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Production of microalgal external organic matter in a *Chlorella*-dominated culture: influence of temperature and stress factors

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Production of microalgal external organic matter in a *Chlorella*-dominated culture: influence of temperature and stress factors

Abstract

Although microalgae are recognised to release external organic matter (EOM), little is known about this phenomenon in microalgae cultivation systems, especially at large scale.

A study was carried out on the effect of microalgae-stressing factors such as temperature, nutrient limitation and ammonium oxidising bacteria (AOB) competition in EOM production by microalgae. The results showed non-statistically significant differences in EOM production at constant temperatures of 25, 30 and 35ºC. However, when the temperature was raised from 25 to 35ºC for 4h a day polysaccharide production increased significantly, indicating microalgae stress. Nutrient limitation also seemed to increased EOM production. No significant differences were found in EOM production under lab conditions when the microalgae competed with AOB for ammonium uptake. However, when EOM concentration was monitored during continuous outdoor operation of a membrane photobioreactor (MPBR) plant, nitrifying bacteria activity was likely to be responsible for the increase in EOM concentration in the culture. Other factors such as high temperatures, ammonium-depletion and low light intensities could also have induced cell deterioration and thus have influenced EOM production in the outdoor MPBR plant. Membrane fouling seemed to depend on the biomass concentration of the culture. However, under the operating conditions tested, the behaviour of fouling rate with respect to EOM concentration was different depending on the initial membrane state.

Water impact

Microalgae bioremediation is attracting increased attention due to their ability of recovering nutrients from wastewater while producing valuable biomass. However, microalgae cultivation has to deal with the production of external organic matter (EOM), which is often

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not considered. The aim of this study is to assess the conditions that increase the production of EOM by microalgae, which still remains unclear.

1 Introduction

The recent interest in developing new sustainable technologies within the circular economy concept has boosted research on novel water resource recovery facilities (WRRF), where sewage is not considered as a waste that has to be treated but as a source of energy, nutrients and reclaimed water, resulting in environmental and economic benefits.[1,2] One possible solution to make this transition to WRRFs is the combination between anaerobic membrane bioreactor (AnMBR) systems with microalgae cultivation technology.[3] AnMBRs have been tested as a promising energy-effective technology to treat sewage since they can obtain biogas from the anaerobic digestion of the organic matter.[4,2] However, AnMBR effluents usually present large nutrient contents[5] that can lead to eutrophication.[6] A post-treatment step is therefore needed when emitting to sensitive areas. In this respect, microalgae have appeared as a suitable option for wastewater remediation[7-9] as they are able to reduce the nutrient content of these AnMBR effluents.[10,11] In addition, microalgae biomass can serve as a renewable source of biofuels, biofertilisers and other valuable products.[12-15] From all the microalgae reported in the literature, the green microalgae *Chlorella* is one of the genus that have shown higher adaptability to wastewater. [16,17,7]

To cultivate microalgae under outdoor conditions, membrane photobioreactors (MPBRs), which consists of the combination of closed PBRs and membrane filtration,[18] have appeared as promising technology.[10] PBRs are designed to attain high photosynthetic efficiencies, biomass productivities and nutrient removal rates,[19] while membrane filtration enables to operate the system at lower hydraulic retention time (HRT), hence reducing the surface area needed to cultivate microalgae.[20,11]

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Filtration entails membrane fouling due to the accumulation of microalgae biomass on the membrane (cake-layer) and the partial block of the internal pores, [21-23] which reduces the filtration efficiency and increases the energy consumption of the process.[24,25] It must be noted that membrane fouling can be more severe due to the release of microalgal external organic matter (EOM) into the medium since it can intensify the cake layer formation or the blockage to the membrane pores.[21,26-28] To remove reversible fouling, back-flushing and air sparging are usually employed.[29] However, the higher attachment of foulants caused by EOM decreases membrane filtration efficiency due to either too frequent back-flushing stages or unsustainable values of specific air demand (SAD) of the membrane.[30] Moreover, irreversible fouling can only be removed by chemical cleaning,[31] which is non-desirable since excessive use of reagents deteriorates the membrane.

EOM production has been extensively assessed in traditional wastewater treatment techniques. However, EOM characterisation in microalgae cultivation technology has been far less investigated, especially in the case of continuous MPBR operation.[23] EOM includes polysaccharides, proteins, nucleic acids, amino acids and peptides, among others[32,33] and is usually excreted in the microalgae culture as a result of cell growth.[23,13] However, the release of EOM has been reported to be boosted under stressing conditions such as unfavourable pH, temperatures, high or low light intensities, nutrient limitation,[34,35] the presence of toxic substances[36] or high biomass content.[37] Biomass (BRT) and hydraulic retention time (HRT) have been also reported to affect EOM production,[26,23] but to the best of our knowledge, stress factors that increase EOM production haves not been previously evaluated in mixed cultures used for wastewater treatment. From all possible factors, temperature variations can be of great interest in outdoor large-scale microalgae cultivation applications due to the variable conditions microalgae are exposed to.[38,39] In addition, the activity of nitrifying bacteria in a microalgae culture has been reported to affect microalgae performance.[16] Nevertheless, the

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influence of microalgae stress due to nitrification on EOM production has not been evaluated previously.

Apart from affecting membrane filtration, EOM increases the organic matter concentration of wastewater,[40] which can hinder microalgae activity by favouring the growth of microalgae-competing organisms such as heterotrophic bacteria and grazers.[41,23] Bacteria can also produce compounds harmful to microalgae such as toxins,[32] while grazers devour the microalgae cells,[42] meaning that EOM production can affect the robustness of the microalgae culture. EOM also increases the aggregation capacity of microalgae to the PBR surface, reducing the light available to the culture [26,12] and can complicate microalgae nutrient uptake.[43] Since EOM can deteriorate both the microalgae culture and the filtration process, it is important to determine the specific conditions and factors which affect EOM production in order to improve outdoor membrane photobioreactor (MPBR) performance.

The aim of this study was adding some useful information related to the factors that influence the production (and release) of excessive amounts of EOM, as well as the possible effects of this EOM on microalgae cultivation and membrane filtration, which still remains unclear in the case of large-scale membrane-based microalgae cultivation systems for wastewater treatment. To achieve this goal, lab-scale experiments were first carried out to analyse the isolated effect of temperature, nutrient limitation and nitrification from other possible stressing factors that could also affect the *Chlorella*-dominated culture. Later, continuous operation of an outdoor flat-panel MPBR plant that treated effluent from an AnMBR was carried out in order to evaluate the behaviour of the microalgae culture, which was affected by several stressing factors simultaneously.

