# Microalgae cultivation in urban wastewater: nutrient removal and biomass production for biodiesel and methane.

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**Abstract**

The freshwater microalgae species *Chlorella kessleri* and *Chlorella vulgaris*, and the marine microalgae species *Nannochloropsis oculata* were cultivated in urban wastewater. The freshwater species demonstrated the possibility of growing in urban wastewater reaching high biomass production and nutrient removal when cultured in batch mode using a flat-panel airlift photobioreactor. Both microalgae species reached high biomass dry weights, 2.70±0.08 g/L and 2.91±0.02 g/L respectively, accompanied by nitrogen concentration reduction around 96% and 95%, and a phosphorous concentration reduction around 99% and 98% respectively. *N. oculata* was able to uptake nutrients from wastewater to grow but with less efficiency, indicating the need of

microalgae acclimation or process optimisation to achieve high nutrient removals. During *C. kessleri* and *C. vulgaris* cultivation, the nitrogen consumption led to a progressive N-starvation process which increased the microalgae potential for biofuels production; both species produced 346±3 $mL_{CH4}/g_{VS}$ and 415±2 $mL_{CH4}/g_{VS}$ during anaerobic digestion, and 7.4±0.2 $g_{Biodiesel}/100g_{VS}$ and 11.3±0.1 $g_{Biodiesel}/100g_{VS}$ respectively.

**Keywords:**

Biodiesel, Methane, Microalgae cultivation, Municipal wastewater, Nutrient removal, Nitrogen starvation.

## 1. INTRODUCTION

Microalgae are able to convert solar energy and carbon dioxide into energy as a result of their photosynthetic activity; when converted into biodiesel or methane, this energy could meet the population energy needs [1]. The microalgae cultivation with energetic purposes started years ago when microalgae, able to accumulate a high amount of lipids, appeared as promising crops substitute in biodiesel production [2]. Currently, crops are the common feedstocks in the biodiesel production industry but also in the food industry, thus the crop-based biofuel production has created increased food prices [3]. A wide variety of microalgae species reaches higher lipid productivities than crops [2], becoming potentially substrates to alleviate the referred to "food-versus-fuel competition" [3]. Despite the efforts made, the industrial biodiesel production from microalgae is not economically viable nowadays due especially to the high costs for drying and lipid extraction among other costs [4]. On the contrary, the anaerobic

digestion process converts wet biomass into methane [5], and energy is recovered not only from the lipid fraction.

The anaerobic digestion process is a widely known technology, currently used in the wastewater treatment plants (WWTPs). The sewage sludge, a waste produced during the wastewater treatment, generates high operating costs in its final disposal [5]; the anaerobic digestion process converts the sludge into a stable product with simultaneous generation of a valuable by-product such us methane. Methane utilisation contributes favourably to reduce the high operating costs generated by the final disposal of sewage sludge [5].

The nutrient level in wastewaters might be another problem in WWTPs. The water discharge with high nutrient levels cause eutrophication problems, thus the Directive 98/15/EEC establishes nutrient levels or minimum percentage of reduction before water discharge [6]. Although the nutrient concentration varies from a WWTP to another, and between the wastewater streams in the process, the stream generated during the digested sludge dewatering process always has higher nitrogen and phosphorus concentrations than in any other streams [7]. This stream, named centrate, is usually recycled for further treatment to avoid environmental problems, but it increases the WWTP costs [7]. Microalgae cultivation offers a solution to reduce the high nutrient content in centrate since microalgae consume nutrients when growing [8]. Moreover, in a waste-to-value approach, the produced biomass constitutes a by-product which could be used for various purposes, including biofuels.

Some freshwater microalgae species have already been cultured in different wastewater streams, with the purpose of reducing the nutrient concentration in wastewater or producing lipids for biofuels [7,9-13]. Similarly, seawater microalgae species have also

been cultured using wastewaters despite the salinity requirements [14,15]. However, the centrate utilisation for microalgae cultivation is scarce; most of the studies were done using wastewater with a low nutrient content compared to the centrate. The use of centrate as nutrient medium allows coupling the wastewater treatment and the microalgae cultivation process with a minimum modification in the WWTP facilities. Wastewater treatment by microalgae cultivation is still limited, and the waste activated sludge (WAS) process is the conventional treatment in WWTP [8]. The substitution of the WAS process implies a fully modification of the WWTP scheme, whereas the centrate utilisation in microalgae cultivation only needs a cultivation unit coupling.

This study analyses the possibility of coupling microalgae cultivation in WWTPs, removing nutrients from the centrate while producing biomass with energy recovery purposes. Due to the high nutrient level in the centrate which can inhibit the cell growth, the first studies aimed to determine the most suitable media for *C. kessleri*, *C. vulgaris* and *N. oculata* cultivation. The microalgae species were cultivated in different dilutions of the centrate; the dilutions were carried out with wastewater before the primary settling tank for the cultivation of freshwater microalgae species, or with natural seawater for the marine microalgae species. After the culture medium screening, the microalgae species were cultured in the most suitable medium, where the nutrient removal and the biomass production were evaluated. Finally, the harvested biomass was used for methane and biodiesel production.

## 2. MATERIALS AND METHODS

### 2.1 Pre-treatments and characteristics of the wastewaters

Wastewater samples from the WWTP of Saint Nazaire (ACCUEIL CARENE, Saint-

Nazaire, France) were used as cultured medium for microalgae cultivation; they consisted of the centrate and the wastewater from a line before the primary settling tank. For marine microalgae cultivation, natural seawater was collected from the coastal area of Saint-Nazaire in France.

Large solid particles were first removed by centrifugation and then, the samples were filtered through a 0.45 μm pore size filter to remove undesirable small particles. The total nitrogen (TN), ammonia nitrogen ($NH_3$–N), nitrate nitrogen ($NO_3$–N), nitrite nitrogen ($NO_2$–N), phosphate phosphorus ($PO_4$-P), and chemical oxygen demand (COD) were determined following the Hach DR 2800 Spectrophotometer Manual using the HACH LANGE cuvette tests and following the procedure specified for each test (Hach, 2008). The characteristics of the wastewaters and the natural seawater can be observed in Table 1.

