1 # Utilization of centrate for the production of the marine microalgae

2 # *Nannochloropsis gaditana*

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4 C. Sepúlveda[1], F.G. Acién[2], C. Gómez[2], N. Jiménez-Ruíz[2], C. Riquelme[1], E. Molina-

5 Grima[2]

6 [1]Department of Applied Microbiology, University of Antofagasta, Chile

7 [2]Department of Chemical Engineering, University of Almería, 04120 Almería, Spain.

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9 Author for correspondence: F. Gabriel Acién Fernández

10 Department of Chemical Engineering, University of Almería

11 Carretera Sacramento s/n, E04120, Almería, Spain

12 Telephone: +34 950015443; Fax: +34 950015484; e-mail: facien@ual.es

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18 **Abstract**

19 In this paper, the production of the microalga *Nannochloropsis gaditana* using centrate

20 from the anaerobic digestion of treated urban wastewater is studied. For this,

21 semicontinuous cultures were performed indoors at laboratory scale, under controlled

22 conditions, supplying seawater with different centrate percentages from a real wastewater

23 treatment plant as the culture medium. It was demonstrated that *N. gaditana* can be

24 produced using solely centrate as the nutrient source but only at percentages below 50%.

25 Above this level, inhibition is caused by an excess of ammonia, thus reducing productivity.

26 In the 30-50% centrate range, biomass productivity was 0.4 $g \cdot l^{-1} \cdot day^{-1}$, equal to that

27 measured when using Algal culture medium. Moreover, the biochemical composition of the

28 biomass was also equal to that measured when using Algal culture medium, with the

29 protein content in the 30-40%d.wt. range; whereas the lipid content ranged from 20 to

30 25%d.wt. Under these conditions, phosphorus depuration from the culture medium was in

31 the 80-90% range while nitrogen depuration was only between 20 and 40%, indicating an

32 excess of nitrogen in the centrate with respect to phosphorus. In spite of this phosphorus

33 limitation, in the optimal centrate range (30-50% in the culture medium), the cells

34 performed under optimal conditions, removing up to 35 $mg_N \cdot l^{-1} \cdot day^{-1}$ and 5.7 $mg_P \cdot l^{-1} \cdot day^{-1}$,

35 with quantum yield values measuring 1.0-1.3 $g \cdot E^{-1}$. By supplying additional phosphorus, it

36 was possible to enhance productivity and increase nitrate and phosphorus depuration to

37 over 80%. The use of centrate is confirmed as a useful method for reducing microalgae

38 production costs while also increasing process sustainability, especially when using

39 biomass for bioenergy applications.

40

1. Introduction

Rising oil prices and global warming, associated with the burning of fossil fuels, has prompted a search for renewable, clean and carbon-neutral biofuels. In this scenario, microalgae have been proposed as a third-generation biofuel source given their high potential energy yield per hectare (Chisti, 2007; Mata et al., 2010). For this reason, considerable effort has been made recently to develop technologies for producing biofuels such as bio-diesel, bio-ethanol, bio-methane and bio-hydrogen from microalgae biomass (Rosenberg et al., 2008; Schenk et al., 2008). However, the process has not yet been exploited industrially as the high cost of microalgae biomass production is still too great to compete in the energy field, especially given the limited availability and cost of nutrients (Acién et al., 2012). When using clean water and artificial fertilizers, algae production costs are still very high, more than 5 €/kg of dry mass (Molina-Grima et al., 2003; Norsker et al., 2011; Acién et al., 2012).

Nitrogen and phosphorus, in addition to CO_2, are the main nutrients required for microalgae production. Approximately 5 t of nitrogen and 1 t of phosphorus are needed to produce 100 t of microalgae biomass. The production of these compounds as fertilizers is limited as well as being associated with high energy consumption and resultant CO_2 emissions - indeed, to produce 1 kg of NH_3, more than 10 kWh of energy is required. Consequently, using fertilizers as the nutrient source reduces the sustainability of microalgae-based processes (Lardon et al., 2009). On the other hand, nitrogen and phosphorus can be obtained from effluents such as wastewaters. Because of this, microalgae production using wastewater as the nutrient source is a very promising alternative, which offers added environmental advantages (Olguín, 2012; Pittman et al., 2011; Dong et al., 2014). As a result, microalgae can be produced from urban or animal wastewater using freshwater strains, at the same time helping to depure the wastewater itself (Olguín, 2003; Muñoz and Guieysse, 2006; Godos et al., 2010; Cabanelas *et al*., 2013). Microalgae production using wastewater, or other contaminated effluents, has additional advantages as microalgae are effective in removing organic matter, heavy metals and xenobiotics as well as inorganic nutrients (Hernández and Olguín, 2002; Olguín, 2003; Muñoz and Guieysse, 2006) thus producing cleaner effluents with high dissolved oxygen concentrations. Moreover, the heavy metal concentrations found in wastewater are many times lower than the toxic levels for most

72 microalgae strains (Dong *et al.*, 2014). Finally, wastewater depuration using microalgae

73 consumes 0.52 MJ/m^3 compared to a value of 3.6 MJ/m^3 when using conventional systems,

74 resulting in both economic and sustainability advantages (personal communication from

75 Aqualia).

76 Nonetheless, the utilization of wastewater limits biofuel production to freshwater

77 microalgae strains even though using seawater strains is actually the most sustainable way

78 to produce biofuels (Yang *et al.*, 2011). As an alternative, centrate from the anaerobic

79 digestion of activated sludge produced in wastewater treatment plants can be used as the

80 nutrient source to produce marine microalgae. There are two main advantages of using

81 centrate: (i) the nutrient content is much higher than in wastewater, and (ii) the presence of

82 aerobic microorganisms is scarce because they are produced under anaerobic conditions.