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2 Material and methods

2.1 Microalgae and substrate

The microalgae substrate, the characteristics of which are shown in Table A.1, was obtained from an AnMBR pilot plant in the Carraixet WWTP.[3] The AnMBR effluent was aerated prior to being fed to the PBRs in order to oxidise the sulphide to sulphate, due to its toxic nature to microalgae.[44] The organic matter loading was mainly inert (Table A.1), thus boosting photoautotrophic metabolism typical of microalgae.[45] However, the presence of EOM in the microalgae culture made the soluble COD concentration to be 144 ± 69 mg COD·L^{-1}.[11] This organic matter favoured the activity of heterotrophic bacteria,[46] which should have degraded some of the EOM produced by microalgae.

Microalgae inoculum was obtained from the walls of the secondary clarifier of the Carraixet WWTP. It consisted of a complex ecosystem which contained green microalgae, cyanobacteria, heterotrophic and autotrophic bacteria amongst others. The inoculum was previously adapted to the substrate as described in González-Camejo et al.[47] Later, microalgae were seeded in an outdoor membrane photobioreactor (MPBR) plant (described in section 2.2.2) in which microalgae evolved to be dominated by green microalgae *Chlorella*, although heterotrophic and autotrophic bacteria were still present in low concentrations.[11]

2.2 Experimental design

Two sets of experiments were conducted using a *Chlorella*-dominated culture obtained from the MPBR plant described in section 2.2.2: i) the first group of experiments was set under lab conditions to isolate the effect of temperature variations, nutrient limitation and nitrification from other possible stressing factors that could affect microalgae under more complex outdoor conditions; ii) the second experiment was up-scaled to a continuously operated outdoor flat-panel MPBR plant that treated effluent from an AnMBR (section 2.1). In this

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case, the *Chlorella*-dominated culture was affected by several stressing factors simultaneously.

2.2.1 Lab experiments

The experimental lab-scale design was based on three stress factors: temperature, nutrient limitation and microalgae-bacteria competition. A total of 5 Experiments were carried out to evaluate the evolution of EOM production: Experiments 1, 2 and 3 focused on analysing the effect of different temperatures (25, 30 and 35ºC); Experiment 4 evaluated the effect of nutrient limitation at 25 and 30ºC; while Experiment 5 analysed the effect of microalgae-nitrifying bacteria competition.

Each experiment lasted 5 days and was conducted in two 2-L Pyrex flasks: R-A and R-B. In both flasks, the culture was mixed and aerated with 0.2 μm pre-filtered air using a membrane air-pump to assure homogenisation and prevent cell sedimentation and biofilm forming on the walls. The airstream was bubbled into the reactors at a flow rate of 0.5-0.6 vvm through fine bubble diffusers placed crosswise on the bottom. Pure CO_2 (99.9%) was injected into the air flow from a cylinder pressurised at 1.5-2 bar to provide both inorganic carbon and maintain pH at 7.5 ± 0.1 in the cultures. Four white LED lamps (18 W, 6000-6500 K) were placed vertically 20 cm away from the flasks to supply a light intensity of 125 $\mu mol \cdot m^{-2} \cdot s^{-1}$ on the PBR surface in 12:12 light:dark cycles.

Both reactors were seeded by 1.5 L of microalgae substrate (section 2.1) and 0.5 L of microalgae culture from the outdoor MPBR plant described in section 2.2.2. As lab experiments were carried out in different time periods, each experiment started-up using microalgae cultures with different nutrient and biomass concentrations (Table A.2). However, R-A and R-B were identical in each experiment. For this reason, R-A was used as reactor control and maintained at 25ºC to compare it with R-B, which was operated at different

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conditions than R-A (temperature or nitrifying bacteria competition) as explained in Table A.3.

Experiment 4 was operated in batch conditions in order to reach nutrient-limited conditions during the experiment. On the other hand, the rest of experiments were fed in semi-continuous mode maintaining an HRT of 3 d. It should be specified that in Experiments 1 and 2, temperatures were maintained constant during all the experiment. On the other hand, in R-B of Experiment 3, temperature was set at 25ºC except for 4 hours a day in which it was risen to 35ºC to simulate the behaviour of temperature under outdoor conditions.[39] In these experiments, 5 $mg \cdot L^{-1}$ of allylthiourea (ATU) were added to the inoculum to inhibit nitrification,[39,48] in both reactors in similar way. In Experiment 5, 10 $mg \cdot L^{-1}$ of ATU were added in R-A to assure complete nitrification inhibition, while R-B was kept without any ATU to allow nitrification to occur (Table A.3). The effect of temperatures lower than 25ºC on EOM evolution was not evaluated as previous study[39] showed no significant differences in microalgae performance when the culture was under temperatures in the range 15-25 ºC. In addition, 35ºC was selected as a representative value of temperature stress according to previous results.[39] Hence, it was not considered necessary to test higher temperatures to evaluate EOM production under microalgae stress.

2.2.2 Pilot plant experiments

The MPBR plant was installed in the Carraixet WWTP and consisted of two flat-plate PBRs connected to a membrane tank (MT). Each PBR had a working volume of 230 L and was continuously stirred by CO_2-enriched air to maintain pH values at 7.5 ± 0.3 and provide carbon-replete conditions. Aeration also prevented wall fouling and ensured culture homogenisation. The 14-L MT contained one hollow-fibre ultrafiltration membrane bundle extracted from an industrial-scale membrane unit (PURON® Koch Membrane Systems

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(PUR-PSH31), 0.03 μm pores) with a filtration area of 3.4 m^2. Further details of the MPBR plant can be found in González-Caamejo et al.[11].

The operation was preceded by a start-up phase[10] (data not shown) and lasted 16 days (Period A), after which culture deterioration occurred. Consequently, another start-up phase was carried out (data not shown) and the operation continued for another 18 days (Period B) to compare MPBR behaviour during both periods. This start-up phase also included a chemical cleaning of the PBRs and membranes following the steps described in González-Camejo et al. [10]. BRT and HRT were maintained at 2 and 1.25 d, respectively.

The membrane was operated continuously at gross 20ºC-standardised transmembrane flux (J_{20}) of around 15-18 LMH and average specific air demand (SAD_P) of around 16-20 $Nm^3 \cdot m^{-3}_{permeate}$ (0.3-0.4 $Nm^3 \cdot m^{-2} \cdot h^{-1}$). Only the amount of permeate needed to maintain hydraulic retention time (HRT) of 1.25 days was taken out of the plant, while the rest was recirculated to the PBRs in order to analyse the filtration process. In addition, the corresponding amount of microalgae culture was purged every day to maintain a biomass retention time (BRT) of 2 days. The membrane followed a sequence of filtration-relaxation (F-R) cycles (i.e. 250 s filtration and 50 s relaxation). Moreover, 40 s of back-flush every 10 F–R cycles, 60 s of ventilation every 20 F–R cycles and 60 s of degasification every 50 F–R cycles were carried out.[10]

In order to evaluate the daily evolution of EOM concentration during the continuous operation of the MPBR plant, grab samples were collected in duplicate at 09:00 (A), 13:00 (B) and 17:00 h (C) on days 9, 10, 12, 16, 24, 25, 27, 31 and 32.