As can be observed, the composition of the wastewater sampled before the primary settling tank composition is given as a range rather than the average value with the standard deviation; two different samples collected in different opportunities were used in the experiments. These differences in the nutrient content were taken into account for the cultured medium preparation.

**Table 1**

## 2.2 Microalgae cultivation

### 2.2.1 Shake flasks

Three different microalgae species were cultured. The freshwater microalgae species *Chlorella kessleri* (strain UTEX2229) and *Chlorella vulgaris* (strain CCAP211/19)

were obtained from the collection of algae at the University of Nantes; and the marine microalgae species *Nannochloropsis oculata*, from the Alphabiotech collection (Asserac, France).

The microalgae were first inoculated in shake flasks. The freshwater microalgae species in 250 mL Erlenmayer flask containing a modified Bold Basal Medium (BBM) and the marine microalgae species, in filtered and sterilised seawater with salinity adjusted at 25‰ and enriched with Conway medium (3 mL/L of seawater). The detailed composition of the modified BBM and Conway medium is given in Pruvost et al. [16,17].

### 2.2.2 Culture medium screening in Efficient Overproducing Screening System - Photobioreactors (EOSS-PBR)

The EOSS-PBR was especially developed for the fast screening of culture media and microalgae species in conditions representative of PBR cultivation. It consisted of six small-scale photobioreactors (bubble columns) run in parallel, each tube having a volume $V_r = 3 \cdot 10^{-5}$ $m^3$, an illuminated area $S_L = 0.008$ $m^2$ and a specific illuminated area $a_{light} = S/V_r$ of 266.7 1/m. A full description of the EOSS-PBR is done in Taleb et al. [18].

The culture medium was prepared by diluting the centrate to reduce the high TN concentration, either with wastewater or with natural seawater (Table 1). Before inoculation, the culture medium was filtered through a 0.45 μm pore size filter to remove undesirable small particles. The TN concentrations in the culture medium for the freshwater microalgae species were 30 mg N/L (0.002 mol/L), 140 mg N/L (0.010 mol/L), 260 mg N/L (0.019 mol/L), 490 mg N/L (0.035 mol/L), 700 mg N/L (0.050

mol/L) and 1200 mg N/L (0.086 mol/L). For the marine microalgae species cultivation, the TN concentrations were 6 mg N/L (<0.001 mol/L), 71 mg N/L (0.005 mol/L), 135 N mg N/L (0.010 mol/L), 265 mg N/L (0.019 mol/L), 524 mg N/L (0.037 mol/L) and 782 mg N/L (0.056 mol/L).

The pH in the culture medium was around 7.5 for the freshwater microalgae species and 8 for the marine microalgae species, according to Pruvost et al. and Taleb et al. [17,18]. The culture agitation was provided by continuous injection of air with 2 vol% $CO_2$ at a flow rate of 3 mL/min; the incident photons flux density (PFD), by a set of 6 fluorescent white tubes was ~150 μmol/$m^2$•s. The temperature was regulated at 25°C by ambient air flow. The reactor was operated in batch mode.

The microalgae growth was evaluated following the evolution of the number of cells as a function of time (*t)*. Cell concentration *N* expressed as number of cells per millilitre of culture was determined under an optical microscope (Axiostar-Plus, Carl Zeiss, Germany) using Malassez counting cell. The chlorophyll fluorescence in the microalgae was observed in the microscope, using the green filter set 530–585 (BP 530–585 as exciter filter, FT 600 as chromatic beam splitter and LP 615 as barrier filter; Zeiss, Oberkochen, Germany). The algal dry weight concentration (Cx) was determined at the end of the experiment, by filtration through a pre-dried and pre-weighed glass-fiber filter (Whatman GF/F). The filters were dried 24 h at 105°C, cooled down in a desiccator and then weighed again. The total pigment content was determined using a spectrophotometric method, following the procedure described in [17].

### 2.2.3 Microalgae cultivation in flat-panel airlift photobioreactor (PBR)

Once the culture medium was defined, microalgae were cultured in batch mode using a

flat-panel airlift PBR with $V_r = 10^{-3}$ $m^3$, and depth of culture of $L_z = 3 \cdot 10^{-2}$ m, a $S_L = 8 \cdot 10^{-3}$ $m^2$ and a $a_{light} = 33.3$ 1/m. Before inoculation, the cells were centrifuged for 3 min at 6000 g at room temperature, and the cell pellets were washed with the decided culture medium twice before finally suspending cells.

The temperature was 25°C, regulated by ambient air flow. The pH was monitored with a sensor inside the reactor (Mettler Toledo SG 3253) and automatically regulated by $CO_2$ injection, at 7.5 for the freshwater microalgae species and 8 for the marine microalgae species. The culture agitation was provided by air bubbling. A constant PFD (~100 $\mu mol/m^2 \cdot s$) was used during the experiments.

The microalgae growth and the Cx were evaluated during cultivation as described in section 2.2.2. The different nutrient concentrations during cultivation were determined as described in section 2.1.; the samples were taken from the reactor, centrifuged and the supernatant analysed. The evolution of proteins, carbohydrates and lipids in the biomass during cultivation was followed with infrared spectra of the microalgae cells; the spectra were obtained in a Bruker Tensor 27 FTIR spectrometer equipped with the ATR platinum module, with a deuterated triglycine sulfate detector RT-DLaTGS and the OPUS v7.0.122 software (Bruker Optics, Germany). After finishing the experiment, the biomass was recovered by centrifugation and dried using freeze-drying equipment (FT33-A Freeze Drier, Armfield Inc.) for 48 hours to avoid major changes on its composition. The carbohydrate content in biomass was quantified by the phenol-sulphuric acid method and protein content, by the Lowry method as described in Caporgno et al. [19].

