# Selecting the most suitable microalgae species to treat the effluent from an anaerobic membrane bioreactor

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**ABSTRACT**

Conventional treatments for nutrient removal in wastewater are shifting to Anaerobic Membrane Bioreactors, which produce a high-quality effluent with minimum sludge production. The effluent resulting contains high nitrogen and phosphorus load that can be eliminated by microalgae culture. The aim of this study is to evaluate the ammonium and phosphorus removal rate of different microalgae species in the effluent of an anaerobic treatment. For that, 4 different microalgae species have been tested (*Chlamydomonas reinhardtii, Scenedesmus obliquus*, *Chlorella vulgaris* and *Monoraphidium braunii*) in batch monoculture and mixed conditions. Results indicate that all species are able to eliminate both P and N in the medium with high removal rates. However a slight interspecies competition may boost these removal rates and productivity values ensuring, the success of the process.

**KEYWORDS**

Microalgae; nutrient removal; productivity; wastewater; submerged anaerobic membrane bioreactor

## Introduction

Nowadays, greater quantities of wastewater both urban and industrial are generated. These effluents contain a large amount of contaminants that must be reduced before the direct discharge to rivers, lakes or the sea. Among the wastewater treatment options, the anaerobic membrane bioreactor (AnMBR) technology can be applied to reduce pollutants to environmentally safe levels. This treatment has several advantages over more traditional aerobic systems, i.e. they consume less energy and produce less sludge whilst generating bio-methane [1]. This technology also achieves a high quality effluent in terms of Total Suspended Solids (TSS) and Chemical Oxygen Demand (COD). Nevertheless, inorganic nutrient removal (mainly nitrogen and phosphorus) cannot be achieved in the anaerobic reactor; hence, the effluent cannot be discharged into the aquatic environment as it may lead to eutrophication.

It must be highlighted that during the anaerobic digestion, all the inorganic nitrogen and some of the organic nitrogen compounds are reduced to ammonium. Consequently, the effluent concentration of ammonium in an AnMBR system is usually higher than the influent concentration. Since ammonium is the preferred nitrogen source for microalgae [2], microalgae cultures offer an ideal solution to decrease the nitrogen concentration of AnMBR effluents. Moreover, microalgae also need phosphorus to grow. Thus, microalgae play an important role during the final treatment easing the established requirements for water discharges: setting Total Nitrogen (TN) in 10 mg $L^{-1}$ and Total Phosphorus (TP) in 1 mg $L^{-1}$, which is considerably lower than the usual AnMBR effluents [3].

In addition, the use of this anaerobic technology for sewage treatment is also increasing in countries all over, which also have good conditions for microalgae growth [4]. Therefore, the applicability of microalgae based technologies as post-treatment of anaerobic effluents is a global challenge.

Several researchers have studied nutrient removal efficiency both in urban wastewater collected in various treatment units and in the agro-industrial wastewater by cultivation of microalgae cultures (Table 1). The main microalgae species applied belong to the Chlorophyta group (*Chlorella, Scenedesmus, Chlamydomonas, Neochlorosis, Botryococcus*) due to their versatile nature to grow in numerous wastewater systems and their effective management [16]. The nutrient removal efficiencies by microalgae cultures mainly depend on several conditions such as the initial effluent nutrient concentration, the light intensity, the nitrogen/phosphorus effluent ratio, the light/dark cycle, temperature, pH and algae species [17]. Between them, one of the main parameters determining the success of the solution offered by microalgae systems is temperature. This is a crucial factor that determines the microalgae growth through its action either on the cells by speeding up or slowing down the different bio-chemical reactions, or on the medium [18]. On the other hand, Pulz and Gross [19] observed that 'successful algal biotechnology mainly depends on choosing theright microalgae with relevant properties for specific cultures conditions and products'. Since there are a large number of microalgae species capable of eliminating nutrient from wastewater, an accurate selection process is required to obtain favourable outcomes. As can be seen in Table 1, the range of ammonium and orthophosphate content in the different wastewater treatments by microalgae are wide, as well as the strain used to treat the effluent. It can be also observed that microalgae can be cultivated in industrial wastewater as remediation technologies. However, nowadays very few studies have coupled AnMBR technology with microalgae post-treatment for nutrient removal and have shown its feasibility [20]. One factor determining the success of this wastewater treatment is the viability of the microalgae culture. Nevertheless, as far as it is known, no studies of species selection or a combination of them in order to maximize nutrient removal rates in AnMBR effluents has been reported. For that, it is important to assess different microalgae species cultures grown in AnMBR effluent, in order to select the faster N and P removal of medium-scale. In addition, after nutrient removal process the waste- grown microalgae culture under hyperconcentrated conditions represents a potential biomass source which may provide a substrate for biofuel production (carbohydrates and lipids) and/or high-value secondary metabolites [21]. For this reason, this research seeks for evaluating the N and P removal rates, as well as the growth rate and biomass productivity of different culture strains (under constant conditions of stirring and pH), with the aim of selecting the microalgae species that yields better performance nutrients, in the tertiary treatment of the effluent of an AnMBR system which treated sewage. Results from this study can provide a useful contribution towards large-scale applications of novel microalgae based technologies.

