristaltic pump controlled by a PC fed the PBR by pulse with 0.25 L of AnMBR effluent. The total volume remained constant since the reactor had an overflow at the top.

2.4 Experimental design

Table 1 shows the operational conditions (light, temperature and dissolved oxygen) in each of the five experiments conducted in the present study. The experimental design was based on two variables: light and temperature. Light was supplied at three different intensities (see Table 1). In these three experiments (L1, L2 and L3), the temperature remained constant. On the other hand, in experiments T1 and T2 temperature was not controlled (see Table 1).

As light source, fluorescent lamps (Sylvania Grolux, 18 W) were placed vertically around the PBR at a distance of 20 cm (see Figure 1b). Four lamps were switched on in Exp. L1 (positions: 1, 2, 11 and 12); eight units in Exp. L2 and T1 (positions: 5, 6, 7 and 8), and the whole set of lamps in Exp. L3 and T2. Lighting was provided 24 hours a day. Photosynthetically active radiation (PAR: 400-700 nm) values were measured by a photo sensor (HOBO_Smart Sensor, s-lia-m003).

In these experiments no inhibition of microalgae by ammonium was expected since *Park et al.* [24] obtained inhibition of *Scenedesmus* sp. with concentrations of ammonium higher than 200 mg $N \cdot L^{-1}$ (the maximum ammonium concentration of the AnMBR effluent was 70.7 $mgN \cdot L^{-1}$). Furthermore, inhibition by high concentration of dissolved oxygen was not expected as it remained under 110% (see Table 1). Vonshak and Torzillo [27] reported that dissolved oxygen could inhibit microalgae growth when it was higher than 400%.

In order to control the temperature in the PBRs, Exp. L1, L2 and L3 were conducted inside a climatic chamber. As previously indicated, in Exp. T1 and T2 the PBR was not run under controlled temperature.

2.5 Sampling and analysis

2.5.1 Analytical Methods

Nutrient removal by microalgae culture was evaluated by recording daily inorganic nitrogen and phosphate levels in the influent (AnMBR effluent) and also in the soluble fraction taken from the PBR. The soluble fraction of the culture was obtained by filtration through 0.45 μm pore size glass fibre filters (Millipore). Volatile suspended solids (VSS) and chlorophyll were determined 3 times a week to verify biomass growth and chlorophyll content. VSS was evaluated according to method 2540-E of Standard Methods [28]. Chlorophyll content was determined by the tricromatic method based on visible spectroscopy [28], using Jeffrey and Humphrey equations [29] to obtain the concentration. Pigment extraction was performed with acetone 90%.

Ammonium, nitrite, nitrate and phosphate were determined according to Standard Methods [25]: 4500-NH3-G, 4500-NO2-B, 4500-NO3-H and 4500-P-F, respectively, in a Smartchem 200 automatic analyzer (WestcoScientific Instruments, Westco).

2.5.2 Calculations

The performance of the PBR and the microalgae–bacteria competition were evaluated through the nutrient removal efficiencies and biomass productivity. All efficiencies were calculated on a daily balance basis considering influent and effluent terms. All nutrients (soluble nitrogen compounds and phosphate) were assumed to be available for biomass growth (as in the case of microalgae and bacteria). Phosphorus precipitation (e.g. as calcium phosphate or struvite) was negligible due to the low solubility of this species in water at neutrality [30]. Moreover, the oxygen concentration maintained in the PBR due to photosynthetic activity (see Table 1) and the very low level of

biodegradable carbon made denitrification unlikely. A nitrogen mass balance made on a few runs confirmed both hypotheses (data not shown). It was therefore assumed that ~~was~~ removal of nutrients was only caused by biological processes.

The *ammonium removal efficiency* (ARE) represents the percentage of ammonium assimilated by both microalgae and nitrifying bacteria (*Eq. 1*). The *ammonium removal efficiency by microalgae* (mARE) shows the ammonium assimilated by microalgae only. This was calculated as ARE, but deducting the ammonium nitrified as nitrite or nitrate (NO_X) (*Eq. 2*). The *phosphorus removal efficiency* (PRE) is the percentage of phosphate assimilated by microalgae. It was calculated similarly to ARE. Since microalgae were the dominant community of the culture, microalgal activity was assumed as solely responsible for P removal in these experiments.

$$ARE(\%) = \frac{I_{NH4} - E_{NH4}}{I_{NH4}} \, (Eq. \, 1)$$

$$mARE(\%) = \frac{I_{NH4} - E_{NH4} - E_{NO2} - E_{NO3}}{I_{NH4}} \, (Eq. \, 2)$$

The biomass productivity was calculated according to Eq. 3:

$$BP(mgVSS \cdot L^{-1} \cdot d^{-1}) = \frac{VSS \cdot V_p}{V} \, (Eq. \, 3)$$

Where BP is the biomass productivity of the PBR (mgVSS·L^{-1}·d^{-1}), VSS is the volatile suspended solids concentration (mgVSS·L^{-1}), V_p is the daily purged volume from the PBR (L·d^{-1}) and V is the volume of the PBR (L).

In order to study the performance in the use of light by the microalgae-bacteria culture, the ammonium removal rate:light irradiance ratio and phosphorus removal rate:light irradiance ratio were calculated according to Eq. 4, Eq. 5 and Eq. 6:

$$ARR/_I = \frac{ARR \cdot V_{PBR} \cdot 10^6}{I \cdot S \cdot 24 \cdot 3600} \qquad (Eq. \, 4)$$

$$\text{mARR}\big/\text{I} = \frac{\text{mARR} \cdot \text{V}_{\text{PBR}} \cdot 10^6}{\text{I} \cdot \text{S} \cdot 24 \cdot 3600} \qquad \text{(Eq. 5)}$$

$$\text{PRR}\big/\text{I} = \frac{\text{PRR} \cdot \text{V}_{\text{PBR}} \cdot 10^6}{\text{I} \cdot \text{S} \cdot 24 \cdot 3600} \qquad \text{(Eq. 6)}$$

Where ARR/I is the ammonium removal rate:light irradiance ratio (mgN·mol photons^{-1}), mARR/I is the ammonium removal rate by microalgae:light irradiance ratio (mgN·mol photons^{-1}), PRR/I is the phosphorus removal rate:light irradiance ratio (mgP·mol photons^{-1}), ARR is the ammonium removal rate (mgN·L^{-1}·d^{-1}), mARR is the ammonium removal rate by microalgae (mgN·L^{-1}·d^{-1}), PRR is the phosphorus removal rate (mgP·L^{-1}·d^{-1}), I is the mean light PAR irradiance applied to the PBRs´ surface (µmol photons·m^{-2}·s^{-1}) and S is the illuminated PBRs surface (m^2).

2.5.3 Microbiological Method

To analyse the microalgae community, 50 µL of sample were filtered with 0.2 µm membranes (Millipore GTTP) twice a week. The filters were washed with distilled water to eliminate the retained salt and then dehydrated with successive 50%, 80%, 90% and 99% ethanol washes. Cell counts were performed by epifluorescence microscopy on a Leica DM2500, using the 100x-oil immersion lens. A minimum of 300 cells were counted and at least 100 cells of the most abundant species or genera were counted with an error of less than 20% [31]. All the reported results were obtained from the above analyses conducted in duplicate.