**2.3 Methane and biodiesel production**

193 Anaerobic digestion was carried out by triplicate, in 120 mL serum bottles sealed with a
194 septum and an aluminium crimp. The inoculum consisted of mesophilic digested sludge
195 taken from an anaerobic semi-continuous plant; the substrates were the freeze-dried
196 microalgae species re-suspended in deionised water. The final volume in the reactors
197 was adjusted to 80 mL before sealing, then the reactors were purged with nitrogen to
198 assure anaerobic conditions and placed into an oven at 33ºC; a detailed procedure is
199 described in Caporgno et al. [19]. The amount of substrate loaded in reactors was
200 calculated based on the organic matter added per inoculum content, the substrate to
201 inoculum ratio was 1:2 $VS_{Substrate}$:$VS_{Inoculum}$. The biogas production and composition,
202 and parameters after anaerobic digestion such as the volatile fatty acid concentration
203 (VFA) were measured as described in Caporgno et al. [19]. The ammonia concentration
204 at the end of the experiment was measured with an ion selective electrode (ISE)
205 (Ammonia Gas Sensing combination electrode, mod. 51927-00, HACH); the alkalinity
206 was analysed according to the standard method 2320B [20]. The theoretical methane
207 production was calculated based on the microalgae composition, and the
208 biodegradability of the biomass, as the fraction of the theoretical methane production
209 reached at the end of the anaerobic digestion experiment as described in Caporgno et al.
210 [19].
211 The freeze-dried microalgae biomass was converted to FAME-biodiesel by direct
212 transesterification procedure according to Johnson and Wen [20]. The FAMEs were
213 analysed by GC-FID according to the Agilent Application Note 228–398 using a HP-
214 INNOWax column (19091N-133), and a 37 component FAME standard mixture
215 (Supelco: 47885-U) was used for calibration of the method.
216

## 3. RESULTS AND DISCUSION

### 3.1. Culture medium screening in EOSS-PBR

The Figure 1a shows the evolution of the number of cells over time for *C. kessleri*, in nutrient media containing from 30 mg N/L to 1200 mg N/L. As can be observed in Table 1, although the differences in the TN concentration in both wastewaters, most of the N content in both wastewaters is in the form of $NH_4^+$.

**Figure 1**

In the nutrient medium with the lowest TN concentration, the curve follows a typical evolution for batch cultivation. There was a lag phase at the beginning, characterised by a period of physiological adjustment due to changes in the nutrient conditions. The cells adapted to the new conditions after 2 days and the growth accelerated; this is the exponential growth phase. Finally, there was no growth after day 9; this is the stationary phase. As observed in Figure 1, when the TN concentration increased from 30 until 140 mg N/L, the lag phase became longer and the growth rate during the exponential growth phase became lower. There was not any microalgae cell growth at a TN concentration higher than 140 mg N/L; in fact, the number of cells decreased and no cells were observed after 3 days cultivation, suggesting inhibition of the cell growth. In the centrate, there was a high level of N in the form of ammonium, and although it can be used by microalgae as source of N, it can also inhibit microalgae growth [8]. Microalgae cells were recovered from the bottom of the reactors, and their appearance was observed in the microscope also using the green filter for chlorophyll fluorescence observation. The cells in the media with 30 mg N/L were smaller than in the media with 140 mg

241 N/L. This result agreed with the dry weight analysis results, which determined similar
242 algal dry weight in both media, in spite of the higher number of cells in the media with
243 30 mg N/L. Regarding the microalgae recovered from the reactors with a nitrogen
244 concentration higher than 140 mg N/L, the cells presented an absence or much less
245 florescence intensity than that recovered from reactors with 30 and 140 mg N/L,
246 indicating that nitrogen concentrations higher than 140 mg N/L affect the cells
247 negatively. Comparable results were reported for *C. vulgaris* cultivation on wastewater
248 with a high concentration of $NH_4^+$-N [12].
249 At the end of the experiment, the algal dry weight was determined in the reactors. The
250 Cx was similar in nutrient media with 30 mg N/L and 140 mg N/L, 1.34±0.10 g/L and
251 1.32±0.06 g/L respectively, in spite of the differences in the number of cells. At TN
252 concentrations higher than 140 mg N/L, microalgae cells settled and Cx was lower than
253 0.05 g/L, as was expected due to inhibition. The total pigment content was analysed at
254 the end of the experiment, due to the more intense green colour observed in the reactor
255 with a TN concentration of 140 mg N/L compared with 30 mg N/L, although the lower
256 number of cells. The results indicated the total pigment content was 15±0.1 μg/mL for
257 140 mg N/L 1.8±0.1 μg/mL for 30 mg N/L, as was also observed elsewhere [12].
258 Based on the results, it was decided that a nutrient medium with a TN concentration
259 around 140 mg N/L is the most suitable for *C. kessleri* species cultivation; this allows
260 the usage of the highest amount of the centrate and produces a high biomass
261 concentration. Furthermore, since similar growth evolution and biomass production was
262 reported for *C. kessleri* and *C. vulgaris* cultivation in wastewater [13], no screening was
263 carried out with *C. vulgaris* species and the same nutrient medium was used for both
264 species.

Regarding the culture medium for the marine microalgae species *N. oculata*, Figure 1b shows microalgae growth in the nutrient media containing from 6 mg N/L to 782 mg N/L. Centrate and seawater mixing affects medium salinity due to a dilution effect; the higher the amount of the centrate in the nutrient medium, the higher the TN concentration but the lower the salinity. The highest salinity was 28‰ in pure seawater with a low TN concentration due to the $NO_3^-$ presence; the lowest was 3‰ with a TN concentration of 782 mg N/L.