83 Inside wastewater treatment plants the centrate is recirculated to depurate it, meaning

84 higher energy consumption and greater cost. Utilizing centrate allows the nitrogen and

85 phosphorus contained within it to be reused and reduces the number of stages required in

86 the wastewater treatment plant, therefore reducing operating costs (Dong et al., 2014).

87 The centrate obtained from filtering the digestate (produced by anaerobic digestion) is the

88 most concentrated stream of ammonium/phosphorus to be found in wastewater treatment

89 plants. This centrate has already been used as the nutrient source to cultivate different

90 microalgae strains such as *Chlorella* sp. *Chlorella vulgaris*, and *Nannochloropsis salina* (Li

91 *et al.*, 2011; Cabanelas *et al.*, 2013; Dong *et al.*, 2014). Within the centrate, typical

92 ammonia and phosphate concentrations range from 400–800 mg·l^{-1} and 20–60 mg·l^{-1},

93 respectively. In addition to the concentration, the N/P ratio is also important because it

94 determines the nutrient, which potentially limits the growth. This ratio should be close to

95 the optimum nitrogen-to-phosphorus stoichiometry encountered in phytoplankton, which

96 has been described as falling within the 8–45 range (Klausmeler *et al.*, 2004). Centrate may

97 also contain certain constituents that inhibit microalgae growth such as urea, organic acids,

98 phenols and pesticides - at high concentrations these might limit the use of such effluents in

99 microalgae production (Kumar *et al.*, 2010). Consequently, research is needed to determine

100 the optimal centrate percentage that can be mixed with seawater to support algae growth for

101 whichever conditions apply. To examine this, a specific study looking at centrate from each

102 wastewater treatment plant should be carried out to evaluate its subsequent use as a nutrient

103 source in microalgae production.

104 The aim of this research is to determine the feasibility of producing *N. gaditana* microalgae

105 using centrate from a real wastewater treatment plant located in Almeria, in which not only

106 the productivity but also the quality of the biomass produced is analysed. To do this,

107 experiments were carried out using Algal culture medium as the standard alongside culture

108 media prepared by adding different centrate percentages to seawater. Mass balances were

109 then performed to determine nutrient yields, and the optical properties of the biomass were

110 analysed to determine the light-use efficiency of the cultures. The quality of the biomass

111 produced was also analysed.

112

113 **2. Materials and methods**

114 **2.1 Microorganism and culture media**

115 The marine microalgae Eustigmatophyceae *Nannochloropsis gaditana* Lubián CCMP 527

116 was selected because of its high growth rate and productivity under outdoor conditions (San

117 Pedro *et al.*, 2014). Culture inoculum was grown under controlled pH (8.0) and temperature

118 (25.0ºC) conditions in a 0.5 l flask, at an irradiance of 150 $\mu E \cdot m^{-2} \cdot s^{-1}$, using Algal medium

119 with 8 mM nitrate (Bionova, Santiago, Spain) in seawater (Fabregas *et al.*, 1984). This

120 medium contains 22.4 $mg \cdot l^{-1}$ of phosphorus and 890 $mg \cdot l^{-1}$ of $NaHCO_3$, in addition to

121 small amounts of iron, calcium, potassium, copper, etc. For the experiments, the culture

122 media were prepared using natural seawater. The control culture medium was prepared by

123 adding chemicals to natural seawater at standard concentrations corresponding to Algal

124 culture medium. Experimental culture media were prepared by mixing natural seawater

125 with different centrate percentages (10 to 80%v/v) taken directly from a real wastewater

126 treatment plant located in Almeria, Spain. The natural seawater was pumped directly from

127 the Mediterranean and filtered through 10, 5 and 1 μm pore-size filters prior to use. No

128 additional treatment was applied to the seawater or culture mediums used. Centrate was

129 obtained directly from the bed filter used in the wastewater treatment plant to separate the

130 solids from the digestate liquid fraction, gathered after the anaerobic digestion of activated

131 sludge produced from wastewater treatment. Therefore, this centrate did not contain solids

132 and was rich in ammonia and phosphorus, in addition to other compounds. A complete

133 analysis of the centrate used is shown in Table 1 while Table 2 shows a summary of the
134 main compounds within the different culture media used.

135

136 **2.2 Photobioreactors and culture conditions**

137 Experiments were carried out indoors in four polymetil-metacrilate bubble-column
138 photobioreactors (0.5 m in height, 0.09 m in diameter). The columns had a medium inlet as
139 well as a harvest valve, together with a pH sensor input at the top. Air was bubbled up from
140 the bottom of the column at 0.2 v/v min to agitate and remove the dissolved oxygen. The
141 temperature was maintained at 20ºC by controlling the air temperature in the chamber
142 within which the reactors were installed. To keep the pH within the optimum range (7.80-
143 7.85), pure CO_2 was injected on demand into the air stream at 0.01 v/v/min. For this, pH
144 5330 probes and an R21 pH-controller from Crison were used. The reactors were
145 artificially illuminated using 28W high-efficiency fluorescent tubes (Philips Daylight T5).
146 The illumination simulated the circadian cycle and two irradiance levels were assayed (300
147 and 500 $\mu E/m^2$ s). The irradiance value was experimentally measured as the mean value at
148 16 different positions; measurements were performed using a spherical SQS-100 Walz
149 GmbH quantum sensor (Effeltrich, Germany).