**Table 1** Nitrogen and phosphorus content in wastewater from different sources reported by different authors

| Effluent category | Description | $NH_4$-N ($mgL^{-1}$) | $PO_4$-P ($mgL^{-1}$) | NT ($mgL^{-1}$) | PT ($mgL^{-1}$) | Species | Ref |
|---|---|---|---|---|---|---|---|
| Municipal wastewater | Treated wastewater, primary settling, activated sludge and secondary settling: | 21 | 5.6 | | | *Scenedesmus obliquus, Chlorella vulgaris, Chlorella kessleri* | [4] |
| | Domestic wastewater obtained from the preliminary sedimentation tank of a sewage plant | 31.38 | 8.19 | 40.02 | 9.24 | *Chlorella vulgaris* | [10] |
| | Primary clarifier effluent | 39 | 2.1 | 51 | | Polyculture [(c)] | [47] |
| | Wastewater before primary settling, Wastewater after primary settling, Wastewater after activated sludge tank Centrate | 33.4±0.6<br>32.2±0.4<br>ND<br>71.8±1.1 | | 40.65±0.07<br>38.95±1.91<br>19.1±0.1<br>131.5±2.1 | 5.66±0.08<br>6.86±0.05<br>0.32±0.04<br>201.5±10.6 | *Chlorella sp.* | [46] |
| | Wastewaters taken from three different stages of the treatment process: influent, effluent, and centrate | 49.92<br>8.78<br>67.00 | | 64.00[(a)]<br>14.30[(a)]<br>128.60[(a)] | 6.92<br>1.25<br>120.60 | *Chlamydomonas reinhardtii* | [20] |
| | Wastewater secondary effluent from an urban wastewater treatment plant | 16.08±0.80 | 2.15±0.002 | 17.45±0.78 | 2.60±.002 | *Scenedesmus obliquus* | [36] |
| | Wastewater after the primary treatment from an urban wastewater treatment plant | | | 28.74±0.26 | 2.34±0.02 | *Chlorella protothecoides* | [42] |
| | Wastewater after the primary treatment from an urban wastewater treatment plant | 41.6±17.1 | 3.1±1.3 | | | *Chlorella vulgaris* | [13] |
| | Wastewater from a sewage secondary-treatment | 27.4 | 11.8 | | | *Scenedesmus obliquus* | [25] |
| Industrial wastewater | Anaerobic sludge bed (UASB) reactor for starch processing wastewater (SPW) treatment. | 217.6-334.7 | 19.3-32.9 | 240.3-382.7 | 22.7-40.2 | *Chlorella pyrenoidosa* | [44] |
| | Anaerobically treated dairy wastewater Dilution 25%-10% | 30.5-16.3 | 2.6-1.8 | 81.0 | 36.6 | Polyculture [(c)] | [47] |
| | Industrial wastewater from carpet and rug mills | 17.58-25.85 | 5.47-13.83 | 32.6-45.9[(a)] | 20.31-35.10[(b)] | Polyculture | [7] |

[a] Total Kjeldahl Nitrogen (TKN)
[b] Total Orthophosphates ($PO_4$-P)
[c] mixture of green algae and diatoms

## Material and methods

### Microalgae strains

The microalgae strains used in this study are *Chlamydomonas reinhardtii* (SAG 11-32b), *Scenedesmus obliquus* (SAG 276-3a), *Chlorella vulgaris* (SAG 211-12) and *Monoraphidium braunii*, supplied by the Algae Culture Collection (SAG) of the University of Göttingen (Germany).

Single species inocula for the experiments were maintained in Combo medium prepared using deionized water [22] incubated at 20 ± 1°C and 250 μE $m^{-2}$ $s^{-1}$ light intensity under 14/10 h light/dark cycle for the pre-inoculum.

### Characterization of AnMBR effluent

The feed medium was obtained from a Submerged Anaerobic Membrane Bioreactor (AnMBR) pilot plant located in Valencia, Spain. It consists of an anaerobic reactor of 1.3 $m^3$ total volume (0.4 $m^3$ head-space volume) connected to two membrane tanks of 0.8 $m^3$ total volume each (0.2 $m^3$ head-space volume). Each membrane tank includes one industrial hollow-fibre ultrafiltration membrane module (PURON® Koch Membrane Systems (PUR-PSH31), 0.03 μm pores). The pilot plant is fed with the effluent of the Carraixet WWTP pre-treatment (screening, degritter, and grease removal). After further pre-treatment in the rotofilter and homogenisation in the equalisation tank, the wastewater is pumped to the anaerobic reactor. The pilot plant was operated at SRT of 100 days and controlled temperature of 33°C. The HRT was maintained at 24 h. The obtained permeate is stored in the CIP (Clean-in-Place) tank. Further details of the characteristics of the AnMBR may be found in Robles et al. [23] and Gimenez et al. [24].

The characteristics of the effluent used in this research are shown in Table 2.

