Cultivation of *Scenedesmus obliquus* in liquid hydrolysate from flash hydrolysis for nutrient recycling

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32 **Abstract**

33 The production of biofuels from microalgae is associated with high demands of
34 nutrients (nitrogen and phosphorus) required for growth. Recycling nutrients from the
35 residual biomass is essential to obtain a sustainable production. In this work, the
36 aqueous phase obtained from flash hydrolysis of *Scenedesmus sp.* was used as
37 cultivation medium for a microalga of the same genus, to assess the feasibility of this
38 technique for nutrient recycling purposes. Batch and continuous cultivations were
39 carried out, to determine growth performances in this substrate compared to standard
40 media, and verify if a stable biomass production could be obtained. In continuous
41 experiments, the effect of hydrolysate inlet concentration and of residence time were
42 assessed to optimize nutrient supply in relation to productivity. Results obtained show
43 that nutrient recycling is feasible by treating biomass with flash hydrolysis, and
44 *Scenedesmus* is capable of recycling large amounts of recovered nutrients.

45 **Keywords**

46 Microalgae, flash hydrolysis, mixotrophy, continuous photobioreactor

1. Introduction

Microalgae have been widely recognized as a very promising feedstock for biofuels and bio-products, aiming at replacing traditional fossil fuels and reducing greenhouse gas emissions, especially for the transportation sector. The numerous advantages that these microorganisms offer compared to terrestrial plants are very well established, such as the possibility of cultivation on non-arable land, the ability of capturing CO₂ from flue-gases, together with higher growth rates, productivities and photosynthetic efficiencies (Mata et al., 2010; Quinn and Davis, 2014; Ullah et al., 2015). Nonetheless, several issues have still to be addressed and solved in order for microalgae-based biofuels production to be sustainable (Chisti, 2013).

Clearly, in order to achieve significant displacement of petroleum-based fuels, very large volumes of microalgal biomass need to be produced. This is associated with the necessity of consistent amounts of nutrients that are required for growth, mainly nitrogen (N) and phosphorus (P). Even though at the beginning nutrients were often neglected when evaluating costs estimates, they are emerging as one of the highest operational costs (Pate et al., 2011). In addition to economic implications, extensive nutrients requirements raise also environmental and sustainability issues. In fact, based on a typical microalgae elemental composition, it can be estimated that, assuming 100% nutrient uptake, roughly 88 kg of N and 12 kg of P are required for producing 1 ton of algal biomass. Pate et al. (Pate et al., 2011) reported that, considering a target biofuels production of 10 billion gallons per year and a biomass oil content of 20%, the nitrogen and phosphorus required for algae cultivation would account for 107% and 51% of overall US consumption, respectively. Moreover, it has to be considered that N and P fertilizers are currently derived from mineral or fossil resources, whose availability is

71 therefore limited. This is particularly critical for phosphorus, since the natural reserves
72 of phosphate rocks are finite, and expected to be depleted in the future (Gifford et al.,
73 2015; Markou et al., 2014; Pate et al., 2011; Ward et al., 2014). Nitrogen is on the
74 contrary more abundant, but its production requires energy intensive processes (Haber-
75 Bosh) (Peccia et al., 2013). Based on these considerations, it is clear that the only way
76 to obtain an economically and environmentally sustainable microalgal biofuels
77 production is to recycle the nutrients, the majority of which is not included in the lipid
78 fraction destined to biofuels, and remains in the residuals. This possibility is clearly
79 highly connected with the method employed for biomass treatment after harvesting. In
80 this regard, different alternatives have been recently proposed (Rösch et al., 2012;
81 Zhang et al., 2014), from anaerobic digestion after lipid extraction (Sialve et al., 2009;
82 Ward et al., 2014), to hydrothermal treatments (Biller et al., 2012; Garcia Alba et al.,
83 2013; Heilmann et al., 2011; Levine et al., 2013; López Barreiro et al., 2013). Among
84 hydrothermal processes, flash hydrolysis (FH) has been proven to be an interesting and
85 environmentally friendly strategy to extract energy dense molecules from biomass: it
86 consists in processing the algae slurry, harvested from the cultivation system, in a
87 continuous-flow reactor under subcritical water conditions (200°C – 300°C) and few
88 seconds of residence time (6s – 10s) (Garcia-Moscoso et al., 2015, 2013). This
89 treatment allows obtaining two phases. The main product of the process is a low-N,
90 high-C solid, that retains the lipid fraction, and is therefore an intermediate for biofuels
91 production (Garcia-Moscoso et al., 2015, 2013). Lipids in this biofuels intermediate are
92 more concentrated and easily extractable compared to the original biomass. The second
93 phase is a liquid hydrolysate that contains the hydrolyzed proteins in the form
94 solubilized peptides and amino acids (up to 66% of the initial N content), as well as

