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Short and long-term experiments on the effect of sulphide on microalgae cultivation in tertiary sewage treatment.

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ABSTRACT

Microalgae cultivation appears to be a promising technology for treating nutrient-rich effluents from anaerobic membrane bioreactors, as microalgae are able to consume nutrients from sewage without an organic carbon source, although the sulphide formed during the anaerobic treatment does have negative effects on microalgae growth. Short and long-term experiments were carried out on the effects of sulphide on a mixed microalgae culture. The short-term experiments showed that the oxygen production rate (OPR) dropped as sulphide concentration increased: a concentration of 5 mg $S \cdot L^{-1}$ reduced OPR by 43%, while a concentration of 50 mg $S \cdot L^{-1}$ came close to completely inhibiting microalgae growth.

The long-term experiments revealed that the presence of sulphide in the influent had inhibitory effects at sulphide concentrations above 20 mg $S \cdot L^{-1}$ in the culture, but not at concentrations below 5 mg $S{\cdot}L^{-1}$. These conditions favoured *Chlorella* growth over that of *Scenedesmus*.

Keywords: *Chlorella*; microalgae; *Scenedesmus*; sewage; sulphide.

1. Introduction

Anaerobic membrane bioreactors (AnMBRs) have been reported as a more promising technology for wastewater treatment than conventional aerobic treatments for their several advantages: i) higher energy recovery from organic matter as biogas, ii) reduced power consumption, and iii) up to 90% reduction in sludge production (Giménez *et al.*, 2011). However, AnMBRs are not able to remove nutrients from wastewater (Aiyuk, 2006), which means some post-treatment is required before discharging wastewater in sensitive areas (European Directive 91/271/CEE). In this respect, microalgae cultivation appears to be a sustainable technology for treating AnMBR effluent, allowing not only nutrient removal but also the possibility of moving towards water resource recovery in the sewage treatment field (Ruiz-Martínez *et al*., 2012; Viruela *et al*., 2016).

Autotrophic microalgae are photosynthetic microorganisms which use light energy and inorganic carbon (CO_2 and HCO_3^-) to grow. They also require high amounts of inorganic compounds, such as ammonium (NH_4^+) and phosphate (PO_4^{3-}), which can be obtained from a nutrient-rich wastewater stream (Tan *et al.*, 2016). The microalgae biomass generated can be used as an energy source, since it can be converted into biogas, biodiesel, biohydrogen, fertilizers and high-value products (Maroneze *et al.*, 2016). The combination of an AnMBR and a microalgae cultivation system is therefore a win-win strategy, since it would be feasible to recover both nutrients and other resources such as energy and water from the wastewater. However, among other issues, it must be taken into account that sulphate is reduced to sulphide in an AnMBR by means of sulphate reducing bacteria (SBR). In acid sulphate soils, such as those typically found in the Mediterranean Basin, water (and therefore wastewater) contains high concentrations of sulphate. AnMBR effluent is thus expected to have high sulphide concentrations but low sulphate concentrations (Giménez, 2014).

Sulphide has been ~~previously~~ reported to inhibit the photosynthesis process of microalgae, as it reduces the electron flow between the photosystem II (PSII) and photosystem I (PSI) (Pearson *et al.*, 1987; Miller and Bebout, 2004). By way of example, Küster *et al.* (2005) studied the toxicity of the *Scenedesmus* microalgae through the inhibition of ~~the~~ cellular reproduction during a one-generation cycle lasting 24 hours. Their results showed ~~a~~ 50% ~~of~~ inhibition when the sulphide concentration was around 2 mg $S\cdot L^{-1}$. González-Sánchez and Posten (2017) studied the deployment of a *Chlorella* sp. culture for biogas upgrading and found that these microalgae were inhibited at sulphide concentrations higher than 16 mg $S\cdot L^{-1}$. However, as sulphur acts as macronutrient for microalgae growth, the absence of sulphide or sulphate in the medium can also limit microalgae growth (González-Sánchez and Posten, 2017). This means that before setting up a microalgae culture to treat sewage on an industrial scale, it will be necessary to analyse the effects of introducing sulphide into the system, such us inhibition, nutrient limitation, species distribution in the culture, etc.

The aim of this work was thus to study the effect of sulphide on mixed microalgae culture in tertiary sewage treatment. Short-term experiments were carried out on a bench-scale and long-term pilot-scale experiments in an outdoor membrane photobioreactor (MPBR) using as growth medium the nutrient-loaded effluent from an AnMBR plant at the Carraixet full-scale WWTP (Giménez *et al.*, 2011).

2. MATERIAL AND METHODS

2.1. Microalgae substrate

The microalgae substrate used for both the short and long-term experiments was the nutrient-rich effluent from an AnMBR plant, which is described in detail in Giménez *et al.* (2011) and Robles *et al.* (2013). The AnMBR influent was from the pre-treatment of the Carraixet WWTP (Valencia, Spain): screening, degritter and grease removal. The average nutrient concentrations of the microalgae substrate during the experimental period were: ammonium ~~of~~ 58.4 ± 4.8 $mgN\cdot L^{-1}$ and phosphate ~~of~~ 7.5 ± 0.5 $mgP\cdot L^{-1}$, with an N:P molar ratio of 17.3 ± 1.3. Nitrite and nitrate concentrations were negligible. The substrate also had a total COD concentration of 57 ± 8 $mg\ COD\cdot L^{-1}$, alkalinity of 810 ± 47 $mg\ CaCO_3\cdot L^{-1}$, VFA of 1.5 ± 0.6 $mg\ HAc\cdot L^{-1}$, and sulphide of 112.7 ± 13.8 $mg\ S\cdot L^{-1}$. Sulphate was detected in negligible concentrations. This microalgae substrate was expected to favour microalgae growth over other organisms as it contained low amounts of COD and TSS but high concentrations of nutrients.

The variability of the nutrient load during the ~~evaluated~~ experimental period was associated with variations in both WWTP and AnMBR performance.

2.2. Microalgae inoculum

The microalgae used in this study were originally collected from the walls of the secondary clarifier in the Carraixet WWTP (Alboraya, Spain). The inoculum consisted of a culture dominated by *Scenedesmus* (>99% ~~of the~~ eukaryotic cells), but it also contained other genera such as *Chlorella*, *Monoraphidium*, as well as diatoms, bacteria and cyanobacteria in negligible concentrations. This inoculum was used because these microalgae had already been adapted to the outdoor conditions (light, temperature, etc.) of the location.

