Utilization of centrate from wastewater treatment for the outdoor production of *Nannochloropsis gaditana* biomass at pilot-scale

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Highlights

- *N. gaditana* can be cultivated outdoor using wastewater centrate as sole nutrient source.
- *N. gaditana* is stressed at NH_4^+ concentration higher than 123 mg L^{-1}.
- *N. gaditana* efficiently removes N and P, being useful for wastewater treatment.
- Adding P to culture medium enhances productivity and nitrogen removal.

Abstract

In this work, the outdoor pilot-scale production of marine microalga *Nannochloropsis gaditana* using centrate from the anaerobic digestion of municipal wastewater was evaluated. For this, outdoor continuous cultures were performed in both tubular and raceways reactors mixing seawater with different centrate percentages (15%, 20% and 30%) as culture medium. It was demonstrated that *N. gaditana* can be produced using centrate as the only nutrients source but at percentages below 30%. At this level inhibition was caused by an excess of ammonium in both photobioreactors, as confirmed by chlorophyll fluorescence and average irradiance data, thus reducing productivity. At 15% and 20% centrate percentages, biomass productivity was equal to that measured when using Algal culture medium, of 0.48 and 0.10 $g \cdot l^{-1} \cdot day^{-1}$ for tubular and raceway reactors respectively. During the experiments nitrogen depuration decreased from 85% to 63% in tubular reactors with the increase of centrate percentage in culture medium and the decrease in biomass productivity, while in raceway reactors an opposite behavior was observed due to ammonia stripping from the cultures. Phosphorus depuration from the culture medium was 85% whatever the system used and the centrate percentage in culture medium indicating a phosphorus limitation into the cultures. By supplying additional phosphorus, to achieve an N:P ratio of 5, it was possible to enhance productivity and increase nitrogen depuration in both systems. The use of centrate is confirmed as a useful method for reducing microalgae production costs and for increasing process sustainability. Consequently, it is demonstrated that for the production of microalgae biomass, centrate from wastewater treatment plants can be used as the exclusive nutrient source, achieving high productivities and nutrient removal rates if using suitable strains and if the system is operated adequately.

1. Introduction

With growing concerns surrounding the rise of oil price and global warming associated with the use of fossil fuels, renewable biofuels have gained much attention during the past decade. In fact, while oil crops biofuels cannot alone meet the existing demand for fuel, microalgae, as a third generation biomass, appear to be a more promising feedstock, because of their high potential energy yield per hectare [1-3]. Although this high potential, an industrial process has not been yet developed and applied because of the high costs of biomass production, in comparison to fossil fuels.

Microalgal biomass contains around 50% of carbon on a dry weight basis, so that approximately 1.8 kg of CO_2 are required to produce 1 kg of biomass; beside this, around 3.8 kg of water, 0.33 kg of nitrogen and 0.71 kg of phosphate are needed to produce 1 kg of algal biodiesel, if clean water is used without recycling [4]. Therefore, the tremendous consumption of water resources, inorganic nutrients and CO_2 is costly for microalgal cultivation, keeping it at more than 5 € per kg of dry biomass [5-7]. Moreover the production of these compounds as fertilizers require finite resources concentrated in few countries [8], or high energy input to be produced and/or transported, causing high GHG emissions [9]. On this way the utilization of chemical fertilizers as nutrients source reduces the sustainability of the microalgae based processes [10]: therefore the possibility to recover nutrients to be employed in new productive processes is becoming mandatory, with wastewaters being an attractive and cheap source of nutrients and water for algae production. Regarding this, microalgae ability to uptake inorganic N and P is well recognized as an efficient bioremediation tool for wastewater treatment so that the use of microalgae for nutrient removal has been considered to be practical, economical and promising [11,12]. Furthermore as an added value of this process, the biomass produced is energy rich and can be further processed to make biofuels or other valuable products such as biofertilizers, biopolymers, bioplastics, lubricants, paints, dyes and colorants [13]. Also, from an energetic point of view, wastewater remediation using microalgae consumes much less than using conventional systems (0.52 MJ m^{-3} versus 3.6 MJ m^{-3} respectively), thus presenting economic and sustainability advantages [14]; notwithstanding these, the utilization

of wastewater limits the production of microalgal biofuels to freshwater strains, although the utilization of seawater is the most sustainable and suitable way to produce them [4].

In this scenario, centrate from anaerobic digestion of wastewater treatment sludge may represents a good supplement to seawater for marine microalgae cultivation, as they contains more nutrients than the starting wastewater, mainly N and P, due to the mineralization processes occurred during anaerobic digestion. Recently, the production of marine strain *Nannochloropsis gaditana* in seawater using centrate from anaerobic digestion of wastewater treatment sludge at laboratory scale has been demonstrated [14]. A key factor in the successful development of this process is the N and P concentration in addition to the N/P ratio into the centrate. This ratio should be close to the optimum nitrogen-to-phosphorus stoichiometry characterizing phytoplankton cells, which has been commonly reported as falling in the 8–45 range [15,16]. Regarding the N and P content, the forms and concentrations of these compounds must never be higher than inhibiting limits, inhibition by ammonium at concentrations higher than 100 $mg \cdot l^{-1}$ being reported for several microalgae strains [17]. In addition, these kinds of wastewaters may also contain compounds that can inhibit microalgae growth such as urea, organic acids, phenols and pesticides, which at high concentrations could have adverse effects and limit the use of these effluents in the process [18]. For this reason, specific applied research is mandatory to determine the optimal percentage of centrate, from each local wastewater treatment plant, that can be mixed with seawater to support algae growth.

The aim of this work was to determine the feasibility of outdoor pilot scale *Nannochloropsis gaditana* production using centrate from municipal wastewater treatment as nutrients source and flue gas as CO_2 source, determining both biomass productivity and quality in addition to nutrients removal from the culture medium. For this, experiments were performed using outdoor raceway and tubular reactors adding different percentages of centrate as only nutrients source to sea water. Experiments were performed in continuous mode at different dilution rates to evaluate the optimal conditions of the process. On this way, the demonstration of outdoor production of marine

100 microalgae strains using only effluents as nutrients source will greatly improve the sustainability
101 and economic profitability of biofuels production from microalgae.
102
103 **2. Materials and methods**
104 *2.1 Microorganism and culture media*
105 Marine microalga *Nannochloropsis gaditana* Lubián CCMP 527 was selected for this work because
106 of its high growth rate and high productivity under outdoor conditions. Inoculum for the cultures
107 was grown indoor under controlled pH (8.0) and temperature (25 ºC) conditions in 5 L glass bottles,
108 at an irradiance of 150 µE m^{-2} s^{-1}, using Algal medium at 8 mM nitrate (Bionova, Santiago, Spain)
109 on seawater. After reaching the stationary phase the cultures were transferred outdoor to 100 L
110 bubble columns, under controlled pH (8.0) by on-demand pure CO_2 injection. During the
111 exponential growth phase the cultures were finally transferred to the reactors for the experiments.
112 Centrate was obtained directly from the Wastewater treatment plant located in Almería (Spain)
113 operated by Aqualia and more specifically after the bed filter used to separate the solids from the
114 liquid fraction of the digestate, obtained after anaerobic digestion of activated sludge produced from
115 wastewater treatment. Thus, this centrate did not contain solids, it being rich in ammonium and
116 phosphorous in addition to other compounds. Composition of the centrate is showed in Table 1. The
117 culture medium was prepared daily by supplementing natural seawater with centrate according to
118 the centrate percentage set for each experiment. The seawater used for medium preparation was
119 pumped directly from the sea. The culture medium was filtered through a set of three sequential
120 filters: 10 µm, 5 µm and 1 µm (3M, France). No additional treatment or sterilization was performed
121 prior to enter to the reactors.
122
123 *2.2 Photobioreactors and culture conditions*

Experiments were carried out outdoors in a set of three fence-type tubular photobioreactors and three raceway reactors. The tubular photobioreactors were built as previously described by Acién *et al*. [19] and Molina *et al*. [20]. Each tubular photobioreactor had a working volume of 340 L while raceway reactors had a working volume of 800 L. Tubular reactors consisted of a vertical tubular solar receiver (125 m length and 0.05 m diameter) and a bubble column for heat exchange and O_2 degassing (1.92 m high and 0.25 m diameter). A centrifugal pump (SE-150-M, Espa, Spain) was used to recirculate the culture through the reactor at 0.5 m s^{-1} [21]. Inter-tube distance was set at 0.05 m to maximize solar radiation capture. The reactors were oriented east–west and the distance between them was 1.6 m so as to minimize shadowing. The temperature during the day was kept under 30.0 °C by circulating seawater through a heat exchanger, and the pH was controlled at 8.0 by on-demand injection of flue-gas CO_2 into the inlet air stream.