95 other inorganic elements (e.g. P, S, K, Na, and Ca), that can be recycled to sustain a new
96 cycle of algal growth. Compared to conventional hydrothermal liquefaction (HTL),
97 which is characterized by longer residence times (generally from few minutes to 1 h),
98 flash hydrolysis offers several advantages. Firstly, lipids are retained and preserved in
99 the solid fraction, while the former produces an organic liquid phase (biocrude), which
100 has to be separated and refined prior to use as fuel for transportation. Also, the amount
101 of phenolic compounds produced, which are known to have inhibitory effects on algae
102 growth when the liquid phase is recycled, is reported to be much lower in the
103 hydrolysate obtained from FH compared to the aqueous phase from HTL (Garcia-
104 Moscoso et al., 2015).

105 This work aims at assessing the feasibility of using residuals from flash hydrolysis for
106 nutrient recycling to improve the sustainability of biofuels production. For this purpose,
107 *Scenedesmus obliquus* was cultivated in the liquid hydrolysate obtained from flash
108 hydrolysis of algae biomass belonging to the same genus. Firstly, batch experiments
109 were carried out under different experimental conditions to compare the growth
110 potential in this substrate with a synthetic cultivation medium where all the nutrients are
111 supplied as soluble inorganic salts. However, despite batch studies are fundamental to
112 determine growth parameters, continuous cultivation is preferable when considering
113 large-scale industrial plants, as it allows reaching steady-state, stable and continuous
114 biomass production (Barbera et al., 2015; Sforza et al., 2014a). For this reason, *S.*
115 *obliquus* was also cultivated in a continuous laboratory-scale photobioreactor (PBR) fed
116 with the hydrolysate, to assess the performances achievable in terms of biomass
117 productivity and efficiency. Specific attention was given to nutrients consumptions and
118 corresponding content in the biomass produced, in order to minimize the amount

119 supplied to the culture while at the same time maintaining an acceptable productivity.
120 Finally, the effect of the residence time of the culture inside the PBR on productivity
121 and on nutrients content was taken into consideration.

122 **2. Materials and Methods**

123 **2.1 Algal strain and culture media**

124 *Scenedesmus obliquus* 276.7 (obtained from SAG-Goettingen, Germany) was
125 maintained in sterile BG11 medium, with 1.5 g L⁻¹ NaNO₃ (247 mg L⁻¹ N) and 30.5 mg
126 L⁻¹ K₂HPO₄ (5.4 mg L⁻¹ P), buffered with 10 mM HEPES pH 8. For all control
127 experiments, BG11 was modified so that N was supplied as NH₄Cl (keeping an
128 equivalent concentration of 247 mg L⁻¹ N). In continuous experiments, BG11 was
129 further modified in order to guarantee non-limiting nutrient concentrations: 494 mg L⁻¹
130 of N (NH₄Cl) and 89 mg L⁻¹ of P (K₂HPO₄) (Sforza et al., 2014b).
131 The hydrolysate used in this study was obtained from FH of *Scenedesmus* sp. at 280°C
132 and 9 s of residence time (Garcia-Moscoso et al., 2013). It was stored, at 4°C, as a
133 freeze-dried powder with the following elemental composition (wt%): 46.5% C, 7.3%
134 H, 9.6% N, 1.37% P. Other inorganic elements, such as S, Ca, K, Fe, Mg, were also
135 present. More detailed analyses on the hydrolysate composition are reported in previous
136 works (Garcia-Moscoso et al., 2015, 2013; Kumar et al., 2014). Nitrogen was available
137 in the medium mainly as soluble peptides and amino acids, with a small fraction (about
138 30% of the total N) present as ammonia. For all batch experiments, an appropriate
139 amount of freeze-dried powder was re-dissolved in distilled water in order to match the
140 same total N concentration of BG11 used as a control (247 mg L⁻¹). This corresponds to
141 a P concentration of 35 mg L⁻¹ (mainly present as phosphate, PO₄-P). In continuous

142 experiments, the hydrolysate concentration was varied as described in Section 3.2. No
143 sterilization was carried out on the hydrolysate prior to inoculation.