Prior to the inoculation of the photobioreactors (PBRs) in the MPBR plant, the culture was adapted to the microalgae substrate (see Section 2.1) under laboratory conditions as described in González-Camejo *et al.* (2017). After this pre-cultivation step, a start-up phase was carried out in the MPBR pilot plant, which consisted of the following: i) inoculation of the PBR with the microalgae culture from the laboratory (pre-cultivation: 10% of the total working volume with a biomass concentration between 300-500 mg VSS·L^{-1} and 90% of the total working volume with microalgae substrate: AnMBR effluent); ii) conditioning stage in batch mode until reaching pseudo-steady state conditions (i.e. reaching stable microalgae biomass concentration); and iii) semi-batch mode maintaining constant biomass retention time (BRT) and hydraulic retention time (HRT) (see Section 2.3.2 for a detailed description).

2.3. Experimental set-up and operation

2.3.1. Short-term experiments

The microalgae photosynthetic activity was determined by respirometric tests (Decostere *et al*., 2013). The oxygen production rate (OPR) was obtained by measuring the dissolved oxygen (DO) slope under well-defined experimental conditions in order to assess the photosynthetic activity of different sulphide concentrations in the microalgae culture.

2.3.1.1. Experimental set-up

The short-term experiments were carried out in a covered 500 mL flask with a magnetic stirrer to homogenise the microalgae culture inside a climatic chamber with air temperature set to 24ºC. 4 LED lamps (Seven ON LED 11 W) continuously illuminated the flask, supplying a light intensity of 300 μE·m^{-2}·s^{-1} measured at the flask surface. In order to determine the OPR, an Orion TM-3 Star Plus portable oximeter (Thermo Scientific TM) was connected to a computer with BioCalibra® software installed (Ribes *et al.*, 2012), which continuously registered dissolved oxygen (DO) concentration and temperature for data monitoring and storage. The short-term experimental assembly is shown in Figure 1.

2.3.1.2. Experimental procedure

Seven different short-term experiments were performed in duplicate with microalgae culture collected from the MPBR plant (see Section 2.3.2) at different sulphide levels. Table 1 gives the sulphide concentrations used. To reach these concentrations, the microalgae culture from MPBR plant was diluted with the appropriate amount of AnMBR effluent (Section 2.1).

Prior to each assay, the samples were kept in darkness to prevent the photosynthetic process from producing oxygen, and were bubbled with nitrogen for 3 minutes to remove any remaining dissolved oxygen.

2.3.2. Long-term experiments

The long-term effect of sulphide on microalgae activity was evaluated on an outdoor pilot-scale microalgae cultivation system for tertiary sewage treatment. This system was fed with the nutrient-loaded effluent from an AnMBR plant that treated the effluent from the pre-treatment of the Carraixet full-scale WWTP as growth medium (see Section 2.1).

2.3.2.1. Experimental set-up

The pilot plant mainly consisted of an outdoor 1.1 m^3 MPBR system located in the Carraixet WWTP (39º30’04.0’’N 0º20’00.1’’W, Valencia, Spain). The MPBR consisted of two outdoor flat-plate PBRs made of transparent methacrylate. Each PBR had total and working volumes of 0.625 m^3 and 0.55 m^3, respectively. Both PBRs were south-facing in order to take full advantage of solar irradiance and both had an additional source of artificial light from twelve LED lamps (Unique Led IP65 WS-TP4S-40W-ME) installed at the rear of the PBRs, offering a continuous light irradiance of 300 $\mu E \cdot m^{-2} \cdot s^{-1}$ (measured on the surface of the reactor) in order to favour night-time microalgae growth over ammonium oxidising bacteria.

The membrane tank (MT) contained an industrial-scale hollow-fibre ultrafiltration membrane unit (PURON® Koch Membrane Systems (PUR-PSH31), 0.03-µm pores) with a filtration area of 3.44 m^2. This MT allowed microalgae biomass filtration and therefore the possibility of decoupling BRT and HRT.

The PBRs and the MT were continuously stirred by CO_2 enriched gas sparging by a blower (C) to prevent wall fouling and ensure~~d~~ adequate CO_2 transference within the broth column. pH was kept at 7.5 ± 0.3 by introducing pure pressurised CO_2 (99.9%) into the system, so that abiotic processes such as ammonia volatilisation and phosphorus precipitation were considered negligible (Whitton *et al.*, 2016). Figure 2 shows the flow diagram of the MPBR plant used, which is further described in Viruela *et al.* (2016).

2.3.2.2. Experimental procedure

During the entire operating period, the MPBR pilot plant was operated under outdoor conditions of variable solar light and temperature. Two different experiments (LT1 and LT2) were carried out in the period of February to May 2015.

Experiment LT1

Experiment 1 lasted 38 days and was carried out without biomass separation, so that HRT was equivalent to BRT. The PBRs were fed in a semi-batch regime, which means that the PBRs were purged with the total amount of culture to maintain a constant BRT of 6 days. The PBRs were then refilled with the AnMBR effluent described in Section 2.1. This experiment was divided into two sub-periods: LT1A and LT1B.

During sub-period LT1A, which lasted 15 days, the AnMBR effluent was pre-aerated before being fed to the MPBR plant in order to oxidise the sulphide to sulphate, for which a pre-aeration step in a regulation tank was applied to the AnMBR effluent through a blower before entering the MPBR plant. An on-off controller was used to keep the DO concentration in the tank at around 2 $mg \cdot L^{-1}$. The controller turned the blower on and off when DO was lower than 1 $mg \cdot L^{-1}$ and higher than 3 $mg \cdot L^{-1}$, respectively. These DO set points achieved complete sulphide oxidation and avoided raising the pH, which remained at values around 7.8, avoiding ammonia volatilisation and phosphorus precipitation (Whitton *et al.,* 2016). After this pre-aeration step, a sulphate concentration of 324.1 ± 51.0 mg $SO_4 \cdot L^{-1}$ was measured in the regulation tank, meanwhile no sulphide was detected. The sulphide was therefore considered to have been completely oxidised in sub-period LT1A.

During LT1B, which lasted 23 days, the AnMBR effluent was fed to the MPBR system with a sulphide concentration of 116.5 ± 2.1 mg $S \cdot L^{-1}$, i.e. the AnMBR effluent was not pre-aerated, so that the sulphide concentration in the culture media reached values around 20 mg $S \cdot L^{-1}$. However, due to the air-stirring, sulphide oxidation did occur inside the PBRs, reaching a sulphate concentration of 332.4 ± 27.3 mg $SO_4 \cdot L^{-1}$.

Experiment LT2

In the 44-days experiment LT2 the BRT and HRT were decoupled through microalgae filtration. The influent was fed to the MPBR plant in continuous mode during daylight hours, maintaining a BRT of 9 days and a HRT of 2.5 days. This long-term experiment was divided into three sub-periods: LT2A, LT2B and LT2C.

