Nutrient removal efficiency of green algal strains at high phosphate concentrations

Jairo Hernan Moreno Osorio, Angelo Del Mondo, Gabriele Pinto, Antonino Pollio, Luigi Frunzo, Piet Nicolaas Luc Lens and Giovanni Esposito

ABSTRACT

The effects of autotrophic and mixotrophic conditions on microalgae growth and nutrient removal efficiency from synthetic wastewater by different microalgae were investigated. Although several studies have demonstrated the suitability of microalgae technologies for ammonia-rich wastewater treatment, only a few have been used for treatment of phosphate-rich industrial wastewaters. In this work, six microalgae were cultivated in batch mode in a growth medium with a high phosphate concentration (0.74 Mm PO₄³⁻/C₀-P) and different carbon sources (ammonium acetate and sodium bicarbonate) without CO₂ supplementation or pH adjustment. Their potential for nutrient removal and biomass generation was estimated. The biomass growth in the reactors was modeled and the data aligned to the Verhulst model with R² > 0.93 in all cases. *Chlorella pyrenoidosa* ACUF_808 showed the highest final biomass productivity of 106.21 and 75.71 mg·L⁻¹·d⁻¹ in media with inorganic and organic carbon sources, respectively. The highest phosphorus removal efficiency was 32% with *Chlorella vulgaris* ACUF_809, while the nitrate removal efficiency in all reactors exceeded 93%. The coupled cultivation of the novel isolated strains of *C. pyrenoidosa* and *C. vulgaris* under mixotrophic conditions supplemented with ammonium acetate might be a promising solution for simultaneous nitrate and phosphate removal from phosphorus-rich wastewaters.

Key words | biomass production, bioremediation, freshwater microalgae, native strains, nutrient removal, wastewater

INTRODUCTION

Population growth, industrialization and rapid urbanization have led to excessive nutrient pollution, presenting a water-quality problem of growing concern (Aslan & Turkman 2006). Industrial wastewaters can contain up to three orders of magnitude higher concentrations of nitrogen (N) and phosphorus (P) than natural water bodies (De la Noüe et al. 1992). Many industrial wastewaters often contain more than 200 mg NO₃⁻·N L⁻¹, while effluents from industries producing explosives, fertilizers, pectin, cellophane and metal finishing contain more than 1000 mg NO₃⁻·N L⁻¹ (Inamori & Fujimoto 2010). Moreover, wastewaters from the mineral fertilizer industry can contain high phosphate concentrations as well; that is, 40–200 mg·L⁻¹ (Yapijakis & Wang 2006; Inamori & Fujimoto 2010). Many technologies are available to remove or reduce pollutants from industrial wastewater (WWAP 2017).
However, most of them are limited mainly by cost-effectiveness in industrial situations (WWAP 2017). Chemical removal is the most used technology to eliminate inorganic P from wastewaters (Nancharaiah et al. 2016). For the removal of inorganic N, bacterial nitrification and denitrification processes are more common. The primary disadvantages of these nutrient removal processes concern the high operational cost of adding chemicals in the case of P removal and aeration in the case of N removal (Nancharaiah et al. 2016).

Microalgae have the flexibility to switch their growth regime to heterotrophic, photoautrophic or mixotrophic by changing the culture medium’s substrate, which stimulates specific metabolic and biosynthetic pathways (Perez-Garcia & Bashan 2015). Many microalgae strains are able to grow in a wide variety of wastewaters including sewage, agricultural runoff and industrial effluents, where they can be used to remove nitrogen, phosphorus and other pollutants (Corpogno et al. 2015). Algae also have the ability to take up more P than required for their growth, known as ‘luxury uptake’, without a prior phosphate starvation stage (Powell et al. 2009). Therefore, it is especially desirable to recover P from wastewater through microalgae metabolism due to the zero chemical addition and possible P reuse from algal biomass as a valuable product.

To date, only a few studies have focused on the performance of microalgae at high phosphate concentrations. Examples of effluents from different nutrient-rich sources treated with microalgae technologies are: citric acid effluent produced by fermentation with 305 mg L\(^{-1}\) TN and 35 mg L\(^{-1}\) TP (Li et al. 2015) and carpet mill industrial effluent with 0.1–28.1 mg L\(^{-1}\) NO\(_3\)-N and 20–35 mg L\(^{-1}\) PO\(_4\)\(^{3-}\)-P (Chinnasamy et al. 2010).

The genera Scenedesmus and Chlorella include many species with similar morphology, but with a hidden molecular diversity (Hegewald et al. 2015). In this study, the nutrient removal capabilities of six microalgae strains from wastewaters were screened. The purpose was to select efficient microalgae strains in terms of successful nutrient removal and algal biomass production in order to develop a process with low addition of electricity and chemical reagents. The biomass growth and nutrient removal performance of the strains at high phosphate concentrations were compared using phosphorus-rich growth media with two different carbon sources.

**METHODS**

**Origin and identification of microalgae strains**

Six microalgae strains from the Algae Culture Collection (ACUF, www.acuf.net) of the Department of Biology, University of Naples ‘Federico II’ (Italy) were used in this study: Scenedesmus dimorphus ACUF_231, Desmodesmus subspicatus ACUF_273, Scenedesmus vacuolatus ACUF_053, Scenedesmus vacuolatus ACUF_298, Chlorella vulgaris ACUF_809 and Chlorella pyrenoidosa ACUF_808 (Figure 1(a)–1(f)). The last two strains were isolated from an MBBR performing nitrification of an industrial wastewater and generating a nitrate-rich (2500 mg NO\(_3\)\(^{-}\)·L\(^{-1}\)) effluent (Moreno Osorio et al. 2018).