Raceway reactors consisted of two 5.00 m long and 0.60 m wide channels connected by 180º bends, the total surdface being 7.2 m^2. The reactors were made of 5 mm thick polyethylene, a stainless steel paddlewheel (0.60 m diameter) was used for the circulation of the culture at a rate of 0.2 m s^{-1}. The paddlewheel was driven by an electric motor with gear reduction (Ebarba, Barcelona, Spain) and the speed was regulated by a frequency inverter (Yaskawa AC Drive V1000, Yaskawa Electric Europe GmbH, Germany). Regarding the culture depth, in the raceway ponds a water depth of 20 cm has been widely reported as optimum; however in the present study both light availability and biomass productivity enhancements of the cultures were sought, therefore the culture depth in all the reactors was set to 0.11 m. Air was constantly supplied to the reactors to reduce dissolved oxygen accumulation through an air sparger placed inside a sump located 0.85 cm upstream of the paddlewheel.

To control the pH and supply CO_2 flue-gas was used instead of pure CO_2. The flue gas was produced on-demand by a diesel-oil boiler connected to a compressor used to store the flue-gas for further utilization. At the outlet of the boiler, flue-gas was necessarily cooled by passing it through a passive stainless steel serpentine. Moreover before being injected in the cultures the gas was filtered

150 by three sequential cartridge filters (1 μm) to reduce the particulate content in the gas stream. The
151 average CO_2 concentration in the flue gas was 10.9%. The air flow rate entering each
152 photobioreactor was 0.1 v v^{-1}·min^{-1} (FR4L72BVBN flow meters, Key Instruments, USA), while the
153 flue gas was injected when required at a constant flow rate of 0.01 v v^{-1}·min^{-1} in all reactors
154 (FR4A41BVBN flow meters, Key Instruments, USA). Dissolved oxygen, pH and temperature
155 values were measured with OD and pH probes (5342 pH electrode and 5120 OD electrode, Crison
156 Instruments S.A., Spain) connected to a MM44 control-transmitter unit (Crison Instruments, Spain).
157 The data were logged in a PC control unit, which allowed the monitoring and control of the culture
158 parameters. The solar radiation received by the facility was measured with a thermoelectric
159 pyranometer connected to an AC-420 adapter (LP-02, Geónica S.A., Spain). The reactors, the data
160 logging system and the control software (DaqFactory 5.0, Azeotech Inc., USA) were designed and
161 built by our research group.
162 The experiments were carried out in semicontinuous mode, by adding fresh medium to the reactors
163 during 5 h in the middle of solar cycle. Tubular photobioreactors were operated at optimal dilution
164 rate of 0.33 day^{-1} whereas raceway reactors were operated at 0.20 day^{-1}, these dilution rates being
165 the optimal for both systems. Only the composition of culture medium used, i.e. the utilization of
166 Algal medium or different percentages of centrate with seawater, being modified. Experiments were
167 performed in the three reactors of each type at the same time, this it being performed in triplicate,
168 average values from the three reactors of each type being used.
169
170 *2.3 Biomass concentration, fluorescence of chlorophylls and quantum yield determination*
171 The dry weight biomass concentration (Cb) was measured by filtering 50 ml of culture through 0.45
172 μm filters and drying it in an oven at 80ºC for 24 h. The cells status was checked daily by
173 measuring the fluorescence of chlorophylls (Fv/Fm) ratio with a fluorometer (AquaPen AP 100,
174 Photon Systems Instruments, Czech Republic). The extinction coefficient (Ka) was calculated by

175 dividing the average absorption by the biomass concentration (Cb) and light path of the cuvette (p)
176 (equation 1).

$$Ka = \frac{Abs}{Cb \cdot p} \quad \text{Eq. 1}$$

177 The average irradiance (in the range of photosynthetically active radiation, PAR) at which cells are
178 exposed inside a culture (Iav) is a function of irradiance in the absence of cells (Io), the biomass
179 extinction coefficient (Ka), the biomass concentration (Cb) and the light path inside the reactor (p).
180 It can be approximated by using Equation 2 [22].

$$Iav = \frac{Io}{(Ka \cdot p \cdot Cb)}\left(1 - \exp(-Ka \cdot p \cdot Cb)\right) \quad \text{Eq. 2}$$

181 Quantum yield (ΨE) is defined in microalgal cultures as the amount of biomass generated by the
182 unit of radiation (usually a mole of photons) absorbed by the culture. Since it represents the ratio of
183 biomass generation to absorbed photon flux, it can be calculated by Equation 3 [22], where Pb
184 stands for the volumetric biomass productivity and Fvol for the photon flux absorbed in the volume
185 unit. The photon flux absorbed through the reactor volume may be obtained from the average
186 irradiance (Iav) on a culture volume basis using Equation 4 [22].

$$\Psi_E = \frac{Pb}{F_{vol}} \quad \text{Eq. 3}$$

$$F_{vol} = Iav \cdot Ka \cdot Cb \quad \text{Eq. 4}$$

187

188 *2.4 Analytical Methods*

189 For analysis of culture medium and supernatant, the standard official methods approved by the
190 Spanish Minister of Agriculture were used [23]. Phosphorus was measured by visible
191 spectrophotometry through the phospho-vanado-molybdate complex [24]. Nitrates were quantified
192 using a spectrophotometer between 220 and 275 nm. Ammonium was measured by the Nessler
193 reactive method [24].

3. Results and discussion

Centrate is a nutrient-rich effluent that can be used as nutrient source to produce microalgae biomass for energy purposes, substituting chemical fertilizers which increase the production costs and decrease environmental sustainability of the whole process. Centrate contains nitrogen but also other major nutrients as phosphorous, calcium, potassium, among others, thus it being a complete culture medium for microalgae [14,25]. Because the nitrogen content of centrate usually exceed that required for microalgae production, it is necessary to dilute the centrate with water to prepare an adequate culture medium. Moreover, as the nitrogen is in the ammonium form, the dilution of centrate is often mandatory: this is due to the fact that, although microalgae assimilate ammonium more easily than nitrate, as its uptake is thermodynamically more favorable, ammonium has been reported to be toxic at concentrations exceeding 100 mg L^{-1} [17], thus the excessive concentration of ammonium can limit the growth. On this sense, to elucidate the appropriate percentage of centrate into the culture medium to be used in the production of *N. gaditana,* experiments were performed using two types of outdoor pilot-scale microalgae reactors, raceway and tubular photobioreactors. Experiments were performed in continuous mode, using Algal medium as reference (0% centrate), and seawater with different percentages of centrate (15%, 20% and 30%) as the only nutrients source. It is important to note that when using standard Algal medium the nitrogen was supplied as nitrate whereas using centrate the nitrogen was supplied as ammonium. Results demonstrate that centrate from a real wastewater treatment plant can be used to produce *N. gaditana* in seawater (Figure 1A). It is observed as in both reactors the volumetric biomass productivity was maximal when using the standard Algal medium, with values of 0.48 and 0.10 g L^{-1} day^{-1} in tubular and raceway reactors respectively. These values do not greatly decrease when using centrate at percentages of 15% and 20%, but when percentage of centrate of 30% was used the biomass productivity greatly reduced to 0.15 and 0.04 g L^{-1} day^{-1}, for tubular and raceway reactors respectively. Biomass productivities obtained using centrate at 15% and 20% are in the