Nitrites and nitrates were not present in the AnMBR effluent. Furthermore, high alkalinity ensured a source of inorganic carbon through the bicarbonate–carbonate buffer system ($CO_2$ – $H_2CO_3$ –$HCO_3^-$ –$CO_3^{-2}$) in the liquid phase. The organic matter loading, soluble COD was inert, thus, photoautotrophic metabolism typical of microalgae was boosted. Finally, the AnMBR effluent was aerated to oxidize the sulphide to sulphate because of its toxic nature to the microalgae growth [25].

| Parameter | Unit | Mean |
|---|---|---|
| pH | | 8 ± 0.1 |
| $NH_4$-N | mg N $L^{-1}$ | 50.0-65.0 |
| $NO_3$-N | mg N $L^{-1}$ | ND |
| $NO_2$-N | mg N $L^{-1}$ | ND |
| $PO_4$-P | mg P $L^{-1}$ | 5.0-6.0 |
| N:P ratio | molar | 18-22 |
| Soluble COD | mg COD $L^{-1}$ | 51 ± 8 |
| Alkalinity | mg $CaCO_3$ $L^{-1}$ | 736.5 ± 78.4 |
| VFA | mg COD $L^{-1}$ | 1.5 ± 3.6 |
| $SO_4$-S | mg S $L^{-1}$ | ND |
| $H_2S$ | mg S $L^{-1}$ | 97.8 ± 8.3 |

ND (Not Detected)

**Table 2** AnMBR-effluent characteristics

## Experimental design

The experiments were conducted in duplicate with four 2 L Pyrex flasks yielding a total volume of 8L in batch mode, fed by the effluent from an Anaerobic Membrane Bioreactor. Pure CO2 (99.9%) from a pressurized cylinder at 1.5-2 bar pressure was injected into the gas flow both to provide inorganic carbon and to maintain a pH of 7.5± 0.1 in the cultures. In addition, four LED lamps (18 W, 6000–6500 K) were placed vertically at a distance of 20 cm to the 2 L-flasks as light source.

The culture was mixed and aerated with 0.2 μm prefiltered air using a membrane air-pump to guarantee homogenization, preventing cell sedimentation and biofilms formation in the walls. The air stream was bubbled into the reactors from the bottom at a flow rate of 1.0–1.2 L $min^{-1}$ through fine bubble diffusers placed crosswise at the bottom.

All experiments were started at the same initial concentration of biomass, as the initial cell density is considered one of the main factors affecting algal growth [26]. The experiments started with an optical density between 0.15 and 0.20, which corresponded to a cell density showed in Table 3.

As it has been said, temperature is a crucial factor that determines the success of the process. For this reason, each experiment was conducted at the optimal species temperature (for example *C. reinhardtii* at 25–30°C; *C. vulgaris* at 30–35°C; *S. obliquus* at 20–25°C; *M. braunii* at 20–25°C) [13,27–30]. Table 3 summarizes the experimental condition performed for the whole set of experiments.

| Species | Exp 1 *Chlamydomonas reinhardtii* | Exp 2 *Chlorella vulgaris* | Exp 3 *Scenedesmus obliquus* | Exp 4 *Monoraphidium braunii* | Exp 5 mix-culture (1) | Exp 6 mix-culture (2) |
|---|---|---|---|---|---|---|
| Aeration ($L_{air}$ $min^{-1}$ $L_{reactor}$) | 1.67 | 1.67 | 1.67 | 1.67 | 1.67 | 1.67 |
| Temperature (ºC) | 25-30 | 30-35 | 20-25 | 20-25 | 20-25 | 20-25 |
| Light/Dark cycle (h) | 14/10 | 14/10 | 14/10 | 14/10 | 14/10 | 14/10 |
| Duration (days) | 18 | 13 | 8 | 9 | 8 | 7 |
| $NH_4$-N (mg $L^{-1}$) initial | 52.26 | 48.74 | 65.64 | 62.37 | 66.85 | 57.15 |
| $NO_3$-N (mg $L^{-1}$) initial | --- | 0.13 | 1.60 | 0.7 | 0.03 | 0.5 |
| $NO_2$-N (mg $L^{-1}$) initial | --- | --- | 0.66 | 0.1 | 0.17 | --- |
| $PO_4$-P (mg $L^{-1}$) initial | 4.94 | 5.35 | 4.56 | 5.60 | 5.57 | 3.86 |
| N:P initial | 23.40 | 20.24 | 32.97 | 24.98 | 26.65 | 33.07 |
| Cell density (Cell $L^{-1}$) initial | 2.60E+07 | 1.16E+08 | 4.7E+07 | 8.48E+08 | 1.35E+08/1.22E+08 (1) | 1.48E+08/7.29E+08 (2) |

(1) Mix-culture *Chlamydomonas reinhardtii* / *Scenedesmus obliquus*
(2) Mix-culture *Monoraphidium braunii* / *Scenedesmus obliquus*

**Table 3** Experimental conditions

## Analytical methods

The biomass concentration was measured indirectly by a Perkin Elmer Lambda 35 spectrophotometer. Since the biomass might be underestimated when the optical density is out of the linear range, samples were diluted to measure getting an absorbance in the range 0.1–1.0 if the optical density was greater than 1.0.