The strains were identified by light microscopy using a Nikon Eclipse E800 (Nikon Instruments Europe B.V. Düsseldorf, Germany) (Figure 1(a)–1(f)). Molecular identification of the isolated strains ACUF_808 and ACUF_809 was done. DNA was extracted from liquid cultures with the protocol followed by Moreno Osorio et al. (2018) and used for a Polymerase Chain Reaction (PCR) with primers targeting the internal transcribed region of rDNA (ITS1_F 5’-TCCGTAGGTGAACCTGCGG-3’; ITS_rev_R5’T– TTCAAGATTCGATGATTCAC-3’). Product amplification and sequencing (PCR) was carried out as described by Moreno Osorio et al. (2018). The amplification primers were used as the sequencing primers. Nucleotide sequence similarity was determined by using BLAST version 2.0 (National Center for Biotechnology Information databases).

To identify the isolates at the species level, rDNA (ITS sequences) was aligned. In total, 69 sequences belonging to different Chlorella species and others that showed a high identity percentage by BLAST analysis were downloaded from the GenBank nucleotide database. A multiple nucleotide alignment was obtained by UGENE software (Okonechnikov et al. 2012) with the addition of the ACUF_808 and ACUF_809 strain sequences. The alignment, trimmed and manually adjusted (by eye) consisted of 187 sites. Manual inspection is a widely-used technique for reducing the number of sequencing errors and improving quality.

Bayesian inference was obtained with MrBayes 3.2.0, running 5 million generations and a sample frequency of 200, and using the general time reversible model (Tavaré 1986) with an invariable four gamma-distributed substitution rate category to correct for among site rate variation.
(GTR + G + I). The analysis was stopped at an average standard deviation of split frequencies of 0.01. The first 25% of the sampled trees were discarded as burn-in before calculating posterior probabilities. Runs were evaluated with Tracer v1.6.0 and the final tree visualized and edited with FigTree v1.4.2 (see Figure S1).

**Culture media**

Two different phosphate-rich culture media were tested to simulate different types of wastewaters and to study the performance of microalgae growth under each condition (Table 1). Bold’s Basal Medium (BBM) is commonly used for green algae cultivation and a modified version was used to grow the microalgae strain stock culture.

The modified version of BBM used was prepared as follows (mg·L⁻¹): CaCl₂ (25), NaCl (25), NaNO₃ (250), MgSO₄ 7H₂O (75), KH₂PO₄ (175), K₂HPO₄ (175), H₃BO₃ (114.2), with the addition of 1 mL of EDTA solution containing (mg·L⁻¹): EDTA (5) and KOH (3), 1 mL of acidifying iron solution, containing in 100 mL: FeSO₄ 7H₂O (498 mg·L⁻¹) and H₂SO₄ (96%) (1 mL), and 1 mL of trace metals solution containing (mg·L⁻¹): MnCl₂ 2H₂O (0.6), CoSO₄ 7H₂O (0.5), CuSO₄ 7H₂O (1.6), Na₂MoO₄ 2H₂O (0.6), and ZnSO₄ 7H₂O (8.8). Nitrate and phosphate concentrations were adjusted in the modified BBM composition (Table 1) to simulate the concentrations in a phosphate-rich industrial wastewater from a mineral fertilizer industry process with a concentration of 217 mg PO₄³⁻·L⁻¹ (0.74 mM PO₄³⁻-P) and 45 mg NO₃⁻·L⁻¹ (0.16 mM NO₃⁻N) (Yapijakis & Wang 2006; Inamori & Fujimoto 2010; Ouchah et al. 2014). The latter simulated the condition of mixing diverse wastewaters resulting in the presence of two different inorganic nitrogen and carbon sources, similarly to the experiments carried out by Kobayashi et al. (2013) for the treatment of the anaerobic digestion effluents, and by Gonzalez et al. (1997) for treating of agroindustrial wastewater in a secondary treatment aerobic stabilization pond with similar P-rich conditions (0.13 mM 16 mM NO₃⁻-N and 0.41 mM PO₄³⁻-P).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Composition of the media used in the experiments: modified Bold’s Basal Medium (BBM), growth medium with sodium bicarbonate (GMSB) and ammonium acetate (GMAA) supplementation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO₃⁻-N (mM)</td>
<td>0.66</td>
</tr>
<tr>
<td>NH₄⁺-N (mM)</td>
<td>–</td>
</tr>
<tr>
<td>PO₄³⁻-P (mM)</td>
<td>0.74</td>
</tr>
<tr>
<td>NaHCO₃ (mM)</td>
<td>–</td>
</tr>
<tr>
<td>CH₃COONH₄ (mM)</td>
<td>–</td>
</tr>
</tbody>
</table>
Experimental design