150 Growth experiments were performed simultaneously in all reactors, which were inoculated
151 with 10% of culture volume from the same standard inoculum. Following this, the reactors
152 were operated in batch mode for 6 days, after which time they were operated in
153 semicontinuous mode. Under these conditions, 25% of culture volume was harvested every
154 day and replaced with fresh culture media. This was carried out using membrane pulse
155 pumps that introduced fresh media into the reactors during the six central hours of daylight,
156 at 0.11 l·h^{-1}. This dilution rate (D) of 0.25 day^{-1} was previously defined as being optimal
157 under these culture conditions using Algal culture medium (Data not shown).
158 Semicontinuous operation was repeated daily until the culture parameters remained
159 constant, which meant for at least three days. In each experiment, the same culture
160 conditions were assayed in two reactors, thus each experimental condition was assayed in
161 duplicate. Measurements of the biomass concentration as well as the biomass and
162 supernatant characteristics were performed by taking fresh culture from the reactor whereas
163 the biochemical composition was determined from harvested biomass.

164

2.3 Biomass concentration, chlorophyll fluorescence, nutrient uptake and quantum yield

The dry-weight biomass concentration (Cb) was measured by filtering 50 ml of culture through 0.45 μm filters and drying it in an oven at 80ºC for 24 h. The cell status was checked daily by measuring the chlorophyll fluorescence (Fv/Fm) ratio with a fluorometer (AquaPen AP 100, Photon System Instruments, The Czech Republic). Nutrient uptake was measured by analysing the nitrogen and phosphorus at the reactor inlet and outlet. The depuration was calculated as the outlet to inlet concentration ratio (Eq. 1). The removal capacity was calculated as the amount of compound removed per time and culture volume unit (Eq. 2).

$$\text{Nutrient Depuration} = \frac{[\text{Nutrient}]_{\text{inlet}} - [\text{Nutrient}]_{\text{outlet}}}{[\text{Nutrient}]_{\text{inlet}}} \qquad \text{Eq. 1}$$

$$\text{Nutrient Removal} = ([\text{Nutrient}]_{\text{inlet}} - [\text{Nutrient}]_{\text{outlet}}) \cdot D \qquad \text{Eq. 2}$$

The optical properties of the biomass were measured in a CM-3500d Minolta spectrophotometer-colorimeter with Spectramagic 3.6 Software (Minolta, Germany). For this, a glass cuvette (3 cm wide, 4 cm high and 1 cm deep) was filled with 12 ml of culture and colour parameters were immediately obtained, in addition to transmittance at wavelengths ranging from 400 to 700 nm. These measurements were carried out directly on the culture within a few seconds, with no pretreatment or operation, such as centrifugation, extraction, etc. The most popular numerical colour–space system is the L*a*b* (also referred to as the CIE-LAB system) originally defined by the CIE in 1976. This system defines L* as a sample's colour lightness measurement, a* measures the red and green components while b* measures the yellow and blue. The extinction coefficient (Ka) was calculated by dividing the average absorption by the biomass concentration (Cb) and light path of the cuvette (p) (Eq. 3).

$$Ka = \frac{Abs}{Cb \cdot p} \qquad \text{Eq. 3}$$

The average irradiance (in the photosynthetically-active radiation range, PAR) at which cells are exposed inside a culture (Iav), is a function of the irradiance in the absence of cells (Io), the biomass extinction coefficient (Ka), the biomass concentration (Cb) and the light

190 path inside the reactor (p). It can be approximated using Equation 4 (Molina-Grima *et al.*,
191 1997).

$$Iav = \frac{Io}{(Ka{\cdot}p{\cdot}Cb)}(1 - \exp(- Ka{\cdot}p{\cdot}Cb))$$

Eq. 4

192 Quantum yield (Ψ_E) is defined in microalgal cultures as the amount of biomass generated
193 by the unit of radiation (usually a mole of photons) absorbed by the culture. Since it
194 represents the ratio of biomass generation to absorbed photon flux, it can be calculated by
195 Equation 5 (Molina-Grima *et al.*, 1997), where Pb stands for the volumetric biomass
196 productivity and Fvol for the photon flux absorbed in the volume unit. The photon flux
197 absorbed through the reactor volume may be obtained from the average irradiance (Iav) on
198 a culture volume basis using Equation 6 (Molina-Grima *et al.*, 1997).

$$\Psi_E = \frac{Pb}{F_{vol}}$$

Eq. 5

$$F_{vol} = Iav{\cdot}Ka{\cdot}Cb$$

Eq. 6

199

200 **2.4 Analytical Methods**

201 For culture medium and supernatant analysis, the standard official methods approved by the
202 Spanish Ministry of Agriculture were used (*Ministerio de Agricultura*, 1982). Phosphorus
203 was measured by visible spectrophotometry through the phospho-vanado-molybdate
204 complex. Nitrates were quantified using a spectrophotometer between 220 and 275 nm.
205 Ammonium was measured by the Nessler reactive method. With regards to biomass,
206 freeze-dried biomass from each steady state was analysed. Lipids were determined
207 gravimetrically from an extract obtained with chloroform:methanol (2:1) (v/v) (Kochert,
208 1978). The protein content was determined using the Lowry method. Fatty acids were
209 determined by gas chromatography (Rodríguez-Ruiz *et al.*, 1998).