To convert the OD 680 values to biomass as dry weight (TSS mg $L^{-1}$), a calibration curve was determined for each experiment. A good correlation is observed between OD 680 and biomass; in all the experiments the regression coefficient was higher than 0.9 (Table 4).

| | TSS=A $OD_{680}$+B | | $R^2$ |
|---|---|---|---|
| | A | B | |
| **Exp [1]** | 316.65 | -18.995 | 0.9924 |
| **Exp [2]** | 280.92 | -18.839 | 0.9172 |
| **Exp [3]** | 195.36 | 43.164 | 0.8912 |
| **Exp [4]** | 248.99 | 27.483 | 0.9503 |
| **Exp [5]** | 361.99 | -56.974 | 0.9645 |
| **Exp [6]** | 297.32 | -5.1952 | 0.9678 |

**Table 4** Biomass and Optical Density relation

Biomass dry weight as suspended solids was determined gravimetrically according to Standard Methods [31]. Microalgae biomass is the portion of solids retained by filtration through a pre-dried and pre-weighed glass fibre filter of 0.7 µm pore diameter (Millipore AP4004705) and Polycarbonate filtering membrane of 0.45 µm pore diameter (Nucleopore 111107). The solids were dried at 105°C until a constant weight was obtained.

Cell density measures were carried out by filtering the samples through a 0.2 µm membrane filter (Millipore GTTP). After this, the material on the filter was dehydrated by successive washings with aqueous ethanol. Each dried filter was placed onto a drop of immersion oil in the center of a slide and 2 more drops were added on the top side of the filter [32]. Finally, a cover glass was placed on the top of the filter. Algal counts were made by epifluorescence microscopy with a Leica DM2500 microscope, using a 100× oil-immersion objective.

Nutrient measures of ammonium (NH4-N), nitrite (NO2-N), nitrate (NO3-N) and phosphate (PO4-P), were determined every day during the experiment, according to Standard Methods: 4500-NH3 G –Automated phenate method; 4500-NO2-B-Colorimetric method; 4500-NO3- H-Automated hydrazine reduction method and 4500-P F-Automated Ascorbic Acid reduction method; respectively [31] in a Smartchem 200 automatic analyzer (West-coScientific Instruments, Westco).

## Calculations

### Specific growth rate

The maximum growth rate (µmax) was determined from the variation in cell concentration in a determined time interval corresponding to the exponential growth phase, as shown in Equation (1).

$$\mu_{max}(d^{-1}) = Ln(N_2/N_1)/(t_2 - t_1) \qquad (1)$$

where $N_1$ and $N_2$ are biomass concentration (Cell $L^{-1}$) at time 1 ($t_1$) and time 2 ($t_2$), respectively.

In addition, the doubling time as the period of time required for a quantity to double in cell concentration (Equation 2) is estimated as follows:

$$Td(d) = (Ln\ 2)/\mu_{max} \qquad (2)$$

### Productivity

The productivity was calculated from the kinetic parameters of the Verhulst model as:

Productivity (mg TSS $L^{-1}$ $d^{-1}$) = $\Delta X / \Delta t = (X_1 - X_0) / (t_1 - t_0)$ (3)

where $X_0$ and $X_1$ are initial and final biomass achieved (mg $L^{-1}$) respectively, $t_1$ is the time required to reach $X_1$ and $t_0 = 0$.

**Nutrient removal rates**

The ammonium removal rate (AR) and phosphorus removal rate (PR) represent the amount of ammonium and phosphate, respectively, assimilated by the microorganisms with respect to the reaction volume. Since microalgae are the dominant community culture, microalgae activity was assumed to be solely responsible for N and P removal in these experiments.

Ammonium and phosphorus removal are calculated by the Equations (4) and (5), respectively.

$$AR(\text{mg } L^{-1} d^{-1}) = (C_{0NH4} - C_{1NH4})/(t_1 - t_0) \quad (4)$$

$$PR(\text{mg } L^{-1} d^{-1}) = (C_{0PO4} - C_{1PO4})/(t_1 - t_0) \quad (5)$$

where $C_0$ and $C_1$ is the inorganic species concentration (mg $L^{-1}$) in the initial time ($t_0$) and time 1, respectively.

## Results and discussion

### Nutrient removal

Figure 1 illustrates the evolution of the $NH_4$-N and $PO_4$-P concentration and cell density in the microalgae culture during the experiments.