The FISH (Fluorescent In Situ Hybridization) technique was performed once a week to detect just the presence of nitrifiers, both ammonia and nitrite-oxidizing bacteria. Since both of them are Gram negative cells, 1 ml aliquots of the sample were fixed in freshly prepared 4% paraformaldehyde (PFA) for 1-3 h at 4 ºC. After fixation, cells were washed with Phosphate Buffered Saline (PBS), resuspended in PBS-absolute ethanol

(1:1, v/v) and stored at 20 ºC [32]. Hybridizations were carried out as described in Amann *et al.* [33]. Oligonucleotide sequences employed in this study are listed in the Appendix (Table A.1). Hybridized samples were observed under epifluorescence microscope (Leica DM2500).

2.6 Statistical analysis

All analytical determinations were performed in duplicate. The results are given as the average with its corresponding standard deviation.

The data were analysed for significance (p-value = 0.05) using an analysis of variance (ANOVA) carried out by SPSS 16.1.

3. Results and Discussion

3.1 Effect of light

The first set of experiments (Exp. L1, Exp. L2 and Exp. L3) were aimed at evaluating the nutrient removal efficiency and the biomass productivity under 3 different light intensities: 40, 85 and 125 $\mu E \cdot m^{-2} \cdot s^{-1}$, respectively; maintaining constant temperature and working conditions. Table 2 shows the results obtained in this section.

Experiment L1 consisted of 17 days during which temperature remained stable (22 ± 1 ºC) and four fluorescent lamps were used to supply a light intensity of 40 $\mu E \cdot m^{-2} \cdot s^{-1}$. As can be seen in Figure 2, ammonium concentration in Exp. L1 started at 6.5 $mgN \cdot L^{-1}$ and the sum of nitrite and nitrate at 4.4 $mgN \cdot L^{-1}$ after the inoculation time. Immediately, the ammonium concentration started to decrease until it was almost depleted (ARE of 97.2 ± 2.3%), and the nitrite and nitrate began to accumulate, exceeding the nitrogen

discharge limit (10 mgN·L^{-1}) at day 5. At the end of the Exp. L1, the concentration of nitrite and nitrate remained approximately stable around 13 mgN·L^{-1}.

The accumulation of these two nitrogen species can be explained by the consumption of ammonium by ammonium-oxidizing bacteria (instead of microalgae). The presence of bacteria in the inoculum and the reduction of light intensity supply from 85 µE·m^{-2}·s^{-1} (during the inoculation) to 40 µE·m^{-2}·s^{-1} (in Exp. L1) limited microalgae growth in Exp.L1. Consequently, bacteria were able to compete with microalgae for ammonium uptake and nitrite and nitrate accumulated until equilibrium was established. Even though it is widely accepted that microalgae consume other nitrogen compounds different than ammonium; for instance, nitrogen oxides [34], the consumption of nitrite and nitrate was not noticeable in this experiment.

Because of the nitrification process, the ammonium removal efficiency only due to microalgae activity was calculated: 73.9 ± 2.9%. It must be highlighted that, even though a bacteria proliferation occurred, the phosphate removal efficiency remained at high values throughout Exp. L1 (98.6% ± 1.4), yielding a low P concentration along Exp. L1 (see Figure 2). Considering only the amount of ammonium consumed by microalgae, the N:P molar ratio remained at 13.3 ± 0.7 in Exp. 1.

The nitrification process also reduced the ammonium removal rate by microalgae, which showed a statistical significant difference (p-value < 0.05) with the total ammonium removal rate: 3.47 ± 0.95 mgN·L^{-1}·d^{-1} and 5.28 ± 0.93 mgN·L^{-1}·d^{-1}, respectively. Regarding phosphorus, PRR was 0.61 ± 0.14 mgP·L^{-1}·d^{-1}. These values are considerably low, since Ruiz-Martínez *et al.* [5] reported significant higher nutrient removal rates: mARR of 19.5 mgN·L^{-1}·d^{-1} and PRR of 3.7 mgP·L^{-1}·d^{-1} in a culture of *Scenedesmus* spp. fed by an AnMBR effluent at BRT of 2 days. This fact suggests the

system could be nutrient-limited due to the relatively high BRT (8 days), since the nutrient efficiency in Exp. 1 was close to 100% for both ARR and PRR (see Table 2).

Exp. L2 consisted of 29 days during which light intensity was the only operational condition modified, increasing from 40 to 85 $\mu E \cdot m^{-2} \cdot s^{-1}$. ARE and PRE remained at high values, similar to the values obtained in Exp. L1 (99.8± 0.2% and 99.2 ± 0.7%, respectively). Nevertheless, a statistically significant increase in mARE from 73.9 ± 2.9% to 99.3 ± 0.2% (p-value < 0.05) occurred. This fact indicates that increasing the light intensity of the system enhanced microalgae performance, giving algae an advantage in the microalgae-bacteria competition. Raising light intensity accelerates the microalgae metabolism as long as it remains under the optimum value. Hence, in these conditions, the microalgae were able to consume ammonium faster than the nitrifying bacteria. This is in accordance with Lydmark [35], who reported a higher ammonium uptake for *Scenedesmus obliquus* than for ammonia-oxidizing bacteria: 18.5 − 82.1 fmolN·cell^{-1}·h^{-1} and 0.03 − 53 fmolN·cell^{-1}·h^{-1}, respectively. The nitrification rate dropped and net nitrogen removal reached high values (99.3 ± 0.2%). As can be seen in Figure 2, from day 21 until the end of Exp. L2, the sum of nitrite and nitrate concentrations stayed lower than 0.6 ppm, which enabled meeting by far the legal requirements (10 mgN·L^{-1}).

Figure 3 shows nitrite and nitrate evolution in the early stages of Exp. L2 (experimental results) and the estimated nitrite and nitrate concentration trends due to nutrient washout only, leaving the other processes (nitrification, nitrite and nitrate uptake) out of consideration (continuous line). As can be observed in Figure 3, the experimental nitrite and nitrate concentrations were fairly lower than those estimated by washout. Thus,

microalgae could have consumed this amount of nitrite and nitrate when ammonium was depleted.

It is known that microalgae prefer ammonium as a nitrogen source [16, 36]. In fact, several studies have pointed out that nitrate consumption by microalgae does not occur until the ammonium is nearly completely removed [37], since the nitrate conversion to ammonium inside the cell requires energy [11]. Hence, it seems that microalgae were able to consume nitrite and nitrate in Exp. L2 because the ammonium concentration was low and because the light received in this case could have provided enough energy for the nitrate conversion to ammonium. On the other hand, in Exp. L1, due to the lack of energy at a low light intensity, algae were unlikely to absorb nitrite and nitrate and these nitrogen species were therefore accumulated (see Figure 2).

In Exp. L2 mARR also increased and reached a similar value than ARR: 6.43 ± 1.49 $mgN \cdot L^{-1} \cdot d^{-1}$ and 6.44 ± 1.47 $mgN \cdot L^{-1} \cdot d^{-1}$, respectively (p-value > 0.05); which reaffirms the dominance of microalgae in the ammonium competition with nitrifying bacteria. In Exp. L2, ARR and PRR were higher than in Exp. L1, because the nutrient load in this case was also higher (see Table 2).