same range of those obtained by Sepulveda *et al*. [14] with the same microalga and type of centrate, although the authors concluded that maximum productivities were achieved using percentages of centrate ranging from 30% to 50%. In that case, the experimental conditions were different, the experiments being performed indoor using bubble column reactors, pure CO_2 and fully controlled culture conditions. Moreover the differences of biomass productivity could be explained also taking into account the different illumination environments as the indoor experiments were performed using a fixed light intensity simulating circadian cycle whereas in this work experiments were performed outdoor according to sunlight availability. This behavior was also observed by Sheets *et al*. [26] who concluded that biomass productivity of *Nannochloropsis salina* cultured using effluent from anaerobic digestion strongly declined when converting cultures from constant to varied illumination conditions.

The same productivity trend is observed when analyzing the areal productivity, maximal biomass productivity of 27 and 14 g m^{-2} day^{-1} being obtained when using standard Algal medium for tubular and raceway reactors respectively. These values decreased to 8 and 5 g m^{-2} day^{-1} when using centrate at 30% in the culture medium (Figure 1B). It is important to note that the differences between tubular and raceway reactors were lower in terms of areal productivity than in terms of volumetric productivity. As in the tubular reactors the surface exposed to light is higher and the control of culture conditions more adequate than in the raceway reactor, it is expected a higher volumetric productivity in the tubular with respect to the raceway reactors. The small differences in terms of areal productivity indicate that the performance of the tubular reactors was not optimal. To clarify this point, measurements of fluorescence of chlorophylls were performed as an index of stress at which the cells were exposed to (Figure 1C). The fluorescence of chlorophylls was higher in the samples from the raceway than from the tubular reactors, thus confirming the existence of adverse culture conditions on the last ones. In both reactors the fluorescence of chlorophylls of the cultures was maximal when using standard Algal medium, of 0.42 and 0.54 for tubular and raceway reactor respectively, and remained constant when using percentages of centrate of 15% and 20%.

However the fluorescence of chlorophylls of the cultures reduced to values of 0.35 and 0.46, for tubular and raceway reactors respectively, when increasing the percentage of centrate to 30%. This behavior is in accordance with the observed reduction on biomass productivity when using 30% of centrate in the culture medium, indicating an adverse effect of centrate when used at high percentages. This is also confirmed when analyzing the average irradiance at which the cells were exposed to into the cultures, as a function of photobioreactor and culture medium used (Figure 1D). In both reactors the average irradiance inside the cultures remained constant when using standard Algal culture medium and percentages of centrate from 15% to 20%, but increased when using 30% of centrate into the culture medium in both reactors: this indicates that the cultures were less efficient and more light was necessary to maintain the growth rate at these conditions. Regarding the average irradiance in both type of reactors, the lower values determined in the tubular photobioreactors demonstrate that the cells were more efficient in the utilization of the light in these reactors than in raceway reactors.

Regarding nitrogen consumption, the nitrogen concentration as ammonium and nitrate, at the inlet and outlet flows from the reactors was determined. The nitrogen concentration into standard Algal culture medium was 112 mg L^{-1}, whereas using centrate the nitrogen concentration at the inlet ranged from 72 to 145 mg L^{-1}, thus the experiments being performed in the same range of nitrogen concentration although supplied as nitrate when using Algal medium and as ammonium when using centrate (Figure 2A). This point is relevant because ammonium is toxic for microalgae cells, then excess of its concentration can reduce the performance of the cultures. A wide range of tolerance has been reported for several microalgae species. For example, *Chlorella sorokiniana* was completely inhibited at ammonium concentration of 210 mg L^{-1} [27] whereas *Spirulina platensis* was only inhibited at 150 mg L^{-1} [28]. Sheets *et al*. [26] optimized semicontinuous cultivation of *Nannochloropsis salina* using a medium containing 7% of anaerobic digestion effluent with 200 mg L^{-1} of ammonium nitrogen. Sepulveda *et al*. [14] reported the absence of inhibition of the same strain *Nannochloropsis gaditana* at an ammonium concentration of up to 334 mg L^{-1}. In this work

the maximal ammonium concentration tested was 145 mg L^{-1}, when using 30% of centrate, this being similar to reported tolerance values. However, on these conditions the data of biomass productivity, fluorescence of chlorophylls and average irradiance indicates that *Nannochloropsis gaditana* cultures were stressed.

Regarding outlet, the nitrogen concentration in exhausted culture medium from tubular reactors was much lower than inlet thus confirming that nitrogen was consumed to produce biomass (Figure 2A). Using standard Algal medium the outlet nitrogen concentration was 16 mg L^{-1}, whereas using centrate the outlet nitrogen concentration ranged from 7 to 47 mg L^{-1}. It is observed as the higher the percentage of centrate in the culture medium the higher the nitrogen concentration at the outlet, thus indicating that the supplied nitrogen overpassed the capacity of the system to fix this nitrogen as biomass. Data from the raceway reactors show a similar trend, although outlet concentrations were much closer to inlet concentrations than in the case of tubular reactors, agreeing with the lower biomass productivity measured in the raceway reactors. In raceway reactors the outlet concentration was 92 mg L^{-1} when using Algal medium, and increased from 46 to 72 mg L^{-1} when using percentage of centrate into the culture medium from 15% to 30% (Figure 2A). In terms of nitrogen depuration, data from the tubular reactors show a high depuration efficiency, of 85% when using standard Algal medium or percentages of centrate from 15 to 20%, but this value reduces to 63% when using 30% of centrate due to the lower biomass productivity on this condition (Figure 2B). These results are in accordance with the results reported by Sepulveda *et al*. [14] working with the same microalga: in that case, it was observed a strong reduction in the nitrogen depuration capacity when percentage of centrate in the culture medium increased upper than 50%. This was related to a great excess of nitrogen as well as to the lower biomass productivity when increasing centrate percentage. On the other hand, data from the raceway reactors show a different trend. In that case nitrogen depuration was only 20% when using Algal standard culture medium, and remained at 35% when using centrate at percentages of 15% and 20%. However, when using 30% of centrate into the culture medium the nitrogen depuration increases up to 49% (Figure 2B). High