As can be observed in Figure 1b, there was not a significant microalgae growth in natural seawater due to the low nutrient content. However, a higher N content after the centrate addition was enough to increase the number of cells. In the medium with 71 mg N/L and 24‰ salinity, *N. oculata* cultivation shows good results. The evolution of the number of cells show a lag phase during the first 2 days and then, the growth accelerates almost continuously. Finally, by the $10^{th}$ day, it seems that microalgae growth is close to the stationary phase. The increases in the TN concentration up to 135 and 265 mg N/L decreased the salinity of the medium, 18‰ and 12.5‰ respectively. As can be seen in the curves, the microalgae did not grow in the medium with 135 mg N/L in spite of the higher salinity of this medium, but they grew in the medium containing 265 mg N/L. In this medium, the evolution of the number of cells followed a 2 day lag phase and an exponential growth and a stationary phase around the 8th day. These results indicated inhibition in the culture medium with 135 mg N/L; however, the inhibition could not be attributed to a high TN concentration or to a low salinity. Comparing the media containing 71 mg N/L and 265 mg N/L, the number of cells decreased by almost 50% due to the low salinity; the optimum salinity for *N. oculata* has been suggested between 22‰ and 25‰ and the changes in the medium salinity affect *N. oculata* cultivation [22].

It was reported that *Nannochloropsis sp.* grew in wastewater with 33‰ and 18.5‰ salinity (11 mg N/L and 55 mg N/L respectively), but the culture failed at lower salinities due to osmotic stress [14]. Furthermore, it was reported that *Nannochloropsis salina* grew in wastewater reaching similar productivities when the salinity was adjusted, and the TN was in a range between 80 mg N/L and 480 mg N/L [15]. Based on this, it was confirmed that the inhibition in the media with 130 mg N/L was not attributable to salinity or TN concentration in the medium. A possible reason was pH; it was reported that pH affects *Nannochloropsis sp.* significantly [~~22~~] [23] and in the experiment, this parameter was not automatically regulated. During ammonia assimilation by microalgae, the pH of the medium decreases and may reach levels near pH 3; the pH shifts might cause growth inhibition [24].

At 71 mg N/L and 24‰ salinity in nutrient medium, the Cx was 1.07±0.07 g/L. The TN concentration increase to 265 N/L decreased the Cx to 0.48±0.02 g/L. In other culture media, the Cx was lower than 0.02 g/L, as was expected due to the inhibition observed.

Assuming that inhibition in the medium with 135 mg N/L was fortuitous, a nutrient medium with 135 mg N/L and 24‰ salinity allows using the higher amount of centrate without affecting Cx, thus these were the characteristics of the culture medium chosen for *N. oculata* cultivation.

### 3.2. Microalgae cultivation in flat-panel airlift PBR

#### 3.2.1 *Chlorella kessleri* cultivation

The microalgae were cultured in 1L PBR to evaluate the microalgae growth, the nutrient removal and the biomass production. Figure 2a shows *C. kessleri* growth as the evolution of the number of cells over time in a culture medium with 130 mg N/L (0.009

mol/L), and Figure 2d shows the N evolution as the percentage of the initial N which remained in the culture medium, and the Cx evolution in the culture.

**Figure 2**

As can be observed in Figure 2a, there was a scarce microalgae growth during the first two days but the algal dry weight slightly increased, indicating microalgae cell size increase. This cell size increase was accompanied by a TN concentration reduction from 130.0±0.3 mg N/L to 108.2±0.3 mg N/L, which represented ~16% TN consumption. After the 2$^{nd}$ day, the microalgae growth started a steady increase until the 7$^{th}$ day. At this time, the Cx in the reactor reached 1.78±0.05 g/L; the total TN consumption was ~95%, the remaining TN concentration was under the limits for the TN discharge [6]. The microalgae growth continued until it reached the stationary phase on the 9$^{th}$ day. Although there was not a significant TN consumption after the 7$^{th}$ day, the Cx reached 2.34±0.01 g/L on the 9$^{th}$ day and 2.70±0.08 g/L at the end of the experiment. The final TN concentration was lower than 5 mg N/L, which meant >96% removal. Regarding P as a nutrient, the initial concentration of TP in growth medium was 5.76±0.08 mg P/L, and at the end of the experiment 0.04±0.01 mg P/L, which indicated more than 99% removal efficiency. Under the culture conditions in this experiment, *C. kessleri* showed a high efficiency at removing TN and TP; the culture conditions affect TN and TP removal differently [7]. The results indicated that *C. kessleri* cultivation can remove TN and TP without a previous acclimation step; a microalgae acclimation step was unnecessary to improve nutrient removal as it is suggested in the literature [25]. Similar results were also reported by Arbib et al. [13]. The N/P ratio in the culture media was

23, higher than the range 6.8-10 indicated as optimal for freshwater microalgae cultivation [9]. As some other freshwater microalgae species [9], *C. kessleri* has demonstrated the ability of growing in nutrient media with an unbalanced N/P ratio.

Based on the stoichiometric needs of biomass and nutrients concentration in medium, the maximal biomass concentration without starvation was calculated. Assuming an elemental composition of biomass equal to $CH_{1.715}O_{0.427}N_{0.148}S_{0.014}P_{0.012}$, where the C-molar mass is 23.45 g C/mol [16], and an initial TN concentration of 130 mg/L, the maximal biomass concentration without starvation resulted 2.18 g/L. As can be seen in Figure 2d (dashed line), this concentration was reached between the $7^{th}$ and $8^{th}$ day of cultivation. In the same period, it remained less than 5% of the initial TN concentration (Figure 2b), indicating that microalgae cells may experience N-starvation.

Figure 3 indicates the microalgae spectra obtained during FTIR analyses, for biomass collected during the $2^{nd}$, $6^{th}$ and $9^{th}$ days, corresponding to the beginning of the exponential phase, when the TN concentration in the culture medium was ~5 mg N/L, and at the beginning of the stationary phase.