144 **2.2 Experimental set-up**

145 Experiments were conducted in both batch and continuous mode. The temperature was
146 kept constant at 23 ± 1 °C for all the experiments, in a refrigerated incubator. Artificial
147 white light was provided by a LED Lamp (Photon System Instruments, SN-SL 3500-
148 22), and the photon flux density (PFD) provided to the culture was measured with a
149 photoradiometer (HD 2101.1 from Delta OHM), which quantifies the PAR
150 (Photosynthetically Active Radiation, 400-700 nm). Except when explicitly specified,
151 CO₂-enriched air (5% v/v, regulated by two flowmeters) was supplied to the cultures.
152 Batch experiments were carried out in Drechsel bottles with 5 cm diameter,
153 continuously mixed by a stirring magnet placed at the bottom of the reactor, at a light
154 intensity of $120 \mu\text{mol m}^{-2} \text{s}^{-1}$. Each batch experiment started with an initial microalgae
155 inoculation of $\text{OD}_{750} = 0.5$, which corresponds to a cell concentration of about $5 \cdot 10^6$
156 cells mL^{-1} , and the culture volume was 100 mL.
157 Continuous experiments were performed in a thin vertical flat-plate polycarbonate (PC)
158 PBR with 100 cm^2 irradiated surface and 1.3 cm depth, to maximize light utilization
159 (Sforza et al., 2014a), at a continuous constant light intensity of $150 \mu\text{mol m}^{-2} \text{s}^{-1}$. CO₂-
160 air mixture was sparged from the bottom of the reactor, ensuring culture mixing, which
161 was also aided by a stirring magnet. Fresh medium was continuously supplied by means
162 of a peristaltic pump (Sci-Q 400, Watson Marlow, USA) that allows to regulate the inlet
163 flow-rate. The reaction volume was kept constant by an overflow tube, through which
164 biomass was withdrawn at the same flow-rate. A scheme of the continuous experiments

165 set-up is shown in Figure 1. The residence time of the culture inside the PBR is
166 regulated by the pump, and calculated according to:

$$167 \quad \tau = \frac{V_R}{Q} \quad (1)$$

168 where V_R is the reactor volume (130 mL) and Q the volumetric flow-rate. After a
169 transient, observed when changing experimental conditions, the biomass concentration
170 was found stable, and a steady state was achieved. As the PBR described can be
171 approximated to a CSTR (Bertucco et al., 2014), steady state productivity P_x was then
172 calculated by:

$$173 \quad P_x = \frac{c_x}{\tau} \quad (2)$$

174 c_x being the average outlet biomass concentration, of at least 4 experimental measures in
175 different days at steady state. The photosynthetic efficiency (PE), referred to the
176 irradiated PAR, is evaluated as:

$$177 \quad \eta_{PAR} \% = \frac{c_x \cdot Q \cdot LHV}{PFD_{in} \cdot E_p \cdot A_{PBR}} \cdot 100 \quad (3)$$

178 where LHV is the lower heating value of the biomass (22 MJ kg⁻¹ (Sforza et al., 2014b)),
179 PFD_{in} the radiation hitting the PBR front surface (μmol m⁻² s⁻¹), E_p the energy of
180 photons (kJ μmol⁻¹), and A_{PBR} the irradiated surface of the flat-panel reactor.

181 **2.3 Analytical procedures**

182 Algal growth was monitored daily in both batch and continuous experiments, by
183 measuring the optical density (OD) at 750 nm with a UV-visible UV 500 double beam
184 spectrophotometer (from Spectronic Unicam, UK). In addition, cell concentration was
185 measured using a Bürker Counting Chamber (HBG, Germany). Specific growth rate of
186 batch experiments were obtained by linear regression of the logarithm of multiple