Batch experiments were performed to assess the potential of six microalgal strains towards nutrient removal (nitrate and phosphate) from an industrial wastewater with two different carbon sources (Table 1); that is, sodium bicarbonate GMSB (NaHCO₃) and ammonium acetate GMAA (CH₃COONH₄), as described by Moreno Osorio et al. (2018). Each growth medium promoted the autotrophic or mixotrophic growth of the strains, respectively. The experiments were carried out in triplicate in 250 mL flasks closed with a cotton plug at 25 (±2) °C for 2 weeks with constant shaking (80 rpm) and continuous illumination at 90 (±10) μmol m⁻² s⁻¹ provided by 36 WT12 fluorescent Cool White (Osram light, Munich, Germany) as higher nutrient uptake rates and biomass production capabilities have been reported with continuous illumination (Taziki et al. 2016). No CO₂ was supplemented to the batch reactors (atmospheric CO₂, ~0.04%). The reactors were inoculated with the same volume of microalgal culture in order to obtain a similar initial biomass concentration in all experiments. The initial cell density was adjusted to 2.5 × 10⁶ cells·mL⁻¹ and no additional pH adjustment was made to the media through the experiments after algae inoculation.

The microalgal strains’ efficiency, in terms of nutrient removal and algal biomass production, was followed during two weeks. The biomass production was measured as biomass growth by spectrophotometry. The nutrient removal performance was analyzed by measuring phosphate and nitrate concentrations through the batch experiment.

Analytical methods

Biomass growth was determined as described by Moheimani et al. (2015) using a SECOMAM s. 250 (Nova Analytics, Ales, France). Samples were diluted by appropriate ratios to ensure that absorbance values were in the range of 0.1–1 (dimensionless). To convert the OD₆₈₀ values to biomass concentration in dry weight (mg·L⁻¹), calibration curves were determined. Biomass dry weight (DW) was measured by filtering an aliquot of an algal sample on pre-weighed glass-fiber filter paper with a pore size of 0.45 μm. The filters were then dried at 105 °C in an oven for 12 h and algal biomass DW was determined by the difference of the two weights. pH values were measured with a pH meter Bench model AD-1030 (Adwa Instruments Inc., Szeged, Hungary).

Experimental biomass productivity Pₓ (mg·L⁻¹·d⁻¹) was calculated following the equation Pₓ = ΔX/Δt, where ΔX is the variation of the biomass concentration (mg·L⁻¹) within a cultivation time Δt (d). To analyze the nutrient removal rate in the media, samples of 2 mL volume were extracted daily from each flask to determine the concentration of phosphorus measured as orthophosphate ions (PO₄³⁻) and nitrogen as nitrate (NO₃⁻). No ammonium measurements were made on the samples. Analyses of nitrate and phosphate were performed with ion chromatography using a 761 compacts IC analyzer (Metrohm, Herisau, Switzerland) as described by APHA (2002). The experiments were carried out in triplicates and the standard deviation was calculated. To analyze the nutrient removal rate in the media, Rᵢ (mg·L⁻¹·d⁻¹) was calculated with the equation Rᵢ = ΔS/Δt, where Rᵢ represents the nutrient removal rate of the substrate (NO₃⁻ or PO₄³⁻), ΔS is the variation of nutrient concentration (mg·L⁻¹) within a cultivation time of Δt (d). Nutrient removal percentages were calculated as described by Acevedo et al. (2017). Every sample was measured in triplicate, means and standard deviations were calculated.

Data analysis

The Verhulst logistic kinetic model (Verhulst 1838) was used to predict the evolution of the experimental biomass concentration in the batch systems. This model can accurately describe the growth of biomass in different culture conditions occurring in many batch bioreactors as a sinusoidal curve (Arbib et al. 2013). The microbial growth can be expressed as described by Equation (1):

\[ X = \frac{X_0 X_m e^{\mu t}}{X_m - X_0 + X_0 e^{\mu t}} \]  

where \( \mu_{\text{max}} \) is the maximum specific growth rate (d⁻¹), \( X_m \), \( X_0 \) and \( X \) are the biomass concentrations (mg·L⁻¹) at an operation time equal to infinite, zero and at a defined time (t), respectively.

The batch biomass productivity (\( P_b \), mg·L⁻¹·d⁻¹) was then calculated as in Equation (2):

\[ P_b = \frac{\mu \cdot (0.9 \cdot X_m - 1.1 \cdot X_0)}{\ln\left(\frac{9 \cdot (X_m - 1.1 \cdot X_0)}{1.1 \cdot X_0}\right)} \]  

Kinetic modeling was performed using the SOLVER tool of Microsoft Excel 2011 (Microsoft®) and the kinetic parameters were estimated by least squares. A confidence interval (C.I.) for \( p \leq 0.05 \) was used for the estimation of the determination coefficient. To evaluate differences between the microalga biomass productivity (\( P_x \) and
nutrient removal rate ($R_m$) of the six strains, one way ANOVA was carried out using OriginPro 2017 (OriginLab, Northampton, MA) with a confidence level of 95%. The null hypothesis stated that the mean productivities were equal. A $p$-value of 0.05 indicates that there is a significant difference between the tested means.

**RESULTS AND DISCUSSION**

**Isolation and identification of the microalgae strain**

The strains *C. pyrenoidosa* ACUF_808 and *C. vulgaris* ACUF_809 were isolated and purified from the effluent of a nitrification reactor. The strains showed tolerance to high nitrate concentrations (0.65 ± 0.02 mM NO$_3$-N) growing spontaneously in this environment. The cells were planktonic and spherical with a diameter range of 3–6 μm, chromatophore single, parietal mantle-shaped covering nearly the whole of the cell wall. A conspicuous round pyrenoid was present in each chromatophore (Figure 1(a)–1(f)).