298 nitrogen depuration rates have been reported with freshwater microalgae cultivated in anaerobic
299 digestion effluents, ranging from 60 to 90% [25,29,30,31]. Consensus exists about that nitrogen
300 depuration is a function of different phenomena taking place as nitrogen uptake by the cells to
301 produce biomass, nitrogen stripping to the atmosphere and including nitrification-denitrification
302 processes carried out by bacteria.
303 Data of nitrogen depuration in tubular reactors agree with biomass productivity thus the uptake by
304 the cells to produce biomass is the major phenomena taking place in this system. On the contrary,
305 data of nitrogen depuration in raceway reactors do not agree with biomass productivity, thus the
306 higher nitrogen depuration measured at the lowest biomass productivity indicates that stripping was
307 highly relevant on these conditions. To study the existence of nitrification-denitrification
308 phenomena the concentration of different nitrogen forms was measured. Using nitrate as nitrogen
309 source (standard Algal medium - 0% of centrate), no ammonium was found into the exhausted
310 medium, nitrogen remaining into the culture being that not uptake by the cells (Figure 2C,D).
311 However, using ammonium as nitrogen source (centrate at percentages from 15% to 30%), both
312 ammonium and nitrate were found into the culture broth. Increasing the percentage of centrate into
313 the culture medium caused the increase of ammonium concentration in the outlet of both tubular
314 and raceway reactors. In the raceway reactors the concentration of ammonium ranged from 23 to 48
315 mg L^{-1}, and it was higher than that measured in the tubular reactors, ranging from 5 to 25 mg L^{-1}.
316 Regarding nitrate, in the raceway reactors its concentration ranged from 22 to 38 mg L^{-1}, higher
317 than that measured into the tubular reactors that ranged from 1 to 18 mg L^{-1}. The presence of nitrate
318 in the cultures that were supplied with ammonium indicates the occurrence of nitrification
319 processes, relative values of nitrate in the raceway and tubular reactors indicating that nitrification
320 was more relevant in the raceway reactors, on which higher ammonium concentrations and lower
321 biomass productivities were measured. In the tubular reactors the nitrate concentration was only
322 relevant when using 30% of centrate into the culture medium, because on these conditions the
323 culture was stressed and the biomass productivity was low, thus the uptake of nitrogen being lower.

These results are also in accordance with the work of Morales-Amaral *et al*. [25] which reported that only under optimal culture conditions using urban wastewater no nitrification was observed, the ammonium being mainly consumed by microalgae although a certain degree of stripping might has also occurred.

To better quantify the efficiency of the process, the nitrogen removal capacity and nitrogen coefficient yields were calculated from nitrogen inlet and outlet measurements, as these parameters more adequately allow comparing different strains/systems. Regarding nitrogen removal capacity data show that using standard Algal medium a maximal value of 31 mg N L^{-1} day^{-1} was measured in the tubular reactors, whereas in the raceway reactors the value was minimal, of 4 mg N L^{-1} day^{-1} (Figure 3A). Using centrate the removal capacity increase in both reactors, up to 32 and 14 mg N L^{-1} day^{-1} at 30% centrate in the culture medium for tubular and raceway reactors respectively, in opposite to that expected from the decreases in biomass productivity in both systems. These data are higher of those reported by other authors; Cabanelas *et al*. [32] obtained a maximal removal capacity of 9.8 mg N L^{-1} day^{-1} using *Chlorella vulgaris* to treat centrate whereas a nitrogen removal capacity of 8.5 mg N L^{-1} day^{-1} was reported for *Chlorella* cultures using ten-fold diluted centrate; this value increasing to 22.7 mg N L^{-1} day^{-1} under optimal conditions [33]. A similar trend was reported for pig manure, with nitrogen removal capacity ranging from 0.5 to 12 mg N L^{-1} day^{-1} [34]. These differences can be due to different phenomena that can take place in microalgae cultures, in fact nitrogen was not only removed from the system by biomass assimilation but also by stripping in spite of controlled pH at 8.0. This is an important aspect of microalgae-based wastewater treatment [35] and although many works conclude that microalgae are able to reduce almost 100% of nitrogen in wastewaters, few studies have focused on the volatilized fractions [36,37]. Moreover, data of nitrogen coefficient yield further confirmed this phenomenon and revealed that stripping was higher in the raceway reactors than in the tubular ones. In fact, using standard Algal medium the nitrogen coefficient yield was 21 $g_{biomass}$ $g_{nitrogen}^{-1}$, that agree with the expected value corresponding to 5% of nitrogen into the biomass (Figure 3B). However, using

centrate in the culture medium the nitrogen coefficient yield reduces, the higher the percentage of centrate in the culture medium the lower the nitrogen coefficient yield value determined. In the tubular reactor the nitrogen coefficient yield reduces from 22 to 5 $g_{biomass}\ g_{nitrogen}^{-1}$ when the percentage of centrate in the culture medium increases from 15% to 30%, whereas in the raceway reactor the nitrogen coefficient yield reduces from 13 to 3 $g_{biomass}\ g_{nitrogen}^{-1}$ on the same conditions. On this way, it was demonstrated that although centrate can be used to produce *Nannochloropsis gaditana* in outdoor pilot-scale reactors, the feasibility of the system is limited by the tolerance of selected microalga to use ammonium as nitrogen source and the biomass productivity achievable into the photobioreactor used. The utilization of closed tubular photobioreactors allows obtaining higher biomass productivities at the same time removing more nitrogen from the culture medium, thus achieving higher nitrogen depuration rates. However, including on these conditions a fraction of nitrogen is lost to the atmosphere due to stripping phenomena, caused by mixing and aeration, and favored by alkaline pH values of the culture medium and to the increase of non-ionized ammonia concentration [37]. This point is crucial as the loss of ammonia to the atmosphere is not environmentally acceptable as it may promote environmental problems such as the formation of particulate matter (PM), water acidification and eutrophication processes [38].

The stripping process occurred in this study may also have been caused by the unbalanced N:P ratio of the centrate, that implies that microalgae cells were not capable of assimilating more nitrogen than that imposed by their biomass N:P ratio. This phenomena has found confirmation in the works of Lee *et al*. [39] and Sepulveda *et al*. [14], who indicated that N removal was higher for P-added media than unbalanced N:P ratio media. To elucidate this aspect the phosphorous balance into the system was performed. The phosphorous concentration of Algal medium was 22 mg P L^{-1}, much higher than that obtained when diluting centrate with seawater, values of 4, 6 and 8 mg P L^{-1} being obtained for 15%, 20% and 30% of centrate into the culture medium (Figure 4A). Thus, the N:P ratio of Algal medium is 5 whereas for medium prepared using centrate the N:P ratio is 17. These data indicates that centrate is poor in phosphorous and the cultures can be limited by this nutrient.

Thus, when using Algal medium the phosphorous concentration outlet the reactors was 5 and 18 mg P L^{-1} for tubular and raceway reactors respectively, indicating that excess of phosphorus was supplied. When using centrate at percentages increasing from 15% to 30% the phosphorous concentration outlet the reactors increases from 0 to 1 mg P L^{-1} in tubular reactors, and from 0 to 2 mg P L^{-1} in raceway reactors. These low concentrations at outlet flows demonstrated that the cultures were phosphorous limited. Thus, using Algal medium the phosphorous depuration was 76% and 20% for tubular and raceway reactors respectively, whereas using centrate the phosphorous depuration was higher than 85% for whatever the percentage of centrate and photobioreactor used (Figure 4B).