experimental points of the exponential phase of growth, taken as the average of two independent biological replicates. In continuous experiments, biomass concentration was measured daily as dry weight (DW, g L⁻¹). DW was measured gravimetrically by filtering 5 mL of culture sample with 0.22 µm cellulose acetate filters, which are then dried for 4 h at 90°C in a laboratory oven. N, P and Chemical Oxygen Demand (COD) concentrations were measured spectrophotometrically, using standard test kits, at initial and final points for batch experiments, and in the inlet and outlet streams, for at least three days of steady state, in continuous experiments. A sample of culture was filtered (0.22 µm) to measure only the dissolved nutrients. Total nitrogen (TN) was measured using HYDROCHECK MONOTEST (provided by Reasol®), based on oxidation of all nitrogen compounds to nitrates. By reaction with 2,6-dimethylphenol a dying molecule is then produced and detected at 340 nm using Spectronic Unicam UV-500 UV-visible spectrometer. Ammonium (NH₄-N) was measured with HYDROCHECK SPECTRATEST (Reasol®), by reaction with Nessler reagent in alkaline conditions, and subsequent absorbance measurement at 445 nm. Phosphorus (PO₄-P) is measured by the absorbance (at 705 nm) of a dying complex between orthophosphate ions and molybdenum under reducing environment. Finally, COD was measured by an analytical kit provided by Sigma-Aldrich, USA (AQUANAL®) and is based on oxidation of organic compounds by potassium dichromate in sulfuric acid solution.

2.4 Statistical analysis

T-student tests were applied to ascertain significant differences in specific growth rates for batch experiments, and in biomass concentration (DW, g L⁻¹) in continuous experiments. The level of statistical significance was assumed for $P < 0.05$.

3. Results and Discussion

3.1 Batch experiments

Batch experiments were conducted in Drechsel bottles using the hydrolysate obtained from flash hydrolysis as culture medium, in order to test the microalgal ability to grow in this substrate, and assess the feasibility of using this technique for nutrient recycling. The growth of *Scenedesmus obliquus* was measured, both in the hydrolysate and in BG11, in different experimental conditions, namely under both a continuous and alternated light (12 h of constant light followed by 12 h of dark) of $120 \mu\text{mol m}^{-2} \text{s}^{-1}$, and with or without bubbling of air enriched with CO_2 (5% v/v), respectively. The growth curves obtained in each condition are reported in Figure 2, and the corresponding specific growth rates (d^{-1}) summarized in Table 1. Under continuous light and bubbling of CO_2 (5% v/v) *S. obliquus* was able to grow efficiently in the hydrolysate, and the specific growth rate resulted to be almost twice the one obtained in the control grown in BG11. Nutrients consumption analyses show that this species was able to uptake the organic nitrogen, available in the medium mainly in the form of amino acids and soluble peptides. In fact, even though roughly 30% of the nitrogen in the hydrolysate is present as ammonium, the consumption of this inorganic form represents only a small fraction of the total nitrogen consumption (Figure 3A). This is consistent with results reported in the literature showing the capability of certain microalgal species to utilize simple organic forms (such as urea but also some amino acids) as nitrogen sources, both in autotrophic and heterotrophic cultivation mode (Markou et al., 2014). In particular, Gu et al. (Gu et al., 2015) also found that *Scenedesmus acutus* was able to assimilate nitrogen contained in amino acids, yeast extracts and proteinaceous algal residuals. Phosphorus consumption was also measured, verifying that its concentration was not limiting (Figure 3B). Besides N and P, the

235 hydrolysate provided also all the other micronutrients required for growth, as proven by
236 the high growth rate obtained. The high growth rate also confirms that the concentration
237 of toxic molecules in the medium is low, as reported by Garcia Moscoso et al. (Garcia-
238 Moscoso et al., 2015). In addition to nutrients, a COD consumption of 19 to 60% with
239 respect to the initial concentration was also observed (Figure 3C). This consumption of
240 organic carbon suggests a mixotrophic growth, which could explain the higher growth
241 rates obtained in the cultures supplied with hydrolysate as compared to the control.
242 The possibility of growing microalgae mixotrophically is very interesting, as the
243 possible exploitation of organic carbon may sustain growth when light is not available
244 (i.e. during night). Thus, some experiments were carried out by exposing the culture to
245 day/night cycles. When the light was provided alternating 12 h of illumination with 12 h
246 of dark, in the presence of CO₂ bubbling, the measured growth rate was lower, both in
247 the hydrolysate and in the control, as a consequence of the reduced light energy input.
248 Comparing the two different cultivation media, they both showed the same growth
249 kinetic k (Table 1), but the final biomass concentration reached in the hydrolysate was
250 significantly higher. In addition, COD consumption was increased (from 19 up to 44%)
251 compared to the case of constant illumination, suggesting that in the dark algal cells
252 consume more organic carbon to compensate the lack of light energy. In a previous
253 work, Sforza et al.(Sforza et al., 2012) verified that CO₂ inhibits mixotrophy in the
254 cultivation of *Chlorella protothecoides* and *Nannochloropsis salina*, likely because of
255 an inhibition on respiration, and therefore experiments were also carried out without
256 supplying the CO₂-enriched air mixture. When no additional CO₂ was provided to the
257 cultivation system, *S. obliquus* growth in the hydrolysate was much higher than in the
258 control, both in the case of continuous illumination and day/night cycles. The reduced