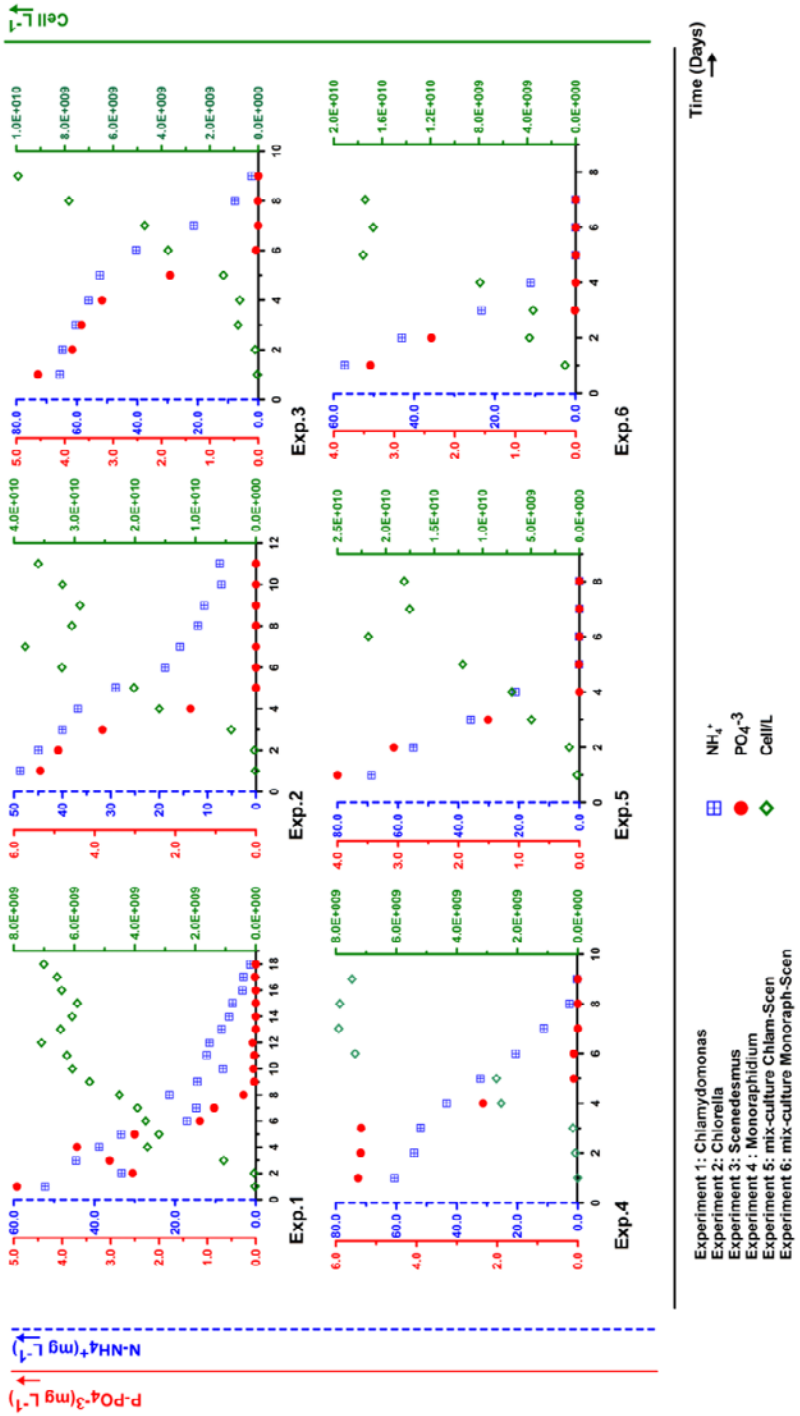


**Figure 1**. Evolution of the $NH_4^+$−N, $PO_4^{-3}$−P concentration and cell density during the experiments.

## Nitrogen removal

In order to study the capability of the different microalgae strains to remove N from the AnMBR effluent, the nitrogen removal rate is calculated (Table 5). As it is known phosphates, as orthophosphates, play a very important role in algae cell growth and metabolism affecting ammonium kinetics [20]. Therefore the ammonium removal rate has been studied in both phosphorous replete and deplete conditions (see Table 5).

| | P replete | | P deplete | | | | Removal (% ) | |
|---|---|---|---|---|---|---|---|---|
| | $NH_4$-N Removal rate (mg $L^{-1}$ $day^{-1}$) | $R^2$ | $NH_4$-N Removal rate (mg $L^{-1}$ $day^{-1}$) | $R^2$ | $PO_4$-P Removal rate (mg $L^{-1}$ $day^{-1}$) | $R^2$ | $NH_4$-N | $PO_4$-P |
| Exp 1 | 4.44±0.7 | 0.83 | 1.60±0.1 | 0.97 | 0.56±0.09 | 0.85 | 97,4 | 100 |
| Exp 2 | 4.07±0.23 | 0.99 | 3.31±0.57 | 0.87 | 1.40±0.2 | 0.95 | 84.6 | 100 |
| Exp 3 | 9.57±1.53 | 0.86 | 9.1 ±0.34 | 0.99 | 1.2 ±0.21 | 0.94 | 96.6 | 100 |
| Exp 4 | 6.7±0.9 | 0.94 | 6.9 ±1.1 | 0.94 | 2.63±0.2 | 0.91 | 99.46 | 100 |
| Exp 5 | 16.2 ±0.6 | 0.99 | -- | -- | 1.35 ±0.1 | 0.98 | 99.96 | 100 |
| Exp 6 | 15.08 ±0.98 | 0.99 | -- | --- | 1.69±0.4 | 0.94 | 100 | 100 |

**Table 5** Ammonium and phosphorus removal rate

In all experiments, high regression coefficients were found. The difference between the highest *(S. obliquus* Exp 3) and the minimum (*C. vulgaris* Exp 2) is of 57.5%, meanwhile, *M. braunii* and *C. reinhardtii* showed intermediate values between.