Exp. L3 consisted of 34 days during which light intensity was further increased, from 85 to 125 $\mu E \cdot m^{-2} \cdot s^{-1}$. The calculated ARE and PRE were slightly higher: $99.9 \pm 0.2\%$ and $99.5 \pm 0.2\%$, respectively; but the differences with Exp. L2 were not statistically significant (p-value > 0.05). Higher mARE was also observed, reaching the maximum mARE value of the three experiments: $99.3 \pm 0.4\%$ (see Table 2). However, the higher mARE was not statistically significant either (p-value > 0.05). The small differences between Exp. L2 and L3 in terms of removal efficiencies could have been due to nutrient limitation. Under the fixed BRT of 8 days, the culture was able to remove

nearly 100% of the nitrogen and phosphorus in both Exp. L2 and Exp. L3. Nevertheless, the light intensity rise caused a significant increase in the biomass concentration from 658 ± 35 to 755 ± 79 mgVSS·L^{-1} (p-value < 0.05). It is possible that this biomass increment was due to the higher nutrient load in Exp L3 compared to Exp. L2 (see Table 2). This higher nutrient load would also explain the higher mARR, ARR and PRR attained in Exp. L3 compared to Exp. L2 (see Table 2).

In order to verify the light effect on nutrient removal, nutrient concentration was monitored during one feed cycle in both experiments. As can be seen in Figure 4, an increase in light intensity from 85 to 125 μE·m^{-2}·s^{-1} raised the one-cycle ammonium removal rate by 73% (from 29.7 to 51.7 mgN·L^{-1}·h^{-1}, respectively). These values correspond to specific one-cycle ammonium removal rates of 42.7 and 75.9 mgN·gVSS^{-1}·d^{-1}, respectively, or 194.3 and 338.6 fmolN·cell^{-1}·h^{-1}, respectively. These values are much higher than those obtained by Lydmark [35] for *Scenedesmus obliquus*: 18.5 – 82.1 fmolN·cell^{-1}·h^{-1}. Phosphorus was consumed very fast and not enough data was available to calculate the corresponding removal rate (data not shown). These results suggest that the system reveals a higher capacity to remove nitrogen and therefore was nutrient-limited. Therefore, the maximum mARR and PRR obtained: 6.92 ± 1.05 mgN·L^{-1}·d^{-1} and 0.90 ± 0.16 mgP·L^{-1}·d^{-1}, respectively, are not representative of the maximum capacity of the system to remove nutrients. Lower BRT should be tested to verify the maximum nutrient load that the system is able to remove from the AnMBR effluent to maintain meeting the effluent standards.

In the appendix, Figure A.1 shows the evolution in biomass productivity in Exp. L1, L2 and L3. Two remarkable increases in the biomass productivity were observed: from 50.5 ± 9.6 to 80.3 ± 6.5 mgVSS·L^{-1}·d^{-1} between Exp. L1 to L2 and from 80.3 ± 6.5 to

94.3 ± 7.9 mgVSS·L^{-1}·d^{-1} between Exp. L2 to L3. These results suggest that biomass productivity increases as light intensity rises, as long as it remains under a light intensity of 125 µE·m^{-2}·s^{-1}. Besides, Figure A.1 shows that the biomass productivity rise from Exp. L1 to L2 is more pronounced than the rise from Exp. L2 to L3. This is maybe due to the nutrient limitation which the culture was being carried out from day 21 on, as it has been already mentioned (see Figure 2). In fact, Pancha *et al.* [38] obtained a significant decrease in biomass productivity from a maximum value of 32.01 mgVSS·L^{-1}·d^{-1} when the nitrogen in the medium was 247 mgN·L^{-1}, to 8.48 mgVSS·L^{-1}·d^{-1} at nitrogen starvation conditions. On their behalf, Sarat Chandra *et al.* [39] reported a biomass productivity of 50 mgVSS·L^{-1}·d^{-1} in a culture of *Scenedesmus obtusus* fed by a modified Bold Basal medium with a light intensity of 60 µE·m^{-2}·s^{-1}, very similar to the value obtained in the present study for Exp. L1: 50.5 ± 9.6 mgVSS·L^{-1}·d^{-1}. Nevertheless, Ruiz-Martínez *et al.* [5] reported considerably higher biomass productivity: 234 mgVSS·L^{-1}·d^{-1}. As already mentioned, the results of Ruiz-Martínez *et al.* [5] were attained at a higher nutrient load. Therefore, it is deduced that higher productivity in Exp. L3 of the present study could have been expected if the nutrient load would have been higher.

Analysing the entire light intensity experimental period (Exp. L1, L2 and L3), it could be concluded that a light intensity increase from 40 to 85 µE·m^{-2}·s^{-1} seriously reduced the nitrification rate and increased the net nitrogen removal by microalgae from 73.9% ± 2.9 in Exp. L1 to 99.3% ± 0.2 in Exp. L2. For this reason, it was assumed that microalgae growth was limited due to the competition of nitrifying bacteria for ammonium in Exp. L1, in which mARE (73.9% ± 2.9) was substantially lower than ARE (97.2% ± 4.9), implying that 23.3% ± 5.1 of the total ammonium consumed was

used by nitrifying bacteria. On the other hand, microalgae were not limited by bacteria after light intensity was raised in Exp. L2 and L3, since the mARE was similar to ARE and close to 100% (see Table 2). It is remarkable that no addition of a nitrification inhibitor was needed for bacteria inhibition, unlike which has been reported by other authors [40]. Hence, supplying the proper light intensity may reduce the use of chemicals in wastewater treatment by microalgae. Regarding phosphorous, high PRE was reached in all the experiments: 98.6% ± 1.4, 99.2%± 0.7 and 99.5% ± 0.2 in Exp. L1, L2 and L3, respectively; thus no significant differences in PRE were registered (p-value>0.05).

Everything mentioned above can be supported by the fact that eukaryotic cell density rose as light intensity increased (6.55 ± 0.04, 11.90 ±1.59 and 14.61 ± 2.91 ($x10^9$) cells·L^{-1}, in Exp. L1, L2 and L3, respectively). Cell counts did not reveal any variation in culture composition since *Scenedesmus* spp. remained as dominant genus (>99% during all the experiments). Regarding the samples observed under the epifluorescence microscope, even though microalgae were dominant, the FISH technique allowed us to identify some groups of nitrifying bacteria in Exp. L1 (see Figure 6a and 6b). On the other hand, the bacteria population was negligible in Exp. L2 (see Figure 6c and 6d).