210

211 **3 Results**

212 The centrate composition used is shown in Table 1. It was observed that, even though the
213 centrate was obtained from wastewater treatment plants, the effluent salinity was high (4.55
214 mmhos/cm), with an approximate total salt content of 3.6 $g{\cdot}l^{-1}$, much higher than
215 freshwater, which typically contains less than 1.0 $g{\cdot}l^{-1}$ of salt. The main compounds

216 contained in this centrate were chloride and bicarbonates, in addition to a high ammonium

217 concentration (up to 615 mg·l^{-1}) whereas the nitrate and phosphorus contents were lower, at

218 29 and 36.0 mg·l^{-1}, respectively. In addition to this, the centrate contained relevant amounts

219 of calcium, potassium, iron, etc., all necessary for microalgae growth. Mixtures prepared

220 solely by adding this centrate to seawater showed a salinity reduction as the centrate

221 percentage increased, because of the lower centrate salinity compared to that of the

222 seawater (Table 2). This is important because a reduction in salinity can be a stress factor in

223 the growth of marine strains such as *N. gaditana*. With respect to the nutrients, by

224 comparing the composition of the Algal culture medium to mixtures that used centrate as

225 the nutrient source, the nitrogen content of the Algal culture medium was observed to be

226 comparable to mixtures obtained using a centrate percentage of 30%; whereas in order to

227 achieve a phosphorus content comparable to that in the Algal culture medium, at least 50%

228 of centrate needed to be mixed with seawater. Due to the variation in these three parameters

229 (salinity, nitrogen and phosphorus), an optimal centrate percentage in the culture medium

230 cannot be defined without experimental determination. Moreover, because nitrogen is in the

231 form of ammonium rather than nitrate, *N. gaditana* tolerance to high concentrations of

232 ammonium also has to be studied.

233 Experiments performed in semicontinuous mode at a 0.25 day^{-1} dilution rate demonstrated

234 that centrate can be used as the nutrient source in the production of *N. gaditana*. Steady

235 states were obtained when using centrate percentages between 20 and 70% (Figure 1A); the

236 biomass productivity being higher the greater the irradiance in the experiments performed.

237 Below 20% of centrate, the nutrient content of the culture medium was insufficient to

238 support growth at the imposed dilution rate, the culture being washed out even at the higher

239 irradiance. At 80% of centrate, the ammonium concentration was so high that inhibition

240 reduced the growth rate to below the imposed dilution rate, meaning the culture was also

241 washed out at both irradiances tested. Moreover, above a 50% centrate level, the

242 ammonium concentration was excessive and biomass productivity was reduced when

243 compared to maximal values obtained at 30 to 50% centrate levels. The fact that maximal

244 biomass productivity was measured at these centrate percentage levels demonstrated that,

245 under these conditions, the reduction in salinity had no adverse effect on *N. gaditana*

246 biomass productivity. Moreover, the biomass productivity measured within this centrate

247 percentage range was equal to that measured using Algal culture medium in seawater at
248 both irradiances tested. The adverse effect of too high a centrate percentage was also
249 observed in the chlorophyll fluorescence measurements (Figure 1B). At centrate
250 percentages higher than 50%, a reduction in chlorophyll fluorescence was clearly observed,
251 dropping from values of 0.65, measured under normal conditions, to 0.45, measured when
252 70% of centrate was used in the culture medium. No measurements were performed at 10%
253 and 80% of centrate because the cultures were washed out. In these cases, no irradiance
254 influence was observed on chlorophyll fluorescence values. To confirm that the adverse
255 behaviour of the culture (when increasing the percentage of centrate) was not due to salinity
256 reduction, an additional set of experiments was performed using Algal medium prepared on
257 seawater diluted with freshwater at different percentages. Results showed that biomass
258 productivity remained constant up to freshwater percentages of 75%; while using only
259 freshwater with no seawater, a notable reduction in biomass productivity occurred (Figure
260 2A). Moreover, a similar trend was observed for chlorophyll fluorescence measurements,
261 experimental values remaining constant (and higher than 0.6) when using percentages of
262 freshwater in the culture medium up to 75%; and only reducing to 0.4 when using Algal
263 medium prepared in freshwater with no seawater (Figure 2B).

264 Regarding the culture medium, nitrogen and phosphorus analysis at the reactor inlets and
265 outlets allowed us to calculate the depuration rate (the percentage of compounds removed
266 compared to the inlet value) and removal capacity (the amount of compounds removed per
267 time and culture volume unit) (Figure 3). The nitrogen depuration values that were
268 measured in Algal culture medium were 55% and 41% at the higher and lower irradiances
269 tested, respectively; thus indicating that this culture medium had an excess of nitrogen
270 allowing *N. gaditana* growth to be maintained under the culture conditions used (Figure
271 3A). In experiments performed using centrate, the results showed that nitrogen depuration
272 was higher, the higher the irradiance, and reduced when the centrate percentage was
273 increased. The maximal value was 80% when using 20% of centrate and high irradiance,
274 but it reduced to 23% when centrate percentages were higher than 50% whatever the
275 irradiance. The reduction in nitrogen depuration occurring when the centrate percentage
276 was increased in the culture medium is related to a greater excess of nitrogen as well as to
277 the lower biomass productivity achieved at centrate percentages in the culture medium