2.3 Analytical methods

A total of 162 samples were analysed from both the lab scale and the outdoor MPBR plant. All the samples were first filtered through a 0.45 μm pore-size glass fibre filters (Millipore) to measure EOM content and nutrient concentrations (NH_4-N, NO_3-N, NO_2-N and PO_4-P). Total

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suspended solids (TSS) were measured as a proxy of biomass.[49] All the measurements were determined from duplicate samples.

2.3.1 EOM polysaccharide (EOM_{-POL})

The polysaccharide content was measured by the phenol/sulfuric acid method[50] with glucose (Panreac) as the standard for the calibration curves to determine polysaccharide concentration. Two mL of filtered sample were pipetted into a colorimetric tube, and 0.05 mL of 80% phenol added. Then, 5 mL of concentrated sulfuric acid was injected onto the sample surface. The tubes were allowed to stand 10 min before readings were taken. The absorbance of the characteristic yellow-orange sample (Fig. A.1c) was measured at 490 nm for hexoses in a Perkin Elmer Lambda 35 spectrophotometer by comparing to the standard to convert to polysaccharide concentration.

It was found that if nitrite concentration of the culture reached values over 2 mg $N \cdot L^{-1}$, the sample got dark (Fig. A.1b). The measurement of the absorbance was thus modified. For this reason, if samples had significant nitrite concentrations, they were diluted with distilled water prior to apply the phenol/sulphuric acid method.

2.3.2 EOM protein (EOM_{-P})

The Lowry method as modified by Peterson[51] was used to measure the protein content of EOM. This method consists of two chemical reactions. The first one is the biuret reaction, in which the alkaline cupric tartrate reagent complexes with the peptide bonds of the protein. And the second one is the reduction of the Folin & Ciocalteu's phenol reagent, which yields a purple color.

1 mL of the filtered sample was placed in a tube with 1 mL of Lowry reagent. The tube was vortexed and 0.5 mL of Folin reagent was added after 20 min at room temperature. After 30 min in darkness at room temperature (to prevent Folin reagent degradation), the absorbance of the sample was measured at a wavelength of 750 nm in a Perkin Elmer Lambda 35

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spectrophotometer. Bovine serum albumin (BSA) was used as the protein standard for the spectrophotometry calibration curves. The absorbance value was converted to protein concentration using the calibration curve.[52]

In this case, if allylthiourea ($C_4H_8N_2S$) is used to inhibit AOB growth in the microalgae culture[48] in concentrations higher than 5 mg·L^{-1}, the sample gets darker (Fig. A.2). Hence, when ATU was present in the microalgae culture in significant concentrations (Experiment 5), the protein concentration of the culture was not measured.

2.3.3 Other measurements

Measurements of ammonium (NH_4-N), nitrite (NO_2-N), nitrate (NO_3-N) and phosphate (PO_4-P) were determined according to Standard Methods[53] 4500-NH_3-G, 4500-NO_2-B, 4500-NO_3-H and 4500-P-F, respectively, in a Smartchem 200 automatic analyser (WestcoScientific Instruments, Westco).

Chemical oxygen demand (COD) and TSS were determined from duplicate samples as described in Standard Methods.[53]

2.4. Calculations

Biomass productivity (mg VSS·L^{-1}·d^{-1}), nitrogen recovery rate (NRR) (mg N·L^{-1}·d^{-1}), phosphorus recovery rate (PRR) (mg P·L^{-1}·d^{-1}) were calculated following the equations shown in González-Camejo et al.[11].

The daily average fouling rate (FR) (mbar·min^{-1}) is defined in Eq. 1:

$$FR = \sum_{j=1}^{z} \left(\frac{\Delta TMP_j}{\Delta t} \right) \Big/ z = \sum_{j=1}^{z} \left(\frac{TMP_j^f - TMP_j^i}{\Delta t} \right) \Big/ z \qquad \text{(Eq. 1)}$$

Where TMP_j^f is the transmembrane pressure at the end of the filtration period j (mbar), TMP_j^i is the transmembrane pressure at the beginning of the filtration period j (mbar), Δt is the time interval of each filtration stage (250s) and z is the number of filtration stages in one day.

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2.5. Statistical analysis

The differences among the experiments were analysed by one-way ANOVA via SPSS software (version 14.0). p-value < 0.05 was considered for statistical significance.

3 Results

It should be noted that the EOM concentration was measured considering only polysaccharide (EOM_{-POL}) and protein (EOM_{-P}) concentrations, since they are the major constituents of the algae EOM.[26,54,13] It should be also considered that microalgae performance was not compared between different experiments since each experiment started with inoculums and substrate with different characteristics (Table A.2) and were thus expected to influence microalgae performance. In addition, it should be bear in mind that the EOM concentrations measured are actually the result of the EOM released by microalgae (EOM released by bacteria is negligible) minus the EOM degraded by heterotrophic bacteria. However, the effect of EOM degradation by heterotrophic bacteria was not considered to significantly alter the results as it should similarly affect all cases in a manner as all inoculums had negligible bacteria concentration.

3.1 Effect of temperature on EOM content

In Experiment 1, similar trend of normalised EOM (i.e. EOM concentration divided by microalgae biomass) was observed in both R-A (25ºC, Fig. 1a) and R-B (30ºC, Fig. 1b). In fact, there were no statistically significant differences between the two temperatures for both normalised $EOM\text{-}_{POL}$ and $EOM\text{-}_{P}$ (p-value > 0.05, n = 9). However, both reactors presented a decrease in the normalised EOM_{-P}, which implied that the EOM_{POL}/EOM_{-P} ratio increased through time from 0.8 to 2.2.

When a higher temperature range between R-A and R-B was tested; i.e. 25 and 35ºC in Experiment 2, the behaviour was similar than Experiment 1; i.e. both normalised $EOM\text{-}_{POL}$ and EOM_{-P} patterns were similar in both reactors (Fig. 1c, 1d), showing no statistically

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significant differences (p-value > 0.05, n = 9). The normalised EOM slope values were positive for polysaccharides and negative for proteins, yielding an EOM-$_{POL}$/EOM-$_{P}$ ratio that increased from 0.5 to 1.7 in both reactors.

Lastly, when temperature increments from 25 to 35ºC were applied to the culture only 4 h a day (Experiment 3), no statistical differences (p-value > 0.05, n = 9) were found between the two reactors for EOM$_{POL}$ and EOM$_{-P}$ concentrations (data not shown). However, when normalised EOM-$_{POL}$ was analysed, the pattern was statistically significantly different (p-value < 0.05, n = 9). At 25ºC (Control, Fig 1e), the normalised EOM-$_{POL}$ increase was less than 10%, while it rose significantly to 42% when temperature peak was applied (Fig. 1f).

In the case of normalised EOM$_{-P}$, no significant differences (p-value > 0.05, n = 9) between both reactors were found (Fig. 1e, 1f). Similarly, to previous experiments, the EOM-$_{POL}$/EOM-$_{P}$ ratio increased in Experiment 3 from 1.6 to 2.6 and 3.8 for R-A and R-B, respectively.