The phosphorus depuration percentage obtained in this work is in the same range of that achieved with other microalgae strains and with a comparable nutrients source: a phosphorous removal of 63% to 75% was previously reported with *Chlorella* sp. grown on digested dairy manure [29], whereas Ruiz-Marin *et al*. [40] reported removal values, in urban wastewaters, of 80% and 83% for *Chlorella vulgaris* and *Scenedesmus obliquus* respectively. These results also demonstrates that phosphorous was limiting the performance of the systems. In terms of phosphorous removal capacity and coefficient yield data confirm the existence of phosphorous limitation during the experiments. Using Algal medium the phosphorous removal capacity was 5.5 and 1.0 mg P L^{-1} day^{-1} for tubular and raceway reactors respectively (Figure 5A). However, using centrate the values were much lower due to the existence of phosphorous limitation. In the tubular photobioreactors the phosphorous removal increases linearly from 1.3 to 2.6 mg P L^{-1} day^{-1} with the increase of percentage of centrate into the culture medium, in the raceway reactors the phosphorous removal increasing from 0.8 to 1.4 mg P L^{-1} day^{-1} on the same conditions (Figure 5A). In terms of phosphorous coefficient yield, the values obtained using Algal medium were equal for tubular and raceway reactors, of 100 $g_{biomass}$ $g_{phosphorous}^{-1}$ (Figure 5B). However, using centrate the phosphorous coefficient yield reduces when increasing the percentage of centrate into the culture medium, from 370 to 60 $g_{biomass}$ $g_{phosphorous}^{-1}$ in the tubular reactors, and from 100 to 26 $g_{biomass}$ $g_{phosphorous}^{-1}$ in the

raceway reactors. These data confirm that centrate imposes the existence of phosphorous limitation, this effect being stronger the lower the percentage of centrate into the culture medium.

To clarify the contribution of phosphorous limitation to the loss of efficiency of the system an additional experiment was performed using culture medium containing 30% of centrate without and with additional phosphorous. In experiment with additional phosphorous phosphate was supplied to achieve a ratio N:P equal to 5, analogous to that of Algal medium. Results demonstrate that supplying additional phosphorous the productivity of the cultures in both reactors increases, from 8.4 to 10.3 g m^{-2} day^{-1} in tubular reactors and from 5.5 to 7.4 g m^{-2} day^{-1} in raceway reactors (Figure 6A). However, these values are still lower than those obtained using 20% of centrate thus indicating that although phosphorous limitation was solved still inhibition by adverse ammonium concentration remained. Regarding nitrogen depuration, the addition of phosphorous also increased the nitrogen depuration in both reactors, up to 80% and 60% in tubular and raceway reactors respectively, thus confirming that nitrogen uptake was limited by phosphorous limitation taking place when using centrate as culture medium (Figure 6B). These results confirm at pilot scale the results of a previous work of our group [14] where it was demonstrated at a laboratory scale that by balancing the N:P ratio adding additional phosphorus to the culture medium it was possible to enhance biomass productivity and nitrogen depuration. Nevertheless, the phosphorous depuration shows an opposite behavior because a fraction of added phosphorous remained into the culture not being uptake by the cultures, and thus the cultures being in excess of phosphorous (Figure 6C).

On this way, although centrate can be used as the only nutrients source for the outdoor production of *Nannochloropsis gaditana* in both tubular and raceway reactors, the percentage of centrate in the culture medium to be used must be accurately defined. Centrate contains ammonium that stresses the cultures at concentrations higher 100 mg L^{-1}. On the other hand is poor in phosphorous thus to depurate most of the nitrogen contained into the culture medium additional phosphorous must be supplied. To maximize the efficiency of the system both nitrogen and phosphorous must be provided to the culture medium according to the final biomass productivity achieved into the

428 reactors. The supply of larger amounts of centrate (ammonium) overpassing the uptake capacity by
429 the cells increases the losses of nitrogen by stripping and reduces the efficiency of the system and of
430 the cells. The analysis of quantum yield values allows confirming that the tubular reactors were
431 more efficient than raceway reactors, the quantum yield on this reactor being 0.7 g E^{-1} in front of
432 0.3 g E^{-1} determined into the raceway reactors when using standard Algal medium or percentages of
433 centrate from 15 to 20% (Figure 7). When using 30% of centrate into the culture medium the
434 quantum yield of the cultures reduces in both reactors down to 0.6 and 0.2 g E^{-1} in tubular and
435 raceway reactors respectively, demonstrating the adverse effect of supplying excess of ammonium
436 to the microalgae cultures. However, the utilization of centrate imposes the existence of
437 phosphorous limitation, thus if phosphorous is added the quantum yield increases including when
438 ammonium was in excess. To optimize the performance of the system the adequate percentage of
439 centrate and the additional phosphorus required must be accurately determined according to the
440 biomass productivity into the reactors.
441
442 **4. Conclusions**
443 It has been demonstrated that marine microalgae strains as *Nannochloropsis gaditana* can be
444 produced outdoor using centrate from anaerobic digestion of wastewater treatment processes as
445 only nutrients source, in spite of low phosphorous content of this effluent. Productivity obtained
446 using this effluent as nutrients source is close to that obtained using standard Algal culture medium
447 when percentages equal or lower than 20% are used, although phosphorous limitation takes place.
448 Upper this percentage the performance of the cultures reduces, the cells being stressed and the
449 quantum yield of the cells reducing by excess of ammonium. Tubular photobioreactors demonstrate
450 to be more productive than raceway reactors including on these conditions, but also more efficient
451 in transforming nitrogen and phosphorous into biomass. However, not all the removed nitrogen is
452 transformed into biomass, a fraction of the inlet nitrogen being lost by stripping in both reactors.
453 Nitrogen losses were higher in the raceway reactor due to its lower biomass productivity. In

addition to stripping it was demonstrated that nitrification takes place, thus a fraction of the inlet ammonium being transformed into nitrate that remains into the culture broth. Balancing the N:P ratio in the culture medium by adding additional phosphorus allow to improve system performance in terms of both biomass productivity and nitrogen depuration. Concluding, the utilization of centrate as nutrients source for the production of marine strain improve the possibility to produce large amounts of microalgae biomass on a more sustainable way, moreover it is possible to apply this concept to the development of wastewater treatment processes for high salinity contaminated waters.

463 Table 1. Composition of centrate obtained from a wastewater treatment plant used to prepare

464 culture medium by mixing with seawater at different proportions.

pH	8.31		
Conductivity	4.55 mmhos/cm 25ºC		
Compound	Concentration, mg L^{-1}	Compound	Concentration, mg L^{-1}
Chloride	1093.76	Carbonate	24.00
Bicarbonate	646.77	Magnesium	19.00
Ammonium	615.48	Iron	0.39
Sodium	358.00	Boron	0.27
Potassium	102.00	Sulphate	0.22
Calcium	96.00	Zinc	0.09
Phosphorus	36.02	Copper	0.03
Nitrate	28.94	Manganese	0.02

