Gonzalez-Camejo, J.; Aparicio Antón, SE.; Ruano, M.; Borrás, L.; Barat, R.; Ferrer, J. (10-2). Effect of ambient temperature variations on an indigenous microalgae-nitrifying bacteria culture dominated by Chlorella. Bioresource Technology. 290:1-10. https://doi.org/10.1016/j.biortech.2019.121788

Effect of ambient temperature variations on an indigenous microalgae-nitrifying bacteria culture dominated by *Chlorella*

J. González-Camejo[a*], S. Aparicio[b], M.V. Ruano[b], L. Borrás[b], R. Barat[a] and J. Ferrer[a]

[a]*CALAGUA – Unidad Mixta UV-UPV, Institut Universitari d'Investigació d'Enginyeria de l'Aigua i Medi Ambient – IIAMA, Universitat Politècnica de València, Camí de Vera s/n, 46022 Valencia, Spain.*

[b]*CALAGUA – Unidad Mixta UV-UPV, Departament d'Enginyeria Química, Universitat de València, Avinguda de la Universitat s/n, 46100 Burjassot, Valencia, Spain.*

**Corresponding author*

Email address: jogonca4@upv.es (J. González-Camejo)

ABSTRACT

Two outdoor photobioreactors were operated to evaluate the effect of variable ambient temperature on an indigenous microalgae-nitrifying bacteria culture dominated by *Chlorella*. Four experiments were carried out in different seasons, maintaining the temperature-controlled PBR at around 25 ºC (by either heating or cooling), while the temperature in the non-temperature-controlled PBR was allowed to vary with the ambient conditions. Temperatures in the range of 15-30 ºC had no significant effect on the microalgae cultivation performance. However, when the temperature rose to 30-35 ºC microalgae viability was significantly reduced. Sudden temperature rises triggered AOB growth in the indigenous microalgae culture, which worsened microalgae performance, especially when AOB activity made the system ammonium-limited. Microalgae activity could be recovered after a short temperature peak over 30 ºC once the temperature dropped, but stopped when the temperature was maintained around 28-30 ºC for several days.

1. INTRODUCTION

Since wastewater contains large amounts of nitrogen and phosphorus, these nutrients have traditionally been removed from water to avoid eutrophication issues (Song et al., 2018). However, classical nitrification-denitrification and phosphorus precipitation

processes release nitrogen into the atmosphere and lose phosphorus with the sludge (Acién et al., 2016). On the other hand, microalgae are able to recover the nutrients present in wastewater (AlMomani et al., 2019; Ledda et al., 2015), while producing valuable microalgae biomass (Acién et al., 2016). Microalgae-based wastewater treatment thus presents as a win-win solution to recover nutrients from water.

Due to their adaptability to wastewater and their striking resistance against protozoa, the green microalgae *Chlorella* is one of the most frequently used to recover nutrients from wastewater (Gupta et al., 2019; Sforza et al., 2014; Yang and Kong, 2011). To achieve maximum growth, microalgae must be maintained at optimum temperature (Huang et al., 2019; Ippoliti et al., 2016). Lower than optimal temperatures limit their growth rate by affecting the kinetics of the cell enzymatic processes (Binnal and Babu, 2017; Huang et al., 2017; Manhaeghe et al., 2019; Serra-Maia et al., 2016). On the other hand, temperatures over the limit deactivate some of the proteins involved in photosynthesis, which reduces the performance of microalgae and can even lead to cell death (Nwoba et al., 2019; Ras et al., 2013; Serra-Maia et al., 2016). In addition, temperature also affects some other parameters related to microalgae growth, e.g. the level of CO_2 solubility in the medium and the pH-value (Binnal and Babu, 2017; Xu et al., 2019). It also affects the light intensity above which microalgae get photoinhibited (Huang et al, 2017), e.g. microalgae tolerate higher light irradiance at temperatures near the optimum (Nwoba et al., 2019). Optimal temperatures of *Chlorella* species have been widely reported in the literature. However, these optimal temperatures are species-specific and results are often controversial. For instance, Sforza et al. (2014) found the optimal temperature of *C. protothecoides* for the treatment of primary effluent to be 30 ºC; while Binnal and Babu (2017) obtained 25 ºC as optimum for the growth of *C. protothecoides* in secondary effluent and Huang et al. (2019) reported 38.7 ºC as the optimum for *C. pyrenoidosa* grown in synthetic water. It should also be borne in mind that all of these studies were carried out in controlled lab conditions However, these lab-scale assays do not reflect the fluctuation of ambient temperatures when microalgae are cultivated outdoors (Gupta et al., 2019; Ling et al., 2019). Temperature variations under outdoor conditions can be especially critical for microalgae growth in closed photobioreactors (PBRs) since there are no evaporation losses that can regulate temperature (Yeo et al., 2018); especially during the summer time in temperate regions (Huang et al., 2017; Nwoba et al., 2019) such as those of the Mediterranean coast. Indeed, Wang et al. (2012) reported that the temperature inside a closed PBR can be around 10-30 ºC higher than the ambient

temperature. Hence, the outdoor evaluation of the appropriate temperature range of indigenous microalgae cultivated in photobioreactors appears to be essential for the application of this technology at industrial scale. However, scarce studies have focused on evaluating the single effect of temperature on the performance of outdoor microalgae PBRs.

It must be also considered that under outdoor conditions, indigenous microalgae tend to dominate the culture since they are better adapted to such conditions, obtaining higher performance than pure cultures (Thomas et al., 2019). Indigenous microalgae coexist with other microorganisms present in wastewater, such as heterotrophic and nitrifying bacteria, protozoa, rotifers, etc. (Sforza et al., 2014), which compete with microalgae for nutrients. In this respect, the competition between microalgae and ammonium-oxidising bacteria (AOB) for ammonium uptake should be controlled, since AOB can reduce microalgae growth by depleting the ammonium concentration in the media (González-Camejo et al., 2018a), hence limiting the performance of the process. Within this microalgae-AOB competition, temperature plays a key role since AOB growth increases sharply at higher temperatures (Jiménez, 2010). This effect has been previously observed under lab conditions of constant temperature (González-Camejo et al., 2018b). However, to the best of our knowledge the effect of variable ambient temperature on microalgae-AOB competition has not been evaluated before. Further research is therefore needed to fully understand the behaviour of an indigenous microalgae culture in outdoor wastewater treatment.

In this context, the aim of this study was to analyse the effect of ambient temperature variations on an indigenous microalgae-nitrifying bacteria culture (dominated by *Chlorella*) which continuously treated the effluent from a sewage-fed AnMBR system. The optimal temperature range of indigenous *Chlorella* growth was first evaluated by operating two flat-panel PBRs during different seasons of the year (without nitrification). Later, the microalgae-AOB competition for ammonium was assessed during the continuous operation of the PBRs under variable ambient temperatures.

2. MATERIAL AND METHODS

2.1. Microalgae substrate and inoculum

The substrate used in this study was the effluent of an AnMBR plant that treated effluent from the primary settler of the Carraixet wastewater treatment plant (WWTP)

(39º30’04.0’’N 0º20’00.1’’W, Valencia, Spain). This plant is described in Seco et al. (2018).

Nitrogen concentration varied in the 35-58 mg $N \cdot L^{-1}$ range, while phosphorus concentration was between 3.5-6.0 mg $P \cdot L^{-1}$. As the AnMBR effluent was aerated in a regulation tank to fully oxidise sulphide into sulphate before being fed to the PBRs, negligible concentrations of sulphide were detected in the PBR influent, thus avoiding microalgae limitation by sulphide (González-Camejo et al., 2017).

Indigenous microalgae were obtained from a mixed culture dominated by green microalgae *Chlorella* (> 99% total eukaryotic cells (TEC)). *Scenedesmus* (< 1% TEC), cyanobacteria, nitrifying and heterotrophic bacteria were also present in lower concentrations.

2.2. PBR pilot plant

Microalgae were cultivated in two outdoor, flat-plate, 1.10-m high x 2-m wide x 0.25-m deep, methacrylate PBRs (PBR-A and PBR-B) with working volumes of 550 L.

The PBRs were continuously sparged by air at a flow rate of 0.10 vvm through two perforated pipes (on the bottom of the PBRs) to homogenise the culture and reduce wall fouling. Oxygen concentrations in the PBRs were in the range of 10-15 mg $O_2 \cdot L^{-1}$, thus avoiding oxygen inhibition of microalgae (Pawlowski et al., 2016). Pure CO_2 (99.9%) was injected into the air system whenever pH was over a set-point of 7.5.

The PBRs were illuminated by twelve LED lamps (Unique Led IP65 WS-TP4S-40W-ME) installed on the rear wall, offering an average light irradiance of 300 $\mu E \cdot m^{-2} \cdot s^{-1}$.

Each PBR incorporated one pH-temperature transmitter (pHD sc Hach Lange), one dissolved oxygen sensor (LDO Hach Lange) and one irradiation sensor (Apogee Quantum) attached to the PBR surface to measure only photosynthetically active radiation (PAR). These on-line sensors allowed continuous data acquisition as explained in Viruela et al. (2018).

PBR temperature was controlled by a water heating and cooling device with a thermostat (Daikin Inverter R410A). Heated or cooled water was supplied to the PBRs by a pump and 20-m long coiled pipe (set inside each PBR). The chosen temperature set-point for heating was 30 ºC and 16 ºC for cooling. The cooling/heating fluid was automatically pumped into the PBRs by opening an electrovalve whenever the temperature went outside the set-point range of 21-25 ºC.

Further information about the PBR plant can be found in González-Camejo et al. (2019).

2.3. Experimental set-up

The effect of temperature on the mixed microalgae culture was assessed in terms of: i) biomass productivity and nutrient recovery, and ii) microalgae-AOB competition. Before each experiment, a start-up phase (described in González-Camejo et al., 2018a) was initiated to reach a consistent culture with a biomass concentration of around 300-400 mg VSS·L^{-1}.

2.3.1. Effect of temperature in nutrient recovery and biomass productivity

The effect on nutrient recovery and biomass productivity was analysed through 4 experiments carried out in different periods of the year: autumn, winter, spring and summer. During this first set of experiments, the PBRs were in semi-continuous operation under the same nutrient loading rate, air sparging flow rate and hydraulic retention time (HRT) of 6 days (i.e. 6-day BRT). They also received the same average solar PAR (Table 1). A concentration of 5 mg·L^{-1} of allylthiourea (ATU) was maintained in both reactors to inhibit AOB growth (González-Camejo et al., 2018a; Krustok et al., 2016). The only parameter that varied was the culture temperature. PBR-A was the temperature-controlled PBR, which was heated up in autumn and winter and cooled down in spring and summer to maintain a culture temperature of around 25 ºC (Table 1). PBR-B was the non-temperature-controlled PBR and thus varied freely with natural temperature variations throughout the year (Gupta et al., 2019).