-3.2 Effect of nutrient limitation on EOM content

In Experiment 4, reactors were operated in batch conditions at 25 (Fig. 2a) and 30ºC (Fig. 2b) in order to reach nutrient-limited conditions; i.e. NH_4-N concentration lower than 10 mg N·L^{-1}.[55] As can be seen in Fig. 2, both EOM-$_{POL}$ and EOM-$_{P}$ concentrations increased over time in batch conditions. At 25ºC (Fig. 1a) the increase was 6.7-fold and 2.6-fold for EOM-$_{POL}$ and EOM-$_{P,}$ respectively, from the beginning to the end of the experiment. At 30 ºC (Fig. 1b), EOM-$_{POL}$ and EOM-$_{P}$ increased by 7.0-fold and 3.1-fold, respectively, presenting no significant differences in comparison to 25ºC (p-value > 0.05, n = 9). This made both reactors reach nutrient limitation on day 4 (Fig. 2). Both experiments revealed a similar gain pattern; i.e. a gradual increase of EOM production rate during the first 4 days of the experiment (0.5-0.7 mg·L^{-1}·d^{-1} for EOM-$_{POL}$ and 0.3-0.4 mg·L^{-1}·d^{-1} for EOM-$_{P}$) and sharp increases when cultures were nutrient-limited (2.4 mg·L^{-1}·d^{-1} and 0.6 mg·L^{-1}·d^{-1} for EOM-$_{POL}$ and EOM-$_{P,}$

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294 respectively, in R-A and 2.1 $mg \cdot L^{-1} \cdot d^{-1}$ and 0.5 $mg \cdot L^{-1} \cdot d^{-1}$ for EOM-$_{POL}$ and EOM-$_P$
295 respectively, in R-B). Since the raise of EOM-$_{POL}$ production rate was significantly higher
296 than that of EOM-$_P$ in both R-A and R-B, the EOM-$_{POL}$/EOM-$_P$ ratio rose throughout
297 Experiment 4 from 1.2 to 2.4.

298 *3.3 Effect of microalgae-AOB competition on EOM content*

299 The competition with AOB was tested at 25ºC in both reactors. As can be seen in Fig. 3,
300 EOM$_{POL}$ evolution throughout Experiment 5 was similar in both cultures with and without
301 AOB competition (p-value > 0.05; n = 8) and finally increased in both reactors by around
302 50%.

303 EOM-$_P$ content was not measured in Experiment 5 since the ATU (added to the culture to
304 inhibit AOB activity) interfered in protein measurement (see Fig. A.2).

305 *3.4 Effect of outdoor conditions on the EOM content*

306 The daily samples taken from the MPBR plant; i.e. samples A, B and C for each day did not
307 show any specific trend in either polysaccharides or proteins for none of the periods analysed
308 (Fig 4). Similar behaviour was found in the normalised EOM concentrations (data not
309 shown).

310 Regarding the evolution of normalised EOM concentration during the continuous operation of
311 the MPBR plant in Period A, both normalised EOM-$_{POL}$ and EOM-$_P$ remained under similar
312 values until day 12, but significantly increased on day 16 (p-value < 0.05; n = 12), as
313 displayed in Fig. 5d. However, this EOM increase on day did not seem to be related to an
314 increase in the transmembrane pressure, which evolution is shown in Figure 6a. It should be
315 noted that the TMP displayed in the graph only corresponds to that measured during filtration
316 stage. The TMP measured during other stages such as relaxation and back-flushing (see
317 Section 2.2.2) is not displayed in Fig. 6a to ease data visualisation. As can be observed in Fig.
318 6a, TMP started Period A with low values around 0.05 bar at the beginning of Period A and

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increased to values in the range of 0.10-0.18 bar on day 9 on. In fact, from day 9 until the end of Period A, the TMP trend was similar, with the exception of day 11 in which maximum value of TMP got close to 0.25 bar (Fig. 6a). On the other hand, the EOM increase on day 16 did coincide with a decrease in NRR and biomass productivity (Fig. 5b,5d). A start-up phase[10] was then carried out after day 16, which reduced the EOM concentration significantly on day 24 (Fig. 5d). The transmembrane pressure of the membrane also decreased to values in the range of 0-0.04 bar (Fig. 6a) due to the membrane chemical cleaning done during this start-up phase (as explained in Section 2.2.2). Once again, the normalised EOM concentrations remained at similar values for around two weeks but rose by the end of Period B (Fig. 5d). However, at this time, only EOM-$_{POL}$ concentration increased significantly (p-value < 0.05; n = 15), while EOM-$_{P}$ concentration remained nearly stable. On the other hand, MPBR performance (in terms of nutrient recovery and biomass productivity) decreased with time in Period B, similarly to what occurred in Period A (Fig. 5b).

Solar light PAR and culture temperature were monitored during the continuous operation of the MPBR plant (Fig. 5a). In the first 10 days, the conditions were favourable for microalgae growth; i.e. solar light intensities of around 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and mid-range temperatures of around 20ºC. However, after day 10, the ambient conditions changed (temperature increased around 5ºC and solar PAR suffered a significant reduction) and probably favoured nitrifying bacteria growth.[16] In addition, the culture was expected to be under ammonium-limited conditions, since NH_4-N concentration was under 10 mg $N \cdot L^{-1}$.[55] This situation made the nitrification rate (NOxR) (which measures the nitrate and nitrite produced through nitrification and is used as an indicator of nitrifying bacteria activity[16,56] increase during Period A to a maximum of 9.3 mg $N \cdot L^{-1} \cdot d^{-1}$ (Fig. 5a). In Period B, after the aforementioned start-up phase, the nitrification rate showed low values, but immediately increased again (Fig.

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5a). A summary of the average results obtained during the continuous operation of the MPBR plant is displayed in Table A.4.

4 Discussion

It has to be considered that EOM products may be classified into different categories according to the phase in which they are released: compounds produced as a result of substrate metabolism are growth-synonymous and growth-associated, while those excreted due to environmental interaction and lysis are growth-independent.[37] Increasing growth-synonymous EOM would entail raised biomass concentrations. Hence, variations of normalised EOM will not consider the evolution of growth-synonymous EOM.[11] On the other hand, growth-independent EOM will not be directly related to microalgae biomass but to microalgae stress. Normalised EOM can thus be used as an indicator of microalgae stress.[11]

4.1 Effect of temperature on the EOM content

According to Experiments 1 and 2, the *Chlorella*-dominated culture did not significantly vary their normalised EOM-$_{POL}$ and EOM-$_{P}$ when the temperature was maintained constant at 25, 30ºC or -35ºC. These results disagrees with those found by other authors who concluded that the EOM content is affected by temperature.[37] It is possible that the microalgae had adapted to the temperatures evaluated in these experiments and were thus not significantly stressed at constant temperatures of 25, 30 and 35ºC.