**Figure 3**

Since the lipids, protein and carbohydrates can be identified due to their characteristic groups [26], the comparison of the three spectra in Figure 3 shows the evolution of the proteins, carbohydrates and lipids in biomass. The protein content decreased during cultivation, due to less intensified absorption bands at 1,500-1,700 $cm^{-1}$ for peptide amide groups of proteins. On the contrary, the absorption bands at 1,000-1,200 $cm^{-1}$, characteristics of C–O and C–O–C groups of carbohydrates, increased. Moreover, more

intensified bands at 1,700-1,750 $cm^{-1}$ and 2,800–3,000 $cm^{-1}$, characteristics for lipids indicated a lipid content increase. These changes in the absorption bands indicated that the microalgae underwent a period of N-starvation; the cell division decreased progressively, the dry weight increase became lower and the lipid content triggers. These facts, visible in Figure 2a, 2d and 3, were also observed in other freshwater microalgae species which experienced progressive N-starvation [16]. The results confirmed that the microalgae cultivation in wastewater follows a progressive N-starvation process, increasing the microalgae lipid content, valuable for biofuels production. The total lipid content was not analysed, and the saponifiable lipid fraction (convertible into biodiesel) was quantified instead; the saponifiable lipid content yielded 7.4±0.2 $g/100g_{VS}$. The content of proteins and carbohydrates in *C. kessleri* were 36.7±1.0 $g/100g_{VS}$ and 44.6±0.1 $g/100g_{VS}$ respectively.

### 3.2.2 *Chlorella vulgaris* cultivation

*C. vulgaris* growth in a culture medium with 130 mg N/L (0.009 mol/L) can be observed in Figure 2b as the evolution of the number of cells over time; the N evolution as the percentage of the initial N which remained in the culture medium and the Cx evolution in the culture can be observed in Figure 2e.

Similar to *C. kessleri*, the number of cells did not change until the second day but the algal dry weight increase revealed a cells size increase. This was accompanied by a 27% consumption of the TN; the TN concentration decreased from 130.0±0.3 mg N/L to 93.4±2.5 mg N/L. From the $2^{nd}$ day until the $6^{th}$ day, the microalgae growth showed a steady increase. After the $6^{th}$ day, the growth rate decreased and on the $11^{th}$ day the culture was near the steady phase. The Cx in the reactor increased daily and it reached

385 2.63±0.09 g/L the 8$^{th}$ day, when the TN consumption was ~94% and the TN level lower
386 than 10 mg N/L required for water discharge [6]. The subsequent number of cell
387 increase was not accompanied by substantial Cx increase or N consumption; in fact, the
388 Cx and the TN consumption reached 2.91±0.02 g/L and ~95% respectively. At the end
389 of the experiment, TP in growth medium was 0.11±0.02 mg P/L, lower than the
390 threshold established by the European Directive (< 1 mg P/L) [6], resulting in a removal
391 efficiency higher than 98%. The results also indicated that *C. vulgaris* grows in
392 wastewater, even when the N/P ratio is unbalanced and higher than the indicated as
393 optimal for freshwater microalgae cultivation [9]. The nutrients removal efficiencies
394 were higher than the reported after *C. vulgaris* cultivation in wastewater sampled in
395 different point of a WWTP, including centrate samples with a similar composition than
396 in the present experiment [9]. Although similar nutrient removals were reported in
397 wastewater mixed with glycerol, the biomass concentration reported at the end of the
398 experiment was considerably lower [10]. Comparable nutrients removals and lower
399 biomass production were reported during the *C. vulgaris* cultivation using centrate
400 obtained from a digester processing cattle slurry and raw cheese whey [11].
401 As during *C. kessleri* cultivation, *C. vulgaris* experienced a progressive N-starvation
402 process, which led biomass to a cell division decrease, a low dry weight increase and a
403 total lipid content accumulation. The maximal biomass concentration without starvation
404 resulted in 2.18 g/L [16] and it is indicated in Figure 2e by a dashed line. This biomass
405 concentration was reached between the 7$^{th}$ and 8$^{th}$ day of cultivation, when the TN
406 concentration was less than the 5% of the initial concentration. After this period, the
407 microalgae increased their lipid content. The saponifiable lipid fraction yielded 11.3±0.1
408 g/100g$_{VS}$ at the end of the experiment, similar to the results reported for the same *C.*

409 *vulgaris* strain cultivation under N-starvation [17]. The content of proteins and
410 carbohydrates in *C. vulgaris* were 35.2±1.4 $g/100g_{VS}$ and 36.2±0.3 $g/100g_{VS}$
411 respectively.
412 A comparison between *C. kessleri* and *C. vulgaris* shows a significant difference in the
413 number of cells. *C. kessleri* reached around $1500 \cdot 10^5$ cells/mL at the end of the
414 experiment, whereas *C. vulgaris* reached almost three times this number (Figure 2a and
415 2b). However, the Cx was in both species, which agrees with the larger size of the
416 *C. kessleri* cells compared with the *C. vulgaris* cells. The higher cell size in *C. kessleri*
417 may increase harvesting efficiency, thus being beneficial from an engineering point of
418 view. The literature comparing *C. kessleri* and *C. vulgaris* for wastewater treatment is
419 scarce, but both species have shown a similar biomass production when cultured under
420 the same conditions [13]. However, the reported biomass production was lower than the
421 2.70±0.08 g/L and the 2.91±0.02 g/L obtained for *C. kessleri* and *C. vulgaris*
422 respectively in the present studio.
423 *Chlorella* microalgae are usually found in wastewater treatment ponds [8], suggesting
424 that can uptake nutrients from wastewater to grow. The species cultured in this study
425 shows a promising future for nutrient removal in wastewaters. Moreover, the biomass
426 cultivation makes possible the centrate utilization as nutrient medium instead of its
427 recirculation to a previous step in the process. The biomass can be used as fertiliser, as
428 animal feed or as biofuel feedstocks, being economically advantageous for the WWTP
429 [8]. Moreover, the progressive N-starvation during cultivation triggers lipid
430 accumulation in biomass, attractive for biofuels production [17].
431
432 **3.2.3 *Nannochloropsis oculata* cultivation**

*N. oculata* was grown in a nutrient medium with ~100 mg N/L (0.007 mol/L) and 25‰ salinity. Due to the higher TN concentration in the centrate collected for this experiment, it was possible to increase the TN concentration in the nutrient medium, keeping salinity in the optimum range [22]. The evolution of the number of cells over time can be observed in Figure 2c. The N evolution and the Cx evolution in the culture can be observed in Figure 2f.