In LT2A, which lasted 22 days, the AnMBR effluent was pre-aerated before entering the MPBR plant following the above-mentioned procedure. In LT2B, which lasted 8 days, the AnMBR effluent was fed to the MPBR system with a sulphide concentration of 102.7 ± 10.8 mg $S \cdot L^{-1}$, i.e. the AnMBR effluent was not pre-aerated. Consequently, the maximum sulphide concentration in the PBRs in sub-period LT2B was around 5 mg $S \cdot L^{-1}$.

In LT2C, which lasted 14 days, the AnMBR effluent was pre-aerated again to determine whether the microalgae culture would return to its initial state. When the substrate was pre-aerated (sub-periods LT2A and LT2C), the sulphide was completely oxidised to sulphate, so that the sulphate concentration in the regulation tank was 319.4 ± 38.1 mg $SO_4 \cdot L^{-1}$. When the AnMBR effluent was not pre-aerated, the sulphide in the substrate fed to the PBRs was oxidised to sulphate due to the PBR air sparging, giving a sulphate concentration in the culture media in sub-period LT2B of 313.0 ± 38.1 mg $SO_4 \cdot L^{-1}$. The outdoor PBR conditions in experiments LT1 and LT2 can be seen in Table 2.

2.4. Sampling and Analytical Methods

2.4.1. Short-term experiments

The sulphide (S^{2-}) and sulphate (SO_4^{2-}) concentrations were measured at the beginning of each short-term experiment just before ~~the~~ DO started to rise after the initial lag phase, i.e., at the initial point of the slope (see Figure 3a). S^{2-} and SO_4^{2-} were also measured at the end of the experiment. Sulphide and sulphate were evaluated at the soluble fraction (filtrate) obtained by vacuum filtration with 0.45 mm pore size filters

(Millipore) according to Standard Methods (APHA *et al.*, 2005): Methods 4500-S^{2-}D and 4500-SO_4^{2-}-F, respectively.

The cell death index was obtained by counting the cells in the counting chamber (Neubauer, LO Laboroptic, Friedrichsdorfs, Germany) and dividing by the number of positive dead cells determined by SYTOX Green nucleic acid stain (Molecular Probes by life technologies TM), (Roth *et al.*, 1997). Algae (50 µL) and SYTOX Green stain (0.1 µL) were mixed and incubated for 5 minutes in darkness. 10 µL of the mixture was then added to the Neubauer counting chamber (in duplicate). The total number of stained cells and algae (excitation 504 nm, emission 523 nm) were determined by means of a Leica DM2500 epifluorescence microscope equipped with a DFC420c digital camera.

2.4.2. Long-term experiments

Grab samples were collected in duplicate from the influent and effluent streams of the MPBR pilot plant three times a week. The soluble fraction (filtrate) was obtained by vacuum filtration with 0.45 mm pore size filters (Millipore). The following parameters were analysed for the influent and the effluent: ammonium (NH_4-N), nitrite (NO_2-N), nitrate (NO_3-N), phosphate (PO_4-P), sulphide (S^{2-}) and sulphate (SO_4^{2-}) according to Standard Methods (APHA *et al.*, 2005): 4500-NH3-G, 4500-NO2-B, 4500-NO3-H and 4500-P-F, respectively, in a Smartchem 200 automatic analyser (Westco Scientific Instruments). The sulphide and sulphate concentrations were also measured according to Methods 4500-S^{2-}D and 4500-SO_4^{2-}-F, respectively (APHA *et al.*, 2005). VSS was analysed according to Method 2540 E (APHA *et al.*, 2005); Total eukaryotic cell number (TE) was obtained by the epifluorescence method~~s~~ (Pachés *et al.*, 2012) and cell death was determined as in the short-term experiments (see Section 2.4.1).

2.5. Calculations

Biomass productivity (mg VSS·L^{-1}·d^{-1}), nitrogen removal rate (NRR) (mg N·L^{-1}·d^{-1}) and phosphorus removal rate (PRR) (mg P·L^{-1}·d^{-1}) were calculated as follows:

$$\text{Biomass productivity} = \frac{\mathrm{X_{VSS}}}{\mathrm{BRT}} \qquad \text{(Eq. 1)}$$

where X_{VSS} (mg VSS·L^{-1}) is the volatile suspended solids concentration in the PBRs and BRT is the biomass retention time (d) of the microalgae culture.

$$\mathrm{NRR} = \frac{N_i - N_e}{\mathrm{t} \cdot \mathrm{V}_{PBR}} \qquad \text{(Eq. 2)}$$

where N_i is the nitrogen concentration of the influent (mg N·L^{-1}), N_e is the nitrogen concentration of the effluent (mg N·L^{-1}), t is the period of time considered (d), and V_{PBR} is the volume of the culture in the PBRs (L).

$$\mathrm{PRR} = \frac{P_i - P_e}{\mathrm{t} \cdot \mathrm{V}_{PBR}} \qquad \text{(Eq. 3)}$$

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

In order to compare different operating periods with variations in solar irradiance~~s~~, the nitrogen removal rate-light irradiance ratio was calculated according to Eq. (4):

$$NRR\!:\!I = \frac{NRR \cdot V_{PBR} \cdot 10^6}{I \cdot S \cdot 24 \cdot 3600} \qquad \text{(Eq. 4)}$$

Where NRR:I is the nitrogen removal rate-light irradiance ratio (mg N·mol photons^{-1}), I is the total light PAR irradiance on the PBR~~s´~~ surface, i.e. the 24-hour average solar irradiance plus the light from the LED lamps (µmol photons·m^{-2}·s^{-1}) and S is the illuminated PBR~~s~~ surface (m^2).

2.6. Statistical analysis

All results are shown as mean ± standard deviation of the duplicates. STATGRAPHICS Centurion XVI.I. was used for conducting ANOVA analysis. P-values < 0.05 were considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Short-term experiments

By way of example, Figure 3a shows the evolution of DO concentration during the short-term experiment conducted at a sulphide concentration of 20 mg $S \cdot L^{-1}$. As can be seen in Figure 3a, a lag phase occurred in all the experiments when the oxygen concentration in the microalgae culture was under the detection limit. It was also noticed that the duration of this lag phase increased as the sulphide concentration rose. This suggests that algae were undergoing photosynthesis, but the oxygen produced was being used to oxidise the sulphide towards sulphate. For example, when the initial sulphide concentration of the culture was 20 mg $S \cdot L^{-1}$, there was a lag of around 420 minutes (Figure 3a).