259 growth in the control is easily explainable by a strong limitation in carbon availability,
260 since the low CO₂ content in atmospheric air is well known to limit productivity. In the
261 hydrolysate, on the contrary, this limitation is less significant because algal cells were
262 able to exploit the organic carbon available in the medium for their growth, reaching, in
263 the case of continuous illumination, satisfactory cell concentrations and specific growth
264 rates. An alternated illumination resulted again in a reduced growth (except in the
265 control, where carbon instead of light was the limiting factor), but in an increased COD
266 consumption (64% instead of 44% measured for continuous light), strongly supporting
267 the hypothesis that *Scenedesmus* cells can consume organic carbon present in the
268 hydrolysate as an energy source, especially during the dark periods.

269 The results obtained show that *Scenedesmus obliquus* grows very well in the
270 hydrolysate obtained from flash hydrolysis of the same genus, without the need of
271 sterilization, and growth kinetics and biomass productivities are even better than in the
272 control medium in the various experimental conditions investigated. This may be due to
273 its capability of exploiting the available organic carbon (and nitrogen) and growing
274 mixotrophically. Although these were not specifically monitored, the high growth
275 measured also allows concluding that the hydrolysate contained all micronutrients (e.g.
276 K, Ca, Fe, and Mg) required for the algal growth. When exposed to long dark periods,
277 the increased consumption of COD suggests that organic molecules are used by the cells
278 to get the energy for their growth. However, the great decrease in the specific growth
279 rate obtained in these light conditions (both with and without CO₂ bubbling), compared
280 to the case of continuous illumination, indicates that photosynthesis provides additional
281 advantages and that mixotrophy is preferred over heterotrophy. Finally, *S. obliquus* was
282 seen to be able to use the sole organic carbon available in the recycled medium as a

source for growth when no inorganic forms were provided. Nonetheless, the inorganic CO₂ is a highly preferred carbon source, as clearly shown by the much higher growth rates obtained with 5% v/v air-CO₂ bubbling (both under continuous and alternated illumination), and by the lower COD consumption measured when inorganic carbon was also present in the medium. This result is also consistent with what reported by Sforza et al. (Sforza et al., 2012), who found that an excess CO₂ supply had an inhibitory effect on the organic substrate consumption in mixotrophic growth. The conditions of CO₂ supply appear to be the most favorable also in terms of nutrients consumption, which, from the recycling point of view, should be as high as possible. In fact, when the inorganic carbon is available, roughly 80% of nitrogen and 55% of phosphorus are consumed (Fig. 3A and B). When instead this inorganic source is absent, both N and P consumption values drop, resulting in a less efficient exploitation of the nutrients recycled by flash hydrolysis.

3.2 Continuous experiments

3.2.1 Effect of inlet concentration

Once verified that the hydrolysate is able to sustain an efficient microalgal growth and provides itself all the nutrients and micronutrients required, continuous experiments were carried out to assess if a stable microalgal production could be obtained in this medium, and subsequently optimize the nutrients supply (i.e., the hydrolysate feed concentration). To this end, *S. obliquus* was cultivated in a flat-panel PBR, under bubbling of CO₂-enriched air (5% v/v), at a residence time $\tau = 4$ d, and under continuous light intensity of 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Since the objective is that of optimizing nutrients consumption, an optimal light intensity for the growth of *S. obliquus* in flat-panel PBRs was chosen (Gris et al., 2013). Since the uptake of nutrients in case of day-