278 higher than 50%. Regarding phosphorus, the phosphorus depuration values measured using
279 Algal culture medium were 60% and 44% at the higher and lower irradiances tested,
280 respectively: indicating that even this culture medium had an excess of phosphorus to
281 maintain *N. gaditana* growth under the culture conditions (Figure 3B). Concerning
282 experiments performed on culture media using centrate, the results showed varying
283 behaviour according to the irradiance. At low irradiance, phosphorus depuration increased
284 with an increase in the centrate percentage up to a value of 50% - a maximal value of 86%
285 being measured. Above 50% of centrate, phosphorus depuration reduced. At high
286 irradiance, phosphorus depuration was high, even at low centrate percentages - with a
287 maximal value of 92%; however, it also reduced when centrate percentages above 50%
288 were used, dropping to 34% when a centrate percentage of 70% was present in the culture
289 medium. The high phosphorus depuration values measured when using low centrate
290 percentages in the culture medium, along with high irradiance, indicate that under these
291 conditions the cultures can be phosphorus limited. It is important to note that European
292 Directive 98/15/EC establishes a water release limit of 10 $mg \cdot l^{-1}$ for nitrogen and 1 $mg \cdot l^{-1}$
293 for phosphorus. In all cases, the outlet nitrogen concentrations present were higher than 10
294 $mg \cdot l^{-1}$, whatever the centrate percentage used in the culture medium; whereas the
295 phosphorus concentration was only lower than 1 $mg \cdot l^{-1}$ when using high irradiance and
296 centrate percentages below 40%. Regarding the removal capacity, results showed that, for
297 Algal medium, the nitrogen removal capacity was 15.5 and 11.5 $mg_N \cdot l^{-1} \cdot day^{-1}$ at the higher
298 and lower irradiances tested, respectively (Figure 3C). When using centrate, the nitrogen
299 removal capacity was constant and equal to that measured for Algal medium up to centrate
300 percentages of 40%. Above this value, the nitrogen removal capacity increased, especially
301 at centrate percentages higher than 50%. Maximal nitrogen removal of 35 $mg_N \cdot l^{-1} \cdot day^{-1}$
302 was measured using 80% of centrate. Regarding phosphorus, results showed that when
303 using Algal medium, the removal capacity was 3.4 and 2.5 $mg_P \cdot l^{-1} \cdot day^{-1}$ at the higher and
304 lower irradiances tested, respectively (Figure 3D). Using centrate, the phosphorus removal
305 capacity increased along with increased centrate percentage in the culture medium, up to
306 50% - with a maximal value of 5.1 $mg_P \cdot l^{-1} \cdot day^{-1}$ being measured. Above 50% of centrate,
307 the phosphorus removal capacity reduced.

308 The optical properties of the biomass were also measured because these affect light
309 availability inside the culture. To do this, the influence of the culture conditions on the
310 extinction coefficient and the biomass colour were studied (Figure 4). The biomass
311 extinction coefficient produced in Algal culture medium was 0.19 and 0.21 $m^2 \cdot g^{-1}$ when
312 using the lower and higher irradiance levels tested, respectively. These values were similar
313 to those obtained with 30 to 50% of centrate in the culture medium. Outside of these values,
314 the biomass extinction coefficient was lower, indicating lower biomass light absorption
315 under these conditions. No irradiance influence was observed on the biomass extinction
316 coefficient. Regarding colour measurements, these were performed to indicate changes in
317 the biochemical composition of the biomass. Colour space CIELAB L*a*b* was used; the
318 a* parameter corresponding to variations in colour from magenta to green whereas the b*
319 parameter corresponded to variations in colour from yellow to blue. Results showed a
320 similar trend to that observed in the extinction coefficient. The colour of the samples
321 obtained from centrate were approximate to the colour obtained using Algal culture
322 medium when centrate percentages were between 30 to 50%. Outside of this range, the
323 colour of the samples changed from green to brown with a* increasing from -7.0 to -4.3
324 while b* reduced from 57.5 to 45.5. Colour measurements are a rapid and precise method
325 to determine a sample's "aspect". For this reason they can be used as a control parameter
326 for determining deviation from optimal culture conditions.

327 The biochemical composition of the biomass was also influenced by the centrate percentage
328 used in the culture medium as well as by the irradiance (Figure 5). Results showed that both
329 the protein and the lipid contents were higher at the higher irradiance, including when Algal
330 culture medium was used. When centrate was used, the protein content reduced along with
331 increased centrate percentage in the culture medium up to 40%, then remained constant.
332 The highest protein content, of 49%d.wt., was measured when using 20% of centrate and at
333 the higher irradiance; this was far greater than the 36%d.wt. measured under the same
334 conditions using Algal culture medium. The biomass protein content agreed with that
335 measured using Algal culture medium at centrate percentages above 40% for both
336 irradiance levels tested, thus indicating a metabolism change when using low centrate
337 percentages due to phosphorus limitation. With regard to lipids, much smaller variations
338 were observed, the mean lipid content measured at both irradiances were in agreement with

339   those measured using Algal culture medium under the same conditions. Mean lipid contents
340   of 24.5% and 22.0%d.w.t were measured at 500 and 300 $\mu E/m^2 \cdot s$, respectively. Fatty acids
341   analysis was also performed. As expected, the results showed that the main fatty acids were
342   20:5n3, 16:1n7 and 16:0 - however, their profile changed according to the composition of
343   the culture medium used (Figure 6). When using Algal medium, 20:5n3 was 33%; whereas
344   when using centrate, its percentage increased from a minimum value of 23% (using 20% of
345   centrate) to a maximum of 38% (with 30-40% of centrate); it reduced again to 25% when
346   using 70% of centrate in the culture medium. Contrary behaviour was observed for 16:0
347   and 16:1n7, starting from high values of 32% and 29%, respectively, when using 20% of
348   centrate, which reduced to 18% and 15%, respectively, when the centrate percentage was
349   increased to 30-50%. Then the percentage of 16:0 and 16:1n7 increased the higher the
350   centrate percentage in the culture medium, up to values of 30% and 22%, respectively,
351   when using 70% of centrate. There was no observed influence of irradiance on this
352   behaviour. From these data, it can be concluded that the fatty acid profile is related to
353   biomass productivity, 20:5n3 increased with biomass productivity whereas 16:0 and 16:1n7
354   increased when biomass productivity fell.
355
356   **4. Discussion**
357   The use of marine strains has been reported as the most sustainable way to produce biofuels
358   from microalgae because no freshwater is required. However, the nitrogen and phosphorus
359   content of seawater is too low to support high microalgae biomass productivity. To solve
360   this problem, nitrogen and phosphorus can be added as fertilizers but this strategy increases
361   production costs and reduces process sustainability. The alternative is to use residuals from
362   other industries as the nutrient source - of these, the utilization of centrate from wastewater
363   treatment plants is an interesting alternative. Centrate is obtained by separating solids from
364   the anaerobic digestion of activated sludge. It contains high concentrations of nitrogen and
365   phosphorus, the nitrogen mainly in the form of ammonium. To demonstrate if centrate is
366   useful as a nutrient source in microalgae biomass production, it is necessary to define the
367   tolerance limits of the selected strain. Two limits must be defined, the minimum centrate
368   percentage that allows productive cultures to be maintained, and the maximum centrate