As it is said, phosphorus plays a critical role in the ammonium uptake in microalgae culture, for that the ammoniums rate is to be higher when phosphorus is present in the medium. However, this rate only underwent significant decreases in Exp 1 and 2 (*C. reinhardtii* and *C. vulgaris*): 64% and 19%, respectively.

The N uptake and its consumption by microalgae cells exhibit different kinetics. Meanwhile the first is related to the media composition between others [33], the second depends on the physiological cell state. These two rates (uptake and consumption) achieve parity at a steady rate of growth [34]. As the rates obtained in these experiments belong to the exponential growth phase, these two rates might be different. In exp 1, the diminish of the ammonium uptake is due to a low external concentration; In fact, when phosphorus is depleted the ammonium concentration was 73% lower (14 mg N $L^{-1}$) and thus, its rate was largely reduced. This is in concordance with Ruiz-Martinez et al. [35], who treated similar AnMBR effluent and reported that ammonium uptake rate decreased when ammonium concentration was reduced to values of around 10–13 mg N $L^{-1}$. Besides, when nitrogen concentration in the medium is low, microalgae have to overcome the concentration gradient, which hinders the ammonium uptake [34].

On the other hand, in exp 2, this decrease is not dependent on the external concentration (>30 mg N $L^{-1}$), but on the sensibility of this species to the lack of phosphorus in the medium. *S. obliquus* and *M. braunii* did not experiment variations in its ammonium uptakes when phosphorous was completely removed although the external concentration of it ranges around 20 mg $L^{-1}$.

However, these ammonium uptake rates increase up to 16.2 ± 0.6 mg $L^{-1}$ $d^{-1}$ when the culture is a combination of two microalgae species (Exp 5: *C. reinhardtii* and *S. obliquus*). Stockenreiter [36] found that diverse communities depleted the inorganic nutrients (PO4-P, NH4-N and NO3-N) faster and more efficiently than monoculture, probably due to niche differentiation. In fact, mixed culture increased up to 41% its AR rates.

Regarding ammonium removal efficiency (%), it is important to highlight that all species tested eliminated more than 95% except for *C. vulgaris* that yielded 85%. Therefore, these four microalgae species are able to efficiently

remove ammonium from an AnMBR effluent although the ammonium load is up to 10 times higher.

At the end of all the experiments, concentration of ammonium was lower than 10 mg N $L^{-1}$ and the percentages of elimination larger than 70-80% concentration and minimum percentage of reduction established, respectively, in the European Commission. *S. obliquus* and *M. brauni* beside the mixed culture achieve the 99% removed. While the pure culture last eight days in achieving this efficiency, the mixed culture only took 5 days (Exp 5 and 6), probably due to the fact that species combination boost the consume of ammonium. This fact would imply that if the system would be scaled up, the reaction volume would be reduced by 37.5%.

The ammonium removal efficiency yields for *S. obliquus* in AnMBR effluent (96.6%) is similar to those found in other studies over real secondary effluent from a wastewater treatment e.g. Martinez et al. [13] higher than 80%; Ruiz-Marin et al. [37] between 97% and100%. Similar efficiency (93%) is also found in research carried out with *S. acutus* in municipal wastewater treatment by Sacristan de Alva et al. [38]. It seems that *Scenedesmus* genera can match a wide range of N:P ratio of the medium supplied [39], which is valuable for wastewater treatment. On the contrary, the efficiency values found for *C. vulgaris* are lower and fluctuate depending on the different effluents studied from 23 to 95% [40].

**Phosphorous removal**

The decrease of $PO_4$-P is detected in all cultures, resulting at the end of the experiment depleted (Figure 1) and 100% elimination. This suggests that phosphorus is the limiting nutrient in the AnMBR effluent for all the species studied. The regression coefficient obtained is higher than 0.9 except for Exp 1 (0.85).

Diminish of phosphorus is due to a combination of growth, adsorption and luxury uptake of this nutrient [41]. It can be assumed that biological activity is carried out by the microalgae culture because of the short time of experiments (mean 10 days). No serious microbiological contamination was noticed. Regarding luxury uptake by microalgae, it is a phenomenon deeply studied in natural environments. Nevertheless, further study is needed about it in photobioreactors cultures for wastewater treatment, although this may affect significantly to phosphorus removal. As it is known between 5 and 30 mg $L^{-1}$ of phosphorus the luxury uptake by microalgae is independent of the concentration in the medium [42] and may account greatly to the amount of phosphorus elimination. Lastly, as the pH is kept constant at 7.5 the decrease by precipitation is not considered.