night irradiation is quite complex (Sforza et al., 2014b), a continuous illumination regime was chosen to focus on a single variable. The various feed concentrations used, always in non-sterile conditions, are summarized in Table 2. Firstly, the system was fed with BG11 modified, as explained in Section 2.1, with large excess of both N and P in order to assure non-limiting concentrations (Sforza et al., 2014b), and the steady-state biomass concentration, productivity and nutrients consumption were measured in these conditions as a control. The biomass concentration resulted to be $3.08 \pm 0.141 \text{ g L}^{-1}$ (Figure 4A) and the corresponding productivity $0.77 \pm 0.035 \text{ g L}^{-1} \text{ d}^{-1}$. N and P difference between inlet and outlet concentration was 320 mg L^{-1} and 62 mg L^{-1} respectively. Considering these values as a reference, the feed was substituted with the hydrolysate properly diluted, in order to obtain a concentration of 350 mg L^{-1} of nitrogen. The phosphorus concentration was adjusted in order to maintain the same conditions of the control. Given the composition of the hydrolysate (Section 2.1), the dilution made to obtain 350 mg L^{-1} of N corresponds to 50 mg L^{-1} of P, therefore the remaining 18 mg L^{-1} were added as K_2HPO_4 , thus reaching a final concentration of 68 mg L^{-1} of elemental phosphorus. In these conditions, the biomass concentration obtained with the hydrolysate was equivalent to the control: $3.11 \pm 0.155 \text{ g L}^{-1}$ of DW, corresponding to a productivity of $0.78 \pm 0.089 \text{ g L}^{-1} \text{ d}^{-1}$. Interestingly, nutrients (total N and P) were not entirely consumed, but instead their consumption percentage with respect to the inlet concentration was more or less the same for all dilutions tested (Figure 4B) and significant concentrations were measured in the outlet stream. Even in this case, a consumption of COD was observed (around 66% of the initial value), confirming mixotrophic growth also in continuous cultivation conditions.

331 In view of an efficient nutrient recycling, it is important to exploit the nutrients present
 332 in the medium as much as possible. Consequently, given the presence of non-consumed
 333 nutrients in the outlet stream, the inlet feed concentration was modified, keeping the
 334 same hydrolysate concentration, but without further addition of phosphate. This
 335 reduction of phosphate concentration, and the consequent increase of the N/P ratio in
 336 the feed, resulted in a significant increase in biomass concentration, up to $3.73 \pm 0.105 \text{ g}$
 337 L^{-1} . The productivity increased, accordingly, to a value of $0.932 \pm 0.026 \text{ g L}^{-1} \text{ d}^{-1}$. The
 338 reason of such an increment in the biomass production could be explained considering a
 339 possibly slowed growth due to high phosphorus concentration, related also to the
 340 phenomenon of luxury up-take. Different authors have reported that some microalgae,
 341 and in particular *Scenedesmus* sp., have the ability to over-uptake phosphorus when the
 342 N/P ratio in the growth medium differs from the normal elementary composition of the
 343 cells (Xin et al., 2010), and to accumulate it in the form of polyphosphate to be used as
 344 an internal source in case of starvation periods (Powell et al., 2009). Polyphosphates
 345 metabolism is quite complex (John and Flynn, 2000), however it is known that the
 346 growth rate tends to decrease with the progressive saturation of internal P pools
 347 (Valiela, 1995). Considering the nutrients content in the biomass (i.e. the uptake
 348 normalized on the dry weight), shown in Table 3 and calculated as:

$$349 \quad Y_{i/x} \% = \frac{c_{i,in} - c_{i,out}}{DW} \cdot 100 \quad (4)$$

350 where i stays for a specific nutrient, and *in* and *out* indicate the inlet and outlet
 351 concentrations respectively, it can be seen that both in the control and in the first
 352 hydrolysate condition the percentage of P is higher than the typical content, which
 353 ranges between 0.3-1% by dry weight, suggesting there is indeed a luxury uptake. In