Bayesian inference (see Figure S1) showing close phylogenetic distance (0.99) between these strains with two other *C. pyrenoidosa* strains and 32 *C. vulgaris* strains was obtained. In the systematics of coccoid green algae, the identification of the *Chlorella* species is a difficult task. There is a large number of algae from various taxonomic groups showing the same morphological characteristics and the classification of coccoid unicellular algae belonging to the *Chlorophyta* needs a molecular approach. However, a recent study carried out on 400 internal transcribed spacer 2 (ITS2) and/or 18S ribosomal RNA sequences of *Chlorella* and related taxa have confirmed that the *Chlorellaceae* consist of a *Parachlorella* and a *Chlorella*-clade (Heeg & Wolf 2015) and that *Chlorella* is polyphyletic (Turmel et al. 2009). Since the genus *Chlorella* has been frequently revised in the past years and taxonomic attribution represents a hard task for phycologists, the trees should be considered with particular attention for the genetic relatedness of unidentified strains, with an approximation that is not 100% resolved for species attribution (Turmel et al. 2009; Heeg & Wolf 2015). For this reason, a clear-cut species designation may be problematic. In the case of the *Chlorella* strains ACUF_808 and ACUF_809, the ITS phylogenetic tree suggests a close (0.99) relation of these strains with strains belonging to *C. pyrenoidosa* and *C. vulgaris* strains, respectively (see Figure S1 in Supplementary Materials). Hence, with caution, our isolates were taxonomically assigned to these two *Chlorella* species.

**Microalgae growth and nutrient removal from different wastewaters**

**Growth medium with inorganic carbon source**

The screened microalgae strains grew in the growth media tested; that is, GMSB and GMAA (Figures 2 and 3). The performance of the strains was studied in batch mode in order to determine their biomass growth. The used growth media simulating high phosphate concentrations could be treated biologically using all microalgae investigated and could be used for algal biomass production.

Algal growth was well characterized by the logistic growth function (Figures 2(a) and 3(a)). Most of the strains growing in GMSB had a short exponential period (2–5 days) after the lag phase and reached the stationary phase after cultivation day 3, except for the *D. subspicatus* strain, which reached the stationary phase on the 7th day (Figure 2(a)). *C. vulgaris* and *S. vacuolatus* ACUF_209 showed a long stationary phase in GMSB starting from day 3 of cultivation until the end of the experiment. A longer lag phase (two days) was recorded for the *D. subspicatus* strain (Figure 2(a)).

In algal batch experiments with limited volume of nutrient supply, a logistic function is usually observed (Schanz & Zahler 1980). The Verhulst kinetic model showed a good fit (lines in Figure 2(a)) with the experimental data. $R^2$ ($p$ ≤ 0.05) was higher than 0.95 in all cases. *C. pyrenoidosa* and *S. dimorphus* showed the highest ($X_m$, 548.2 mg·L$^{-1}$) and lowest ($X_m$, 238.1 mg·L$^{-1}$) maximum final biomass concentration, respectively (Table 2). *D. subspicatus* ACUF_273 and *S. dimorphus* ACUF_231 achieved the highest $X_m$ values (1.62 d$^{-1}$); these values are comparable with the specific growth rate range reported in the literature from 0.1 to 0.9 d$^{-1}$ (Gonzalez et al. 1997; de-Bashan & Bashan 2004; Chinnasamy et al. 2010), which confirms the good growth tolerance of these strains to the phosphorus-rich growth media tested. Biomass productivities of *S. vacuolatus* reported by Mata et al. (2010) (between 36 and 41 mg·L$^{-1}$d$^{-1}$) are similar to those obtained by *S. vacuolatus* ACUF_053 (34.05 mg·L$^{-1}$d$^{-1}$) and *S. vacuolatus* ACUF_298 (67 mg·L$^{-1}$d$^{-1}$) in GMSB.

A fast nitrate removal was observed (Figure 2(d)), although significant differences in nitrate removal rate between the six microalgae strains were observed ($p$ < 0.05). The highest nitrate removal percentage was
obtained by *S. dimorphus* (99.5 ± 0.6%). *C. vulgaris* grows in nitrogen and phosphorus-rich wastewaters and can exhibit phototrophic and mixotrophic growth, showing differences in biomass production (*Wu et al. 2014*). When *C. vulgaris* grows phototrophically, the biomass concentration can vary from 700 to 1500 mg·L⁻¹ (*Wu et al. 2014*). On the contrary, in this study *C. vulgaris* ACUF_809 grown in GMSB showed a twice lower biomass concentration (390 ± 7.06 mg·L⁻¹). However, another study where *C. vulgaris* was grown in a wastewater with low nitrogen concentrations (0.25 mgN·L⁻¹) reported higher biomass concentrations (Table 3), in a range of 100–600 mg·L⁻¹ (*Fadeyi et al. 2016*), similar to the values obtained in GMSB (390 ± 7.1 mg·L⁻¹) in this study (Figure 2(a)). Conversely, no significant difference in phosphate removal rate was observed between the strains (*p* ≤ 0.05). The maximum phosphate removal percentages were low for all the strains investigated and the highest was obtained by *C. vulgaris* (9.1 ± 6.5%) (Table 4).