Figure 2c shows that there was not a significant lag phase at the beginning of the experiment; the number of cells increased at an almost constant rate from the beginning until the $8^{th}$ day. After the $8^{th}$ day, it started a stationary phase followed by a declination phase derive from the biomass precipitation observed. The stationary phase started the $8^{th}$ day; the TN concentration was 67.0±0.8 mg N/L, which barely represented ~35% consumption of the initial 102.8±0.1 mg N/L. On the $11^{th}$ day, the TN concentration reached 55.9±2.0 mg N/L, ~47% consumption; the TN concentration in the culture medium exceeded the limit required for water discharge [6] but to prolong the cultivation beyond did not increase the N consumption significantly. The experiment was conducted until the $14^{th}$ day due to the low N consumption observed; however, the number of cells was not counted beyond the $11^{th}$ day. The Cx increased at an almost constant rate from the beginning of the experiment until the end of the experiment; the $11^{th}$ day, the Cx reached 0.66±0.02 g/L and on the $14^{th}$ day, 1.05±0.06 g/L despite there was not any N consumption in this period. The Cx values were considerably lower compared with the Cx obtained in the freshwater microalgae cultivation (Figures 2d and 2e). The TP concentration at the end of the experiment was 0.04±0.01 mg P/L, lower than the threshold established by the European Directive (< 1 mg P/L), resulting in a removal efficiency higher than 96% [6].

*Nannochloropsis* species have shown the capability to grow in different nutrient media varying nitrogen concentration, salinity, ph, light intensities, between some other factors [14,15,23]. It has been reported that the microalgae grows without major changes in a medium with N/P ratio of 32, although the optimal ratio is 16 [27]. The initial N/P ratio was considerably higher than the optimal, around 100. The excess or lack of P influences negatively the cell division and the biomass productivity [27]; thus affecting the microalgae growth and the N consumption.

Although *N. oculata* grows in wastewater, the results suggest that the aim of the wastewater utilisation should be established in advance. If cultivation is aimed to reduce nutrient content, the freshwater microalgae species are preferable; the nitrogen level at the end of *N. oculata* cultivation still exceeds the threshold established by the European Directive for water discharge. However, if cultivation is aimed at biofuels production or valuable compound production from marine microalgae species instead, centrate can replace nutrient addition reducing the production costs. In this case, the salinity needs to be adjusted in order to obtain high biomass productivities.

### 3.3. Methane production from microalgae

*C. kessleri* and *C. vulgaris* grown in wastewater were recovered from the PBR and anaerobically digested in order to evaluate their potential as substrate for methane production. The methane production curves from *C. kessleri* and *C. vulgaris* are shown in Figure 4.

**Figure 4**

481 The methane production was similar for both microalgae species during the first four
482 days, becoming different after this period. On the 6$^{th}$ day, the methane production from
483 *C. vulgaris* was 11% higher than from *C. kessleri*. This difference progressively
484 increased until the end of the experiment, when it reached 20%. The final methane
485 production from *C. kessleri* and *C. vulgaris* were 346±3 $mL_{CH4}/g_{VS}$ and 415±2
486 $mL_{CH4}/g_{VS}$ respectively. For *C. kessleri*, it has been reported 218 $mL_{CH4}/g_{VS}$ under
487 mesophilic conditions; for *C. vulgaris* digestion, 240 $mL_{CH4}/g_{VS}$ and 286 $mL_{CH4}/g_{VS}$
488 were reported after 28 days and 49 days mesophilic conditions respectively [4];
489 however, there is no literature relating to the anaerobic digestion of microalgae
490 cultivated in wastewaters. These methane productions were clearly exceeded in the
491 present experiment. What is more, the methane productions obtained are higher than
492 those reported for several microalgae species [4]. Even more interesting results appear
493 when comparing the methane production from microalgae with substrates commonly
494 used in anaerobic digestion, such as sewage sludge, which under similar conditions
495 produced around 350 $mL_{CH4}/g_{VS}$ [19]. The methane production from several substrates
496 such as municipal solid wastes, swine manure, maize silage and straw, etc., was also
497 exceeded [4].
498 The theoretical methane production resulted in 533±5 $mL_{CH4}/g_{VS}$ and 567±1 $mL_{CH4}/g_{VS}$
499 for *C. kessleri* and *C. vulgaris* respectively. The biodegradability resulted in 65% and
500 66% for *C. kessleri* and *C. vulgaris* respectively, higher than the values reported for
501 other microalgae species and similar to the values reported for sewage sludge
502 mesophilic digestion [4]. The values of pH, alkalinity, and concentration of ammonia
503 and VFA were measured at the end of the experiments corroborated that the digesters
504 operated at optimum conditions. The biogas composition, analysed several times during

505 the experiments, indicated that the methane percentage ranged between 66% and 71% in
506 all reactors.
507 The methane production from both microalgae species shows that biomass cultivation in
508 wastewater coupled to anaerobic digesters allows wastewater treatment and energy
509 recovery simultaneously. During the wastewater treatment process, the TN
510 concentration reduction can lead to high lipid content in biomass; a high lipid content
511 increases the methane production potential of the biomass.
512
513 **3.4. Biodiesel production from microalgae**
514 The FAME content in freeze-dried *C. kessleri* and *C. vulgaris* grown in wastewater was
515 quantified, to evaluate suitability of the microalgae for biodiesel application.
516 The biodiesel yields obtained from *C. kessleri* and *C. vulgaris* amounted to
517 7.4±0.2 g/100$g_{VS}$ and 11.3±0.1 g/100$g_{VS}$ respectively. These results are comparable to
518 the results obtained from *Chlorella sp.* reported elsewhere [7,28]. Although the
519 biodiesel yields for both microalgae strains were not high enough for industrial
520 biodiesel production, changes in microalgae cultivation conditions can enhance the
521 accumulation of lipids and the biodiesel yields [29]. The biodiesel yield in *C. vulgaris*
522 was also similar to results found during the cultivation of the same strain under N-
523 starvation [17], confirming that the N-starvation increased the lipid content during
524 cultivation in wastewater. Moreover, the fatty acid profiles of both microalgae strains
525 were dominated by palmitic (C16:0), oleic (18:1), linoleic (C18:2) and linolenic (C18:3)
526 acids; these fatty acids are the most common fatty acids contained in biodiesel produced
527 from vegetable oils [30]. Fatty acid profile of *C. kessleri* consisted of 20.3%, 10.4%,
528 32.2% and 12.9% of palmitic, oleic, linoleic and linolenic acid, respectively. *C. vulgaris*