Table 1. Operating conditions in the evaluation of the effect of temperature in nutrient recovery and biomass productivity.

Exp.	**Days of operation**	**Light intensity (µmol·m^{-2}·s^{-1})**	**Temperature (ºC)**		**Temperature control**	
			PBR-A	**PBR-B**	**PBR-A**	**PBR-B**
1.1	29	254 ± 147	24.0 ± 1.4	20.6 ± 1.6	H	NC
1.2	14	184 ± 130	22.8 ± 2.4	16.4 ± 2.7	H	NC
1.3	16	225 ± 40	25.0 ± 1.5	28.8 ± 1.5	C	NC
1.4	25	262 ± 85	25.6 ± 1.4	31.5 ± 1.8	C	NC

H: heating; NC: no control of temperature; C: cooling.

2.3.2. Effect of temperature in microalgae-AOB bacteria competition

In a second set of experiments (2.1 and 2.2) PBR-A and PBR-B were operated in the same conditions (BRT = HRT = 6 days) in which temperature was allowed to vary but was the same in both PBRs. However, ATU concentration was kept at 5 $mg \cdot L^{-1}$ in PBR-A to inhibit AOB growth (González-Camejo et al., 2018a), thus being the nitrification-inhibited PBR. On the other hand, no AOB inhibitor was added to PBR-B. PBR-B was hence the non-nitrification-inhibited PBR.

2.4. Sampling and calculations

Duplicate grab samples were collected from the microalgae substrate (influent) and PBR effluent three times a week. Ammonium (NH_4), nitrite (NO_2), nitrate (NO_3) and phosphate (PO_4) were analysed according to Standard Methods (APHA, 2005): 4500-NH3-G, 4500-NO2-B, 4500-NO3-H and 4500-P-F, respectively, on an automatic analyser (Smartchem 200, WestcoScientific Instruments, Westco). Volatile suspended solids (VSS) concentration was also measured three times a week in duplicate according to method 2540 E of the Standard Methods (APHA, 2005).

Nitrogen recovery efficiency (NRE), phosphorus recovery efficiency (PRE) and biomass productivity (BP) were calculated according to Eq.1, Eq. 2 and Eq. 3, respectively:

$$\text{NRE}\ (\%) = \frac{N_i - N_e}{\text{N}_\text{i}} \cdot 100 \qquad \text{(Eq. 1)}$$

where N_i is the nitrogen concentration of the influent (mg $N \cdot L^{-1}$) and N_e is the nitrogen concentration of the effluent (mg $N \cdot L^{-1}$).

$$\text{PRE}\ (\%) = \frac{\text{P}_i - \text{P}_e}{\text{P}_\text{i}} \cdot 100 \qquad \text{(Eq. 2)}$$

where P_i is the phosphorus concentration of the influent (mg $P \cdot L^{-1}$) and P_e is the phosphorus concentration of the effluent (mg $P \cdot L^{-1}$).

$$\text{BP} = \frac{\text{VSS}}{\text{HRT}} \qquad \text{(Eq. 3)}$$

where BP (mg $VSS \cdot L^{-1} \cdot d^{-1}$) is biomass productivity, VSS (mg $VSS \cdot L^{-1}$) is the PBR volatile suspended solids concentration and HRT is the microalgae culture hydraulic retention time (d).

To compare between experiments operating under different solar PAR, the biomass productivity:light irradiance ratio (BP:I, g VSS·mol^{-1}) was calculated according to Eq. 4.

$$\text{BP:I} = \frac{\text{BP}\cdot V_{PBR}\cdot 1000}{\text{TP}\cdot t\cdot S\cdot 24\cdot 3600} \qquad \text{(Eq. 4)}$$

where TP is the total photon flux applied to the PBR surface (i.e. solar irradiance plus artificial lighting, µmol·m^{-2}·s^{-1}); t is the period of time considered (d) and S is the PBR surface (m^2).

In order to assess the growth of nitrifying bacteria, the nitrification rate (NOxR) (mg N·L^{-1}·d^{-1}) was obtained by Eq. 5:

$$\text{NOxR} = \frac{F\cdot(NOx_e - NOx_i)}{V_{PBR}} \qquad \text{(Eq. 5)}$$

where F is the treatment flow rate (m^3·d^{-1}); NOx_e is the concentration of nitrite plus nitrate of the effluent (mg N·L^{-1}); N_i is the concentration of nitrite plus nitrate of the influent (mg N·L^{-1}); and V_{PBR} is the volume of the culture in the PBRs (m^3).

SYTOX Green DNA staining dye (Invitrogen S7020) was used to monitor cell viability (Sato et al., 2004). 0.1µL of SYTOX Green 5mM was added to 50µL of 250-400 mg·L^{-1} suspended solids concentration of microalgae culture. As SYTOX Green is light-sensitive, the samples were incubated in darkness for 5 minutes. After the given reaction time had elapsed, the samples were excited by fluorescence microscope (DM2500, Leica, Germany) equipped with a filter set at 450 – 490 nm for excitation and 515 nm for emission. More than 400 cells were counted in duplicate for viability calculation in a Neubauer counting chamber in each experiment.

2.5. Statistical analysis

All results are shown as mean ± standard deviation of the duplicates. To determine the effect of temperature on microalgae performance, productivity, nitrogen and phosphorus removal efficiencies of R-A (temperature control) and R-B (non-temperature control) were compared. A t-test was carried out between the means values obtained for each reactor. In the case of comparing different seasons, an analysis of variance (ANOVA) was performed to evaluate statistical significant differences. Statistical analysis was assessed by STATGRAPHICS Centurion XVI.I. p-values < 0.05 were considered statistically significant with a level of significance of 95%.

3. RESULTS AND DISCUSSION

3.1. Effect of temperature on biomass productivity and nutrient recovery

In the first set of experiments, the temperature-controlled PBR was kept at a mean value of around 25 ºC (Table 1).

Average NRE, PRE and biomass productivity values are shown in Figure 1.

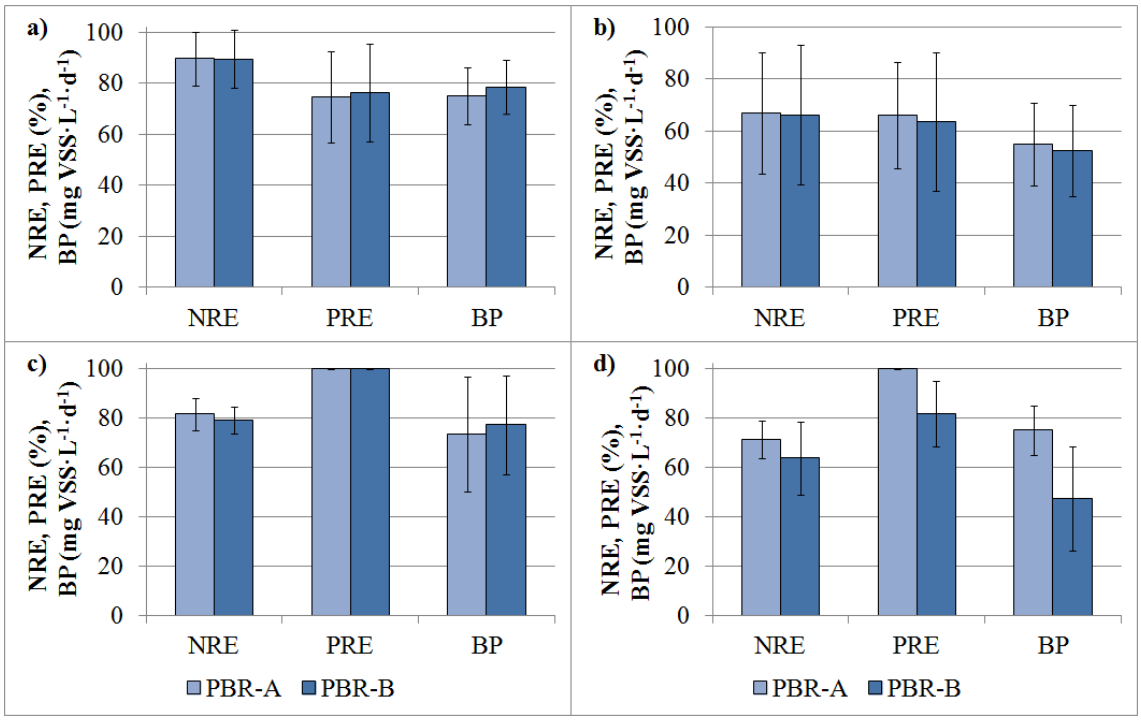

Figure 1. Effect of temperature in biomass productivity and nutrient recovery. Mean values of NRE, PRE and productivity. PBR-A: temperature controlled at around 25 ºC; PBR-B: free temperature. a) Experiment 1.1 (autumn); b) Experiment 1.2 (winter);c) Experiment 1.3 (spring); d) Experiment 1.4 (summer).

Experiments in autumn, winter and spring did not show any significant differences in terms of NRE, PRE and biomass productivity between the temperature-controlled and the non-temperature-controlled PBR; i.e., p-values were higher than 0.05. Microalgae cell viability was also similar in both PBRs, being in the range of 95-99% of viable cells. The results obtained in autumn and spring were as expected, since the temperatures remained within moderate ranges between 20-30 ºC (Figure 2). In fact, Suthar and Verma (2018) reported this temperature range of 20-30 ºC as optimum for the growth *C. vulgaris*.