The faster removal rate corresponds to *M. braunii* 2.63± 0.2 mg $L^{-1}$ $d^{-1}$ although its behaviour differs from the rest of the species which consumed phosphorus from the beginning of the experiment, having a longer lag phase to the AnMBR effluent. However, *M. braunii* consumed all the phosphorus within 5 days, which was the fastest of the pure cultures (Figure 1). C. vulgaris and *S. obliquus* show similar rates 1.40 ± 0.2 and 1.2 ± 0.21 mg $L^{-1}$ $d^{-1}$ respectively, and finally, the lowest rate corresponds to *C. reinhardtii* of 0.56 ± 0.09 mg $L^{-1}$ $d^{-1}$.

Some microalgae species might be adapted to the rapid rate of nutrient uptake while others are storage adapted [34]. Our results suggest that *M. braunii* reveals the first strategy for the phosphorus uptake; therefore, it might be suitable for the purpose. Moreover, this agrees with Patel et al. [43] that found the freshwater species of *Monoraphidium* a good candidate for the purpose of phosphorus removal due to its high efficiency across all P loading (5–15 mg $L^{-1}$).

All species tested show 100% of phosphorus elimination over the AnMBR effluent. These results are significantly higher than 59% of P removal reported for *C. vulgaris* in an influent of 21–30 N:P ratio in municipal wastewater [6]. Moreover, the phosphate removal for *Scenedesmus* is also higher than that of 64.3% found by Sacristan de Alva et al. [38] in an influent of 7.3 ± 0.3 mg $L^{-1}$. Nevertheless, microalgae cell density continued increasing once phosphorus had been depleted in all the experiments. This is because of luxury uptake, which permits the storage of phosphorus. The ability of phosphorus storage is specific for each microalgae species and it seems related to the receptor transport system [34]. In fact, the affinity of the system determines the capability to storage several times the minimum needed to sustain 3 or 4 cell doubling cycles and therefore to obtain high yields when external conditions vary.

Regarding mixed culture, its removal rates are in concordance of those found in pure cultures and the higher rate is found in the *M. braunii- S. obliquus*: 1.69 ± 0.4 (Exp 6), due to the presence of the former. However, unlike the results of ammonium removal rate, mixed culture (Exp 5 and 6) do not exhibit higher P removal rates. This suggests that the phosphorus uptake is not enhanced by species competition, but internal total concentration, as well as algal physiology. Nonetheless, the time needed to deplete the phosphorus was only 4 and 3 days for experiments 5 and 6, respectively, which were considerably lower than the time needed for pure cultures (Figure 1). This suggests that mixed cultures adapt better to the medium than pure cultures, which is an important quality regarding the scalability of this technology.

**Specific growth rate**

Table 6 shows the specific growth rate, doubling time and productivities values obtained for the six experiments. The specific growth rates obtained indicate differences between the species tested (Table 6) since values ranges between 0.45 and 0.98 $d^{-1}$. However, these differences cannot be attributed to monoculture and mixed cultures.

All species studied in this research present the doubling time comparable to values reported by other authors, since the average doubling time for green algae is 24 h, corresponding to a μ of 0.69 $d^{-1}$ [44]. However, at best conditions, some microalgae can even double their biomass in 3.5 h [45]. Regarding to maximum growth rate, *M. braunii* presented the highest (0.98 $d^{-1}$) and *S. obliquus* the lowest (0.45 $d^{-1}$) respectively. In fact, the former double the specific growth rate of *Scenedesmus*. In relatively high velocity adapted species like *M. braunii*, cellular growth and replications are high and match by also fast nutrient uptake. However, *S. obliquus* which is more storage adapted species, growth rates are lower permitting a net accumulation of an intracellular reserve that might provide better competitor skills.

Regarding to mixed culture, Exp 5 reveals the highest growth rate (0.63/0.56 $d^{-1}$ to *C. reinhardtii* / *S. obliquus*, respectively).

Results reveal that the growth rate of *S. obliquus*, which is the lowest in monoculture experiments increase up to 19.6% or stay similar in mixed cultures (Exp 5 and 6, respectively), where interspecific competitions occur. Moreover, *C. reinhardtii* also undergoes growth rate increments up to 20.3% under competitive pressure. As it has been said, this suggests that both microalgae are good competitors since they are able to adapt, modifying its growth rate, morphology, size and metabolic pathways. In fact, in Exp 5, the species involved *C. reinhardtii – S. obliquus*) increased their growth rates (faster reproduction) in detriment of its size (observed under microscopy). Nevertheless, *M. braunii* is capable of growing rapidly (maximum μmax and PR rate) when environmental conditions are favourable (out competitions and nutrients replete) suggesting that this species exhibit an R-strategy but under interspecific competitions against *S. obliquus* (Exp 6) and unfavourable conditions (lower nutrient content), its growth rate is reduced drastically, 55%.