465

466

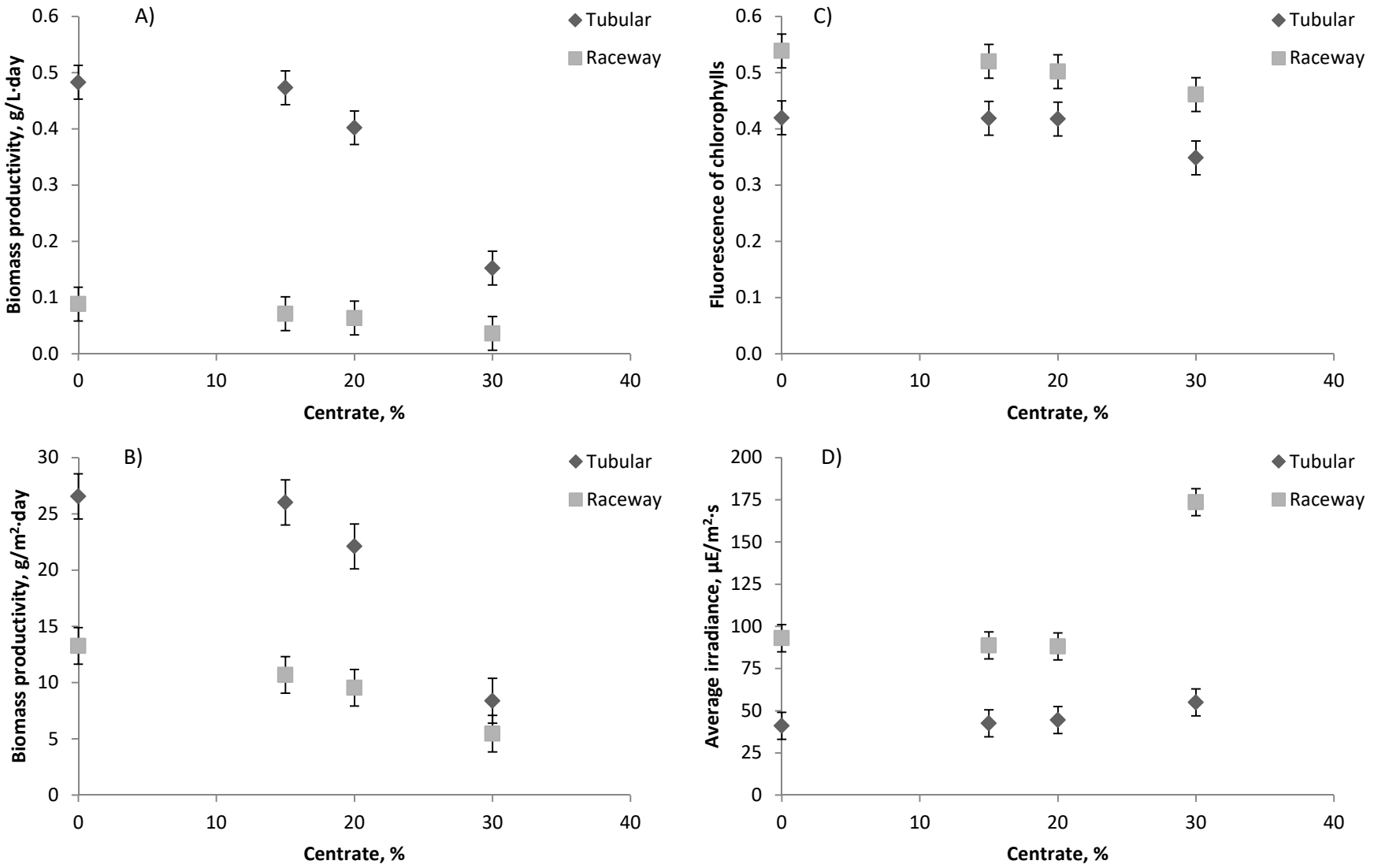


Figure 1.- Influence of centrate percentage in the culture medium on the performance of *Nannochloropsis gaditana* cultures carried out in raceway and tubular photobioreactors. A) Volumetric biomass productivity, B) Areal biomass productivity, C) Fluorescence of chlorophylls, D) Average irradiance at which the cells are exposed to.

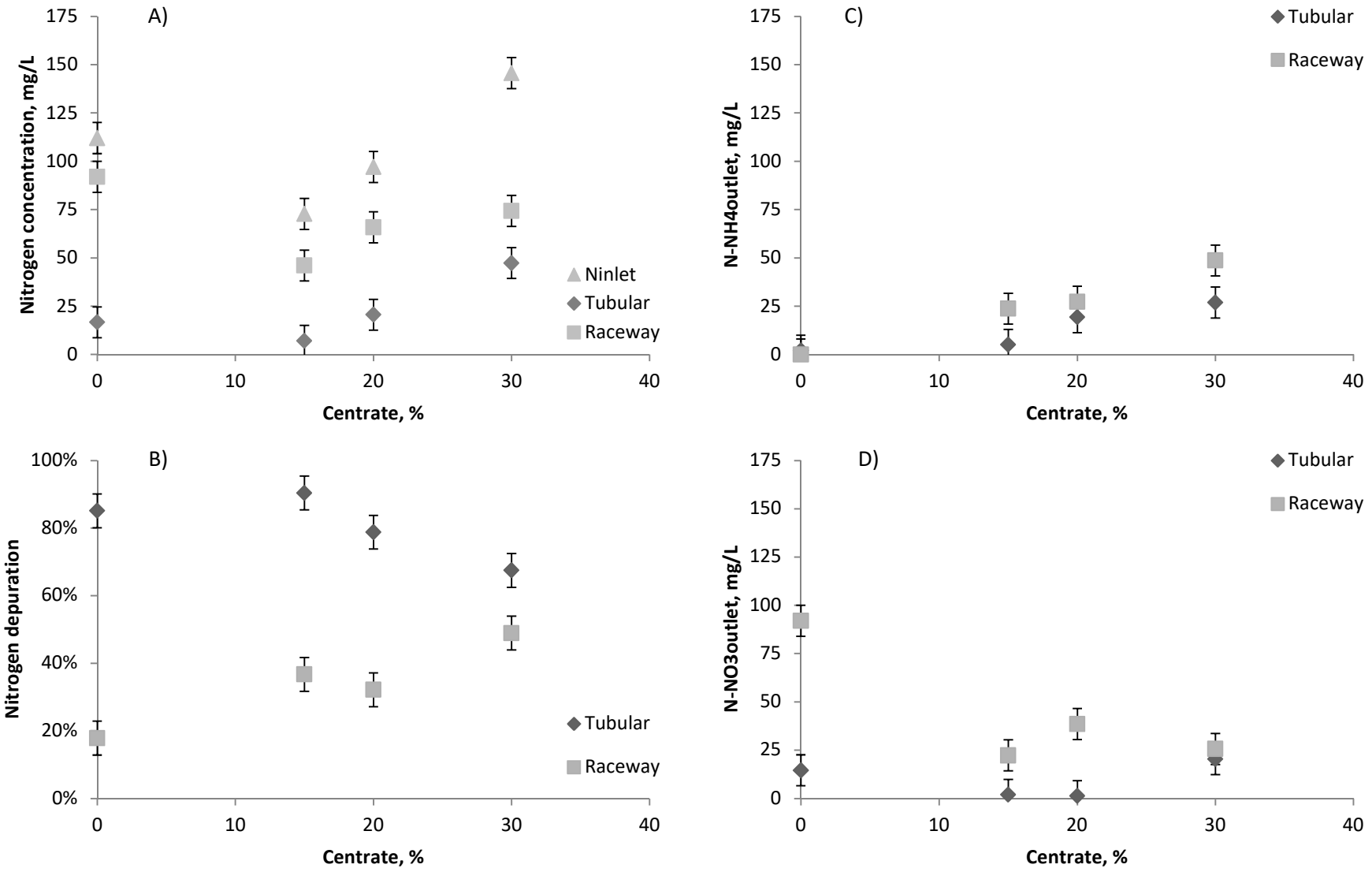


Figure 2.- Influence of centrate percentage in the culture medium on the nitrogen consumption and nitrogen forms during *Nannochloropsis gaditana* cultures carried out in raceway and tubular photobioreactors. A) Nitrogen concentration in the liquid phase, B) Nitrogen depuration, C) $N\text{-}NH_4$ concentration in the liquid phase, D) $N\text{-}NO_3$ concentration in the liquid phase.