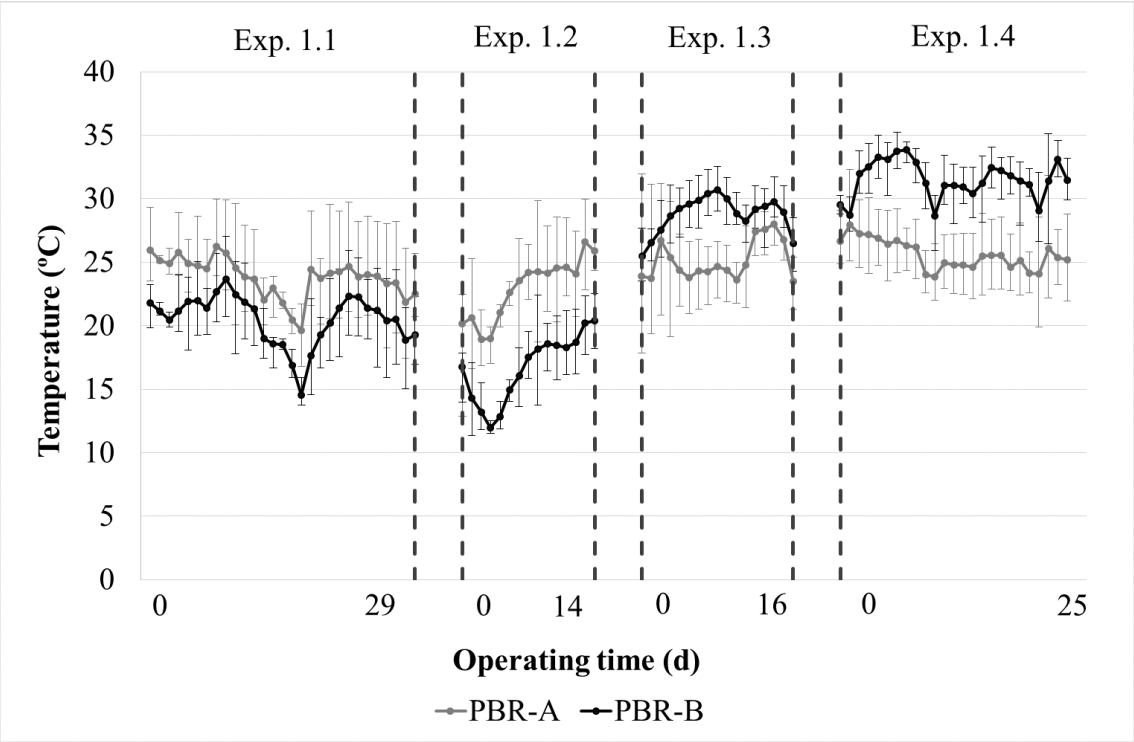

Figure 2. Evolution of average temperatures (with minimum and maximum intervals) during the first set of experiments.

On the other hand, in winter experiment, when temperatures in the non-temperature-controlled PBR varied between 12-20 ºC (Figure 2), surprisingly, there were non-significant differences between both PBRs (p-value > 0.05, see Figure 1b). These results disagree with other authors who reported lower microalgae performance when temperature falls to moderate values; i.e., under 15 ºC (Gupta et al., 2019; Sforza et al., 2014; Xu et al., 2019). According to Bussotti (2004), reducing the temperature slows down the electron transfer in photosynthesis. Several factors could have been responsible for this unexpected behaviour: i) the minimum temperature of around 12 ºC in the non-temperature-controlled PBR (Figure 2) may not have been low enough to significantly affect this indigenous culture. In this respect, Posadas et al. (2015) reported efficient nutrient removal of *Scenedesmus* sp. in raceways at average temperatures of 10-11 ºC; ii) the temperature reached values below 15 ºC only during 50% of the winter experiment. In this respect, Serra-Maia et al. (2016) reported that microalgae productivity could recover when temperature rises again after a significant reduction; iii) other factors such as daily light variations, PBR orientation, light gradients, etc. (Slegers et al., 2011) could have had a stronger influence on microalgae performance, lessening the temperature effect. In fact, Ferro et al. (2018) reported that adapted

microalgae strains could grow at 5 ºC as long as they had enough light irradiance, but did not proliferate when light intensity was low.

On the contrary, experiment in summer did show significant differences (p-value < 0.05) between the temperature-controlled and the non-temperature-controlled PBR, although both reactors started at similar nutrient and VSS concentrations. In addition, when comparing the light-normalised biomass productivity (BP:I) between different experiments no significant differences (p-value > 0.05) were observed in all cases, with the exception of the BP:I of the non-temperature-controlled PBR during summer, which was the lowest (Table 2).

Table 2. Biomass productivity:light irradiance ratio (BP:I) for the first set of experiments.

Exp.	**BP:I**	
	PBR-A	**PBR-B**
1.1	0.39 ± 0.10	0.41 ± 0.11
1.2	0.35 ± 0.10	0.33 ± 0.08
1.3	0.44 ± 0.12	0.42 ± 0.09
1.4	0.36 ± 0.04	0.22 ± 0.10[(1)]

[(1)] *Showed significant differences (p-value < 0.05).*

During summer, the non-temperature-controlled PBR remained at the highest mean temperatures of 31.5 ± 1.8 ºC, reaching peak values over 35 ºC for several days. As a consequence, cell viability dropped to 69 ± 1% in this PBR but remained at 96 ± 2% in the temperature-controlled PBR, which suggests that a culture deterioration occurred in the non-temperature-controlled PBR due to heat stress (Manhaeghe et al., 2019; Nwoba et al., 2019). Dead microalgae cells can release their nutrient content into the medium, as reported by Serra-Maia et al. (2016). In fact, from day 16 onwards nutrients started to accumulate in the non-temperature-controlled PBR, especially phosphorus, which remained at negligible values in the temperature-controlled PBR, but reached over 2 mg $P \cdot L^{-1}$ in the non-temperature-controlled PBR at the end of summer experiment (Figure 3a).

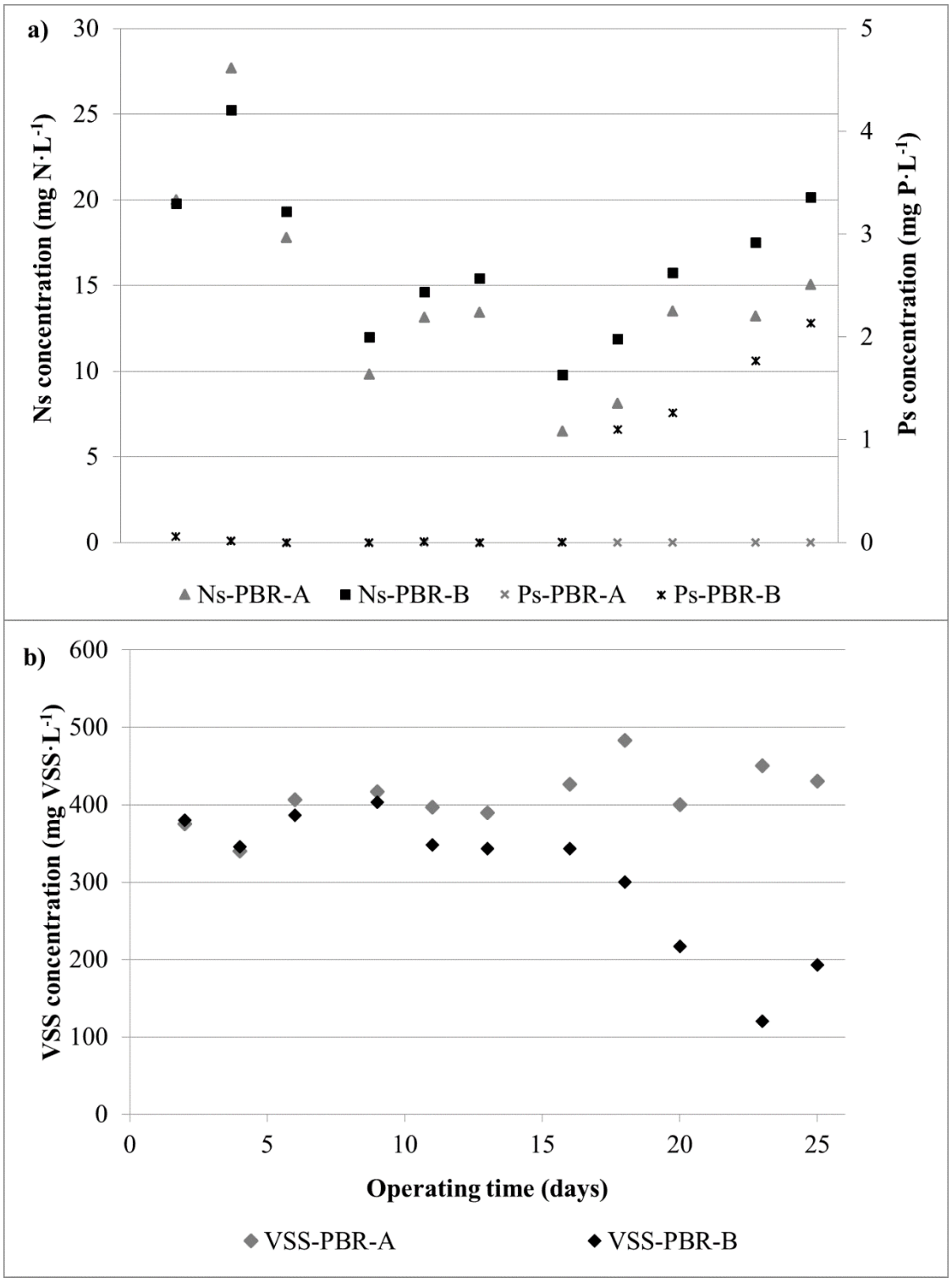

Figure 3. Evolution during Experiment 1.4 in PBR-A and PBR-B of: a) nitrogen (Ns) and phosphorus (Ps) concentrations; b) volatile suspended solids (VSS) concentration.

Other authors have also reported the unequal effects of high and low temperatures on the microalgae culture (Almomani et al., 2019; Ras et al., 2013; Serra-Maia et al., 2016). Microalgae growth drops much more abruptly at high than low temperatures. In

fact, most microalgae strains can tolerate temperatures around 15 ºC below the optimum, but exceeding the optimum temperature by only 2-4 ºC can be detrimental for algae growth (Venkata Subhash et al., 2014). Hence, it is essential to find out the maximum tolerable temperature of the microalgae culture in order to obtain an optimal performance in the microalgae cultivation process. In this respect, Binnal and Babu (2017) observed a noticeable decrease in the performance of *Chlorella protothecoides* when temperature attained 30 ºC. Similarly, García-Cubero et al. (2018) obtained lower biomass productivity of *Chlorella vulgaris* at 30 ºC but no microalgae growth was observed at 35 ºC.

It can thus be concluded that the indigenous microalgae used in this study (mainly composed of *Chlorella*) can be processed without temperature limits or inhibition in the range of around 15-30 ºC. Further research is needed to determine the lowest temperature at which microalgae restrictions begin. This optimum temperature range of the indigenous microalgae culture is wider than those reported for pure cultures grown in synthetic media. For instance, Suthar and Verma (2018) reported maximum growth of *Chlorella vulgaris* in the range of 20-30 ºC, while Babel et al. (2002) obtained 28-35 ºC as the optimal for *Chlorella* sp. growth. In the study of García-Cubero et al. (2018), *C. vulgaris* obtained the highest biomass productivity in the temperature range of 15-25 ºC.