The analysis of the sulphide concentration in the microalgae culture throughout the experiments confirmed that the sulphide concentration was negligible when the oxygen concentration in the culture started to rise, i.e. at the end of the lag phase, so that OPR could only be measured when all sulphide had been oxidised.

Figure 3b shows the oxygen production rates obtained from the short-term experiments (ST1-ST7) at different sulphide concentrations and it can be seen that OPR drops at higher sulphide concentrations. The microalgae could not produce oxygen at the same rate when sulphide content rose because of reduced photosynthetic capacity (Küster *et* *al.*, 2005). This indicates that the low sulphide concentration (5 mg S·L^{-1}) markedly reduced OPR (43%); ~~mean~~while concentrations between 5 and 30 mg S·L^{-1} reduced OPR by 60-72%; those above 40 mg S·L^{-1} were close to completely inhibiting microalgal photosynthetic activity: OPR decreased by 87 and 94% with sulphide concentrations of 40 and 50 mg S·L^{-1}, respectively. These results suggest that the microalgae evaluated in these assays, which grew in the effluent of an AnMBR system (Giménez *et al.*, 2011), were sensitive to very low sulphide concentrations, which indicates that the presence of sulphide limited the photosynthetic capacity of a culture in which *Scenedesmus* and *Chlorella* were the predominant genera (80% and 16% of total eukaryotic cells, respectively). Previous studies have also reported algae restricted by sulphide in natural water, e.g. Küster *et al.* (2005) found strongly inhibited *Scenedesmus* reproduction with hydrogen sulphide concentrations above 2 mg S·L^{-1}.

In order to model this inhibition of photosynthetic activity by sulphide, the OPR values were adjusted to an inhibition function, as shows in Eq. (5):

$$\mathrm{OPR} = OPR_{max}\frac{K_I}{K_{I+}[S^{2-}]} \qquad \text{(Eq. 5)}$$

Where $\mathrm{OPR}_{\mathrm{max}}$ (g O_2·L^{-1}·d^{-1}) is the OPR value with no sulphide effect on the culture and K_I is the sulphide inhibition constant.

Figure 3b shows that the proposed kinetic function accurately predicts the inhibition effect of sulphide on microalgae during photosynthesis. The K_I obtained from these experimental values was 8.7 mg S L^{-1}, which suggests that a sulphide concentration of 8.7 mg S L^{-1} was enough to reduce the microalgae oxygen production rate by half.

The microalgae viability study showed that cell viability decreased as sulphide concentration increased. Differences of less than 5% were observed in assays at low sulphide concentrations (0, 5, and 10 mg S·L^{-1}). At higher concentrations (20, 30, 40 and 50 mg S·L^{-1}), there were significant differences: microalgae viability dropped by

44, 50, 56 and 58% at concentrations of 20, 30, 40 and 50 mg $S\cdot L^{-1}$, respectively, at the end of the experiment. The cell viability study indicated that higher sulphide concentration implies higher mortality.

The results of the short-term experiments suggest that increasing the culture sulphide concentration negatively affects the microalgae´s photosynthetic capacity. These results agree with the findings of Miller and Bebout (2004), who observed that the refill of electrons in the PSII reaction centres during photosynthesis was reduced if sulphide was present. The results also show~~ed~~ that high concentrations of sulphide reduce culture performance. In fact, the maximum sulphide concentration studied (50 mg $S\cdot L^{-1}$) reduced OPR by 94% and mortality by 58%.

3.2. Long-term experiments

3.2.1. Experiment LT1

Figure 4.a shows the evolution of nutrient~~s~~ removal values in experiment LT1. This figure shows that in sub-period LT1A (no sulphide in the influent), the NRR reached higher values than in LT1B (116.5 ± 2.1 mg $S\cdot L^{-1}$ influent sulphide). In fact, the mean values of NRR were 7.4 ± 1.5 and 6.0 ± 1.8 mg $N\cdot L^{-1}\cdot d^{-1}$ for LT1A and LT1B, respectively. The NRR values obtained in experiment LT1 were similar to the findings of other studies concerning the application of microalgae cultivation for wastewater treatment. For instance, Park and Jin (2010) attained a nitrogen removal rate of 5-6 mg $N\cdot L^{-1}\cdot d^{-1}$ by *Scenedesmus* sp. when treating the effluent from an anaerobic digester fed with piggery wastewater and applying cycles of artificial light (PAR of 200 $\mu E\cdot m^{-2}\cdot s^{-1}$ for 12 hours per day). Marcilhac *et al*. (2014) obtained a maximum nitrogen removal rate of 8.5 mg $N\cdot L^{-1}\cdot d^{-1}$ at lab-scale using a green microalgae culture dominated by

Scenedesmus sp. for treating digestate supernatant (PAR of 244 $\mu E \cdot m^{-2} \cdot s^{-1}$ for 12 hours per day).

With regard to phosphorus, no significant differences ($p\text{-value} > 0.05$) in PRR were found between sub-periods LT1A and LT1B: 1.1 ± 0.2 mg $P \cdot L^{-1} \cdot d^{-1}$ and 1.3 ± 0.3 mg $P \cdot L^{-1} \cdot d^{-1}$, respectively. Rasoul-Amini *et al.* (2014) reported similar PRR values for *Chlorella* sp. fed by wastewater from a secondary effluent: 1.1-1.4 mg $P \cdot L^{-1} \cdot d^{-1}$. However, it should be remembered that the performance of an outdoor PBR strongly depends on environmental factors such as solar radiation and temperature. Many authors have reported that the higher the light irradiance ~~is~~, the higher the nitrogen removal rate, as long as it remains below the light saturation level (Anbalagan *et al*., 2015; Viruela *et al*., 2016; Yan *et al*., 2016). However, the average solar PAR during LT1A (NRR of 7.4 ± 1.5 mg $N \cdot L^{-1} \cdot d^{-1}$) was lower than LT1B (NRR of 6.0 ± 1.8 mg $N \cdot L^{-1} \cdot d^{-1}$): 270 ± 149 and 350 ± 81 ($\mu mol \cdot m^{-2} \cdot s^{-1}$), which disagrees with the aforementioned findings, probably due to the sulphide effect, which will be discussed below.

The NRR-light irradiance ratio was calculated to compare NRR values in LT1A and LT1B, and gave mean values of NRR:I of 20.7 ± 6.4 and 13.6 ± 4.3 mg $N \cdot mol\ photons^{-1}$ for LT1A and LT1B, respectively. There was thus a significantly higher NRR:I value in LT1A than in LT1B ($p\text{-value} < 0.05$). Temperature remained fairly constant throughout experiment LT1. Other authors have found that temperature can affect biomass productivity more than the nutrient removal rates (Viruela *et al*., 2016). According to these results, it can be concluded that the presence of sulphide in the influent affected ~~the~~ PBRs~~´~~ performance when the maximum sulphide concentration in the PBRs was 20 mg $S \cdot L^{-1}$.