contained 22.2%, 27.2%, 11.6% and 10.6% of palmitic, oleic, linoleic and linolenic acid, respectively.

Both microalgae cultivated in wastewater are suitable sources of lipids for biodiesel production, contrary to some other microalgae species with a high content of polyunsaturated fatty acids, which decreases oxidative stability of biodiesel [29,31]. The anaerobic digestion of microalgae wastes from biodiesel production was not evaluated. However, the idea of coupling biodiesel production and anaerobic digestion of the microalgae wastes might be a way to improve the global efficiency of biodiesel production.

## 4. CONCLUSIONS

The centrate from a WWTP was found interesting to supply nutrients for microalgae cultivation. The freshwater microalgae species *Chlorella kessleri* and *Chlorella vulgaris* removed N and P almost completely (>95%); the progressive N-starvation derived from N consumption lead to a biomass with a high potential for methane production. Moreover, the biodiesel produced from both species showed interesting properties, indicating the possibility of producing this biofuel. Under the cultivation condition evaluated, *Nannochloropsis oculata* showed a low efficiency at nutrient removal; in order to obtain higher nutrient removal or higher biomass production for valuable compounds, cultivation must be optimised.

## REFERENCES

[1] L. Brennan and P. Owende, Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products,

553 Renew. Sust. Energ. Rev. 14 (2010) 557-577.
554 [2] T.M. Mata, A.A. Martins, N.S. Caetano, Microalgae for biodiesel production
555 and other applications: A review, Renew. Sust. Energ. Rev. 14 (2010) 217-232.
556 [3] I. Rawat, R.R Kumar, T. Mutanda, F. Bux, Biodiesel from microalgae: A critical
557 evaluation from laboratory to large scale production, Appl. Energ. 103 (2013) 444-467.
558 [4] E. Kwietniewska and J. Tys, Process characteristics, inhibition factors and
559 methane yields of anaerobic digestion process, with particular focus on microalgal
560 biomass fermentation, Renew. Sust. Energ. Rev. 34 (2014) 491-500.
561 [5] L. Appels, J. Baeyens, J. Degrève, R. Dewil, Principles and potential of the
562 anaerobic digestion of waste-activated sludge, Prog. Energ. Combust. 34 (2008) 755-
563 781.
564 [6] European Union law, 1998. Commission Directive 98/15/EC. Official Journal of
565 the European Communities. http://eur-lex.europa.eu/legal-
566 content/EN/TXT/PDF/?uri=CELEX:31998L0015&from=EN
567 [7] Y. Li, W. Zhou, B. Hu, M. Min, P. Chen, R.R. Ruan, Integration of algae
568 cultivation as biodiesel production feedstock with municipal wastewater treatment:
569 Strains screening and significance evaluation of environmental factors, Bioresource
570 Technol. 102 (2011) 10861-10867.
571 [8] J.K. Pittman, A.P. Dean, O. Osundeko, The potential of sustainable algal biofuel
572 production using wastewater resources, Bioresource Technol. 102 (2011) 17-25.
573 [9] L. Wang, M. Min, Y. Li, P. Chen, Y. Chen, Y. Liu, Y. Wang, R. Ruan,
574 Cultivation of green algae *Chlorella sp.* in different wastewaters from municipal
575 wastewater treatment plant, Appl. Biochem. Biotechnol. 162 (2010)1174-1186.
576 [10] I.T. Dominguez Cabanelas, Z. Arbib, F.A. Chinalia, C. Oliveira Souza, J.A.

577 Perales, P.F. Almeida, J. Izabel Druzian, I. Andrade Nascimento, From waste to energy:
578 Microalgae production in wastewater and glycerol Appl. Energ. 109 (2013) 283-290.
579 [11] M. Franchino, E. Comino, F. Bona, V.A. Riggio, Growth of three microalgae
580 strains and nutrient removal from an agro-zootechnical digestate, Chemosphere 92
581 (2013) 738-744.
582 [12] P.J. He, B. Mao, C.M. Shen, L.M. Shao, D.J. Lee, J.S. Chang, Cultivation of
583 *Chlorella vulgaris* on wastewater containing high levels of ammonia for biodiesel
584 production, Bioresource Technol. 129 (2013) 177-181.
585 [13] Z. Arbib, J. Ruiz, P. Álvarez-Díaz, C. Garrido-Pérez, J.A.Perales, Capability of
586 different microalgae species for phytoremediation processes: Wastewater tertiary
587 treatment, $CO_2$ bio-fixation and low cost biofuels production, Water Res. 49 (2014)
588 465-474.
589 [14] L. Jiang, S. Luo, X. Fan, Z. Yang, R. Guo, Biomass and lipid production of
590 marine microalgae using municipal wastewater and high concentration of $CO_2$, Appl.
591 Energ. 88 (2011) 3336-3341.
592 [15] T.Cai, S.Y. Park, R. Racharaks, Y. Li, Cultivation of *Nannochloropsis salina*
593 using anaerobic digestion effluent as a nutrient source for biofuel production, Appl.
594 Energ. 108 (2013) 486-492.
595 [16] J. Pruvost, G. Van Vooren, G. Cogne, J. Legrand, Investigation of biomass and
596 lipids production with *Neochloris oleoabundans* in photobioreactor, Bioresource
597 Technol. 100 (2009) 5988–5995.
598 [17] J. Pruvost, G. Van Vooren, B. Le Gouic, A. Couzinet-Mossion, J. Legrand,
599 Systematic investigation of biomass and lipid productivity by microalgae in
600 photobioreactors for biodiesel application, Bioresource Technol. 102 (2011) 150–158.