369 percentage that inhibits growth. Between these two limits, the optimal percentage selection
370 must be defined as a function of biomass productivity and depuration efficiency.

371 The results reported here demonstrate that centrate from a real wastewater treatment plant
372 can be used to produce *N. gaditana* in seawater. The productivity values obtained were
373 similar to those measured using Algal culture medium (Figure 1), with no stress observed
374 caused by centrate dilution with seawater (Figure 2). It was demonstrated that *N. gaditana*
375 can utilize ammonium as the nitrogen source although concentrations higher than 300 mg·l^{-1}
376 $^{-1}$ in the inlet medium (corresponding to 50% of centrate) cause stress to the cells and reduce
377 productivity; meaning a maximum centrate percentage of 50% can be used. Ammonium
378 has been reported as toxic for microalgae strains when above 100 mg·l^{-1} (Collos and
379 Harrison, 2014). For instance, *C. sorokiniana* was completely inhibited at an ammonium
380 concentration of 210 mg·l^{-1} (Muñoz *et al.*, 2005) whereas *Spirulina platensis* was nearly
381 completely inhibited at 150 mg·l^{-1} (Ogbonna *et al.*, 2000). *Chlorella sorokiniana* presented
382 similar growth to the artificial medium in 4 to 8-times-diluted pig slurry, whilst severe
383 biodegradation process inhibition was recorded in undiluted and twice-diluted wastewater
384 (González *et al.*, 2008). Using *N. Salina,* it was demonstrated that nitrogen can be supplied
385 by adding up to 75% of centrate to a culture medium; above this value, productivity
386 decreases (Dong *et al.*, 2014). Conversely, if the centrate percentage is lower than 20%, the
387 culture is nutrient limited, and productivity is reduced. The limiting nutrient is not nitrogen,
388 as might be expected, but phosphorus, as observed in the nutrient removal results. The N/P
389 centrate ratio is 13.3, in the 8-45 ratio range encountered in phytoplankton (Klausmeler *et*
390 *al.*, 2004). It has been reported that this range can be modified depending on the culture
391 conditions and the species involved, a factor which determines the most suitable species
392 under different conditions (Klausmeler *et al.*, 2004). Although the centrate composition
393 can change according to the particular wastewater treatment plant's operating conditions,
394 no great variations in the nitrogen/phosphorus ratio are expected, meaning that the centrate
395 percentage range that can be incorporated into the culture medium is defined as being
396 between 30% and 50%. Given that 30% of centrate in the culture medium allows one to
397 maintain high productivity (Figure 1) along with higher depuration efficiency (Figure 3),
398 compared to using 50% of centrate, the former percentage is recommended.

399   Biomass production is accomplished by taking up nutrients from the culture medium, the

400   depuration ranging from 80 to 20% and from 92 to 34% for phosphorus and nitrogen,

401   respectively. Similarly high depuration rates have also been reported with freshwater

402   microalgae (Craggs *et al*., 1997; Sydney *et al*., 2011). With settled domestic sewage and

403   secondary-treated domestic effluent, supplemented with settled swine wastewater, the

404   nitrogen depuration was in the 92–95% range although the phosphate depuration was

405   lower, at approximately 62–80% (Wang *et al*., 2010). However, as depuration is a function

406   of the net concentration supplied, then removal capacity is a more adequate parameter to

407   compare different strains/systems. Data here reported demonstrate that the nitrogen

408   removal capacity using Algal medium (hence the use of nitrate as the nitrogen source) was

409   equal to that measured using centrate. Accordingly, ammonium was used as the nitrogen

410   source when centrate levels below 50% were used. Under these conditions, the cultures

411   were phosphorus limited, the biomass productivity and the phosphorus removal capacity

412   increased when increasing the centrate percentage in the culture medium up to 50%. Above

413   this value, biomass productivity reduced because of ammonium inhibition and,

414   consequently, the phosphorus removal capacity reduced. Nonetheless, nitrogen removal

415   still increased, indicating there was a relevant contribution from ammonium stripping when

416   operating at such high ammonium concentrations - in spite of the pH being controlled at

417   8.0. The maximal nitrogen and phosphorus removal capacity values were 35 $mg_N \cdot l^{-1} \cdot day^{-1}$

418   and 5.7 $mg_P \cdot l^{-1} \cdot day^{-1}$, respectively. Using *C. Vulgaris,* a maximal removal capacity of 9.8

419     $mg_N \cdot l^{-1} \cdot day^{-1}$ and 3.0 $mg_P \cdot l^{-1} \cdot day^{-1}$ were reported using centrate (Cabanelas *et al*., 2013).