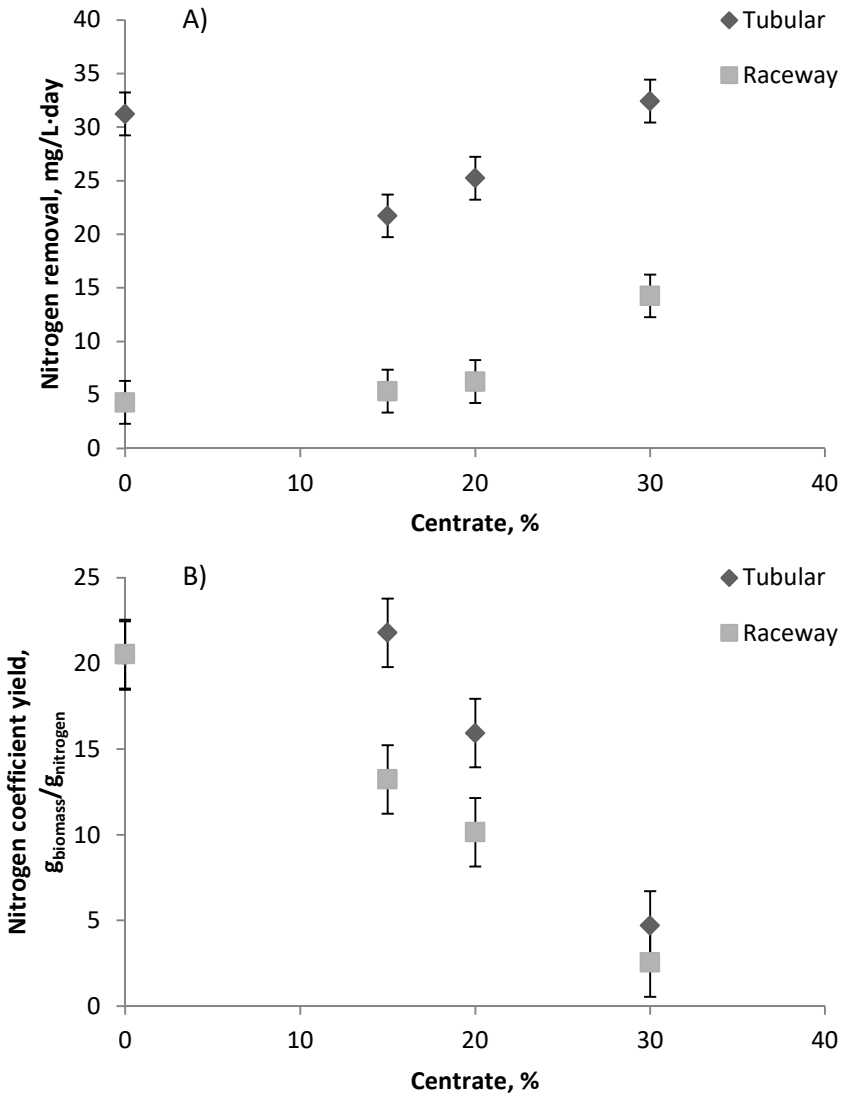
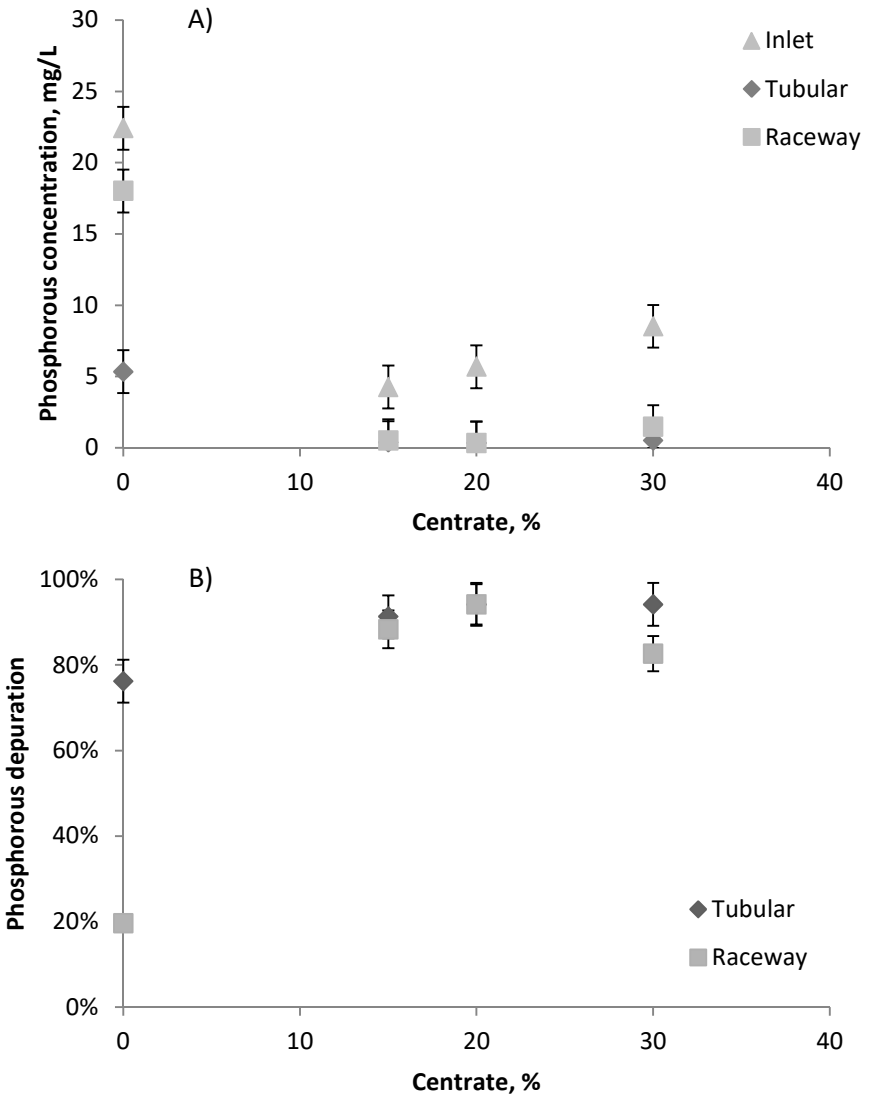


Figure 3.- Influence of centrate percentage in the culture medium on (A) the nitrogen removal capacity and (B) nitrogen coefficient yield of *Nannochloropsis gaditana* cultures carried out in raceway and tubular photobioreactors.



481

482 Figure 4.- Influence of centrate percentage in the culture medium on the phosphorous consumption
483 during *Nannochloropsis gaditana* cultures carried out in raceway and tubular photobioreactors. A)
484 Phosphorous concentration in the liquid phase, B) Phosphorous depuration.
485