601 [18] A. Taleb, J. Pruvost, J. Legrand, H. Marec, B. Le-Gouic, B. Mirabella, B.
602 Legeret, S. Bouvet, G. Peltier, Y. Li-Beisson, S. Taha, H. Takache, Development and
603 validation of a screening procedure of microalgae for biodiesel production: Application
604 to the genus of marine microalgae Nannochloropsis, Bioresource Technol. 177 (2015)
605 224–232.
606 [19] M.P. Caporgno, R. Trobajo, N. Caiola, C. Ibáñez, A. Fabregat, C. Bengoa,
607 Biogas production from sewage sludge and microalgae co-digestion under mesophilic
608 and thermophilic conditions, Renew. Energ. 75 (2015) 374-380.
609 [20] E.W. Rice, R.B. Baird, A.D. Eaton, L.S. Clesceri, Standard methods for the
610 examination of water and wastewater. 22nd ed. APHA, AWWA, WEF (2012).
611 [21] M.B. Johnson and Z. Wen, Production of biodiesel fuel from the microalga
612 *Schizochytrium limacinum* by direct transesterification of algal biomass, Energ. Fuel 23
613 (2009) 5179–5183.
614 [22] N. Gu, Q. Lin, G. Li, G. Qin, J. Lin, L. Huang, Effect of Salinity Change on
615 Biomass and Biochemical Composition of *Nannochloropsis oculata*, J. World Aquac.
616 Soc. 43 (2012) 97–106.
617 [23] H. Khatoon, N.A. Rahman, S. Banerjee, N. Harun, S.S. Suleiman, N.H. Zakaria,
618 F. Lananan, S.H.A. Hamid, A. Endut, Effects of different salinities and pH on the
619 growth and proximate composition of *Nannochloropsis sp*. and *Tetraselmis sp.* isolated
620 from South China Sea cultured under control and natural condition, Int. Biodeter.
621 Biodegr. 95, Part A (2014) 11–18.
622 [24] E.W. Becker, Microalgae: biotechnology and microbiology, Cambridge
623 University Press, 2008.
624 [25] O. Osundeko, A.P. Dean, H. Davies, J.K. Pittman, Acclimation of microalgae to

625 wastewater environments involves increased oxidative stress tolerance activity. Plant
626 Cell Physiol. 55 (2014) 1848-1857.
627 [26] M.A. Pistorius, W.J. DeGrip, T.A. Egorova-Zachernyuk, Monitoring of biomass
628 composition from microbiological sources by means of FT-IR spectroscopy,
629 Biotechnol. Bioeng. 103 (2008) 123–129.
630 [27] J.J. Mayers, K.J. Flynn, R.J. Shields, Influence of the N:P supply ratio on
631 biomass productivity and time-resolved changes in elemental and bulk biochemical
632 composition of *Nannochloropsis sp*, Bioresource Technol. 169 (2014) 588-595.
633 [28] Y. Li, Y. Chen, P. Chen, M. Min, W. Zhou, B. Martinez, J. Zhu, R. Ruan,
634 Characterization of a microalga *Chlorella sp.* well adapted to highly concentrated
635 municipal wastewater for nutrient removal and biodiesel production, Bioresource
636 Technol. 102 (2011) 5138-5144.
637 [29] G. Van Vooren, F. Le Grand, J. Legrand, S. Cuiné, G. Peltier, J. Pruvost,
638 Investigation of fatty acids accumulation in *Nannochloropsis oculata* for biodiesel
639 application, Bioresource Technol. 124 (2012) 421–432.
640 [30] G. Knothe, "Designer" biodiesel: optimizing fatty ester composition to improve
641 fuel properties, Energ. Fuel. 22 (2008) 1358–1364.
642 [31] S.D. Ríos, J. Castañeda, C. Torras, X. Farriol, J. Salvadó, Lipid extraction
643 methods from microalgal biomass harvested by two different paths: Screening studies
644 toward biodiesel production, Bioresource Technol. 133 (2013) 378–388.
645

**Figure captions**

**Figure 1:** Microalgae growth as the evolution of the number of cells over time for the freshwater microalgae specie *C. kessleri* (a) in the culture media with 30 mg N/L, 140 mg N/L, 260 mg N/L, 490 mg N/L, 700 mg N/L and 1200 mg N/L, referred as FM30, FM140, FM260, FM490, FM700 and FM1200. In part (b), the marine microalgae specie *N. oculata*, in the culture media with 6 mg N/L, 71 mg N/L, 135 N mg N/L, 265 mg N/L, 524 mg N/L and 782 mg N/L, referred as SM6, SM71, SM135, SM265, SM524 and SM782. **2-column fitting image.**

**Figure 2:** In the upper part, the microalgae growth as the evolution of the number of cells over time for (a) *C. kessleri*, (b) *C. vulgaris* and (c) *N. oculata* respectively. In the lower part, the N evolution as the percentage of the initial N which remains in the culture medium and the Cx evolution during cultivation for (d) *C. kessleri*, (e) *C. vulgaris* and (f) *N. oculata* respectively. **2-column fitting image.**

**Figure 3:** FTIR spectra of *C. kessleri* microalgae collected during the $2^{nd}$, $6^{th}$ and $9^{th}$ day. **Single-column fitting image.**

**Figure 4:** Methane production curves from *C. kessleri* and *C. vulgaris* cultivated in wastewater, batch reactors and 33ºC. **Single-column fitting image.**