420   On the other hand, nitrogen removal of 8.5 mg_N $l^{-1} \cdot day^{-1}$ was reported for *Chlorella*

421   cultures using ten-fold diluted centrate; this value increasing to 22.7 mg_N $l^{-1} \cdot day^{-1}$ under

422   optimal conditions (Marcilhac *et al*., 2014). A similar trend was reported for pig manure,

423   with nitrogen removal ranging from 0.5 to 12 mg_N $l^{-1} \cdot day^{-1}$ (Sevrin-Reyssac, 1998).

424   The most intensive parameter for the design and operation of any bioprocess is the

425   coefficient yield. This is the amount of biomass produced per mass unit of nutrient removed

426   from the culture medium. In our study, this parameter was calculated (Figure 7) using

427   biomass productivity values and nutrient concentrations (nitrogen and phosphorus) entering

428   and leaving the reactor. It can be observed that the nitrogen coefficient yield when using

429   Algal culture medium was equal to the expected value of 20 $g_b \cdot g_N^{-1}$, which correspond to a

430   nitrogen content in the biomass of 5%d.wt. Using centrate, the same value was obtained

431   when the centrate percentage was in the optimal value range for maximizing biomass

432   productivity, namely from 30% to 50%. However, outside this range, the nitrogen

433   coefficient yield reduced, so less biomass was produced with the same amount of nitrogen

434   removed from the culture. With respect to phosphorus, the coefficient yield measured using

435   Algal culture medium was in the 90 $g_b \cdot g_P^{-1}$ range. However, using centrate, the coefficient

436   yield varied significantly from 150 to 20 $g_b \cdot g_P^{-1}$ when the centrate percentage in the culture

437   medium was modified. The optimal phosphorus coefficient yield value was obtained at the

438   optimal centrate percentage in the culture medium - previously defined at 30%, meaning

439   125 and 150 $g_b \cdot g_P^{-1}$ for the lower and higher irradiances tested, respectively. These values

440   correspond to the expected approximate biomass composition, containing 5%d.wt. of

441   nitrogen and 1%d.wt. of phosphorus. Reported values for *Nannochloropsis salina* were 20

442    $g_b \cdot g_N^{-1}$ and 143 $g_b \cdot g_P^{-1}$ (Dong *et al*., 2014), whereas for *Chlorella vulgaris* values of 20

443    $g_b \cdot g_N^{-1}$ and 65 $g_b \cdot g_P^{-1}$ (Cabanelas *et al*., 2013) were reported; centrate was also used as the

444   culture medium in this case. Using secondary-treated wastewater for the production of

445   *Muriellopsis* sp., it was observed that the nitrogen and phosphorus coefficient yields

446   approached 20 $g_b \cdot g_N^{-1}$ and 100 $g_b \cdot g_P^{-1}$, respectively, when operating under nutrient-

447   sufficient conditions, whereas under severe nitrogen conditions, they decreased to 10 $g_b \cdot g_N^{-1}$

448   and 6 $g_b \cdot g_P^{-1}$ (Gómez *et al*., 2012). Values of 15 $g_b \cdot g_N^{-1}$ and 14 $g_b \cdot g_P^{-1}$ were reported using

449   *C. vulgaris* and artificial culture mediums with up to 400 $mg \cdot l^{-1}$ of $N-NH_4^+$ (Aslan and

450   Kapdan, 2006). Large variations exist in the reported coefficient yield values due to

451   different strains and culture conditions being used, but also due to different phenomena

452   taking place in each one.

453   The culture medium composition also influenced the optical properties of the biomass and,

454   consequently, the average irradiance inside the culture and the quantum yield. It is

455   important to study these variables because, in addition to nutrients, light-use efficiency

456   must be maximized in whichever microalgae production system used. Analysis of the

457   extinction coefficient and the biomass colour demonstrated that, outside the optimal

458   centrate range in the culture medium, the extinction coefficient diminished meaning more

459   light had to be made available to the cells. Moreover, the light quality inside the reactor was

460   also altered when the centrate percentage was modified because the biomass colour changes

461 from green to brown when the centrate percentage in the culture medium is outside the

462 optimal range. In terms of light availability, results showed that within the optimal centrate

463 percentage range in the culture medium, light availability inside the culture was the lowest,

464 with values of 25-30 $\mu E \cdot m^{-2} \cdot s^{-1}$, and equal to those when using Algal culture medium

465 (Figure 8A). Outside this optimal range, light availability increased but biomass

466 productivity decreased, thus indicating that cells were not capable of utilising this increased

467 light availability. This is because, at 20% of centrate, the culture is phosphorus-limited

468 whereas above 50%, the culture is inhibited by an excess of ammonium. These phenomena

469 were better observed when analysing the quantum yield values obtained (Figure 8B). It was

470 shown that at the optimal centrate range in the culture medium, the quantum yield was

471 maximal, with values up to 1.16 $g \cdot E^{-1}$, comparable to the 0.9 $g \cdot E^{-1}$ value obtained using the

472 Algal culture medium. However, when using centrate percentages outside the optimal

473 range, the quantum yield reduced to values of 0.4 and 0.3 $g \cdot E^{-1}$, thus demonstrating lower

474 light-use efficiency under these conditions. In cultures performed with *Muriellopsis* sp., the

475 quantum yield reduced from 0.6-0.7 $g \cdot E^{-1}$ under no, or low, nitrogen limitation to 0.38 $g \cdot E^{-1}$

476 under severe nitrogen limitation (Gómez *et al*., 2012), meaning this strain is less energy

477 efficient under these conditions. The quantum yield has been reported as reaching

478 maximum values of 0.65 $g \cdot E^{-1}$ when culturing *Isochrysis galbana* at low light under

479 optimal conditions (Molina-Grima *et al*., 1997), reducing to 0.1 $g \cdot E^{-1}$ under high light

480 conditions - to the point of causing photoinhibition. Values reported here were higher,

481 indicating this strain's greater efficiency in using light and that the cultures were highly

482 photolimited.