The~~ presence of~~ sulphide in the PBR~~s~~ influent not only had an inhibitory effect, as observed in the short-term experiments, but also changed the culture population. In

LT1A, the total eukaryotic cells concentration was fairly stable and *Scenedesmus* (Sc) remained the predominant genus (> 99% of total eukaryotic cells); whereas *Chlorella* (Chl) presented a negligible concentration (see Figure 4b). Nevertheless, in LT1B, when aeration stopped in the AnMBR effluent (at a sulphide concentration of 116.5 ± 2.1 mg $S·L^{-1}$ in the influent), *Chlorella* growth increased dramatically and there was a shift in the population of the microalgae culture: *Chlorella* replaced *Scenedesmus* as the predominant genus (see Figure 4b), which suggests that *Chlorella* is more resistant to sulphide inhibition than *Scenedesmus.* According to Küster *et al.* (2005), *Scenedesmus* is strongly inhibited at sulphide concentrations of around 2 mg $S·L^{-1}$. On the other hand, González-Sanchez and Posten (2017) obtained *Chlorella* sp. inhibition at sulphide concentrations higher than 16 mg $S·L^{-1}$, which agrees with the results obtained in the present study. The microalgae viability of both *Scenedesmus* and *Chlorella* in experiment LT1 was always above 87%.

Another consequence of the culture shift was the lack of phosphorus for microalgae growth in sub-period LT1B. In LT1A, the phosphorus concentration in the effluent remained at 0.90 ± 0.62 mg $P·L^{-1}$. However, once the microalgae population changed from *Scenedesmus* to *Chlorella* (from day 20), the effluent phosphorous concentration was negligible (see Figure 4c). This agrees with the findings of Sommer (1986), who reported a competitive advantage of *Chlorella* over *Scenedesmus* at low phosphorus concentrations.

The microalgae population shift was also reflected in the N:P molar ratio consumed in both sub-periodsLT1A and LT1B. In particular, in sub-period LT1A, the average N:P molar ratio was 14.4 ± 3.2, whereas in LT1B it dropped to 12.4 ± 3.4. *Chlorella* thus consumed a proportionally higher amount of phosphorus than *Scenedesmus*, which could have caused the lack of phosphorus in LT1B (see Figure 4c). According to Arbib *et al.* (2013), the optimal molar N:P ratio of *Scenedesmus obliquus* is in the range 9-13; ~~mean~~while Kapdan and Aslan (2008) and Silva *et al.* (2015) reported a lower optimal N:P molar ratio of around 8 for *Chlorella* sp.

VSS and TE significantly decreased at the end of LT1B. As can be seen in Figure 4c, MPBR effluent phosphorous content reached negligible values from day 20 to the end of LT1B, suggesting that the absence of phosphorus in the culture could have caused the decay of microalgae, as reported by Ruiz-Martinez *et al.* (2014). The lack of phosphorus could also have been responsible for the cyanobacteria proliferation in the microalgae culture at the end of the long-term experiment LT1 (data not shown). According to Arias *et al.* (2017), cyanobacteria proliferation is favoured at low nutrient concentrations, in contrast to green microalgae. The cyanobacteria could therefore have affected the microalgae culture (see e.g. Kim *et al.*, 2007; Leão *et al.*, 2009; Zak *et al.*, 2011) since there was a significant drop in total eukaryotic cells after day 33 (see Figure 4b). Further research is needed to clarify long-term culture behaviour.

3.2.2. Experiment LT2

Among the physical factors that affect microalgae cultivation performance (besides sulphide concentration), solar irradiance varied significantly throughout LT2, as can be seen in Figure 5a and Table 2. NRR in sub-periods LT2A, LT2B and LT2C thus could not be directly compared because of the strong influence of solar irradiance on the nitrogen removal rate. The NRR-light irradiance ratio was found to be 33.3 ± 3.0, 39.2 ± 4.8 and 37.1 ± 3.7 mg N·mol photons^{-1} in LT2A, LT2B and LT2C, respectively. Even though these values apparently differ, the ANOVA analysis found no statistical differences between the~~se~~ mean values (p-value > 0.05). It can thus be concluded that the microalgae culture did not suffer from significant sulphide inhibition in experiment

LT2 at an influent sulphide concentration of 102.7 ± 10.8 mg $S\cdot L^{-1}$ and that sulphide inhibition of the microalgae culture in the MPBR studied is not significant at concentrations below 5 mg $S\cdot L^{-1}$.

In Figure 5b it can be seen that Experiment LT2 started with a mixed culture of *Scenedesmus* and *Chlorella*. During sub-period LT2A, *Scenedesmus* became the predominant genus, especially after day 16, when there was a significant increase in TE, probably due to increased solar irradiance after several days with little sunlight (see Figure 5a). However, once the AnMBR effluent ceased to be aerated (in LT2B), TE rose due to the proliferation of *Chlorella* (see Figure 5b). This behaviour was also observed in LT1B, which would be in agreement with Küster *et al.* (2005), and González-Sanchez and Posten (2017), who reported that *Chlorella* sp. resist ~~a~~ higher sulphide concentrations than *Scenedesmus.* It should be noted that when AnMBR effluent aeration was restored and the sulphide was oxidised to sulphate in the regulation tank, *Scenedesmus* again became the predominant eukaryotic algae genus (see Figure 5b). In this experiment, the microalgae viability of both *Scenedesmus* and *Chlorella* remained higher than 85%.

Unlike in experiment LT1, in LT2 no significant cyanobacteria proliferation took place in the microalgae culture, probably because phosphate concentration in the culture media was always above 2.90 $mgP\cdot L^{-1}$ (see Figure 5c).

The results obtained in experiments LT1 and LT2 suggest that *Scenedesmus* was the predominant genus under the given outdoor conditions when the PBRs were fed with AnMBR effluent without sulphide. Viruela *et al.* (2016) also found *Scenedesmus* to be the main genus of the microalgae culture in similar working conditions. On the other hand, when a sulphide concentration of around 112.7 ± 13.8 mg $S\cdot L^{-1}$ was introduced with the influent, *Chlorella* became the predominant microalgae genus, since they are known to support a higher sulphide concentrations than *Scenedesmus* (Küster *et al.* 2005; González-Sanchez and Posten, 2017). This situation did not negatively affect microalgae growth when there was no nutrient limitation and the sulphide concentration remained under 5 mg S·L^{-1} in the PBRs (experiment LT2). However, in LT1, with higher sulphide concentrations in the PBRs (20 mg S·L^{-1}), the system became phosphorus-limited when *Chlorella* proliferated and led to the appearance of cyanobacteria. This was an unfavourable situation because cyanobacteria compete for nutrients with eukaryotic microalgae and can damage microalgae cells (Rajneesh *et al.*, 2017). It can therefore be concluded that in outdoor conditions, oxidising the AnMBR effluent sulphide to sulphate plays an important role in avoiding microalgae sulphide inhibition and cyanobacteria proliferation, especially at low phosphorus concentrations.