At higher temperatures peaks of around 35 ºC, microalgae could be cultivated but its performance was significantly reduced. Hence, in this microalgae-based system, temperature has to be kept under 35 ºC to reduce microalgae mortality and avoid culture collapse. Cooling microalgae in summer can be challenging (Huang et al., 2019) since, apart from the ambient temperature, the culture can be heated by the excess of light energy received by algae, emitted as fluorescence or heat through non-photochemical pathways (Huang et al., 2017; Nwoba et al., 2019). Efforts will thus have to be made to look for efficient ways of cooling microalgae on hot days to make the transition of this technology feasible on a large scale. By way of example, Almomani et al. (2019) reported a net energy benefit from cooling the culture in summer by using flue gas as the carbon source for microalgae growth.

3.2 Effect of temperature in microalgae-AOB bacteria competition

Temperature affects not only microalgae metabolism but also other organisms present in the culture, such as nitrifying bacteria (Jiménez, 2010). AOB proliferation is not

desirable, since they compete with microalgae for ammonium uptake and can worsen microalgae performance (González-Camejo et al., 2018a). Another set of experiments (2.1 and 2.2) was thus carried out to assess the effect of temperature on microalgae-AOB competition.

In these experiments, the same ambient and operating conditions were maintained in both PBRs, except for ATU concentration, which was added only to the nitrification-inhibited PBR. The main difference between Experiments 2.1 and 2.2 was the mean temperature of both PBRs, which was 18.5 ± 2.5 and 26.7 ± 1.1 ºC, respectively.

The NOxR, i.e. the production of nitrite and nitrate in the mixed microalgae-nitrifying bacteria culture, was used to assess nitrifying bacteria activity (Rossi et al, 2018). It should be noted that NOxR is an approximate value since it does not include the nitrate and nitrite consumed by algae. These nitrite and nitrate absorbed by microalgae were expected to be low, since the ammonium uptake is far higher than that of nitrate (Eze et al., 2018). However, if the nitrate uptake rate were to be higher than the nitrification rate, negative NOxR values would be obtained.

3.2.1 Experiment 2.1

This experiment lasted 81 days and was carried out in autumn-winter, so that temperature presented a mean value of 18.5 ± 2.5 ºC. It was divided into two periods: Period 2.1.I (41 days) and Period 2.1.II (40 days). Figure 4 shows the evolution of the nutrient concentrations and the nitrification rate during this experiment. The high variability of nutrient concentrations can be seen in Figure 4a. This was due not only to PBR performance, but also to the large variations in the nutrient load (data not shown).

In Period 2.1.I mean temperatures remained under 25 ºC and no significant differences were observed between the nitrification-inhibited and the non-nitrification-inhibited PBR in terms of nutrient concentrations (Figure 4a) and nitrification rates, which were in the range of -1/+1 mg $N \cdot L^{-1} \cdot d^{-1}$ (Figure 4b). Microalgae cell viability was also similar; i.e. 94 ± 7% in the nitrification-inhibited PBR and 92 ± 4% in the non-nitrification-inhibited PBR. This suggests that AOB activity was not significant in Period 2.1.I in either reactor.

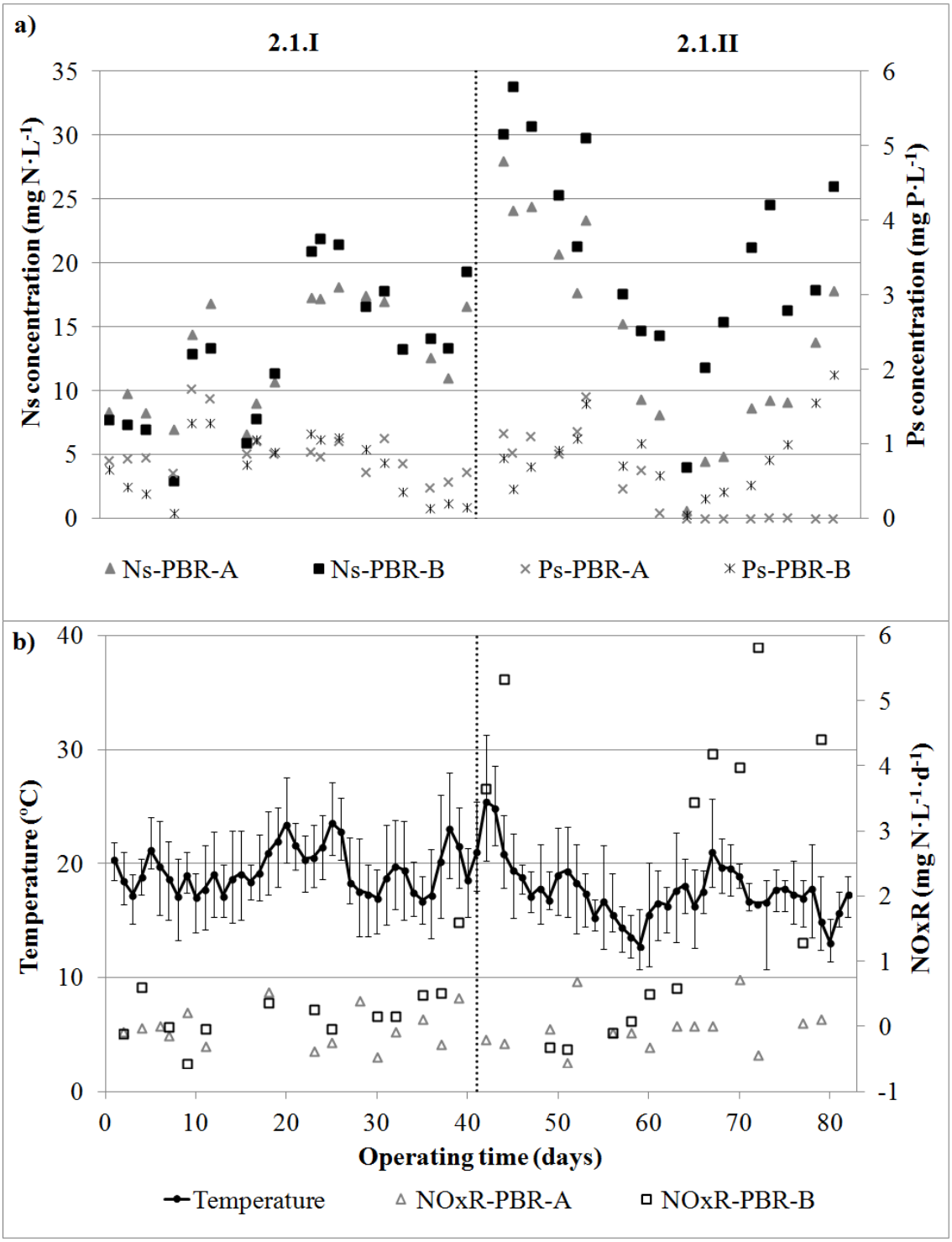

Figure 4. Evolution during experiment 2.1 in PBR-A (inhibited nitrification) and PBR-B (free nitrification) of: a) nitrogen (Ns), and phosphorus (Ps) concentrations; b) temperature and nitrification rate (NOxR).

However, on days 42 and 43 (beginning of Period 2.1.II) it presented average values over 25 ºC with peaks over 30 ºC (Figure 4b), which sharply increased nitrifying bacteria activity, reaching NOxR values in the range of 3-6 mg $N \cdot L^{-1} \cdot d^{-1}$. On the other

hand, when the temperature dropped steadily on days 44-60, the nitrification rates returned to negligible values (Figure 4b). It is well known that AOB growth is strongly favoured at high temperatures and is around 0.77 d^{-1} at 18 ºC, which is similar to that of *Chlorella*; i.e. 0.65-0.87 d^{-1} (Ledda et al., 2015; Xu et al., 2015). However, at 25 ºC it can reach up to 1.61 d^{-1} (Jiménez, 2010), while *Chlorella* remain in the former range.

After day 60, nitrifying bacteria activity again started to rise, with a sharp peak on day 64. This time the temperature stayed at mean values in the range of 15-18 ºC (Figure 4b), so that AOB activity had to be theoretically low (Jiménez, 2010), as previously mentioned. However, at this time, the non-nitrification-inhibited PBR had nitrogen concentrations under 10 mg $N \cdot L^{-1}$ (Figure 4a). It has previously been reported that microalgae activity is significantly reduced at nitrogen concentrations below 10 mg $N \cdot L^{-1}$ (Pachés et al., 2018). Under these conditions, the microalgae growth rate in the non-nitrification-inhibited PBR was therefore reduced because of limiting nitrogen, and AOB activity was favoured when the ammonium load increased after day 65, reaching an NOxR of 3.9 ± 2.1 mg $N \cdot L^{-1} \cdot d^{-1}$.

The higher nitrifying bacteria activity worsened microalgae performance in the non-nitrification-inhibited PBR after day 65. In fact, both nitrogen and phosphorus concentrations accumulated in this PBR, which meant lower nutrient recovery rates than the nitrification-inhibited PBR. In addition, microalgae cell viability fell slightly, reaching values of 84 ± 3% in the non-nitrification-inhibited PBR, while it remained at 93 ± 2% in the nitrification-inhibited PBR during Period 2.1.II.

Another factor that could have favoured nitrifying activity in Period 2.1.II was light intensity, since it was significantly higher in Period 2.1.I (308 ± 110 $\mu mol \cdot m^{-2} \cdot s^{-1}$) than in Period 2.1.II; i.e., 256 ± 152 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Light irradiance has been reported to inhibit nitrifying bacteria growth (Guerrero and Jones, 1996), especially under conditions of high oxygen concentrations (Prosser, 1990), as in this case. A previous lab study (González-Camejo et al., 2018b) has also shown that the threshold temperature at which AOB growth is favoured increases with higher light intensity; i.e. AOB rose at 22 ± 1ºC and 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$, but at 85 $\mu mol \cdot m^{-2} \cdot s^{-1}$, AOB activity did not significantly notice until 27-28 ºC was reached. Lastly, at a light irradiance of 125 $\mu mol \cdot m^{-2} \cdot s^{-1}$, negligible AOB activity was seen below 32 ºC. These results suggest that AOB activity is significant only when their growth rate is considerably higher than that of microalgae.

3.2.2 Experiment 2.2

As Experiment 2.2 was carried out in spring and summer, culture temperatures were considerably higher than in Experiment 2.1 (i.e., mean value of 26.7 ± 1.1 ºC), and remained fairly stable (Figure 5b).