On the other hand, statistically significant differences (p-value < 0.05, n = 9) were found in the culture subjected to a sharp temperature increase from 25 to 35ºC for 4h a day (R-B in Experiment 3). This changes in temperature greatly boosted the release of normalised EOM-$_{POL}$ over that of the reactor control (R-A), which suggested that the culture should have suffered stress due to those temperature variations. This stress factor must be thus considered when operating large-scale microalgae cultivation systems since temperature variations over 10ºC are easily reached outdoors.[39]

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4.2 Effect of nutrient concentrations on EOM content

Since nutrient levels have been reported to play a significant role on EOM production and composition.[32,57] batch cultures (Experiment 4) made it possible to analyse the behaviour of EOM production under nutrient-replete and nutrient-deplete conditions. In nutrient-replete conditions (days 1-4), EOM increased as a consequence of the biomass accumulating in the system and hence must have been growth-synonymous.[37,11] However, when the microalgae reached nutrient-deplete conditions at NH_4-N < 10 mg N·L^{-1}, [55] by the end of the experiments, there was a sudden increase in EOM_{-POL} production in both reactors (Fig. 2), which suggests that under nutrient-deplete conditions EOM_{-POL} production was not only due to microalgae growth (growth-synonymous), but also that nutrient depletion was likely to have stressed the culture. As some authors have pointed out, the lack of nutrients (especially nitrogen) may redirect the carbon metabolism towards incorporation into polymers, increasing the sugar accumulated in the cells[32] and consequently, higher amounts of EOM-$_{POL}$ were likely to be released in the medium. This statement is also interesting regarding the up-scaling of microalgae cultivation. It suggests that if EOM concentration wants to be maintained low in order to avoid culture deterioration, nutrient-deplete conditions should be avoided.

Although some studies found EOM-$_P$ to be more important than EOM-$_{POL}$ in both wastewater aerobic or anaerobic sludge[58,28] and microalgae cultivation experiments,[13] in the present study with microalgae fed with AnMBR effluent, EOM-$_{POL}$ production was higher than that of EOM-$_P$. In fact, the EOM-$_{POL}$/EOM-$_P$ ratio increased in all the lab experiments by as much as 3-fold. It therefore seems that products of a polysaccharide nature are preferentially released into the medium over proteins. Similar results were obtained by Felipe Novoa et al.[26], who reported EOM-$_{POL}$/EOM-$_P$ values in the range of 1.9-4.9.

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4.3 Effect of nitrifying bacteria-microalgae competition on EOM content

Bacteria have been suggested to have a significant effect on the EOM secretion process.[35] The interspecies competition between microalgae and nitrifying bacteria for nutrients may thus affect both the uptake and the release of EOM. For this reason, the other stress factor tested under lab conditions was the microalgae-AOB competition at the optimal temperature in nutrient-replete conditions since this competition can play a significant role when treating effluents from anaerobic digestion.[39,16,59]

No significant differences were observed in EOM production in the lab-scale experiments. These results could be explained by two possible hypotheses: i) either the microalgae-AOB competition did not significantly stress the microalgae; or ii) the operating conditions of this lab-scale experiment (experimental time, HRT, etc.) did not produce significant changes in the culture with respect to microalgae-nitrifying bacteria competition.

4.4 MPBR plant

4.4.1 Daily evolution of EOM concentration

Since EOM production has been reported as a light-dependent process.[32] the daily trend of EOM concentration was expected to be similar to that of the solar PAR measurements; i.e. lower values in the morning (Sample A) and evening (Sample C) and the highest value at midday (Sample B). However, neither the EOM_{-POL} nor EOM_{-P} concentrations followed the same pattern as light intensity in the continuous operation. Moreover, $EOM\text{-}_{POL}$ concentration was variable (Fig. 4a), while $EOM\text{-}_{P}$ remained fairly constant (Fig. 4b). In this respect, Period A started with an $EOM\text{-}_{POL}/EOM\text{-}_{P}$ ratio of 1.2 and finished it with 1.7, while Period B started presenting an $EOM\text{-}_{POL}/EOM\text{-}_{P}$ ratio of 0.7 but it rose to 1.7 at the end. Hence, $EOM\text{-}_{POL}$ was likely to be more affected by stressing factors. Similar behaviour was observed in the lab experiments (Sections 3.1, 3.2).

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These results suggest that EOM production in the outdoor MPBR plant is not directly proportional to microalgae activity (i.e. growth-synonymous and growth-associated EOM[37]) and that increasing EOM production could have been related to stress factors, such as higher temperature, light limitations, ammonium depletion or competition with nitrifying bacteria.

4.4.2. Continuous operation of microalgae cultivation

EOM concentration raised for both polysaccharides and proteins during Period A (Fig. 5d), probably because several stress factors affected microalgae at the end of this Period (day 16): i) the average culture temperature increased by around 5ºC at the end of Period A (Fig. 5a), reaching maximum values over 30ºC. Previous study with similar substrate and culture showed microalgae performance to decrease at temperatures over 30ºC;[39] ii) ammonium-deplete conditions were reached, obtaining NH_4-N values lower than 10 mg $N \cdot L^{-1}$ at the end of Period A (Fig. 5c); iii) solar PAR reduced significantly to values under 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ on days 14-15 (Fig. 5a); iv) nitrifying bacteria activity (measured by NOxR) increased during Period A reaching a maximum value of 9.3 mg $N \cdot L^{-1} \cdot d^{-1}$ on day 16 (Fig. 5a). All these factors could have induced cell deterioration and so could have led to higher EOM release to the culture,[33] obtaining significantly higher EOM_{-POL} and EOM_{-P} concentrations on day 16 than on days 9, 10 and 12 (Fig. 5d).

The trend of Period B regarding EOM production was similar than Period A as it increased at the end of the period. However, this increase only affected EOM_{-POL}, while EOM_{-P} remained at similar values (Fig. 5d). Unlike Period A, the temperature in Period B only reached 17.2 ± 1.3ºC, which was lower than Period A (Table A.4). Moreover, ammonium and phosphorus were in replete conditions from day 24 on (Fig. 5c). However, the nitrification rate increased with time (Fig. 5a). These results therefore suggest that EOM_{-POL} production in Period B must have been highly influenced by the stress caused by the presence of nitrifying bacteria in the culture. This behaviour was the opposite of that observed in Experiment 5 under lab

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conditions, in which no significant differences were found in EOM-$_{POL}$ concentrations between cultures with and without nitrification. There are several factors that could be responsible for this different behaviour: i) nitrifying bacteria activity highly depends on the nitrogen load,[60] which was significantly higher in the MPBR plant (HRT = 1.25 d) than in the lab-scale Experiment (HRT = 3 d); ii) the MPBR plant achieved significantly higher biomass concentration than lab-scale reactors, therefore suffering more significant shadow effect.[61,62] Microalgae were thus likely to be more limited in the pilot plant than at lab-scale; iii) in the lab-scale experiment the culture only lasted 5 d while under outdoor conditions the operation was lengthened to 16-18 days. The age of the culture could have also affected the nitrifying bacteria proliferation as microalgae are usually better adapters to the microalgae substrate used in this study than nitrifying bacteria, according to previous results.[39]

As aforementioned, EOM-$_P$ stayed at similar values during Period B unlike Period A (Fig. 5d). It was hypothesised that EOM-$_P$ increased only at the end of Period A because there were several stress factors in this period that could have affected EOM production, while in Period B microalgae-nitrifying bacteria competition was the only noticeable stress factor (Fig. 5). This confirms that polysaccharides are used by microalgae to interact with the environment in preference to proteins, as observed in the lab-scale experiments (Sections 3.1, 3.2) and the outdoor MPBR plant (Section 4.4.1), where the EOM-$_{POL}$/ EOM-$_P$ ratio of the culture always increased at the end of the Experiment/Period.