Similarly to the results obtained for cell density, VSS rose as light intensity increased: 429 ± 15, 658 ± 35 and 755 ± 79 mg·VSS L^{-1}, in Exp. L1, L2 and L3, respectively, which is an indicator that most of the biomass corresponded to eukaryotic microalgae. Moreover, the fact that the chlorophyll content dropped from Exp.L1 to Exp. L2 and Exp. L3 (4.18 ± 0.99, 2.44 ± 0.59 and 1.44± 0.44 mg·gVSS^{-1}, respectively) confirmed the results observed by Reynolds [22] and Chen *et al*. [41]. Reynolds [22] stated that at low levels of irradiance, one of the mechanisms used to enhance photosynthetic

potential is to increase cell-specific light-harvesting capacity, which involves synthesis in higher quantities of chlorophyll. On the other hand, Chen *et al.* [41] noticed that at higher light intensities, microalgae reduce chlorophyll content in order to reduce photon absorption and to protect themselves against photoinhibition, which agrees with the results obtained in the present study. Sarat Chandra *et al.* [39] also obtained a higher chlorophyll concentration with a light intensity of 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ compared to 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for *Scenedesmus obtusus.*

In Exp. L1, L2 and. L3, the N:P ratio obtained for microalgae was 13.3 ± 0.7, 18.5 ± 1.1 and 17.6 ± 1.7, respectively. However, the culture did not suffer a significant change in microalgae composition during the entire experimental period as *Scenedesmus* spp. remained as dominant genus (>99%). It is therefore possible that in Exp. L1 the microalgae consumed higher amount of phosphorus in proportion, accumulating it as internal polyphosphate reserves. According to Powell *et al.* [42], microalgae are able to store large amounts of phosphorus in order to accumulate intracellular polyphosphate. Theoretically, this polyphosphate is able to sustain three or even four cell doublings without taking up any more phosphorus [22]. It was assumed that no phosphorus precipitation took place, since the culture pH values (7.4 ± 0.1) and low ions concentration made this precipitation unlikely.

It has been shown that the operating conditions (temperature, pH, nutrient loading and BRT) clearly have an effect on the microalgae-bacteria competition. In Exp. L1, the light intensity supplied was too low, and the system was unable to remove nitrogen under the discharge limits. On the other hand, Exp. L2 achieved ARE and PRE values close to 100%. In Exp. L3 (with considerably higher light intensity) the rise in these values was not significant (p-value > 0.05). In order to choose the most efficient PBR

configuration, the nutrient removal rate:light irradiance ratio (both mARR/I and PRR/I) was calculated. This parameter informs about the amount of nutrients that microalgae absorb for every mole of photons that they receive. In Exp. L2 and. L3, the mARR/I obtained was 76.7 ± 17.8 and 59.0 ± 8.9 mgN·molphoton^{-1}, respectively; meanwhile the PRR/I resulted in Exp. L2 and. L3 were 15.5 ± 3.5, 9.3 ± 1.9 and 7.6 ± 1.4 mgP·molphoton^{-1}, respectively. These results suggest that the use of light by the culture was more efficient in Exp. L2, when lower light intensity was supplied to the system. For this reason, Exp. L2 might be the most cost-effective configuration to achieve an efficient nutrient removal, which implies meeting the discharge limits for both nitrogen and phosphorus. Regarding biomass productivity, Exp. L3 showed a significant increase compared to Exp. L2; although this increment could have been influenced by the higher nutrient load received in Exp. L3 (57.3 ± 5.0 mgN·d^{-1} and 7.4 ± 0.7 mgP·d^{-1}) compared to Exp. L2 (56.2 ± 8.4 mgN·d^{-1} and 6.8 ± 0.7 mgP·d^{-1}). Thus, it cannot be assured that light intensity is the main factor that made obtain higher biomass productivity when the light supplied was increased from 85 µE·m^{-2}·s^{-1} to 125 µE·m^{-2}·s^{-1}.

3.2 Effect of temperature

The goal of this second set of experiments was to check whether microalgae were able to dominate the ammonium competition under different temperatures with the same light intensities that were previously studied and where microalgae took advantage over ammonium oxidizing bacteria: 85 µE·m^{-2}·s^{-1} and 125 µE·m^{-2}·s^{-1} (see Exp. L2 and L3). The other operational parameters (pH, BRT, HRT) remained at the same values than in Exp. L1, L2 and L3.

Figure 5 shows the nutrient concentration and the temperature evolution with daily maximum and minimum temperatures during Exp. T1 and T2.

As can be seen in Figure 5a, Exp. T1 had maximum daily temperatures (marked as vertical lines) of 27 and 28 ºC, respectively, the first two days of the experiment. From the second day on, the nitrite-nitrate concentration started to increase, suggesting that a proliferation of nitrifying bacteria was taking place although the temperature remained almost constant until day 11 (with the exception of day 6, see Figure 5a). It is important to notice that ammonium concentration remained negligible during all Exp. T1. Hence, it can be deduced that punctual variations of the culture temperature at a light intensity of 85 $\mu E \cdot m^{-2} \cdot s^{-1}$ are enough to favour ammonium oxidizing bacteria growth with regard to microalgae growth. This caused a remarkable decreased in mARE, which was 73.6% at the end of Exp. T1; meanwhile, in Exp. L2, with the same light intensity and controlled temperature, mARE had high values: $98.3 \pm 1.9\%$. A decrease in mARR/I was also observed: from 76.7 ± 17.8 mgN·mol^{-1} in Exp. L2 to 59.8 ± 12.8 mgN·mol^{-1}.

On the other hand, the removal of phosphorus during this period was not affected by the AOB proliferation, since it remained at negligible concentrations during the entire Exp. T1 (see Figure 5a).

Regarding Exp. T2, it can be observed that a continuous rise in nitrite and nitrate concentration occurred after day 8 (see Figure 5b), reaching a concentration of nitrite and nitrate of 36.1 mgN·L^{-1} the day 23. It is remarkable that these compounds patterns are similar to those shown in Exp. T1, suggesting that nitrifying bacteria proliferation started at that moment and continued until the end of Exp. T2 (see Figure 5b). By the end of Exp. T2, the culture was dominated by bacteria, which implied, apart from the high concentration of nitrite and nitrate, that the concentration of phosphate in the

culture was not negligible as occurred in the rest of experiments. In fact, in day 29 of Exp. T2, the phosphate concentration was higher than 2 mgP·L^{-1}. This provoked that mARE and PRE decreased from nearly 100% when the temperature was controlled (Exp. L3), to 24.2% and 63.7%, respectively at the end of Exp. T2. A decrease in mARR/I was also observed: from 59.0 ± 8.9 mgN·mol^{-1} in Exp. L3 to 36.2 ± 17.6 mgN·mol^{-1} in Exp. T2. Thus, when temperature rose above a limit temperature, microalgae were not able to use the light energy for nitrogen removal as efficiently as they did when temperature was under control.

It must be highlighted that in Exp. T2 (light intensity of 125 µE·m^{-2}·s^{-1}), the proliferation of nitrifying bacteria (inferred through the nitrite and nitrate accumulation) started to take place from a maximum temperature of 32 ºC; meanwhile in Exp. T1 (light intensity of 85 µE·m^{-2}·s^{-1}), the proliferation of bacteria started to occur from a maximum temperature of 28 ºC. On the other hand, in Exp. L1 (light intensity of 40 µE·m^{-2}·s^{-1}), the proliferation of bacteria occurred at a maximum temperature of 23 ºC. This suggests that the higher the light intensity supplied to the PBR is, the higher the temperature is needed for the nitrifying bacteria to proliferate.

Cabello *et al.* [43] verified that the optimum temperature for *Scenedesmus obtusiusculus* was 35 ºC under normal growth conditions and 28.5 ºC under nitrogen-limiting conditions. It therefore seems that a pure microalgae culture is able to grow successfully within the temperature range registered in Exp. T1 and T2. However, in the present study, due to the algae-bacteria competition, these temperatures reported by Cabello *et al.* [43] were over the optimum value for microalgae growth.