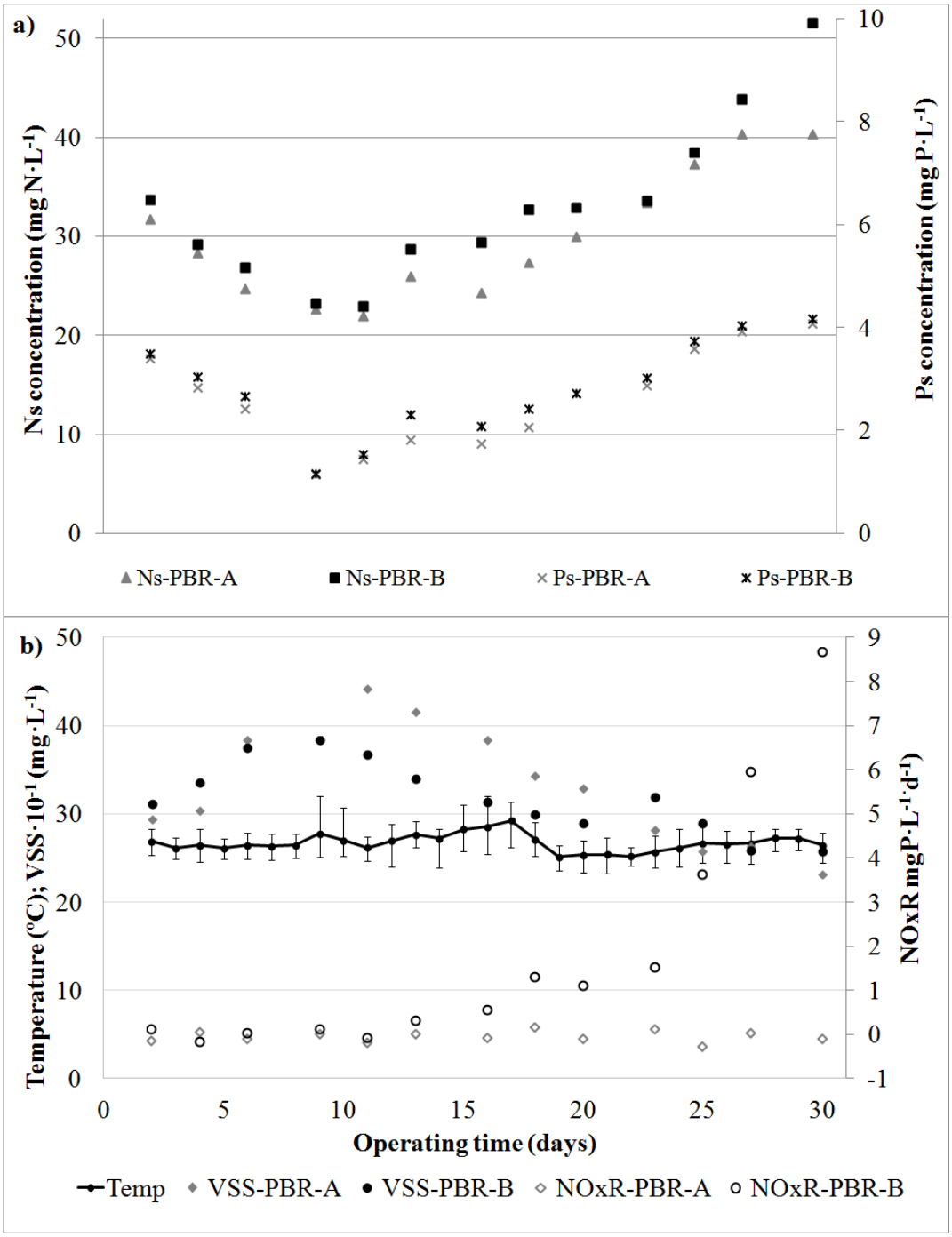

Figure 5. Evolution during experiment 2.2 in PBR-A (inhibited nitrification) and PBR-B (free nitrification) of: a) nitrogen (Ns), and phosphorus (Ps) concentrations; b) temperature, volatile suspended solids (VSS) concentration and nitrification rate (NOxR).

At these high temperatures, AOB growth was expected to rapidly surpass that of the microalgae, due to their theoretically higher growth rate than *Chlorella*, as mentioned in Section 3.2.1. However, there were negligible differences in the nitrification rates of both PBRs at the beginning of the experiment, even after maximum temperatures over 30 ºC on days 9-10 (Figure 5b). As reported by other authors (Lau et al., 2019; Ras et al., 2013; Yadav and Sen, 2017), it is possible that this indigenous microalgae could have been adapted to high temperatures since the start-up phase of Experiment 2.2 was performed at similar temperatures to those of its continuous operation (data not shown), thus being more competitive than AOB and reaching a consistent microalgae biomass of 384 mg $VSS \cdot L^{-1}$ at day 10 (in Period 2.1.I, the VSS concentration prior to nitrification only achieved 299 ± 22 mg $VSS \cdot L^{-1}$). However, after 3 days of temperatures over 30 ºC (days 16-18), NOxR rose steadily in the non-nitrification-inhibited PBR (Figure 5b), probably because of two simultaneous effects: i) the increasing AOB activity at higher temperatures (Jiménez, 2010) as explained in section 3.2.1; ii) the reduction of the microalgae performance under temperatures of 30-35 ºC, as already stated in section 3.1. Consequently, nitrifying bacteria outcompeted the microalgae from day 25 on, which implied that nitrogen concentration in the non-nitrification-inhibited PBR was higher than in the nitrification-inhibited PBR at the end of Experiment 2.2 (Figure 5b) and viability in the non-nitrification-inhibited PBR fell to 80 ± 17%.

It is possible that sudden temperature rises also had an influence on microalgae-AOB competition. It seems that under normal light and mild temperature situations, microalgae growth is higher than AOB (Marcilhac et al., 2014; Risgaard-Petersen et al., 2004), therefore increasing their biomass concentration and outcompeting nitrifying bacteria. However, sudden temperature rises can prompt accelerate AOB growth, making them able to compete with microalgae for ammonium uptake. After this sharp increase in AOB, if the ambient conditions such as high temperatures are maintained favourable for nitrifying bacteria growth (as in Experiment 2.2), nitrification will rise steadily and the nitrifiers will outcompete the microalgae, as occurred at the end of Experiment 2.2 (Figure 5). This suggests that the competition between microalgae and nitrifying bacteria leads to competitive exclusion (Passarge et al., 2006). On the other hand, if the temperature is re-established after its peak, the nitrification rate will drop and microalgae performance can recover, as was seen in Experiment 2.1 (Section 3.2.1).

To sum up, variability of temperature plays an important role in the competition between microalgae and AOB. Temperature peaks over 30 ºC and the maintenance of

the culture high temperatures can make nitrifying bacteria outcompete microalgae, which can imply the culture collapse.

4 Conclusions

The optimal temperature range for the growth of indigenous microalgae was around 15-30 ºC. Within this range, no significant differences were found in microalgae cultivation performance. However, microalgae viability was significantly reduced at temperatures over 30-35 ºC.

Sudden temperature rises favoured AOB activity within the indigenous microalgae culture, after which the microalgae could recover when the ambient temperature fell as the nitrification rate was reduced. However, when ambient temperatures stayed high, the nitrifying bacteria could outcompete the microalgae, collapsing the culture.

Since nitrifiers can exhaust the ammonium in the culture, it seems essential to keep nitrifying bacteria activity low.

E-supplementary data of this work can be found in online version of the paper.

Acknowledgments

This research work was supported by the Spanish Ministry of Economy and Competitiveness (MINECO, Projects CTM2014-54980-C2-1-R and CTM2014-54980-C2-2-R) jointly with the European Regional Development Fund (ERDF), both of which are gratefully acknowledged. It also received support from the Spanish Ministry of Education, Culture and Sport via a pre-doctoral FPU fellowship to authors J. González-Camejo (FPU14/05082) and S. Aparicio (FPU/15/02595).

References

1. Acién, F.G., Gómez-Serrano, C., Morales-Amaral, M.M., Fernández-Sevilla, J.M., Molina-Grima E., 2016. Wastewater treatment using microalgae: how realistic a contribution might it be to significant urban wastewater treatment? Appl. Microbiol. Biotechnol. 100, 9013–9022. http://dx.doi.org/10.1007/s00253-016-7835-7
2. Almomani, F., Al Ketife, A.M.D., Judd, S., Shurair, M., Bhosale, R., Znad, H., Tawalbeh, M., 2019. Impact of CO_2 concentration and ambient conditions on microalgal growth and nutrient removal from wastewater by a photobioreactor. Sci. Total Environ. 662, 662-671. https://doi.org/10.1016/j.scitotenv.2019.01.144

3. A.P.H.A., A.W.W.A., W.P.C.F., Standard methods for the examination of water and wastewater, 21st edition, American Public Health Association, American Water Works Association, Water Pollution Control Federation, Washington DC, 2005.
4. Babel, S., Takizawa, S., Ozaki, H., 2002. Factors affecting seasonal variation of membrane filtration resistance caused by *Chlorella* algae. Water Res. 36(5), 1193-1202
5. Binnal, P., Babu, P.N., 2017. Optimization of environmental factors affecting tertiary treatment of municipal wastewater by *Chlorella protothecoides* in a lab scale photobioreactor. J. Water Process Eng. 17, 290-298. http://dx.doi.org/10.1016/j.jwpe.2017.05.003
6. Bussotti, F., 2004. Assessment of stress conditions in *Quercus ilex* L. leaves by O-J-I-P chlorophyll *a* fluorescence analysis, Plant Biosystems 138, 101-109.
7. Eze, V.C, Velasquez-Orta, S.B., Hernández-García, A., Monje-Ramírez, I., Orta-Ledesma, M.T., 2018. Kinetic modelling of microalgae cultivation for wastewater treatment and carbon dioxide sequestration. Algal Res. 32, 131–141. https://doi.org/10.1016/j.algal.2018.03.015
8. Ferro, L., Gorzsás, A., Gentili, F.G., Funk, C., 2018. Subarctic microalgal strains treat wastewater and produce biomass at low temperature and short photoperiod. Algal Res. 35, 160-167. https://doi.org/10.1016/j.algal.2018.08.031
9. García-Cubero, R., Moreno-Fernández, J., Acién-Fernández, F.G., García-González, M., 2018. How to combine CO_2 abatement and starch production in *Chlorella vulgaris*. Algal Res. 32, 270-279. https://doi.org/10.1016/j.algal.2018.04.006
10. González-Camejo, J., Viruela, A., Ruano, M.V., Barat, R., Seco, A., Ferrer, J., 2019. Effect of light intensity, light duration and photoperiods in the performance of an outdoor photobioreactor for urban wastewater treatment. Algal Res. 40, 101511. https://doi.org/10.1016/j.algal.2019.101511
11. Gonzalez-Camejo, J., Barat, R., Ruano, M.V., Seco, A., Ferrer, J., 2018a. Outdoor flat-panel membrane photobioreactor to treat the effluent of an anaerobic membrane bioreactor. Influence of operating, design and environmental conditions. Water Sci. Technol. 78(1) 195-206. http://dx.doi.org/10.2166/wst.2018.259
12. González-Camejo, J., Barat, R., Pachés, M., Murgui, M., Ferrer, J., Seco, A., 2018b. Wastewater Nutrient Removal in a Mixed Microalgae-bacteria Culture: Effect of Light and Temperature on the Microalgae-bacteria Competition. Environ. Technol. 39(4), 503-515. http://dx.doi.org/10.1080/09593330.2017.1305001
13. González-Camejo, J., Serna-García, R., Viruela, A., Pachés, M., Durán, F., Robles, A., Ruano, M.V., Barat R., Seco, A., 2017. Short and long-term experiments on the effect of sulphide on microalgae cultivation in tertiary sewage treatment. Bioresour. Technol. 244, 15-22. http://dx.doi.org/10.1016/j.biortech.2017.07.126
14. Guerrero, M.A., Jones, R.D., Photoinhibition of marine nitrifying bacteria. I. Wavelength-dependent response. Mar. Ecol. Prog. Ser. 141 (1996) 183-192.
15. Gupta S., Pawar, S.B., Pandey, R.A., 2019. Current practices and challenges in using microalgae for treatment of nutrient rich wastewater from agro-based industries. Sci. Total Environ. 687, 1107–1126. https://doi.org/10.1016/j.scitotenv.2019.06.115