Nitrate assimilation is a key process for nitrogen acquisition in green microalgae (*Sanz-Luque et al. 2015*). The concentrations and availability of inorganic nitrogen sources change depending on the environment and limit the growth and productivity (*Combres et al. 1994*) as it triggers a rapid decline in photosynthetic pigments, inducing complete loss of photosynthetic efficiency (*Wang et al. 2014*) which could explain the decreasing biomass growth behavior observed in this study.

No additional pH adjustment was done to the media along the experiments after algae inoculation, as shown by *Eustance et al. 2015* the growth on nitrate caused the pH to increase, which could regulate the decrease of pH reported to microalgae growth on ammonium (as GMAA) without additional aeration or chemical buffering.

The highest pH values (10.2–10.8) were measured at day six of incubation, (Figure 2(b)). The pH affects the microalgae growth in a strain-dependent way. Different
species and strains have different optimal pH at which the fastest growth is achieved (Kong et al. 2009). During the first five days, fast growth of the algae strains and consumption of nutrients was observed. Metabolites from the algal growth generated a rapid increase in the pH values, which in turn could cause the reduction of the availability of the carbon source and phosphate precipitation in the medium.

Table 2 | Kinetic growth parameters for six microalgae strains obtained by the Verhulst logistic model for the different types of synthetic wastewater tested

<table>
<thead>
<tr>
<th>Synthetic wastewater</th>
<th>Kinetics parameter</th>
<th>S. dimorphus</th>
<th>C. pyrenoidosa</th>
<th>D. subspicatus</th>
<th>S. vacuolatus ACUF_053</th>
<th>S. vacuolatus ACUF_298</th>
<th>C. vulgaris</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMSB</td>
<td>$X_m$ (mg L$^{-1}$)</td>
<td>238.11</td>
<td>548.21</td>
<td>511.96</td>
<td>433.63</td>
<td>298.77</td>
<td>347.98</td>
</tr>
<tr>
<td></td>
<td>$\mu_m$ (d$^{-1}$)</td>
<td>0.53</td>
<td>1.17</td>
<td>1.62</td>
<td>0.40</td>
<td>1.45</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>$R^2$</td>
<td>0.998</td>
<td>0.979</td>
<td>0.950</td>
<td>0.951</td>
<td>0.938</td>
<td>0.939</td>
</tr>
<tr>
<td></td>
<td>$P_b$ (mg L$^{-1}$·d$^{-1}$)</td>
<td>21.77</td>
<td>106.61</td>
<td>96.20</td>
<td>34.05</td>
<td>76.61</td>
<td>63.33</td>
</tr>
<tr>
<td>GMAA</td>
<td>$X_m$ (mg L$^{-1}$)</td>
<td>493.42</td>
<td>983.63</td>
<td>1200.57</td>
<td>980.14</td>
<td>1109.57</td>
<td>1003.91</td>
</tr>
<tr>
<td></td>
<td>$\mu_m$ (d$^{-1}$)</td>
<td>0.46</td>
<td>0.45</td>
<td>0.36</td>
<td>0.42</td>
<td>0.34</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>$R^2$</td>
<td>0.999</td>
<td>0.994</td>
<td>0.993</td>
<td>0.995</td>
<td>0.990</td>
<td>0.986</td>
</tr>
<tr>
<td></td>
<td>$P_b$ (mg L$^{-1}$·d$^{-1}$)</td>
<td>35.99</td>
<td>75.71</td>
<td>71.48</td>
<td>72.28</td>
<td>67.22</td>
<td>70.90</td>
</tr>
</tbody>
</table>

$X_m$ (mg biomass L$^{-1}$): maximum final biomass concentration; $\mu_m$ (d$^{-1}$): maximum specific growth rate and $P_b$ (mg L$^{-1}$·d$^{-1}$): batch productivity. GMSB: growth medium with sodium bicarbonate, GMAA: growth medium with ammonium acetate.
Table 3 | Experimental maximum biomass concentration ($X_m$) and biomass productivity ($P_b$) of microalgae strains grown under different conditions