It should be noted that nutrient recovery rates and biomass productivity decreased at the end of both Periods A and B (Fig. 5b) when normalised EOM were the highest (Fig. 5d). Similar behaviour has been observed by other authors.[43,33] However, in this study, the reduction in nutrient recovery and biomass productivity could also have been due to other factors such as lower solar radiation and ~~a~~ higher nitrification rate (Fig. 5). In fact, light and competition with nitrifying bacteria have been reported to be key factors in microalgae cultivation

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systems.[63,16,38,64] Hence, the higher normalised EOM in the culture might not have been the main factor in the lower microalgae cultivation performance observed by the end of both Periods A and B. It will thus be necessary to monitor the system for longer operating periods and to relate all the possible factors which influence nutrient recovery and biomass productivity to properly assess the weight of each individual factor on MPBR performance.

4.4.3. Continuous membrane filtration

Fig. 6a shows the evolution of TMP along Period A and B. It should be remembered that TMP is the pressure that the system has to overcome due to the membrane resistance.[65] On the other hand, FR measures the rate which this resistance increases during operation. The aim of membrane filtration operation will thus focus on decreasing the FR as it would increase operating costs.[3]

At the beginning of Period A (days 1-5), TMP started at low values of around 0.05 bar (Fig 6a). It must be noted that there were oscillations in these parameters (Fig 6a) due to relaxation and back-flushing stages which helped to reduce the cake layer in the membrane.[21,22,29] This is a common behaviour that has been observed in previous operations of the MPBR plant.[10,11] As continuous membrane operation goes on, TMP continuously is expected to rise due to the accumulation of foulants on the membrane. However, from day 5 until the end of Period A, TMP remained quite stable with the exception of day 11 in which a significant TMP rise was observed (Fig. 5a). With respect to Period B, TMP was maintained under 0.05 bar during all Period (Fig. 5a) since it was preceded by a chemical cleaning of the membranes. Due to this cleaning, the behaviour of the membrane concerning to FR was different for both Periods, showing higher fouling rate in Period A (in the range of 6.5-7.5 mbar), where the membrane started at higher TMP than in Period B: 0.6-2.7 $mbar^{-1}$. These FR values are considerably low, [65] probably due to limited transmembrane flux that was operated: 15-18 LMH. [11]

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It should be highlighted that for both Periods A and B, FR was significantly correlated to TSS concentration (Fig. 6b). In fact, coefficient of determination (R^2) accounted for 0.482 and 0.772 for Period A and B, respectively. This behaviour of membrane fouling has been widely reported in previous studies, not only for MPBR systems,[26,10,57] but also in sludge-based systems.[28] On the other hand, total EOM concentration (EOM_{-Total}; i.e. the sum of EOM_{-POL} and EOM_{-P}) was only correlated to FR in Period B ($R^2 = 0.623$) but it was not in Period A (Fig. 6c). These results seem contradictory, but literature with regards to this topic is also unclear. For instance, some authors have reported the correlation between EOM concentration and membrane fouling,[27,25] but others[23] did not observe a link between EOM and membrane.

The different relation between EOM and FR in Periods A and B was hypothesised to be related to the different fouling state of the membrane at the beginning of each Period. In Period A, where TMP was higher (Fig. 6a), FR was mainly dominated by the TSS concentration as there was no significant correlation between EOM_{-Total} and FR (Fig. 6b, 6c). Maybe in this Period there was a thicker cake layer on the membrane so that the effect of EOM was negligible as much of EOM could deposit on the cake layer instead of the membrane surface itself, reducing its global impact on fouling rate. In fact, cake layer retention has been reports as the main removal mechanism of EOM in a microalgae culture.[26,66] On the other hand, in Period B both TSS and EOM were correlated, which suggested that both microalgae biomass and EOM released by microalgae had significant influence on FR, probably because the membrane started perfectly clean, which implied that EOM was more likely to block not only the membrane surface but also membrane pores.[26,67] It should also be highlighted that the correlation of EOM_{-Total} and FR found in Period B was mainly due to polysaccharides. Indeed, EOM_{-POL} and FR showed good correlation, i.e. R^2 of 0.593; while EOM_{-P} showed no significant changes with FR ($R^2 = 0.032$). Similar behaviour was found by Felipe Novoa et al.[26]. However, as data obtained during the continuous

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operation of the MPBR plant was scarce, longer operating periods should be tested to corroborate these statements.

5 Conclusions

The lab-scale experiments showed that sudden temperature rises from 25 to 35ºC and nutrient limitation are stress factors and increased polysaccharide release, although protein production remained stable. On the other hand, there were no significant differences with constant temperatures in the range of 25-35ºC and competition with nitrifying bacteria. In outdoor operation the sharp variations in the culture temperature should be thus reduced at minimum during continuous operation to avoid microalgae stress and EOM production. In addition, the competition with nitrifying bacteria seemed to produce a certain degree of stress in the microalgae culture, since nitrification rate increases were related to increasing EOM production. However, this rise was also affected by a combination of several stress factors, such as excessive temperature, reduced solar light and ammonium depletion. On the other hand, lower microalgae performance in terms of nutrient recovery and biomass productivity was observed in the MPBR plant at higher EOM concentrations, although this decay could also have been influenced by other factors. Membrane fouling was found to be related to the biomass concentration of the culture. However, fouling rate obtained under the operating conditions tested showed different behaviour concerning to EOM concentration depending on the initial transmembrane pressure (TMP).

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

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Figure captions

Figure 1: EOM_{-POL}, EOM_{-P}, NH_4-N and PO_4-P concentrations in lab-scale continuous mode. Experiment 1: a) 25ºC, b) 30ºC; Experiment 2: c) 25ºC, d) 35ºC; Experiment 3: e) 25ºC; f) intervals of 10ºC increment from 25 to 35ºC.

Figure 2: EOM_{-POL}, EOM_{-P}, NH_4-N and PO_4-P concentrations in lab-scale batch conditions (Experiment 4) at: a) 25ºC; and b) 30ºC.