4. Conclusions

The short-term results showed that sulphide reduces microalgae´s photosynthetic capacity and viability. A low sulphide concentration (5 mg S·L^{-1}) reduced OPR by 43% and sulphide concentrations above 40 mg S·L^{-1} almost inhibited microalgae growth, reaching maximum mortality (58%) and minimum OPR at 50 mg S·L^{-1}.

The long-term experiments revealed that the presence of sulphide had inhibitory effects when the sulphide concentration reached 20 mg S·L^{-1}, but not when less than 5 mg S·L^{-1}. The presence of sulphide was responsible for *Chlorella* replacing *Scenedesmus* as the predominant genus due to its higher resistance to sulphide.

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Appendix A. Supplementary material.

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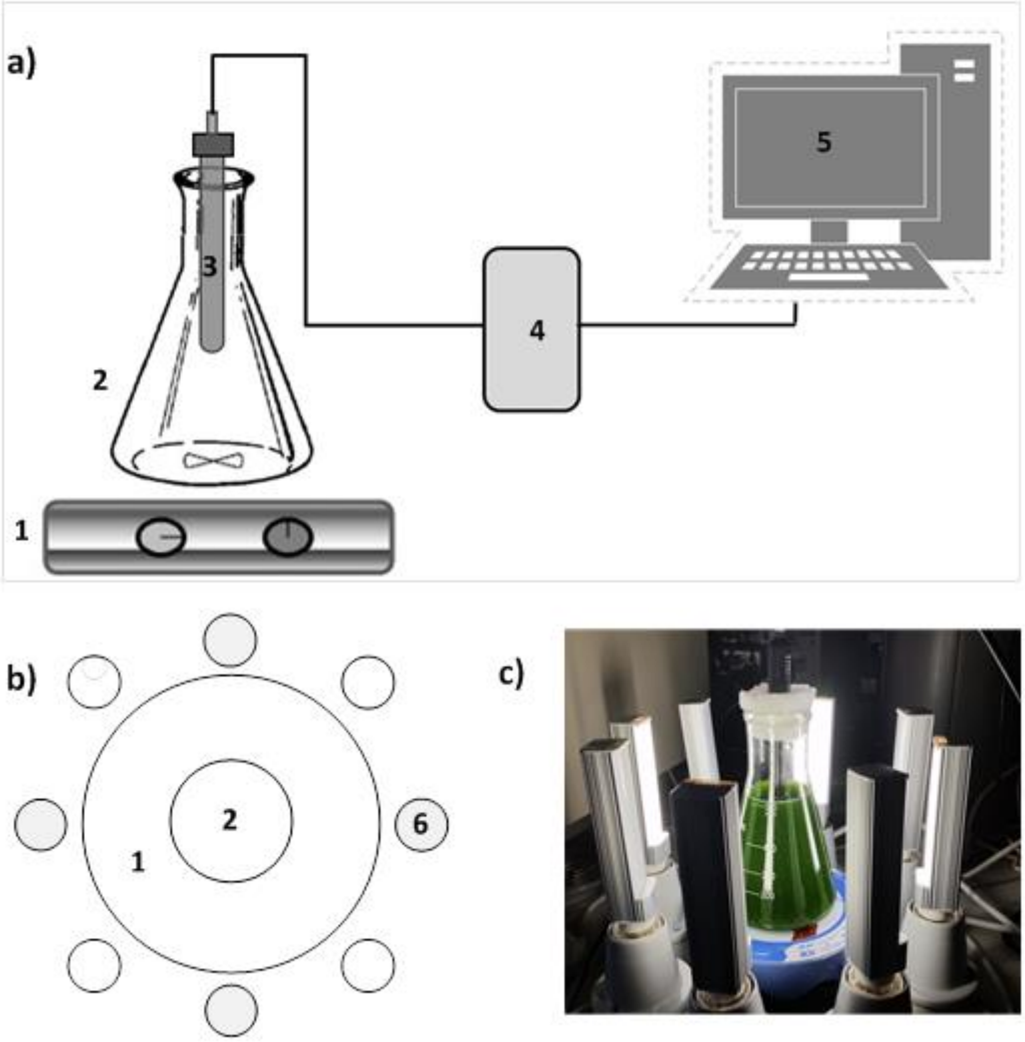

Figure 1. General view: a) Front view; b) Top view; c) Experimental set-up. Nomenclature: 1: Magnetic stirrer; 2: Erlenmeyer flask; 3: Oxygen and temperature probe; 4: Oximeter; 5: Biocalibra software; 6: Led lamp on.

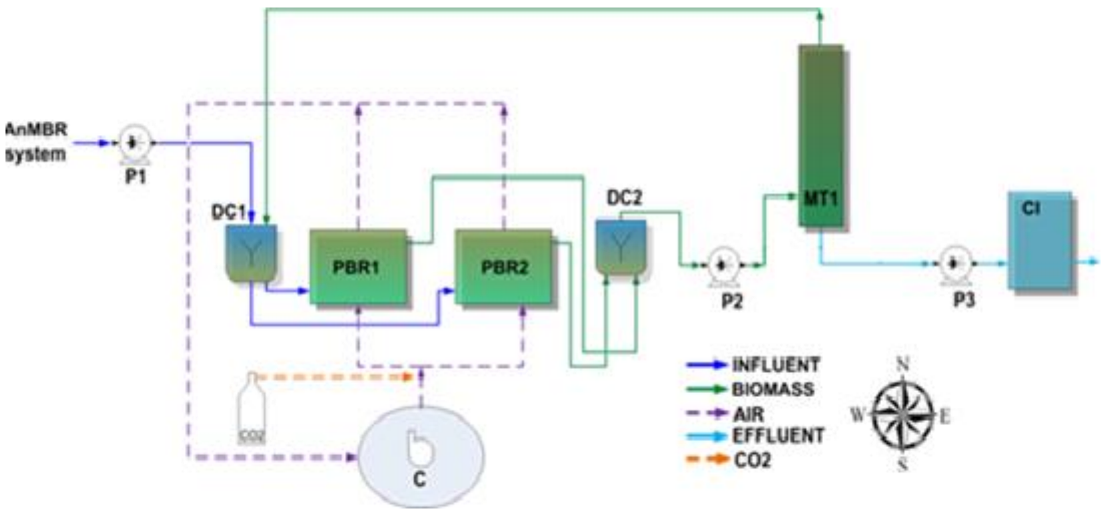

Figure 2. Flow diagram of the PBR pilot plant. Nomenclature: P: pumps; DC: distribution chambers; PBR: photobioreactors; MT1: membrane tank; CI: clean-in-place; C: blower.