Summarising, it can be concluded that the operational conditions in these experiments of 85 and 125 µE·m^{-2}·s^{-1} and achieving temperatures above 28 ºC in the case of 85

$\mu E \cdot m^{-2} \cdot s^{-1}$ (Exp. T1), and above 32 ºC in the case of 125 $\mu E \cdot m^{-2} \cdot s^{-1}$ (Exp. T2) reduce the microalgae activity and allow the nitrifying bacteria growth; making the culture unable to remove nitrogen efficiently.

To verify that nitrifying bacteria proliferated in both Exp. T1 and T2, the FISH technique was used. The FISH technique identified nitrifying bacteria in Exp. T1 (see Figure 6e), as well as detecting the presence of other bacteria in T1 and T2 (see Figure 6f and 6g). The rise in filamentous *Cyanobacteria* was observed in Exp. T2 (see Figure 6h), which is in concordance with Zhu *et al.* [44], who reported that high temperatures stimulated the growth of cyanobacteria in water bodies, especially at low nutrient levels.

4. Conclusions

It has been demonstrated that using a microalgae-bacteria culture as tertiary treatment allows nutrient effluent standards to be met, as long as nitrification is controlled and the culture is not dominated by bacteria. These limits were not exceeded in Exp. L2 and L3, when microalgae were the main organism in the culture. On the other hand, the presence of nitrifying bacteria in the PBR has been shown to clearly reduce the nitrogen removal rate of the system, since they compete with microalgae for ammonium. In Exp. L1, T1 and T2, when nitrifying bacteria grew remarkably, the discharge limit for nitrogen was not achieved, and for phosphorus was met only in Exp. L1 and T1. Hence, if maximum performance of microalgae is required, the optimal conditions for their growth must be supplied in order for microalgae to dominate the competition.

Increasing light intensity favoured microalgae growth and the advantage of algae over the nitrifying bacteria. In fact, biomass productivity raised from 50.5 ± 9.6 mgVSS·L^{-1}·d^{-1} in Exp. L1 to 80.3 ± 6.5 mgVSS·L^{-1}·d^{-1} in Exp. L2 and 94.3 ± 7.9 mgVSS·L^{-1}·d^{-1}

in Exp. L3; which implied that nitrogen removal efficiency by microalgae reached high values (over 99.0 %) in Exp. L2 and L3.

It was observed that the PBR removed nitrogen and phosphorus efficiently when the temperature was around 22 ºC. In this situation, no significant proliferation of nitrifying bacteria was observed. When the temperature rose, bacteria took advantage for ammonium uptake and thus the microalgal culture could not remove enough nutrients to meet the effluent limits established in the European Union Council Directive 91/271/EEC.

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Table 1. Operational conditions of the semi-continuous photo-bioreactor during the experiments.

Experiment		Operational days	PBR light intensity ($\mu E \cdot m^{-2} \cdot s^{-1}$)	Temperature (ºC)	Dissolved oxygen (% saturation)
Light effect	L1	17	40	22 ± 1	102 ± 4%
	L2	29	85	22 ± 1	105 ± 2 %
	L3	34	125	22 ± 1	110 ± 2 %
Temperature effect	T1	12	85	23 ± 2	108 ± 6%
	T2	30	125	28 ± 2	106 ± 5%

Table 2. Mean values ± standard deviation on steady stage (Exp. L1, L2 and L3).