Figure 3: EOM_{-POL}, NH_4-N and PO_4-P in lab-scale Experiment 5: a) nitrification inhibited; and b) nitrification non-inhibited.

Figure 4. EOM concentrations and solar photosynthetically active radiation (PAR) during the continuous operation of the MPBR plant: a) $EOM\text{-}_{POL}$ (red); and b) $EOM\text{-}_{P}$ (blue).

Figure 5. Continuous operation of the MPBR plant: a) Temperature (T), solar photosynthetically active radiation (PAR) and nitrification rate (NOxR); b) nitrogen recovery rate (NRR); phosphorus recovery rate (PRR) and biomass productivity (BP); c) ammonium (NH_4-N) and phosphate (PO_4-P) concentration ; d) normalised EOM_{-POL} and EOM_{-P}.

Figure 6. Continuous operation of the MPBR plant: a) Time evolution of transmembrane pressure (TMP); b) Fouling rate (FR) vs total suspended solids (TSS) concentrations in Periods A (blue) and B (red); c) Fouling rate (FR) vs total EOM ($EOM\text{-}_{Total}$) concentrations in Periods A (blue) and B (red).

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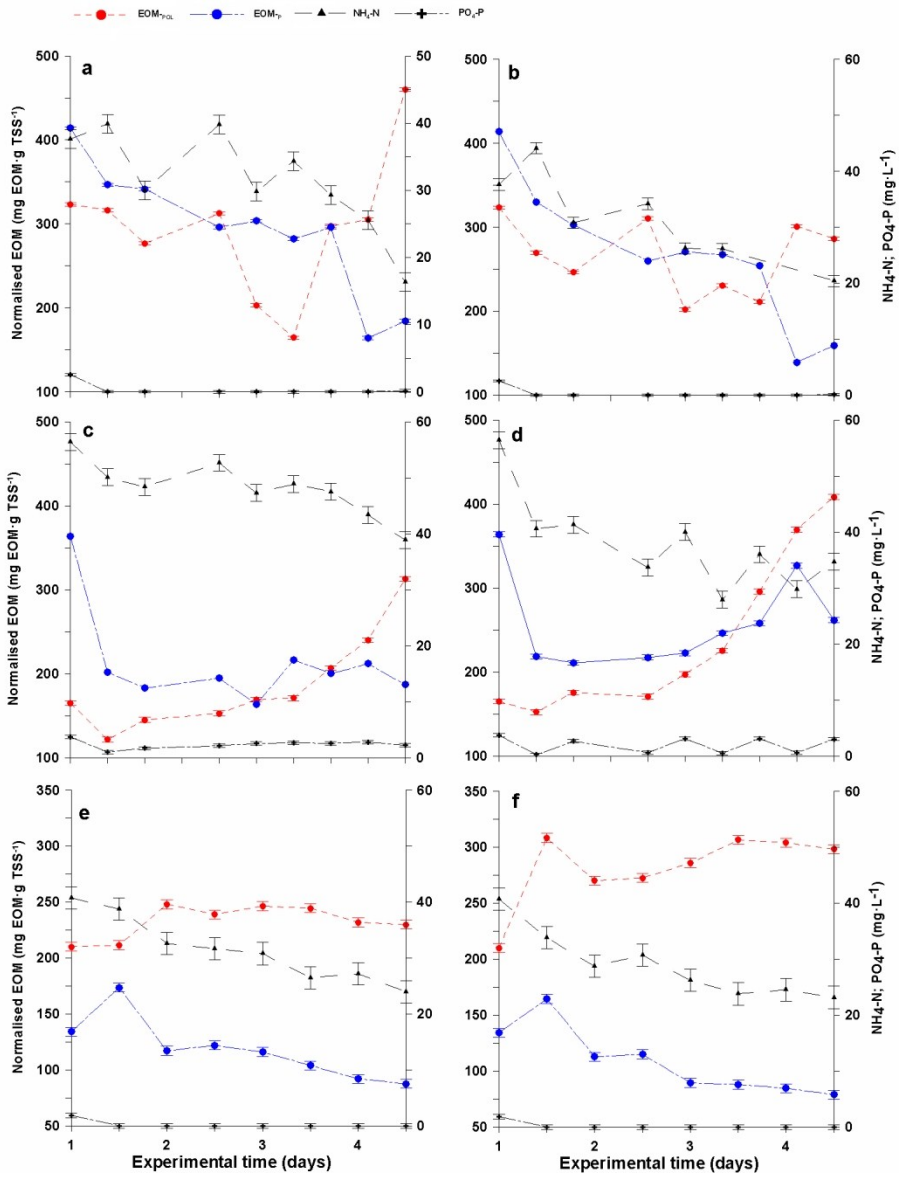

Figure 1: EOM-POL, EOM-P, NH4-N and PO4-P concentrations in lab-scale continuous mode. Experiment 1: a) 25ºC, b) 30ºC; Experiment 2: c) 25ºC, d) 35ºC; Experiment 3: e) 25ºC; f) intervals of 10ºC increment from 25 to 35ºC.

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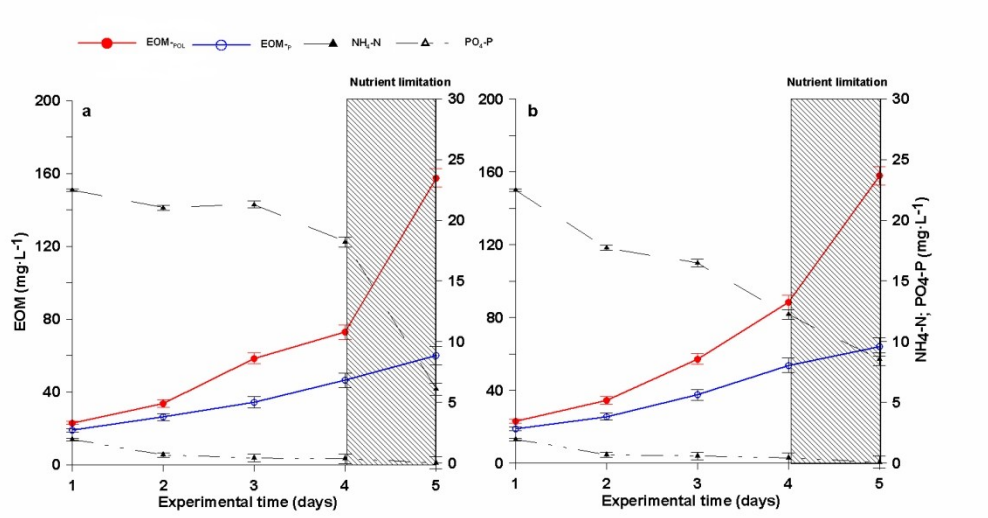

Figure 2: EOM-POL, EOM-P, NH4-N and PO4-P concentrations in lab-scale batch conditions (Experiment 4) at: a) 25ºC; and b) 30ºC.