354 both cases, in fact, the N/P ratio of the feed was lower than the typical microalgal
 355 elementary composition (N/P 7.2:1 by weight) (Redfield, 1934), as reported in Table 2.
 356 When the phosphorus concentration was reduced, also $Y_{P/X}$ decreased to a value of 0.77
 357 %.
 358 Despite the increase in productivity, both N and P were still present in the outlet stream.
 359 Thus, the hydrolysate was diluted to a level corresponding to 300 mg L⁻¹ of N and 43
 360 mg L⁻¹ of P. Note that the N/P ratio is now fixed by the hydrolysate composition (7:1).
 361 In this case, the biomass production was comparable to the previous conditions (Figure
 362 3A). A further feed concentration reduction (250 mg L⁻¹ of N and 36 mg L⁻¹ of P)
 363 resulted instead in a severe decrease in biomass concentration and productivity, to 2.51
 364 ± 0.05 g L⁻¹ and 0.628 ± 0.012 g L⁻¹ d⁻¹ respectively, meaning that in these conditions
 365 nutrients become limiting for growth. It has to be noted that even in this case N and P
 366 were not entirely consumed (Figure 4B). This might suggest that the actual limitation
 367 could be due not to nitrogen or phosphorous but to other micronutrients that became too
 368 diluted. Hence, the optimum hydrolysate concentration to obtain the maximum
 369 productivity (0.932 ± 0.026 g L⁻¹ d⁻¹) with minimum nutrient supply was identified as
 370 300 mg L⁻¹ of N and 43 mg L⁻¹ of P. The corresponding photosynthetic efficiency PE
 371 resulted to be 9.14 ± 0.26 % of the irradiated PAR.
 372 Concerning the nutrients consumptions, it is worth noticing that N and P are never
 373 entirely consumed. Instead, the consumption percentage of both nutrients appears quite
 374 unvaried for all the conditions investigated and ranges between 60% and 70% of inlet
 375 concentration, regardless the hydrolysate feed concentration, while the absolute values
 376 (ΔN and ΔP) decrease with decreasing inlet concentration. This is also reflected in the
 377 fact that the nutrient contents in the biomass are seen to decrease together with the feed

378 concentration, meaning that microalgal cells use smaller amounts per gram of biomass
379 produced. However, the N and P content in the biomass is also influenced by the N/P
380 ratio of the feed: in fact, as reported in Table 2 and 3 the ratio between $Y_{N/X}$ and $Y_{P/X}$
381 matches the inlet N/P ratio, as was also reported by Xin et al. (Xin et al., 2010) for batch
382 cultivation of *Scenedesmus* sp.
383 Therefore, it is essential to minimize the nutrients supply to avoid luxury-uptake
384 phenomena that, from the recycling point of view, represent a waste. In addition, also
385 the N/P ratio of the feed plays a major role, both in relation to luxury-uptake and actual
386 biomass productivity. The hydrolysate considered in this study, having a N/P ratio of
387 7:1, which is very close to the Redfield value (Redfield, 1934), proved to be a good
388 substrate for continuous cultivation of *Scenedesmus obliquus*, allowing satisfactory
389 productivities once the inlet concentration is optimized.

390 3.2.2 Effect of residence time

391 In addition to the inlet concentration of the hydrolysate (i.e. of nutrients), another
392 operating parameter that is known to strongly influence the nutrients content in the
393 biomass in a continuous reactor is the residence time (Sforza et al., 2014b). For this
394 reason, two other values of τ were investigated, respectively 2.5 d and 1.5 d, keeping the
395 inlet hydrolysate concentration fixed at 250 mg L⁻¹ of N and 36 mg L⁻¹ of P, with the
396 aim of seeing if better consumptions could be achieved. The biomass concentration
397 decreased with the residence time τ (Figure 5A), while the productivity showed an
398 increasing trend, up to a maximum (Figure 5B), in agreement with what is already
399 published in the literature (Barbera et al., 2015; Takache et al., 2010). However, the
400 difference in productivity between a residence time of 2.5 d and 1.5 d is not statistically
401 significant and, considering the results obtained reported in other works (Barbera et al.,

2015; Sforza et al., 2014b), the optimum residence time is likely to be between these values.

When considering the consumption percentage of nitrogen and phosphorus, it appears not to change significantly with the residence time (Figure 6). On the other hand, the nutrients content in the biomass is significantly affected by this operating parameter for both N and P, and in particular the value increases when the residence time is lower (Figure 7). Thus, while the biomass concentration decreases with τ , the amount of nutrients consumed per gram of biomass produced is higher. This behavior was also found by Sforza et al., (Sforza et al., 2014b), and explained considering that at lower residence times the respiration rate and maintenance requirements of microalgal cells were found to increase.

However, since the global nutrients consumption remains quite unvaried, even though it never reaches 100%, it is clear that the photobioreactor should be operated at the value of residence time that guarantees the maximum productivity in the specific experimental conditions.