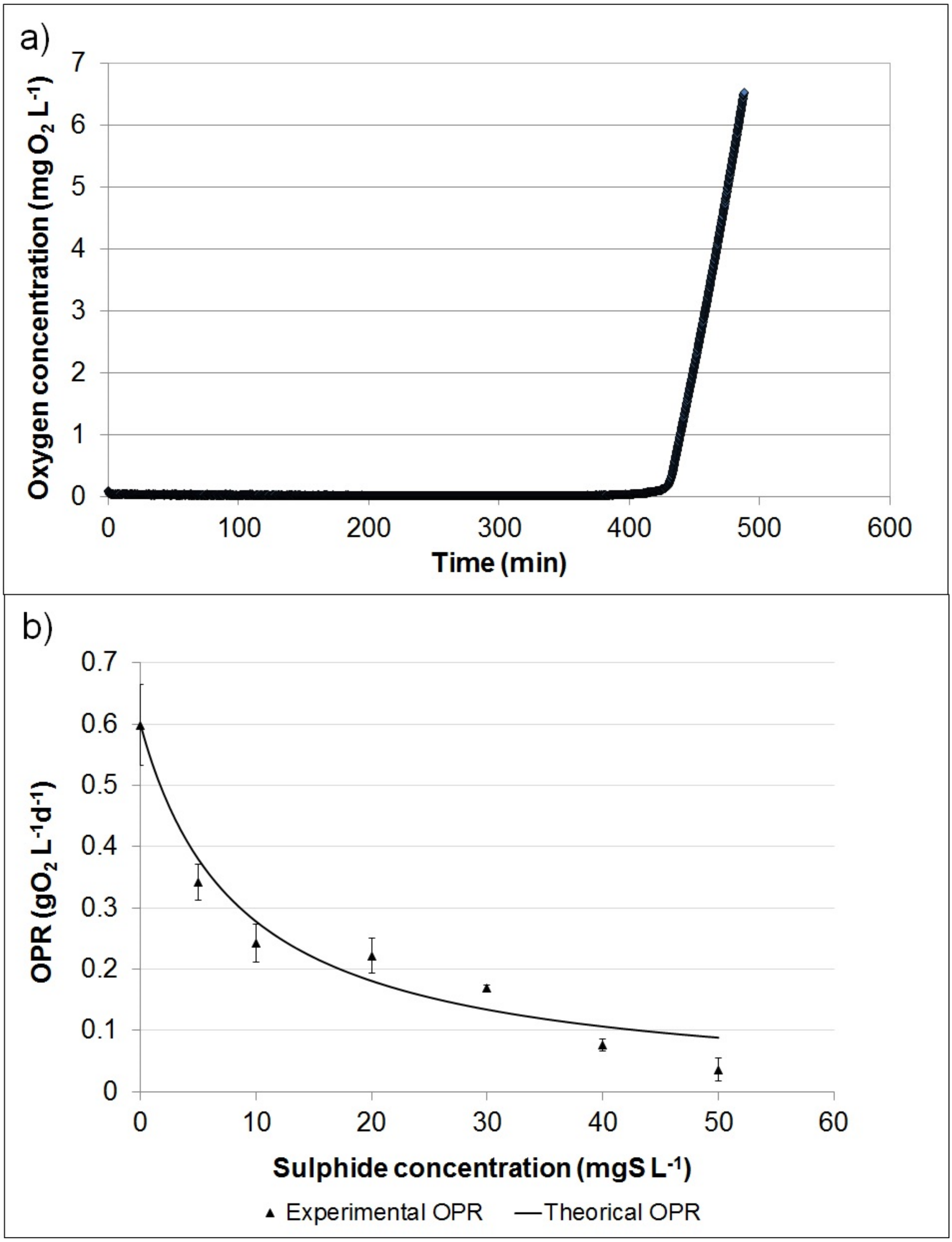

Figure 3. a) Time evolution of the oxygen concentration at a sulphide concentration of 20 mg S·L^{-1}. b) Oxygen production rates obtained at different sulphide concentrations in the microalgae culture.