| Parameter	Units	Exp. L1	Exp. L2	Exp. L3
VSS	$mgVSS \cdot L^{-1}$	429 ± 15	658 ± 35	755 ± 79
Eukaryotic cells (x10^9)	$cells \cdot L^{-1}$	6.55 ± 0.04	11.90 ± 1.59	14.61 ± 2.91
N-load	$mg \cdot d^{-1}$	48.2 ± 5.9	56.2 ± 8.4	57.3 ± 5.0
P-load	$mg \cdot d^{-1}$	5.9 ±0.7	6.8 ± 0.7	7.4 ± 0.7
N:P ratio	molar	13.3 ± 0.7	18.5 ± 1.1	17.6 ± 1.7
mARE	%	73.9 ± 2.9	98.3 ± 1.9	99.3 ± 0.4
ARE	%	97.2 ± 2.3	99.8 ± 0.2	99.9 ± 0.2
PRE	%	98.6 ± 1.4	99.2 ± 0.7	99.5 ± 0.2
mARR	$mgN \cdot L^{-1} \cdot d^{-1}$	3.47 ± 0.95	6.43 ± 1.49	6.92 ± 1.05
ARR	$mgN \cdot L^{-1} \cdot d^{-1}$	5.28 ± 0.93	6.44 ± 1.47	6.96 ± 1.03
PRR	$mgP \cdot L^{-1} \cdot d^{-1}$	0.61 ± 0.14	0.78 ± 0.16	0.90 ± 0.16
mARE/I	$mgN \cdot molphoton^{-1}$	88.1 ± 24.2	76.7 ± 17.8	59.0 ± 8.9
ARE/I	$mgN \cdot molphoton^{-1}$	134.1 ± 23.7	76.8 ± 17.6	59.3 ± 8.8
PRE/I	$mgP \cdot molphoton^{-1}$	15.5 ± 3.5	9.3 ± 1.9	7.6 ± 1.4
Chlorophyll content	$mg \cdot gVSS^{-1}$	4.18 ± 0.99	2.44 ± 0.69	1.88 ± 0.44
Productivity	$mgVSS \cdot L^{-1} \cdot d^{-1}$	50.5 ± 9.6	80.3 ± 6.5	94.3 ± 7.9
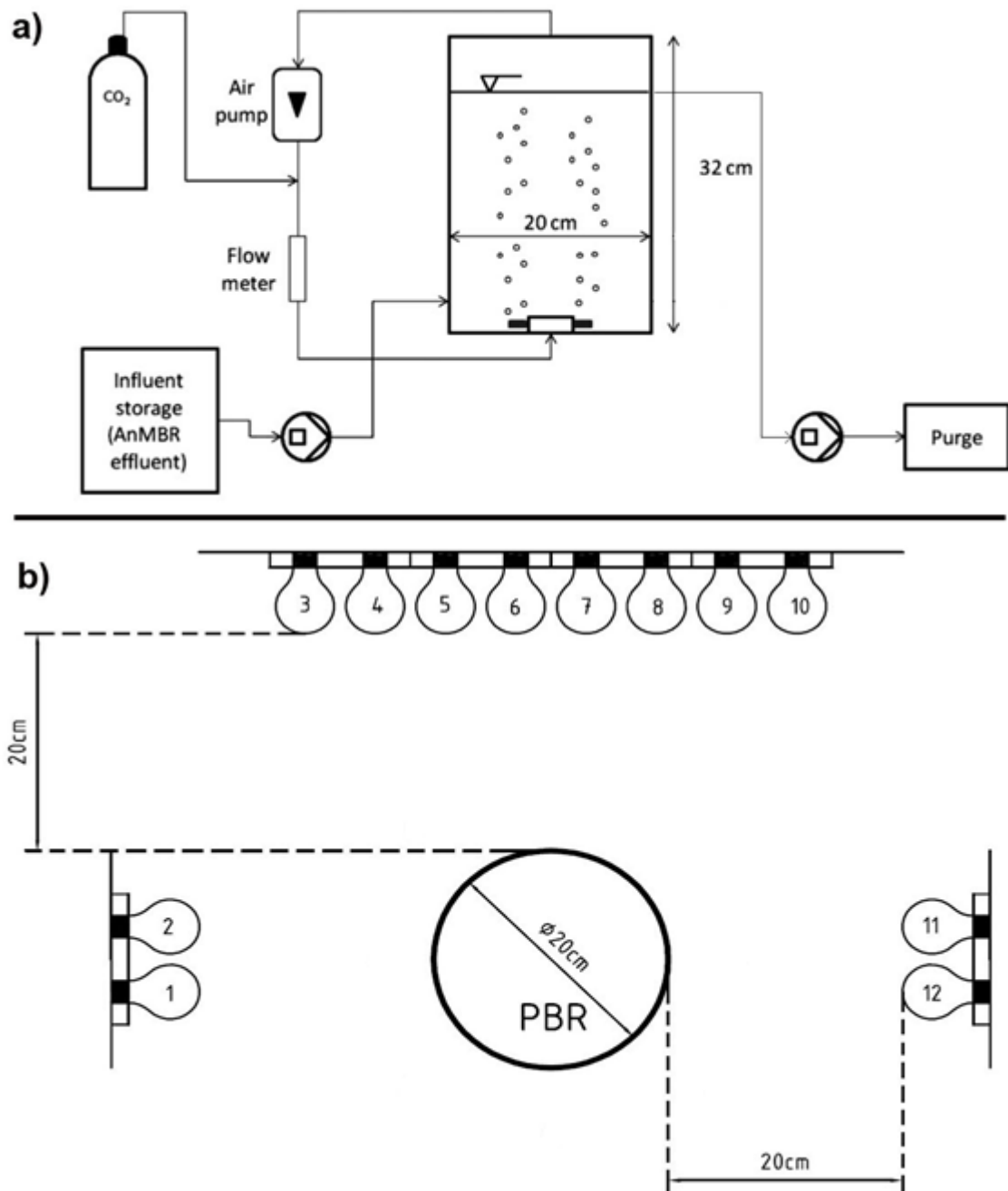
Figure 1. a) Lab-scale cylindrical photobioreactor [5]; and b) lamps distribution around the PBR.
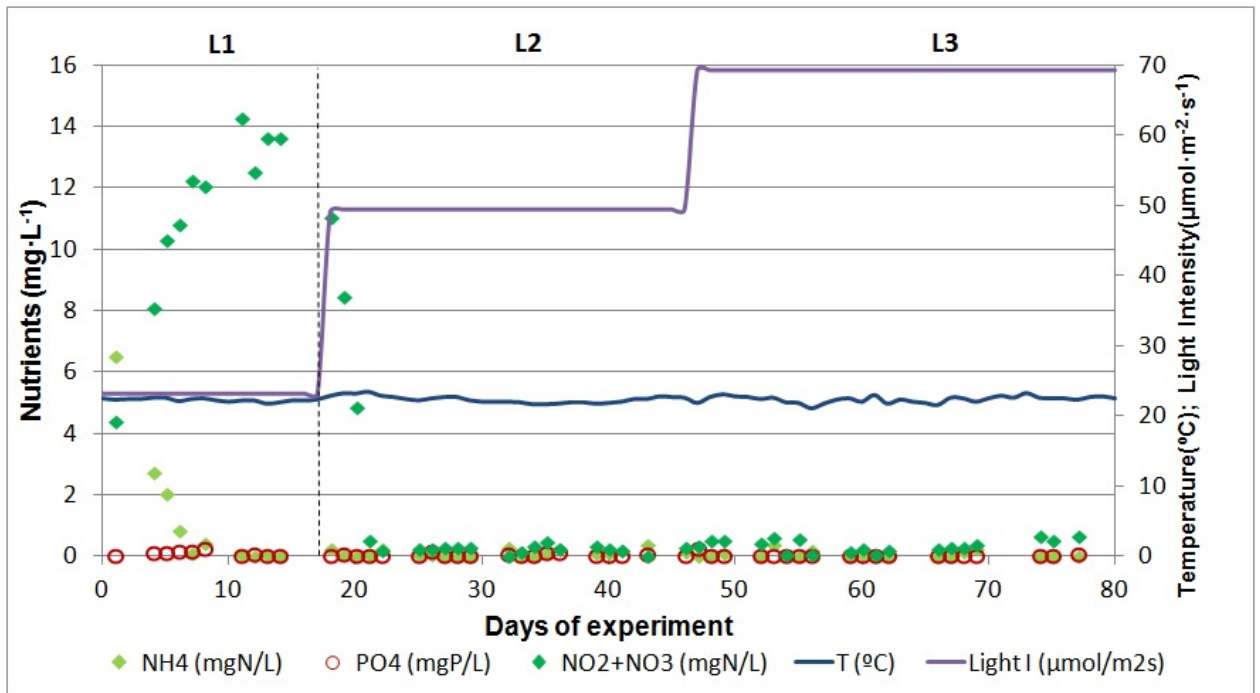
Figure 2. Evolution of nutrients concentration, temperature and light intensity in the PBR during Exp. L1, L2, and L3.
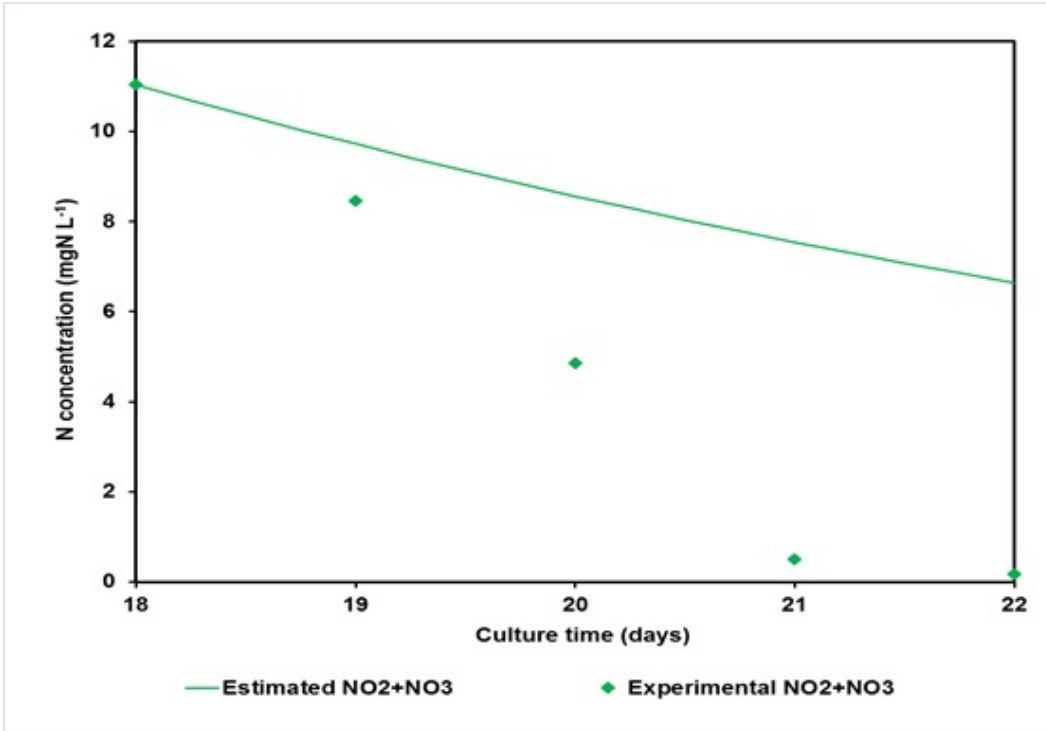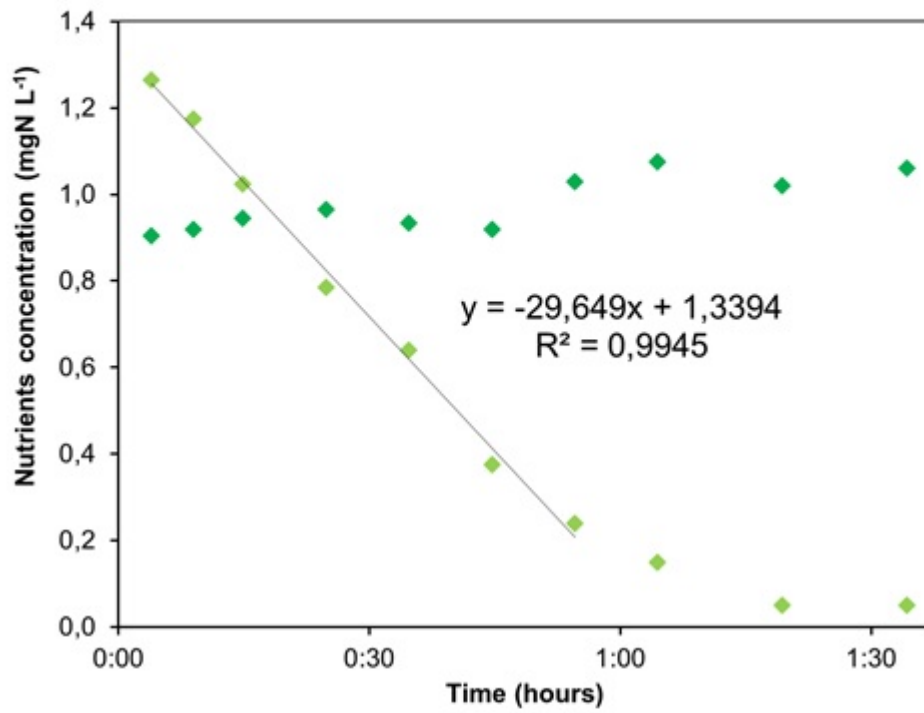
Figure 3. Nitrite and nitrate evolution during the first days of the Exp. L2: experimental