4. Conclusions

This work evaluates the growth of *Scenedesmus obliquus* in the hydrolysate obtained from flash hydrolysis of the same microalgal genus, from a nutrient recycling point of view.

Batch experiments showed that the hydrolysate was able to efficiently sustain microalgal growth providing all necessary nutrients. *S. obliquus* was able to assimilate organic nitrogen from the medium. In continuous cultivation, satisfactory productivities were obtained. Particular attention was dedicated to nutrients consumptions,

425 highlighting that inlet concentrations should be optimized to avoid luxury up-take
426 phenomena.
427 The residence time should also be optimized to guarantee maximum productivity.

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431 and PRIN 2012XSAWYM to TM.

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526 **Figure captions**

527 **Figure 1** Scheme of the flat-panel photobioreactor used in continuous experiments

528 **Figure 2** Growth of *Scenedesmus obliquus* in hydrolysate (full circles), and BG11
529 (open circles), under different conditions: continuous light and CO₂ bubbling (A),
530 alternated light and CO₂ bubbling (B), continuous light and no CO₂ bubbling (C),
531 alternated light and no CO₂ bubbling (D)

532 **Figure 3** Nitrogen (A), phosphorus (B) and COD (C) consumption of the hydrolysate
533 cultures (grey) and BG11 controls (white) in different experimental conditions. 24 h
534 stays for continuous light, while 12 h indicates alternated light. In Figure 3A light grey
535 bars indicate the fraction of ammonium consumed with respect to the total initial
536 concentration

537 **Figure 4** Biomass concentration (A) and nutrients consumption (B) at steady state for
538 the different feed conditions. Dashed asterisks indicate statistically significant
539 differences between two consecutive steady-states

540 **Figure 5** Biomass concentration (A) and productivity (B) as a function of the residence
541 time. In Figure 5B also the photosynthetic efficiency values are reported

542 **Figure 6** Nitrogen (dark grey) and phosphorus (light grey) consumptions at steady state
543 for different residence times

544 **Figure 7** Nitrogen (white) and phosphorus (black) content in the biomass produced as a
545 function of residence time

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547 **Table captions**

548 **Table 1** Specific growth rate of *Scenedesmus obliquus* in the hydrolysate and in BG11
549 (control), under different experimental conditions. Statistically different results are
550 marked with the same letter.

551 **Table 2** Nutrients inlet concentrations of the different experimental conditions, and
552 corresponding N/P ratio

553 **Table 3** N and P content in biomass produced for the different feed concentrations
554 tested

555 **Figures and Tables**

556 **Figure 1**

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Figure 2

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Figure 3

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Figure 4

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Figure 6

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Figure 7

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Table 1

Specific growth rate k (d⁻¹)	Hydrolysate	BG11 (control)
<i>Continuous light, CO₂ (5% v/v)</i>	1.05 ± 0.141^a	0.566 ± 0.031^a
<i>Alternated light, CO₂ (5% v/v)</i>	0.347 ± 0.004	0.358 ± 0.030
<i>Continuous light, no CO₂</i>	0.343 ± 0.030^b	0.096 ± 0.025^b
<i>Alternated light, no CO₂</i>	0.223 ± 0.061^c	0.100 ± 0.020^c

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Table 2

Concentration (mg L⁻¹)	N	P	N/P
<i>Control (BG11)</i>	494	89	5.55
<i>Hydrolysate 1</i>	350	68	5.15
<i>Hydrolysate 2</i>	350	50	7
<i>Hydrolysate 3</i>	300	43	7
<i>Hydrolysate 4</i>	250	36	7

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Table 3

	$Y_{N/X} \%$	$Y_{P/X} \%$	$Y_{N/X} / Y_{P/X}$
<i>Control (BG11)</i>	11.01 ± 0.66	2.14 ± 0.08	5.16 ± 0.36
<i>Hydrolysate 1</i>	6.96 ± 0.66	1.36 ± 0.03	5.12 ± 0.51
<i>Hydrolysate 2</i>	6.09 ± 0.02	0.77 ± 0.07	7.84 ± 0.64
<i>Hydrolysate 3</i>	4.92 ± 0.09	0.632 ± 0.03	7.78 ± 0.39
<i>Hydrolysate 4</i>	4.84 ± 0.04	0.67 ± 0.05	7.18 ± 0.53

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