| | $\mu_{max}$ (1) ($d^{-1}$) | Td (2) (d) | Productivity (mg TSS $L^{-1}$ $d^{-1}$) |
|---|---|---|---|
| Exp 1 | 0.50 | 1.39 | 38.93 |
| Exp 2 | 0.56 | 1.24 | 42.17 |
| Exp 3 | 0.45 | 1.54 | 47.70 |
| Exp 4 | 0.98 | 0.71 | 65.05 |
| Exp 5 (3) | 0.63 / 0.56 | 1.16 | 68.45 |
| Exp 6 (4) | 0.46 / 0.44 | 2.31 | 98.79 |

(1) specific growth rate (cellular) (2) growth rate doubling time
(3) (C.reinhardtii / S. obliquus) (4) (M. braunii / S. obliquus

**Table 6** Growth rate parameters

## Biomass productivity

The productivity values obtained in monocultures varies between 38.93 and 65.05 mg $L^{-1}$ $d^{-1}$ in *C. reinhardtii* and *M. braunii* respectively. However, the productivity doubled their values when mixed cultures are used, especially in experiment 6 with 98.79 mg $L^{-1}$ $d^{-1}$.

Under interspecific competition, species may increment their growth rates higher than the size-cell reduction undergoes, yielding a global increase in productivity values. For that, the use of mixed culture for nutrient removal aims may fit perfectly, arising out higher biomass productivity, which can be beneficial at a larger scale if the biomass produced may be used for biomethane or biofuel production.

The productivity achieved in this study for monoculture experiments (Exp 1–4) are similar to the those obtained by some consulted authors; e.g. Scragg et al. [46] for *C. vulgaris* achieved 24 and 40 mg $L^{-1}$ $d^{-1}$ in the low nitrogen medium and Watanabe's medium respectively. For *S. obliquus* De Morais and Costa [47], obtained maximum productivity of 64–85 mg $L^{-1}$ $d^{-1}$ and 61–90 mg $L^{-1}$ $d^{-1}$ for *C. kessleri* both cultivated with Bristol medium and with $CO_2$ at different concentrations (0.038 to 18% v/v).

However, Arbib et al. [5], shows higher productivity values than our results for *C. vulgaris* (116.0 mg $L^{-1}$ $d^{-1}$) and *S. obliquus* (201.4 mg $L^{-1}$ $d^{-1}$) using wastewater with low content of NH4-N. Also, Yoo et al. [48] reported higher values for *C. vulgaris* (104.76 ± 10.73 mg $L^{-1}$ $d^{-1}$) and *Scenedesmus sp*. (217.50 ± 11.24 mg $L^{-1}$ $d^{-1}$) cultivated with BG11 medium and 10% $CO_2$.

It must be taken into account when comparing biomass productivity values reported by other authors that the dispersion of data is mainly owed to physical factors such as pH, light intensity, temperature, etc. and the complex interactions among them [49].

To sum up, our results indicate that phosphorus removal is not so dependent on the species since microalgae have been adapted for gathering this essential and scarce nutrient taking up as much as possible of this element. In addition, the phosphorus concentration in the effluent allows luxury uptake by microalgae. Nevertheless, ammonium removal rate is more dependent on species; therefore this is the removal rate to focus on.

When scaling the process, the behavior of this nutrient will be the critical point to consider. The faster micro-algae in eliminating ammonium from the AnMBR effluent is *S. obliquus* (9.57 ± 1.53 mg $L^{-1}$ $d^{-1}$), which also show productivity values of 47.70 mg $L^{-1}$ $d^{-1}$. However the mixed culture yields an increase up to 41%, suggesting the kind of two-species culture may fit better for the purpose.

On the other hand, the highest phosphorous removal rate (2.63 ± 0.2 mg $L^{-1}$ $d^{-1}$) corresponds to *M. braunii* with the highest productivity value of 65.05 mg $L^{-1}$ $d^{-1}$. These two species are known to be dominant species [50] and fit properly to the aim of the study. In fact, in Exp 6 testing these two species reach the highest productivity values (98.79 mg TSS $L^{-1}$ $d^{-1}$) and faster ammonium and phosphorus removal rates, thus being this combination good dual purpose candidate.

## Conclusions

All strains tested (*C. reinhardtii*, *C. vulgaris*, *S. obliquus* and *M. braunii*) achieved 100% of phosphorus and higher than 85% of ammonium elimination, obtaining final concentrations which meet the legal requirements imposed in the European Directive (98/15/CE). In this respect, *M. braunii* shows the highest phosphorus removal rates and the optimal species to remove ammonium is *S. obliquus* that is not affected by the lack of phosphorus.

On the other hand, *C. reinhardtii*, *C. vulgaris* show a decrease in their ammonium removal rates when the system was phosphorus depleted. However, the best results in the treatment of this AnMBR effluent in terms of

ammonium removal rate and biomass productivity were obtained by mixed cultures. In addition, in the experiments with mixed cultures, phosphorus was depleted faster than in pure cultures. This suggests that the interspecific competition fosters nutrient removal rates and biomass productivity, especially, when the species forming the culture are *M. braunii* and *S. obliquus*. These results can be useful at a larger scale since the time needed to remove ammonium in mixed cultures is reduced by 37.5% and the biomass productivity is increased by 41%.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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