<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium</th>
<th>Mode</th>
<th>Reactor</th>
<th>Biomass concentration (mg·L$^{-1}$)</th>
<th>Biomass productivity (mg·L$^{-1}$ d$^{-1}$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. obliquus</td>
<td>Detmers medium</td>
<td>continuous</td>
<td>1 L glass vessel</td>
<td>2100 ± 80</td>
<td>440.68 ± 15.8</td>
<td>Ho et al. (2012)</td>
</tr>
<tr>
<td>S. obliquus</td>
<td>SW</td>
<td>batch</td>
<td>Erlenmeyer flask 2 L</td>
<td>1810 ± 194</td>
<td>193.2</td>
<td>Arbib et al. (2013)</td>
</tr>
<tr>
<td>S. obliquus</td>
<td>Real wastewater</td>
<td>batch</td>
<td>Erlenmeyer flask 250 ml</td>
<td>1270</td>
<td>–</td>
<td>Fadeyi et al. (2016)</td>
</tr>
<tr>
<td>S. obliquus</td>
<td>BG11</td>
<td>batch</td>
<td>Erlenmeyer flask 250 ml</td>
<td>3370</td>
<td>–</td>
<td>Fadeyi et al. (2016)</td>
</tr>
<tr>
<td>S. dimorphus</td>
<td>GMSB</td>
<td>batch</td>
<td>Erlenmeyer flask 100 ml</td>
<td>234.1 ± 48.3</td>
<td>19.71 ± 3.9</td>
<td>This study</td>
</tr>
<tr>
<td>S. dimorphus</td>
<td>GMAA</td>
<td>batch</td>
<td>Erlenmeyer flask 100 ml</td>
<td>419.7 ± 10.7</td>
<td>36.65 ± 0.5</td>
<td>This study</td>
</tr>
<tr>
<td>D. subspicatus</td>
<td>SGMSB</td>
<td>batch</td>
<td>Erlenmeyer flask 100 ml</td>
<td>555.2 ± 10.2</td>
<td>39.57 ± 0.7</td>
<td>This study</td>
</tr>
<tr>
<td>D. subspicatus</td>
<td>GMMA</td>
<td>batch</td>
<td>Erlenmeyer flask 100 ml</td>
<td>1017 ± 45.0</td>
<td>62.98 ± 4.2</td>
<td>This study</td>
</tr>
<tr>
<td>S. vacuolatus</td>
<td>GMSB</td>
<td>batch</td>
<td>Erlenmeyer flask 100 ml</td>
<td>444 ± 11.04</td>
<td>31.31 ± 1.07</td>
<td>This study</td>
</tr>
<tr>
<td>S. vacuolatus</td>
<td>ACUF_053</td>
<td>batch</td>
<td>Erlenmeyer flask 100 ml</td>
<td>948 ± 49.8</td>
<td>70.08 ± 4.0</td>
<td>This study</td>
</tr>
<tr>
<td>S. vacuolatus</td>
<td>ACUF_053</td>
<td>batch</td>
<td>Erlenmeyer flask 100 ml</td>
<td>369.2 ± 59.3</td>
<td>33.85 ± 5.8</td>
<td>This study</td>
</tr>
<tr>
<td>S. vacuolatus</td>
<td>ACUF_298</td>
<td>batch</td>
<td>Erlenmeyer flask 100 ml</td>
<td>985.8 ± 76.2</td>
<td>76.02 ± 10.9</td>
<td>This study</td>
</tr>
<tr>
<td>C. vulgaris</td>
<td>SW</td>
<td>batch</td>
<td>Erlenmeyer flask 2 L</td>
<td>821 ± 88</td>
<td>94.1</td>
<td>Arbib et al. (2015)</td>
</tr>
<tr>
<td>C. vulgaris</td>
<td>Real wastewater</td>
<td>batch</td>
<td>Erlenmeyer flask 250 ml</td>
<td>2310</td>
<td>–</td>
<td>Fadeyi et al. (2016)</td>
</tr>
<tr>
<td>C. vulgaris</td>
<td>BG11</td>
<td>batch</td>
<td>Erlenmeyer flask 250 ml</td>
<td>3640</td>
<td>–</td>
<td>Fadeyi et al. (2016)</td>
</tr>
<tr>
<td>C. vulgaris</td>
<td>GMSB</td>
<td>batch</td>
<td>Erlenmeyer flask 100 ml</td>
<td>390 ± 7.06</td>
<td>28.14 ± 1.4</td>
<td>This study</td>
</tr>
<tr>
<td>C. vulgaris</td>
<td>GMMA</td>
<td>batch</td>
<td>Erlenmeyer flask 100 ml</td>
<td>949.6 ± 88.1</td>
<td>61.62 ± 7.2</td>
<td>This study</td>
</tr>
<tr>
<td>C. pyrenoidosa</td>
<td>GMSB</td>
<td>batch</td>
<td>Erlenmeyer flask 100 ml</td>
<td>561.6 ± 8.7</td>
<td>68.40 ± 0.5</td>
<td>This study</td>
</tr>
<tr>
<td>C. pyrenoidosa</td>
<td>GMMA</td>
<td>batch</td>
<td>Erlenmeyer flask 100 ml</td>
<td>932 ± 86.4</td>
<td>68.24 ± 6.4</td>
<td>This study</td>
</tr>
</tbody>
</table>

GMSB: growth medium with sodium bicarbonate, GMAA: growth medium with ammonium acetate, SW: synthetic wastewater.

Growth medium with organic carbon source

The absence of a stationary phase was recorded for the D. subspicatus strain (Figure 3(a)). The best fitting of the Verhulst model to the biomass growth was observed in these experiments with an $R^2$ of 0.99 (Table 2). D. subspicatus showed the highest maximum final biomass concentration ($X_m$, 1200.6 mg·L$^{-1}$) and the lowest was

Table 4 | Maximum phosphorus and nitrogen removal percentage of the six strains growing in the growth media tested