16. Huang, J., Hankame, B., Yarnold, J., 2019. Design scenarios of outdoor arrayed cylindrical photobioreactors for microalgae cultivation considering solar radiation and temperature. Algal Res. 41, 101515. https://doi.org/10.1016/j.algal.2019.101515
17. Huang, Q., Jiang, F., Wang, L., Yang, C., 2017. Design of Photobioreactors for Mass Cultivation of Photosynthetic Organisms. Engineering 3, 318-329. http://dx.doi.org/10.1016/J.ENG.2017.03.020
18. Ippoliti, D., Gómez, C., Morales-Amaral, M.M., Pistocchi, R., Fernández-Sevilla, J.M., Acién, F.G., 2016. Modeling of photosynthesis and respiration rate for *Isochrysis galbana* (T-Iso) and its influence on the production of this strain. Bioresour. Technol. 203, 71–79. http://dx.doi.org/10.1016/j.biortech.2015.12.050
19. Jiménez, E., 2010. Mathematical modelling of the two-stage nitrification process. Developement of modelling calibration methodologies for a SHARON reactor and activated sludge process (Modelación matemática del proceso nitrificación en dos etapas. Desarrollo de metodologías de calibración del modelo para un reactor SHARON y un proceso de fangos activados). PhD Thesis, Polytechnic University of Valencia, Spain.
20. Krustok, I., Odlare, M., Truu, J., Nehrenheim, E., 2016. Inhibition of nitrification in municipal wastewater-treating photobioreactors: Effect on algal growth and nutrient uptake. Bioresour Technol. 202, 238-243. http://dx.doi.org/10.1016/j.biortech.2015.12.020
21. Lau, A.K.S., Bilad, M.R., Osman, N.B., Marbelia, L., Putra, Z.A., Nordin, N.A.H.M., Wirzal, M.D.H., Jaafar, J., Khan, A.L., 2019. Sequencing batch membrane photobioreactor for simultaneous cultivation of aquaculture feed and polishing of real secondary effluent, Journal of Water Process Engineering 29, 100779. https://doi.org/10.1016/j.jwpe.2019.100779
22. Ledda, C., Idà, A., Allemand, D., Mariani, P., Adani, F., 2015. Production of wild *Chlorella* sp. cultivated in digested and membrane-pretreated swine manure derived from a full-scale operation plant. Algal Res. 12, 68-73. http://dx.doi.org/10.1016/j.algal.2015.08.010
23. Ling, Y., Sun, L.P., Wang, S.Y., Lin, C.S.K., Sun, Z., Zhou, Z.G., 2019. Cultivation of oleaginous microalga *Scenedesmus obliquus* coupled with wastewater treatment for enhanced biomass and lipid production. Biochem. Eng. J. 148, 162–169. https://doi.org/10.1016/j.bej.2019.05.012
24. Manhaeghe, D., Michels, S., Rousseau, D.P.L., Van Hulle, S.W.H., 2019. A semi-mechanistic model describing the influence of light and temperature on the respiration and photosynthetic growth of *Chlorella vulgaris.* Bioresour. Technol. 274, 361-370. https://doi.org/10.1016/j.biortech.2018.11.097
25. Marcilhac, C., Sialve B., Pourcher A.M., Ziebal C., Bernet N., Béline F., 2014. Digestate color and light intensity affect nutrient removal and competition phenomena in a microalgal-bacterial ecosystem, Water Res. 64, 278-287. http://dx.doi.org/10.1016/j.watres.2014.07.012
26. Nwoba, E.G., Parlevliet, D.A., Laird, D.W., Alameh, K., Moheimani, N.R., 2019. Light management technologies for increasing algal photobioreactor efficiency. Algal Res. 39, 101433. https://doi.org/10.1016/j.algal.2019.101433

27. Pachés, M., Martínez-Guijarro, R., González-Camejo, J., Seco, A., Barat, R., 2018. Selecting the most suitable microalgae species to treat the effluent from an anaerobic membrane bioreactor. Environ. Technol. (in press). https://doi.org/10.1080/09593330.2018.1496148
28. Passarge, J., Hol, S., Escher, M., Huisman, J., 2006. Competition for nutrients and light: stable coexistence, alternative stable states, or competitive exclusion? Ecological Monographs, 76(1), 57–72.
29. Pawlowski, A., Frenández, I., Guzmán, J.L., Berenguel, M., Acién, F.G., Dormido, S., 2016. Event-based selective control strategy for raceway reactor: A simulation study. IFAC-Papers OnLine 49(7), 478–483. https://doi.org/10.1016/j.ifacol.2016.07.388
30. Posadas, E., Morales, M.M., Gomez, C., Acién, F.G., Muñoz, R., 2015. Influence of pH and CO_2 source on the performance of microalgae-based secondary domestic wastewater treatment in outdoors pilot raceways. Chem. Eng. J. 265, 239–248. http://dx.doi.org/10.1016/j.cej.2014.12.059
31. Prosser, J.I., 1990. Autotrophic nitrification in bacteria. In *Advances in microbial physiology* (Vol. 30, pp. 125-181). Academic Press.
32. Ras, M., Steyer, J.P., Bernard, O., 2013. Temperature effect on microalgae: a crucial factor for outdoor production, Rev. Environ. Sci. Biotechnol. 12, 153-164. https://doi.org/10.1007/s11157-013-9310-6
33. Risgaard-Petersen, N., Nicolaisen, M.H., Revsbech, N.P., Lomstein, B.A., 2004. Competition between ammonia oxidizing bacteria and benthic microalgae. Appl. Environ. Microb. 70(9), 5528-5537. https://doi.org/10.1128/AEM.70.9.5528-5537.2004
34. Rossi, S., Bellucci, M., Marazzi, F., Mezzanotte, V., Ficara, E., 2018. Activity assessment of microalgal-bacterial consortia based on respirometric tests. Water Sci Technol. 78(1-2), 207-215. https://doi.org/10.2166/wst.2018.078
35. Sato, M., Murata, Y., Mizusawa, M., Iwahashi, H., Oka, S.I., 2004. A simple and rapid dual-fluorescence viability assay for microalgae. Microbiol. Cult. Coll. 20(2), 53-59.
36. Seco, A., Aparicio, S., González-Camejo, J., Jiménez-Benítez, A., Mateo, O., Mora, J.F., Noriega-Hevia, G., Sanchis-Perucho, P., Serna-García, R., Zamorano-López, N., Giménez, J.B., Ruiz-Martinez, A., Aguado, D., Barat, R., Borrás, L., Bouzas, A., Martí, N., Pachés, M., Ribes, J., Robles, A., Ruano, M.V., Serralta, J. and Ferrer, J., 2018. Resource recovery from sulphate-rich sewage through an innovative anaerobic-based water resource recovery facility (WRRF). Water Science and Technology 78 (9), 1925-1936. https://doi.org/10.2166/wst.2018.492
37. Serra-Maia, R., Bernard, O., Gonçalves, A., Bensalem, S., Lopes, F., 2016. Influence of temperature on *Chlorella vulgaris* growth and mortality rates in a photobioreactor. Algal Res. 18, 352–359. http://dx.doi.org/10.1016/j.algal.2016.06.016
38. Sforza, E., Ramos-Tercero, E.A., Gris, B., Bettin, F., Milani, A., Bertucco, A., 2014. Integration of *Chlorella protothecoides* production in wastewater treatment plant: From lab measurements to process design. Algal Res. 6, 223–233. http://dx.doi.org/10.1016/j.algal.2014.06.002