results and theoretical washout calculated not considering nitrification.

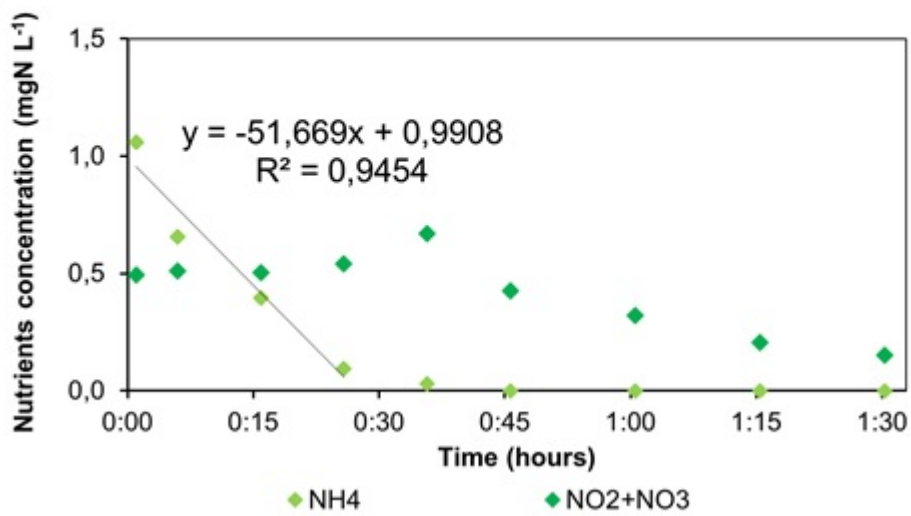Figure 4. Nutrient removal rate monitoring during one cycle feed: a) in Exp. L2; and b) in Exp. L3.
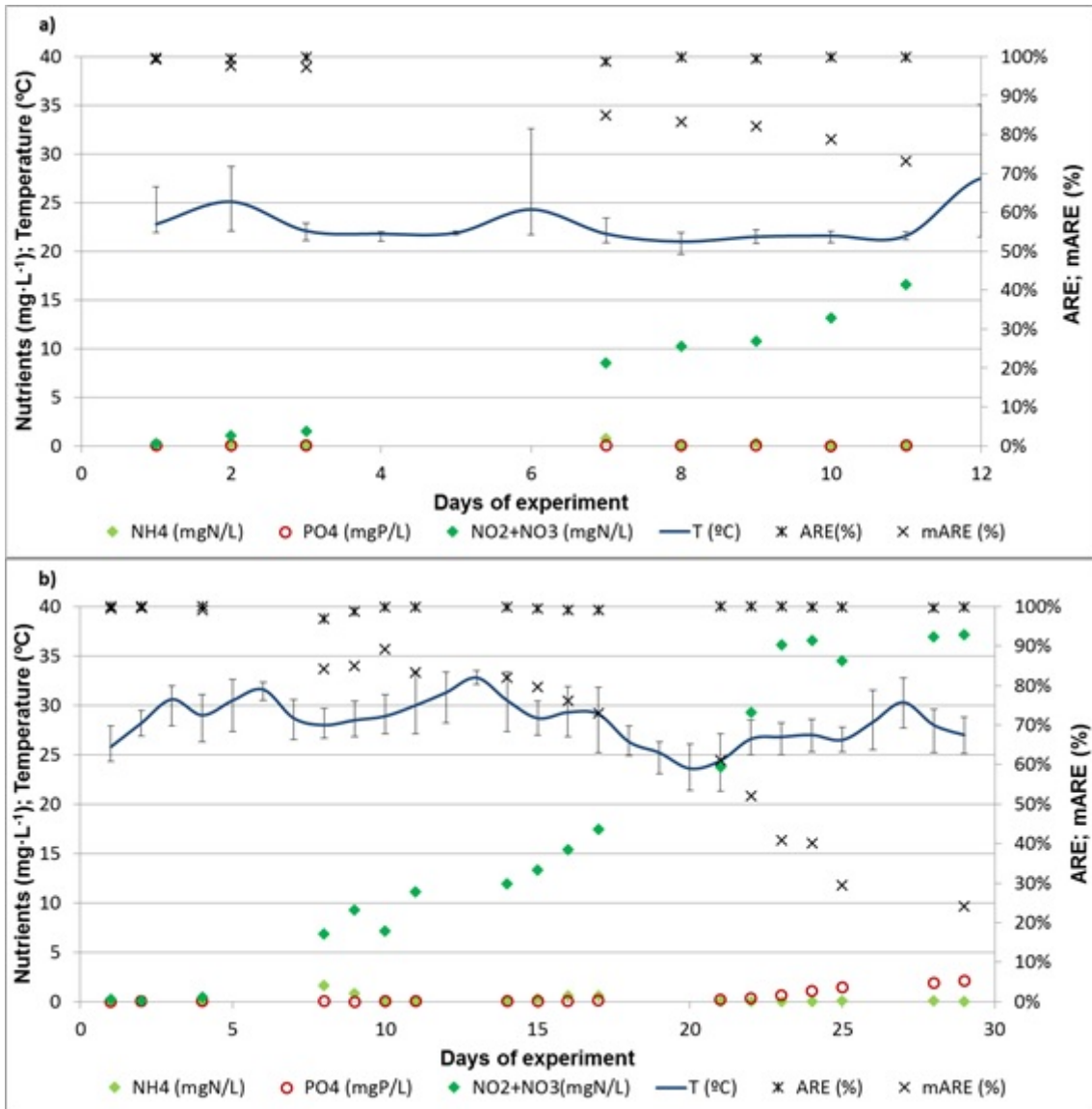
Figure 5. Evolution of nutrients concentration and temperature in the PBR: a) In Exp. T1; b) in Exp. T2.
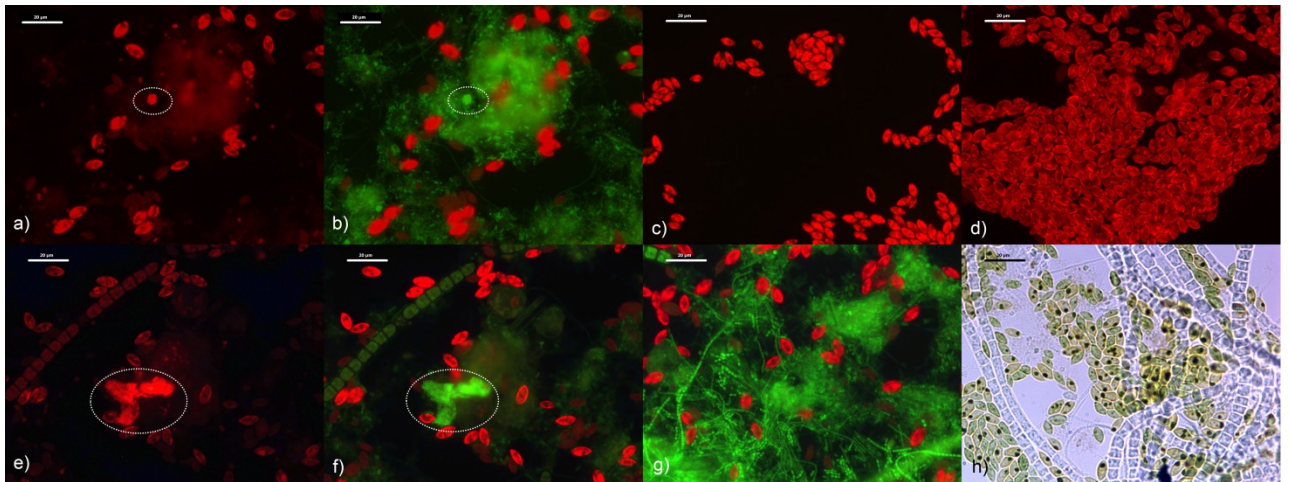
Figure 6. Samples observed under epifluorescence microscope (Leica DM2500/ DFC420c digital camera) using a 63x objective. Scale bar = 20µm. (a, b) Exp. L1, the same fields of view are shown: a [hybridized with NSO1225 probe (red), a little group of AOB indicated by a white circle] and b [hybridized with EUBMIX probe (green)]; (c, d) Exp. L2, *Scenedesmus* spp. as dominant genus; (e, f) Exp. T1, the same fields of view are shown: e [hybridized with NSO1225 probe (red), a higher group of AOB indicated by a white circle] and f [hybridized with EUBMIX probe (green)]; (g) Exp. T2, sample hybridized with EUBMIX probe (green) allows to identify diverse groups of bacteria; (h) Exp. T2, bright-field image showing *Scendesmus* spp. and *Cyanobacteria*.