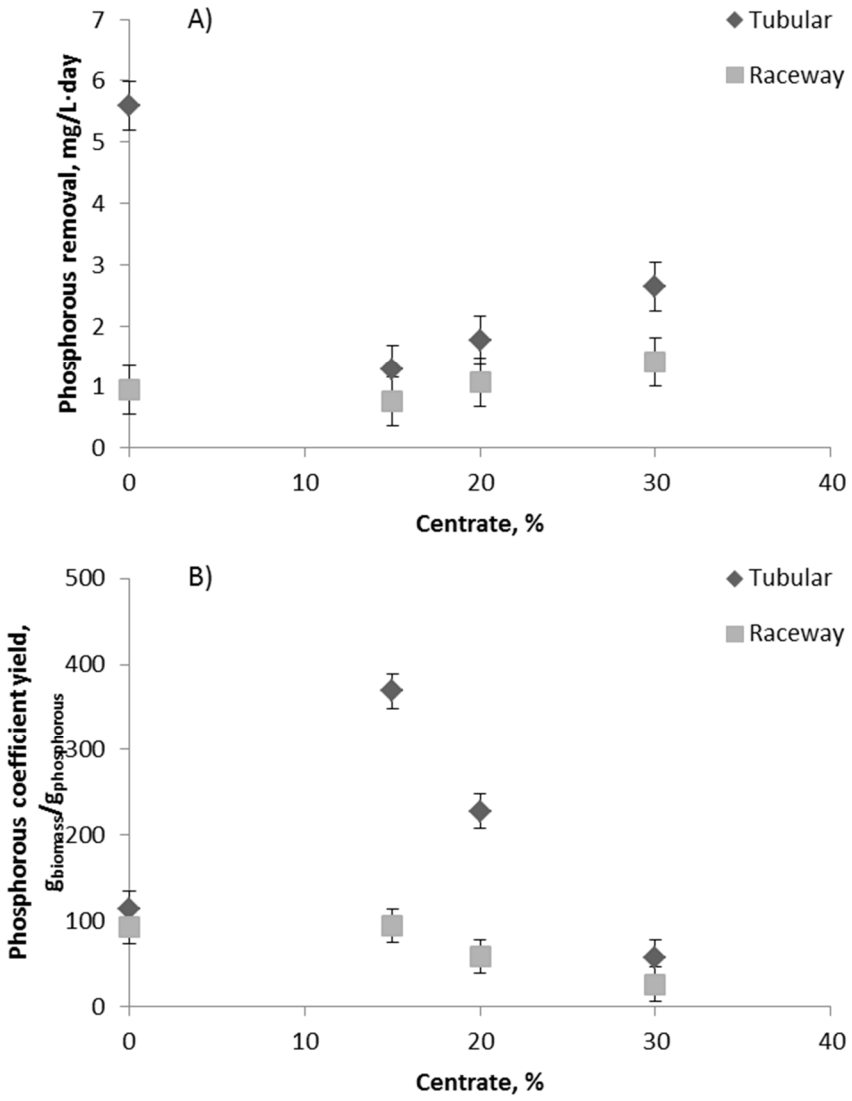


Figure 5.- Influence of centrate percentage in the culture medium on (A) the phosphorous removal capacity and (B) phosphorous coefficient yield of *Nannochloropsis gaditana* cultures carried out in raceway and tubular photobioreactors.

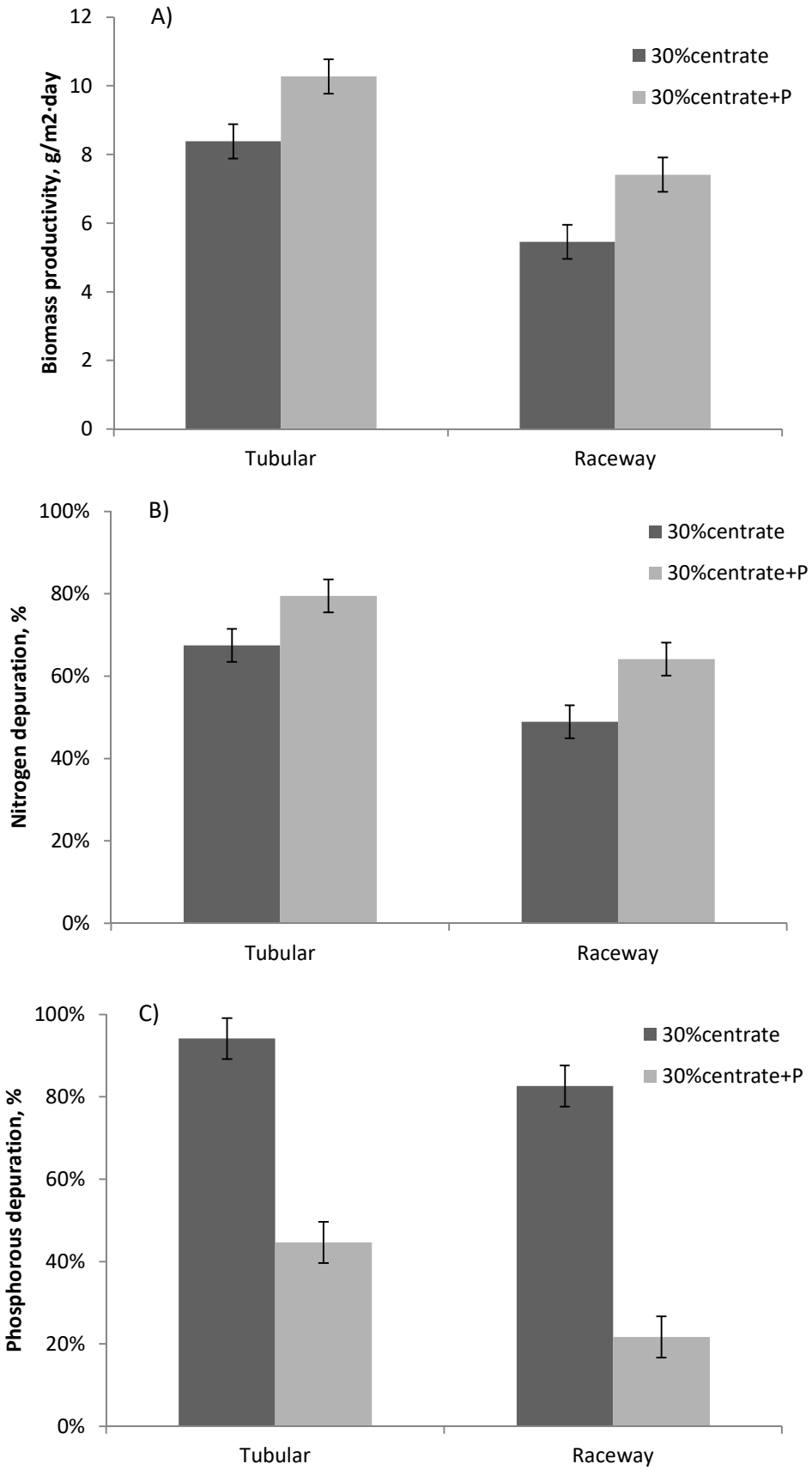


Figure 6.- Influence of addition of phosphorous to culture medium containing 30% of centrate on (A) the biomass productivity, (B) the nitrogen depuration and (C) the phosphorous depuration, of *Nannochloropsis gaditana* cultures carried out in raceway and tubular photobioreactors.

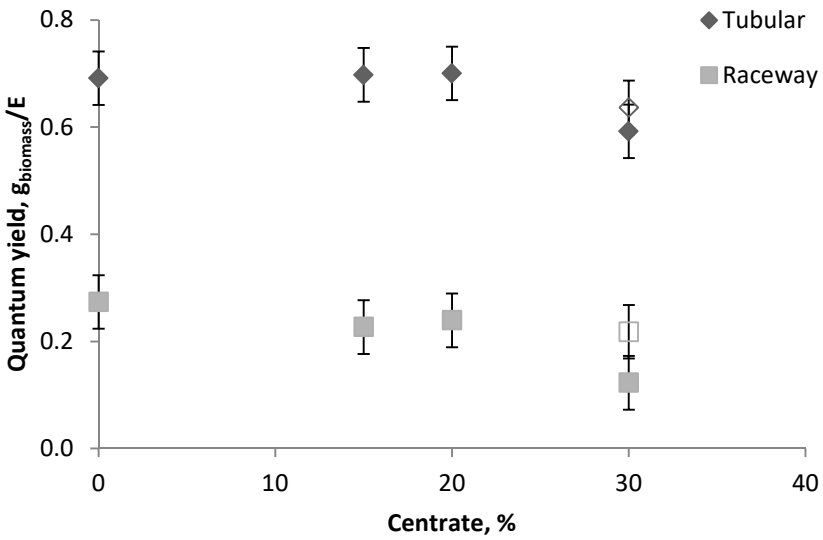


Figure 7.- Influence of centrate percentage in the culture medium on the quantum yield of *Nannochloropsis gaditana* cultures carried out in raceway and tubular photobioreactors. Open symbols correspond to experiments performed adding phosphorous to achieve a N:P ratio equal to 5.

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