<table>
<thead>
<tr>
<th>Culture media</th>
<th>Nutrient</th>
<th>S. dimorphus</th>
<th>C. pyrenoidosa</th>
<th>D. subspicatus</th>
<th>S. vacuolatus ACUF_053</th>
<th>S. vacuolatus ACUF_298</th>
<th>C. vulgaris</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMSB</td>
<td>NO$_3$-N % removal</td>
<td>99.5 ± 0.4</td>
<td>97.3 ± 3.1</td>
<td>99.5 ± 0.4</td>
<td>93.4 ± 9.8</td>
<td>96.4 ± 3</td>
<td>95.7 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>NO$_2$-N removal rate (d$^{-1}$)</td>
<td>19.3 ± 1.4</td>
<td>19.3 ± 1.4</td>
<td>19.3 ± 1.4</td>
<td>13 ± 0.8</td>
<td>22.1 ± 1.4</td>
<td>18.1 ± 6</td>
</tr>
<tr>
<td></td>
<td>PO$_4^3$-P % removal</td>
<td>9.1 ± 3.8</td>
<td>0 ± 5.7</td>
<td>3.4 ± 4</td>
<td>3.4 ± 12</td>
<td>3.7 ± 1.2</td>
<td>3.4 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>PO$_4^3$-P removal rate (d$^{-1}$)</td>
<td>7.4 ± 3.4</td>
<td>4.7 ± 4.1</td>
<td>4.7 ± 4.1</td>
<td>0.12 ± 12</td>
<td>3.7 ± 1.2</td>
<td>3.4 ± 3.1</td>
</tr>
<tr>
<td>GMAA</td>
<td>NO$_3$-N % removal</td>
<td>99.8 ± 1.7</td>
<td>99.4 ± 0.31</td>
<td>99.3 ± 0.94</td>
<td>99.8 ± 0.27</td>
<td>98.1 ± 1.0</td>
<td>99.5 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>NO$_2$-N removal rate (d$^{-1}$)</td>
<td>5.3 ± 1.3</td>
<td>5.3 ± 1.3</td>
<td>5.3 ± 1.3</td>
<td>2.3 ± 4.8</td>
<td>7.3 ± 0.1</td>
<td>5.6 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>PO$_4^3$-P % removal</td>
<td>16.5 ± 2.1</td>
<td>0 ± 11.7</td>
<td>23.6 ± 5.85</td>
<td>17.8 ± 1.86</td>
<td>9.0 ± 11.24</td>
<td>32.5 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>PO$_4^3$-P removal rate (d$^{-1}$)</td>
<td>0.76 ± 1.3</td>
<td>0.7 ± 1.3</td>
<td>8.8 ± 7.7</td>
<td>6.1 ± 0.8</td>
<td>8.6 ± 4.4</td>
<td>5.7 ± 0.7</td>
</tr>
</tbody>
</table>

GMSB: growth medium with sodium bicarbonate, GMAA: growth medium with ammonium acetate.
displayed by *S. dimorphus* (*Xₘₚ*, 493.4 mg·L⁻¹). This latter was twice lower than the values observed for the other strains screened (Table 2). The *C. pyrenoidosa* strain reached the highest productivity (75.1 mg·L⁻¹·d⁻¹) (Table 2). When *C. vulgaris* grows mixotrophically, the growth can vary from 400 to 2000 mg·L⁻¹ (Wu et al. 2014). These values are similar to those observed for the experimental data for *C. vulgaris* ACUF0_298, i.e. 949.6 (± 88.1) mg·L⁻¹ (Table 3).

The low biomass concentration achieved by the microalgae strains (Figure 3(a)) can be due to the low initial N:P ratio, since phosphorus was present in excess. The increase in nitrogen concentration (higher N:P ratio) enhanced the maximum final biomass concentration achieved, while the increase in phosphorus concentration did not have the same enhancing effect as reported previously (Arbib et al. 2013; Moreno Osorio et al. 2018). The presence of extra nitrogen (0.83 mM NH₄-N) in GMAA provided a mixotrophic growth mode with the assimilation of organic carbon and additionally CO₂ fixation to promote algae growth (Wang et al. 2012), as observed for most of the algae strains screened in this study. When phosphate is not the limiting nutrient, the N:P ratios can affect the ability of *C. vulgaris* to remove nutrients from wastewater (Wu et al. 2014). Therefore, after days 6 and 7 of cultivation, when nitrate depletion was obtained by all strains (Figure 3(d)), the microalgae biomass growth decreased during the rest of the experimental time.

*Scenedesmus obliquus* cultivated in a nitrogen-limited culture medium had a maximum biomass productivity of 440.68 (±15.79) mg·L⁻¹·d⁻¹ (Ho et al. 2012), which was about twofold higher than the productivities (263 mg·L⁻¹·d⁻¹) obtained by Arbib et al. (2013) under no limitation of nitrogen (N:P 1.0) and five times higher than the productivities obtained for the *Scenedesmus* strains tested in this study (56–72 mg·L⁻¹·d⁻¹) with GMAA. The difference in productivity values among different studies can be due to the criteria used to terminate experiments. As commented by Arbib et al. (2013), not all the authors interrupt the microalgae culturing experiments at the same time or they do not indicate the criteria used. Some authors terminate the experiments before reaching the stationary phase. In those cases, the productivities are partial, as the maximum biomass that can be reached in the reactors is unknown. This could be the case for the *D. subspicatus* ACUF_273 strain grown in GMAA, which was the only strain that showed an exponential growth at day 13 of incubation (Figure 3(a)).

The experimental data showed that *D. subspicatus* reached the highest biomass concentration (1017 ± 45 mg·L⁻¹) and *S. vacuolatus* ACUF0_298 reached the highest biomass productivity (76.0 ± 10.9 mg·L⁻¹·d⁻¹) (Table 3). The highest pH value recorded in this experiment was 10.7 for *C. vulgaris*, while the other strains achieved pH values below 9.7 (Figure 3(b)). A general trend observed among all the strains in this growth medium was the reduction of the pH value after day 6 of incubation (Figure 3(b)).