39. Slegers, P.M., Wijffels, R.H., van Straten, G., van Boxtel, A.J.B., 2011. Design scenarios for flat panel photobioreactors. Appl. Energy 88, 3342–3353. https://doi.org/10.1016/j.apenergy.2010.12.037
40. Song, X., Luo, W., Hai, F.I., Price, W. E., Guo, W., Ngo, H.H., Nghiem, L.D., 2018. Resource recovery from wastewater by anaerobic membrane bioreactors: Opportunities and challenges. Bioresour. Technol. 270, 669-677. https://doi.org/10.1016/j.biortech.2018.09.001
41. Suthar, S., Verma, R., 2018. Production of *Chlorella vulgaris* under varying nutrient and abiotic conditions: A potential microalga for bioenergy feedstock. Process Safety and Environmental Protection 113, 141–148. https://doi.org/10.1016/j.psep.2017.09.018
42. Thomas, P.K., Dunn, G.P., Good, A.R., Callahan, M.P., Coats, E.R., Newby, D.T., Feris, K.P., 2019. A natural algal polyculture outperforms an assembled polyculture in wastewater-based open pond biofuel production. Algal Res. 40, 101488. https://doi.org/10.1016/j.algal.2019.101488
43. Venkata Subhash, G., Rohit, M.V., Prathima Devi, M., Swamy, Y.V., Venkata Mohan, S., 2014. Temperature induced stress influence on biodiesel productivity during mixotrophic microalgae cultivation with wastewater. Bioresour Technol. 169, 789–93. http://dx.doi.org/10.1016/j.biortech.2014.07.019
44. Viruela A., Robles A., Durán F., Ruano M.V., Barat R., Ferrer J., Seco A., 2018. Performance of an outdoor membrane photobioreactor for resource recovery from anaerobically treated sewage. J. Clean. Prod. 178, 665-674. https://doi.org/10.1016/j.jclepro.2017.12.223
45. Wang, B., Lan, C.Q., Horsman, M., 2012. Closed photobioreactors for production of microalgal biomasses. Biotechnol. Adv. 30(4), 904-912. https://doi.org/10.1016/j.biotechadv.2012.01.019.
46. Xu, X., Gu, X., Wang, Z., Shatner, W., Wang, Z., 2019. Progress, challenges and solutions of research on photosynthetic carbon sequestration efficiency of microalgae. Renew. Sust. Energy Rev. 110, 65–82. https://doi.org/10.1016/j.rser.2019.04.050
47. Xu, M., Li, P., Tang, T., Hu, Z., 2015. Roles of SRT and HRT of an algal membrane bioreactor system with a tanks-in-series configuration for secondary wastewater effluent polishing. Ecol. Eng. 85, 257-264. http://dx.doi.org/10.1016/j.ecoleng.2015.09.064
48. Yadav, G., Sen, R., 2017. Microalgal green refinery concept for biosequestration of carbon-dioxide vis-à-vis wastewater remediation and bioenergy production: Recent technological advances in climate research. J. CO_2 Util. 17, 188–206. http://dx.doi.org/10.1016/j.jcou.2016.12.006
49. Yang, Z., Kong, F., 2011. Enhanced growth and esterase activity of *Chlorella pyrenoidosa* (Chlorophyta) in response to short-term direct grazing and grazing-associated infochemicals from *Daphnia carinata*. J. Freshw. Ecol. 26, 553–561. https://doi.org/10.1080/02705060.2011.577973
50. Yeo, U.H., Lee, I.B., Seo, I.H., Kim, R.W., 2018. Identification of the key structural parameters for the design of a large-scale PBR. Biosist. Eng. 171, 165-178. https://doi.org/10.1016/j.biosystemseng.2018.04.012

APPENDIX A. ASSESSMENT OF THE MICROALGAE-NITRIFYING BACTERIA COMPETITION FOR AMMONIUM UPTAKE IN LAB-CONDITIONS

When microalgae cultivation systems are used to treat the effluent of anaerobic membrane bioreactors (AnMBRs) (Robles et al., 2018), the ammonium competition between microalgae and ammonium oxidising bacteria (AOB) is likely to occur (González-Camejo et al., 2018a; Molinuevo-Salces et al., 2010). AOB are autotrophic bacteria which oxidises ammonium to nitrite (i.e., first step of the nitrification process). Nitrite oxidising bacteria (NOB) can in turn oxidise this nitrite to nitrate, carrying out the second step of nitrification (Risgaard-Petersen et al., 2004; Winkler and Straka, 2019). Hence, the nitrifying bacteria (both AOB and NOB) activity is not usually desirable in microalgae cultivation systems since they reduce the amount of ammonium (González-Camejo et al., 2018), which is the main nitrogen source for microalgae (Eze et al., 2018; Najm et al., 2017), therefore decreasing its recovery in the microalgae biomass.

Since AOB activity is highly influenced by temperature (Jiménez, 2010; Weon et al., 2004), AOB are likely to grow significantly in closed PBRs operated in warm regions (for instance, Valencia, Spain). Hence, evaluating the affection of AOB on a mixed microalgae culture would help to understand the role of these microorganisms in the application of this technology for industrial purposes.

EXPERIMENTAL DESIGN, MATERIALS AND METHODS

3 lab-scale assays (i.e., A1, A2 and A3) were elaborated. Each of them was carried out by using microalgae samples taken from PBR-B of the PBR plant (see section 2.2).

For each assay, two 8-L vertical reactors (i.e., R-A and R-B) were used. Both of them were placed in a climatic chamber which maintained the culture in temperatures around 25-27 ºC. They were air-stirred in order to homogenise the culture and avoid biofilm formation. CO_2 was added to maintain the culture pH at a maximum set-point value of 7.5. Five LED lamps (Trilux 9w) were placed vertically around each reactor to supply a light PAR of 125 $\mu mol \cdot m^{-2}s^{-1}$ (measured at the reactor´s surface).

Both reactors were filled with 50% of substrate (i.e., AnMBR effluent, see section 2.1) and 50% of the microalgae culture from the aforementioned PBR plant. The

characteristics of each media; i.e., ammonium (NH_4), soluble nitrogen (N_s) and volatile suspended solids (VSS) concentration, are shown in Table A.1.

Table A.1. Characteristics of the microalgae culture and substrate of the lab assays.

	Substrate			Culture		
Assay	NH_4 (mg $N \cdot L^{-1}$)	N_s (mg $N \cdot L^{-1}$)	VSS (mg $VSS \cdot L^{-1}$)	NH_4 (mg $N \cdot L^{-1}$)	N_s (mg $N \cdot L^{-1}$)	VSS (mg $VSS \cdot L^{-1}$)
A1	46.6	56.1	< LOD*	16.9	38.5	214
A2	42.3	57.8	< LOD*	22.6	27.9	390
A3	45.7	46.9	< LOD*	0.5	21.3	413

*LOD: Limit of detection

The difference between the reactors was their allylthiourea (ATU) content. In R-A the nitrification process was free to occur because ATU was not injected (similar to the operation in the MPBR plant). On the contrary, ATU was added in R-B until reaching 10 $mg \cdot L^{-1}$. Consequently, AOB activity in R-B was inhibited (González-Camejo et al., 2018a).

Ammonium (NH_4), nitrite (NO_2) and nitrate (NO_3) were analysed according to Standard Methods (APHA, 2005): 4500-NH3-G, 4500-NO2-B and 4500-NO3-H, respectively, using an automatic analyser (Smartchem 200, WestcoScientific Instruments, Westco). Soluble nitrogen (N_s) was calculated as the sum of all the nitrogen species measured; i.e., NH_4 NO_2 and NO_3. The volatile suspended solids (VSS) concentration was measured according to Standard Methods (APHA, 2005): method 2540 E.

The performance of both reactors was compared in terms of nitrogen removal rate and biomass productivity along one-day batch operation.

Data

From the evolution of the concentration of nutrients and VSS during Assays A1, A2 and A3 (Figures A.1., A.2 and A.3, respectively), ammonium, nitrate and nitrogen recovery rates, nitrification rate (measured as the production of nitrite and nitrate as an approximation) and biomass productivity of both reactors were obtained (Tables A.2, A.3 and A.4). It must be noted that negative values of slope represent consumption of nutrients, while positive values mean production of nutrients or biomass.

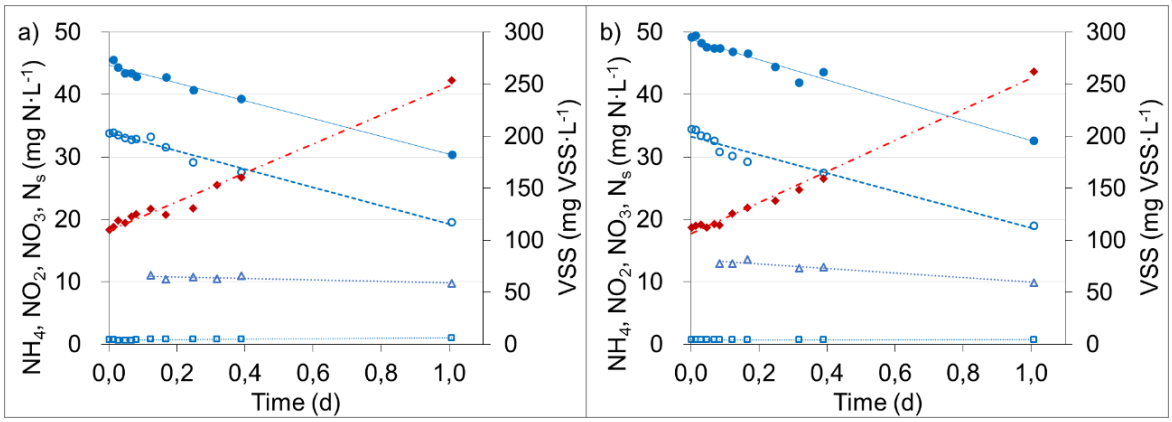

Figure A.1. Evolution of NH_4 (○), NO_2 (□), NO_3 (△), N_s (●) and VSS (♦) concentration during Assay A1: a) R-A; b) R-B.

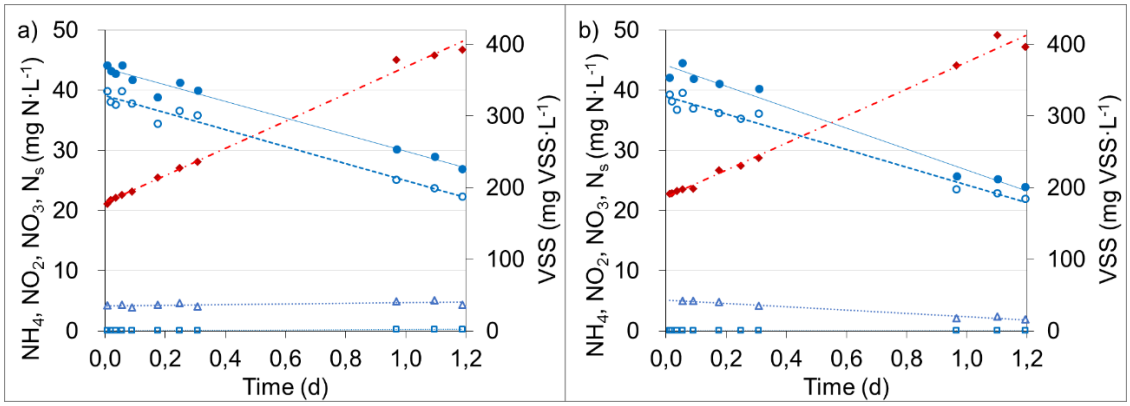

Figure A.2. Evolution of NH_4 (○), NO_2 (□), NO_3 (△), N_s (●) and VSS (♦) concentration during Assay A2: a) R-A; b) R-B.