Table A1. Oligonucleotide sequences employed in this study.

| Probe		Sequence (5'→3')	Specificity	% FA	Ref.
EUBmix	EUB 338 I	GCT GCC TCC CGT AGG AGT	Eubacteria	0-50	[33]
	EUB 338 II	GCA GCC ACC CGT AGG TGT	Planctomycetes	0-50	[45]
	EUB 338 III	GCT GCC ACC CGT AGG TGT	Verrucomicrobiales	0-50	[45]
NSO1225		CGC CAT TGT ATT ACG TGT GA	Betaproteobacterial ammonia-oxidizing bacteria	45	[46]
Ntspa712		CGC CTT CGC CAC CGG CCT TCC	Most members of the phylum Nitrospirae	50	[47]
NIT 3		CCT GTG CTC CAT GCT CCG	*Nitrobacter spp*	40	[48]
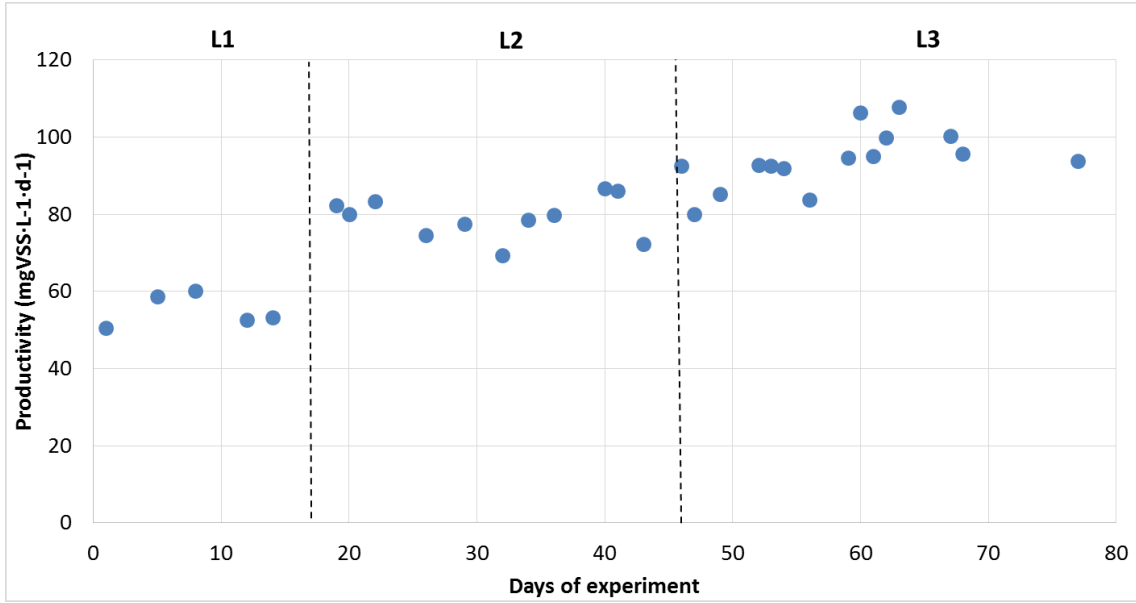
Figure A.1. Evolution of biomass productivity during Exp. L1, L2, and L3.