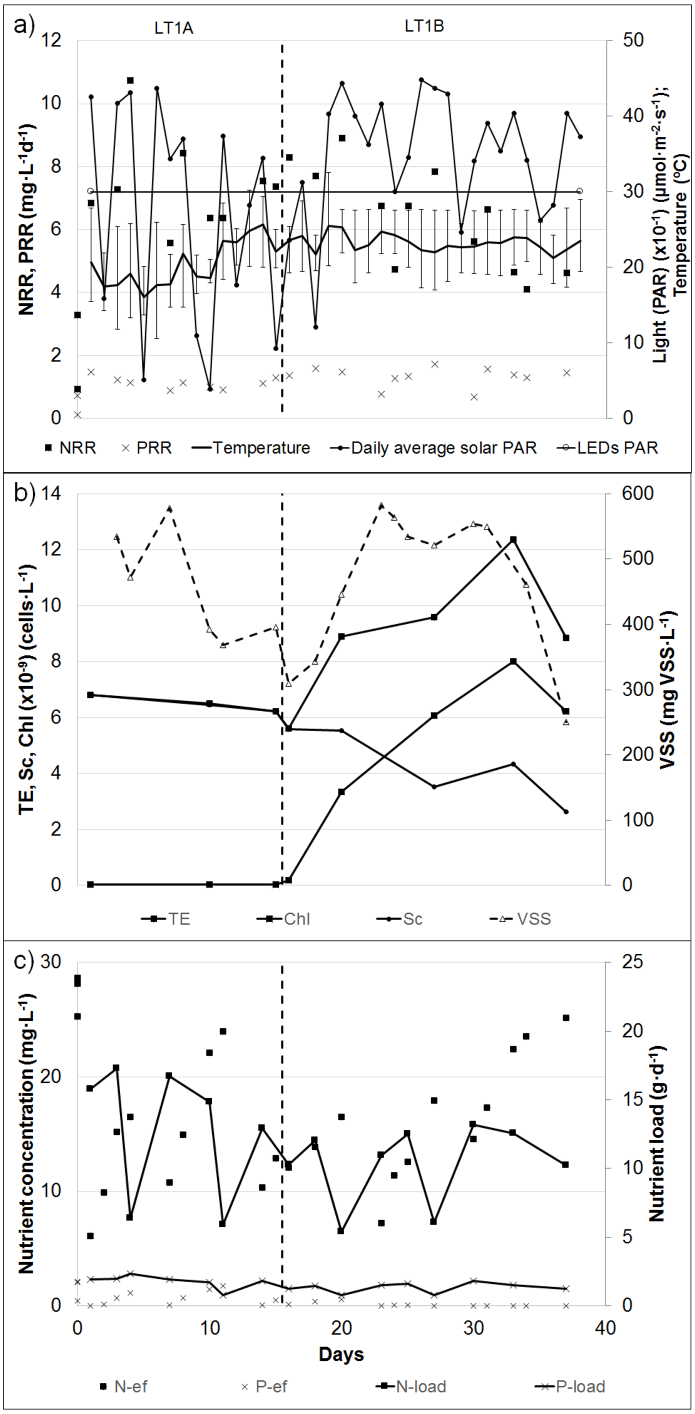

Figure 4: Experiment LT1: Time evolution of: a) Nitrogen removal rate (mg N·L^{-1}·d^{-1}), phosphorus removal rate (mg P·L^{-1}·d^{-1}), light (PAR) (x10^{-1}) (µmol·m^{-2}·s^{-1}) and temperature (ºC); b) cell concentration (cells·L^{-1}) of total eukaryotic cells (TE), *Scenedesmus* (Sc) and *Chlorella* (Chl) and volatile suspended solids concentration (mg VSS·L^{-1}); c) nutrient concentration (mg·L^{-1}) and nutrient load (g·d^{-1}).

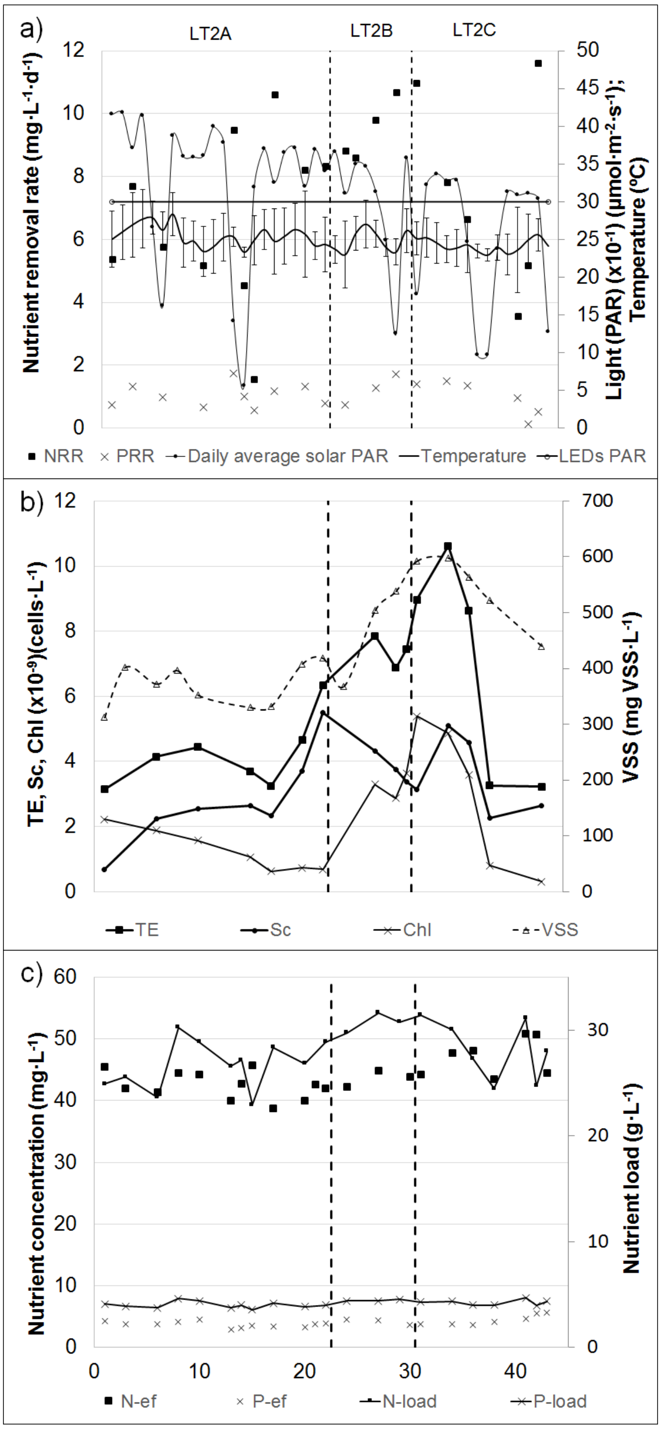

Figure 5: Experiment LT2: Time evolution of: a) Nitrogen removal rate (mg N·L^{-1}·d^{-1}), phosphorus removal rate (mg P·L^{-1}·d^{-1}), light (PAR) (x10^{-1}) (µmol·m^{-2}·s^{-1}) and temperature (ºC); b) cell concentration (cells·L^{-1}) of total eukaryotic cells (TE), *Scenedesmus* (Sc) and *Chlorella* (Chl) and volatile suspended solids concentration (mg VSS·L^{-1}); c) nutrient concentration (mg·L^{-1}) and nutrient load (g·d^{-1}).

Table 1. Sulphide concentration in each short-term experiment.

Experiment	Sulphide concentration (mg S L^{-1})
ST1	0
ST2	5
ST3	10
ST4	20
ST5	30
ST6	40
ST7	50

Table 2. Operation conditions of long-term experiments LT1 and LT2.

Experiment	Sub-period	Days of operation	Daily natural average light intensity ($\mu E \cdot m^{-2} \cdot s^{-1}$)	Temperature (ºC)	Max. [HS] in PBR culture ($mg\ S \cdot L^{-1}$)	BRT (d)	HRT (d)
Exp. **LT**1	Sub-period LT1A	15	270 ± 149	20.3 ± 3.0	< LD	6	6
	Sub-period LT1B	23	350 ± 82	23.2 ± 1.1	20	6	6
Exp. **LT**2	Sub-period LT2A	22	326 ± 94	25.5 ± 1.4	< LD	9	2.5
	Sub-period LT2B	8	288 ± 86	24.9 ± 1.4	5	9	2.5
	Sub-period LT2C	14	252 ± 90	24.2 ± 0.8	< LD	9	2.5