In this study, all the screened strains except *C. vulgaris* showed low phosphate removal efficiencies between 3–23% (Table 4). This efficiency falls in the range reported for *C. vulgaris* and *S. dimorphus* cultivated in synthetic industrial wastewater with high phosphorus concentrations (112 mg·L⁻¹, 0.41 mM ·P) in batch, showing removal efficiencies of only 20–55% (Gonzalez et al. 1997). In both cases, the removal rate and efficiency values could be explained by the limiting nitrogen availability (Acevedo et al. 2017). The phosphate removal rate was not significantly (*p < 0.05*) different between the microalgae strains (Table 4). However, the *C. vulgaris* strain showed the highest phosphate removal efficiency with 32.5% (Figure 3(c)). These results are similar to those observed by Moreno Osorio et al. (2018) under the same simulated wastewater N and P concentrations as this study. They showed for *Chlorella* sp. ACUF_802 a low phosphate removal efficiency (17.8% and 11.4% for synthetic wastewater with sodium bicarbonate and ammonium acetate, respectively). Conversely, different results were observed when the effect of the N:P ratio was studied; they showed that *Chlorella* sp. ACUF_802, growth in synthetic wastewater with a N:P higher than 5:1 (7.4–7.5) completely removed the nitrate and phosphate present in the phosphate-rich synthetic wastewater. Overall, total inhibition of the growth rates due to the phosphate concentration tested was not observed. The nitrate removal efficiency from GMAA was ≥ 99% by the microalgal strains (Table 4).

Figures 2(c), 2(d), 3(c) and 3(d) show that phosphorus concentrations become higher after complete nitrogen depletion, which could be due to the phosphate release by the senescent or dead algal cells to the growth medium (Arbib et al. 2015). However, all removed nutrients are not always taken up by the microalgae. For instance, phosphate removal under alkaline conditions, as observed in GMAA that achieved a pH higher than 10 (Figure 3(b)), can be caused by the formation of insoluble precipitates (Jiang et al. 2016).
Competitive N and P removal was obtained by the isolated strains among the strains screened from these particular conditions simulated. Ammonium acetate was found to be a promising carbon source in the microalgae systems used for wastewater treatment, because a lower concentration was used compared to sodium bicarbonate (Table 1). This advantage is related to the fact that ammonium acetate offers both organic carbon and extra nitrogen to the microalgae, which allows the continuous removal of P along the experiment (Combres et al. 1994). However, it is not like this for all microalgae strains, some Chlorella spp., Botryococcus braunii and Dunaliella tertiolecta prefer nitrate rather than ammonium for growth and they can also utilize organic nitrogen sources (Muthuraj et al. 2014) and microalgae grown on nitrate have shown high biotechnological potential for biomass use (Eustance et al. 2013) as an important feature of wastewater treatment.

Supply of ammonium acetate can be a cost-effective option to increase the nutrient removal efficiency in phosphate-rich wastewaters. Supplementation with acetate as the carbon source has been reported as the most cost-effective substrate for wastewater treatment (de-Bashan & Bashan 2004). The presence of ammonium and magnesium can stimulate the formation of recovery forms of phosphates (like struvite) under alkaline conditions, which could lead to new research in a combined biological/chemical option for treatment of fertilizer industry effluents like that mimicked by GMAA. Additionally, the combination of the newly isolated freshwater microalgae strains (C. pyrenoidosa ACUF_808 and C. vulgaris ACUF_809) might represent an efficient solution for nitrate and phosphate removal from wastewaters with high P concentrations, since C. pyrenoidosa and C. vulgaris showed a high removal efficiency of, respectively, nitrate and phosphate.

CONCLUSION

The six strains investigated could adapt to the synthetic wastewater with both carbon sources, showing a short or no lag phase, except for S. dimorphus, which showed the lowest growth rates. C. pyrenoidosa ACUF_808 obtained the highest biomass productivity of 106.61 mg·L⁻¹·d⁻¹ in GMSB and 75.71 mg·L⁻¹·d⁻¹ in GMAA. All strains exhibited high nitrate (>90%), but low phosphate (<33%) removal efficiencies under both conditions. Strains grown in GMAA had a maximum nitrate removal efficiency of >95%. C. vulgaris ACUF_809 showed the highest phosphate removal efficiency. This strain also showed the highest simultaneous nitrogen and phosphorus removal efficiency among all the strains tested. Therefore, it is the most suitable strain for microalgal treatment of phosphate-rich industrial wastewaters that also have a high organic carbon load (mimicked by GMAA).

ACKNOWLEDGEMENT

The authors thank Dr. Ludovico Pontoni from the University of Naples ‘Federico II’ (Naples, Italy) for laboratory and instrument assistance and Federica Calabrese and Langping Wu for their proofreading of the manuscript, and pertinent comments. The authors would also like to thank the EU for providing financial support through the Erasmus Mundus Joint Doctorate Programme, Environmental Technologies for Contaminated Solids, Soils and Sediments, grant agreement FPA no. 2010-0009 (ETeCoS³).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this paper is available online at https://dx.doi.org/10.2166/wst.2019.431.

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First received 5 February 2019; accepted in revised form 14 December 2019. Available online 9 January 2020