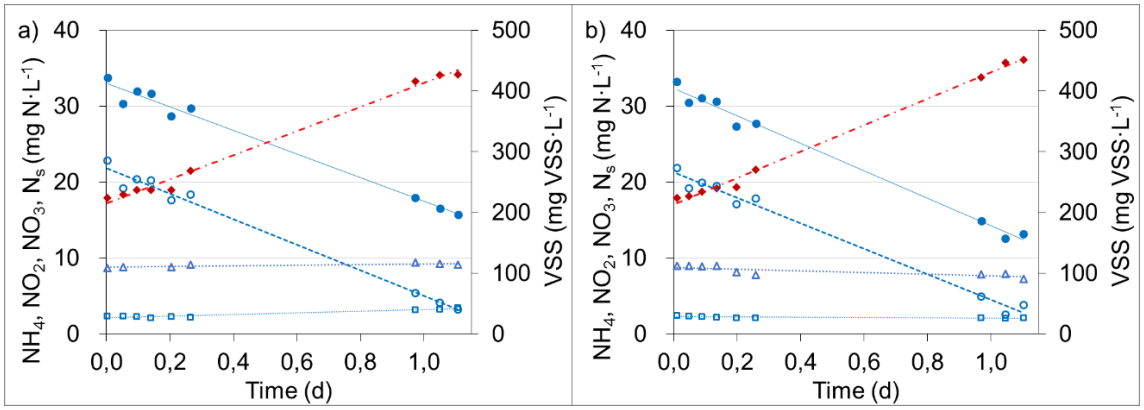

Figure A.3. Evolution of NH_4 (○), NO_2 (□), NO_3 (△), N_s (●) and VSS (♦) concentration during Assay A3: a) R-A; b) R-B.

Table A.2. Nitrogen recovery rates, biomass production and nitrification rates obtained in both reactors during Assay A1.

	R-A		R-B	
	Slope ($mg \cdot L^{-1} \cdot d^{-1}$)	R^2	Slope ($mg \cdot L^{-1} \cdot d^{-1}$)	R^2
NH_4	-14.6	0.982	-14.7	0.989
NO_2	0.3	0.895	0.0	0.137
NO_3	-1.2	0.645	-3.6	0.927
N_s	-14.2	0.986	-16.3	0.975
VSS	139	0.979	150	0.989

Table A.3. Nitrogen recovery rates, biomass production and nitrification rates obtained in both reactors during Assay A2.

	R-A		R-B	
	Slope ($mg \cdot L^{-1} \cdot d^{-1}$)	R^2	Slope ($mg \cdot L^{-1} \cdot d^{-1}$)	R^2
NH_4	-14.2	0.974	-14.7	0.979
NO_2	0.2	0.926	0.0	0.258
NO_3	0.5	0.433	-2.8	0.977
N_s	-13.7	0.976	-17.4	0.981
VSS	189	0.995	189	0.992

Table A.4. Nitrogen recovery rates, biomass production and nitrification rates obtained in both reactors during Assay A3.

	R-A		R-B	
	Slope ($mg \cdot L^{-1} \cdot d^{-1}$)	R^2	Slope ($mg \cdot L^{-1} \cdot d^{-1}$)	R^2
NH_4	-16.9	0.987	-16.8	0.987
NO_2	1.1	0.929	-0.2	0.574
NO_3	0.4	0.659	-1.1	0.643
N_s	-15.5	0.983	-18.1	0.989
VSS	198	0.991	218	0.996

As expected, the nitrifying bacteria activity in the three assays in reactor R-B was negligible since AOB were inhibited by the ATU addition. On the other hand, the AOB activity in reactor R-A (no nitrification inhibition) accounted for 0.3, 0.7 and 1.5 mg $N \cdot L^{-1} \cdot d^{-1}$ in Assays A1, A2 and A3, respectively. The soluble nitrogen recovery rates of R-B in Assays A1, A2 and A3 were 14.8%, 27.0% and 16.8% higher than those of R-A. Regarding biomass production, it was also higher in R-B than in R-A for Assays A1 and A3 (7.9% and 9.9%, respectively), but similar in Assay A2 (Table A3). This data therefore confirms that the nitrification process worsen the microalgae performance as was suggested in previous studies at lab-scale (González-Camejo et al., 2018b) and pilot-scale (González-Camejo et al., 2018a). However, in these previous studies, microalgae affection was also influenced by nutrient limitation, but in these lab Assays, nutrient did not get depleted. These results contradicts those of Rada-Ariza et al. (2017), who did not observe any negatively affection of microalgae due to nitrification in flat-panel sequencing batch photo-bioreactors.

It must be noted that the differences in the ammonium consumption were not significant between R-A and R-B, even during Assay A3, where nitrification rate was the highest (Table A.4). Hence, the lower microalgae activity in R-A had to be compensated with the AOB activity so that both R-A and R-B had similar ammonium recovery rates.

As aforementioned, ammonium is the main nitrogen source of microalgae (Eze et al., 2018; Najm et al., 2017). In fact, some authors have stated that other nitrogen compounds such as nitrate and nitrite are not consumed by microalgae until ammonium is completely depleted (Jebali et al., 2018; Ramanna et al., 2014) since microalgae need to reduce these compounds to ammonium prior to use them (Gupta et al., 2019; Reynolds, 2006; Shoener et al., 2019). However, R-B showed nitrate recovery rates in all the assays in spite of not being nitrogen-limited (Figures A.1, A.2, and A.3); although they were 4.1-15.3-fold lower than their corresponding ammonium recovery rates, which corroborated that ammonium is the preferred nitrogen source of this culture. On the contrary, R-A displayed nitrate production in Assays A2 and A3 because of their nitrifying bacteria activity (Tables A.3 and A.4), only obtaining a nitrate consumption in Assay A1, where the activity of nitrifiers was the lowest (Table A.2). It was therefore considered that the activity of AOB limits microalgae, reducing not only the microalgae biomass production and ammonium uptake, but also the nitrate consumption.

References

1. A.P.H.A., A.W.W.A., W.P.C.F., 2005. Standard methods for the examination of water and wastewater, 21st edition, American Public Health Association, American Water Works Association, Water Pollution Control Federation, Washington DC, USA.
2. Eze, V.C, Velasquez-Orta, S.B., Hernández-García, A., Monje-Ramírez, I., Orta-Ledesma, M.T., 2018. Kinetic modelling of microalgae cultivation for wastewater treatment and carbon dioxide sequestration. Algal Res. 32, 131–141. https://doi.org/10.1016/j.algal.2018.03.015
3. González-Camejo, J., Barat, R., Ruano, M.V., Seco, A., Ferrer, J., 2018a. Outdoor flat-panel membrane photobioreactor to treat the effluent of an anaerobic membrane bioreactor. Influence of operating, design and environmental conditions, Water Sci. Technol. 78(1) 195-206. http://dx.doi.org/10.2166/wst.2018.259
4. González-Camejo, J., Barat, R., Pachés, M., Murgui, M., Ferrer, J., Seco, A., 2018b. Wastewater Nutrient Removal in a Mixed Microalgae-bacteria Culture: Effect of Light and Temperature on the Microalgae-bacteria Competition. Environ. Technol. 39 (4), 503–515. https://doi.org/10.1080/09593330.2017.1305001
5. Gupta S., Pawar, S.B., Pandey, R.A., 2019. Current practices and challenges in using microalgae for treatment of nutrient rich wastewater from agro-based industries. Sci. Total Environ. 687, 1107–1126. https://doi.org/10.1016/j.scitotenv.2019.06.115
6. Jebali, A., Acién, F.G., Rodriguez Barradas, E., Olguín, E.J., Sayadi, S., Molina Grima, E., 2018. Pilot-scale outdoor production of *Scenedesmus* sp. in raceways using flue gases and centrate from anaerobic digestion as the sole culture medium. Bioresour. Technol. 262, 1-8. https://doi.org/10.1016/j.biortech.2018.04.057
7. Jiménez, E., 2010. Mathematical modelling of the two-stage nitrification process. Developement of modelling calibration methodologies for a SHARON reactor and activated sludge process (Modelación matemática del proceso nitrificación en dos etapas. Desarrollo de metodologías de calibración del modelo para un reactor SHARON y un proceso de fangos activados). PhD Thesis, Polytechnic University of Valencia, Spain.
8. Molinuevo-Salces, B., García-González, M.C., González-Fernández, C., 2010. Performance comparison of two photobioreactors configurations (open and closed to the atmosphere) treating anaerobically degraded swine slurry. Bioresour. Technol. 101, 5144–5149. https://doi.org/10.1016/j.biortech.2010.02.006
9. Najm, Y., Jeong, S., Leiknes, T., 2017. Nutrient utilization and oxygen production by *Chlorella vulgaris* in a hybrid membrane bioreactor and algal membrane photobioreactor system. Bioresour. Technol. 237, 64-71. http://dx.doi.org/10.1016/j.biortech.2017.02.057
10. Rada-Ariza, A.M., Lopez-Vazquez, C.M., van der Steen, N.P., Lens, P.N.L., 2017. Nitrification by microalgal-bacterial consortia for ammonium removal in flat panel sequencing batch photo-bioreactors. Bioresour. Technol. 245, 81–89. http://dx.doi.org/10.1016/j.biortech.2017.08.019
11. Ramanna, L., Guldhe, A., Rawat, I., Bux, F., 2014. The optimization of biomass and lipid yields of *Chlorella sorokiniana* when using wastewater supplemented with different nitrogen sources. Bioresour. Technol. 168, 127–135. https://doi.org/10.1016/j.biortech.2014.03.064.

12. Reynolds, C.S., 2006. The Ecology of Phytoplankton (Ecology, Biodiversity and Conservation). Cambridge: Cambridge University Press, UK.
13. Risgaard-Petersen, N., Nicolaisen, M.H., Revsbech, N.P., Lomstein, B.A., 2004. Competition between ammonia oxidizing bacteria and benthic microalgae, Appl. Environ. Microb. 70(9), 5528-5537. https://doi.org/10.1128/AEM.70.9.5528-5537.2004
14. Robles, A., Ruano, M.V., Charfi, A., Lesage, G., Heran, M., Harmand, J., Seco, A., Steyer, J.P., Batstone, D.J., Kim, J., Ferrer, J., 2018. A review on anaerobic membrane bioreactors (AnMBRs) focused on modeling and control aspects, Bioresour. Technol. 270, 612-626. https://doi.org/10.1016/j.biortech.2018.09.049
15. Shoener, B.D., Schramm, S.M., Béline, F., Bernard, O., Martínez, C., Plósz, B.G., Snowling, S., Steyer, J.P., Valverde-Pérez, B., Wágner, D., Guest, J.S., 2019. Microalgae and cyanobacteria modeling in water resource recovery facilities: A critical review. Water Res. X 2, 100024. https://doi.org/10.1016/j.wroa.2018.100024
16. Weon, S.Y., Lee, S.I., Koopman, B., 2004. Effect of Temperature and Dissolved Oxygen on Biological Nitrification at High Ammonia Concentrations. Environ. Technol. 25(11), 1211-1219. https://doi.org/10.1080/09593332508618369
17. Winkler, M.K.H., Straka, L., 2019. New directions in biological nitrogen removal and recovery from wastewater, Current Opinion in Biotechnology 57C, 50–55. https://doi.org/10.1016/j.copbio.2018.12.007