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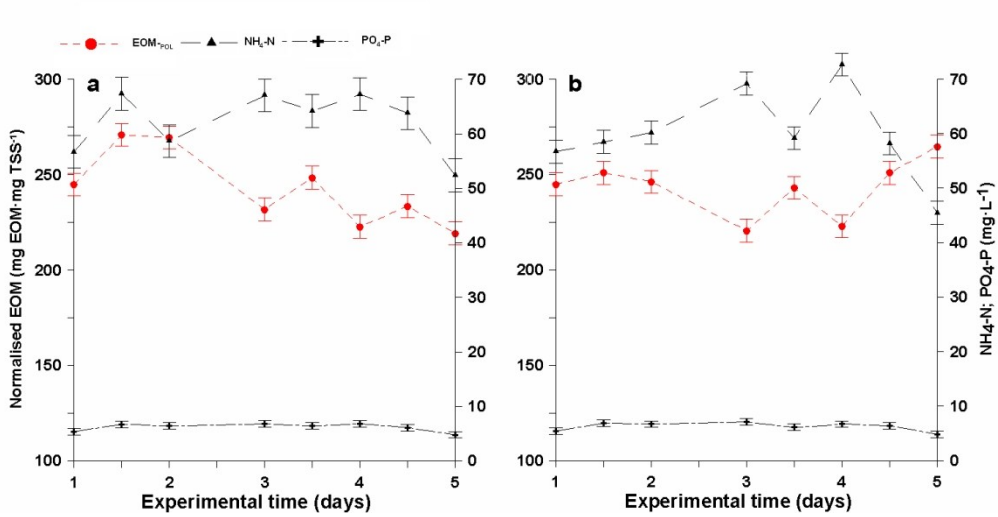

Figure 3: EOM-POL, NH4-N and PO4-P in lab-scale Experiment 5: a) nitrification inhibited; and b) nitrification non-inhibited.

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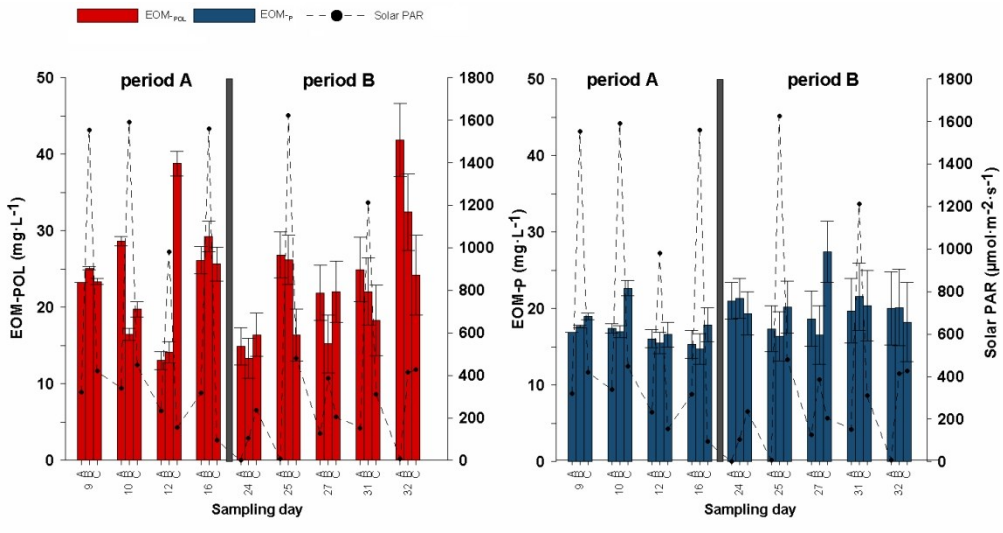

Figure 4. EOM concentrations and solar photosynthetically active radiation (PAR) during the continuous operation of the MPBR plant: a) EOM-POL (red); and b) EOM-P (blue).

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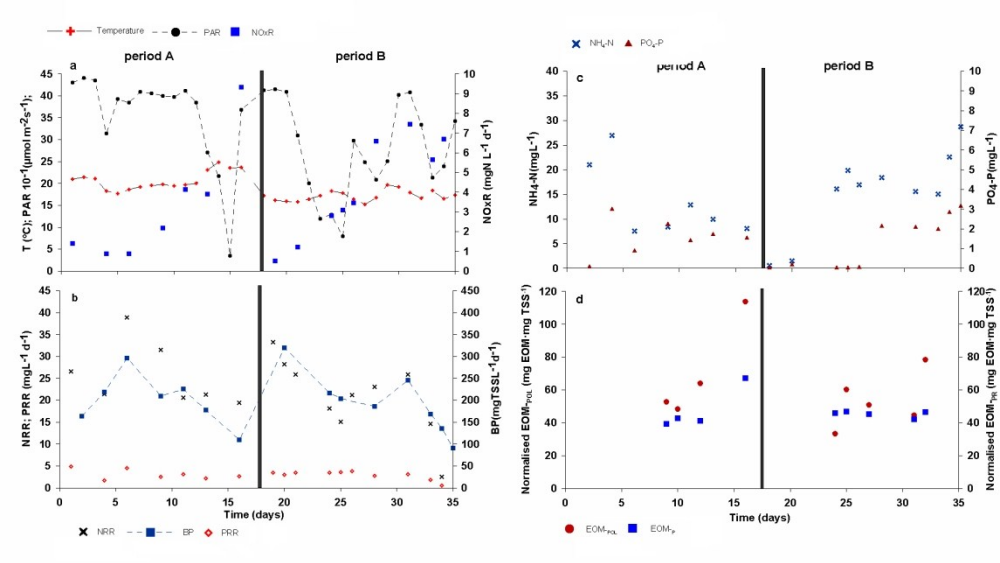

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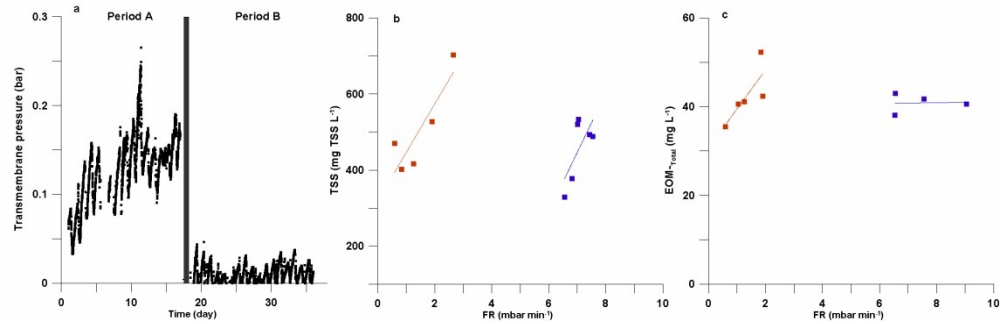

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