483 With respect to the quality of the biomass produced, results demonstrated that the

484 utilization of centrate as the culture medium in the optimal percentage range had little

485 influence on the biomass's protein and lipid content when compared to values using the

486 Algal culture medium. No lipid enhancement was determined by modifying the centrate

487 percentage in the culture medium, a mean lipid content of 23%d.wt. being measured. Only

488 an increase in the protein content using low centrate percentages was determined, with

489 maximal values of 50% d.wt., whereas under optimal production conditions, the mean

490 protein content was 34% d.wt. Because an increase in protein content is not accomplished

491 by a decrease in the lipid content, it is believed that the carbohydrate content is reduced

492   under these conditions although this was not measured. Such behaviour indicates that

493   phosphorus limitation might limit the production of carbohydrates, thus enhancing the

494   accumulation of proteins in the biomass. Whatever the reason, as there is little interest in

495   carbohydrate production, this variation has no great consequence from an application point

496   of view. Variation in the fatty acid profile is more relevant when the centrate percentage is

497   modified in the culture medium. Results demonstrated that the 20:5n3 fatty acid profile was

498   richest when using optimal centrate percentages in the culture medium, concurrent with

499   maximal biomass productivity, thus confirming the role of polyunsaturated fatty acids as

500   structural lipids and, consequently, as primary metabolites in this strain. Conversely, under

501   these optimal growth conditions, the proportion of 16:0 and 16:1n7 reduced, indicating

502   lower storage lipid accumulation. It was previously reported that under nitrogen-limited

503   conditions, the percentage of saturated fatty acids increases with respect to values obtained

504   under nitrogen-saturated conditions (San Pedro *et al.*, 2014). Similarly, it was previously

505   reported (Sukenik *et al.*, 1993) that saturated fatty acids increase when irradiance

506   availability increases; with average irradiance inside the culture increasing under nitrogen-

507   limited conditions. Variation in the fatty acids profile is relevant when producing biodiesel,

508   given that saturated and monounsaturated fatty acids are preferred, and *N. gaditana*

509   production using centrate does not favour these fatty acids and thus may be a disadvantage

510   for biofuel production under these conditions.

511   Finally, to demonstrate the existence of phosphorus limitation when centrate is used as the

512   sole nutrient source, an additional set of experiments was carried out using 20% of centrate

513   as the culture medium (N-NH$_4$=95.7 mg·l^{-1}, P-PO$_4$=7 mg·l^{-1}), but supplying additional

514   NaH$_2$PO$_4$ to achieve a N/P ratio equal to 5, the same as for Algal medium. Results obtained

515   under these conditions are summarized in Figure 9. It was shown that by supplying

516   additional phosphorus, the productivity increased (even when using only 20% of centrate

517   with lower biomass productivity than at higher centrate percentages). Biomass productivity

518   measured under these conditions was 0.23 and 0.33 g·l^{-1}·day^{-1}, approaching that measured

519   using the Algal culture medium (0.27 and 0.37 g·l^{-1}·day^{-1}), at the lower and higher

520   irradiance levels tested, respectively. Moreover, phosphorus removal remained in the 80%

521   range when using both 20% of centrate and 20% of centrate enriched with phosphorus as

522   the culture medium; whereas nitrogen removal greatly increased when supplying additional

phosphorus. Therefore, when using only centrate, nitrogen removal was 46 and 51% for the lower and higher irradiance levels tested, respectively; whereas by supplying additional phosphorus, the nitrogen removal increased to 79 and 85% for the same levels tested.

In summary, although centrate can be used to produce *N. gaditana* biomass for biofuel production, this needs to be supplemented with additional phosphorus. Ideally, residual phosphorus can be used although this compound is scarce. The utilization of commercial fertilizers rich in phosphorus can only be acceptable for minimizing the supernatant nitrogen content if the phosphorus is released into the environment; otherwise, the utilization of external phosphorus sources reduces both the sustainability and economic viability of the overall biofuel production process.

5. Conclusions

It has been demonstrated that the marine microalgae *N. gaditana* can be produced using centrate as the sole nutrient source. The enrichment of seawater with up to 50% of centrate allows one to achieve higher biomass productivity, comparable to that obtained using standard Algal culture medium, although the cultures become phosphorus limited. Likewise, the quality of the biomass produced is comparable to that obtained using standard Algal culture medium; although regulations concerning the utilization of centrate as the nutrient source might limit its commercial application in producing biomass. Nonetheless, for biofuel production (or related compounds), these types of extraction regulations are not envisaged, meaning the utilization of centrate to produce microalgae would be a cheap and sustainable method for third-generation biofuel production.

6. Acknowledgements

7. References

Acién FG, Fernández JM, Magán JJ, Molina-Grima E. 2012. Production cost of a real microalgae production plant and strategies to reduce it. Biotechnol Adv. 30(6): 1344–1353.

Aslan S, Kapdan IK. 2006. Batch kinetics of nitrogen and phosphorus removal from synthetic wastewater by algae. Ecol.Eng. 28(1): 64-70.

Cabanelas ITD, Ruiz J, Arbib Z, Chinalia FA, Garrido-Pérez C, Rogalla F, Nascimento IA, Perales JA. 2013. Comparing the use of different domestic wastewaters for coupling microalgal production and nutrient removal. Bioresour Technol 131:429-436.

Chisti Y. 2007. Biodiesel from microalgae. Biotechnol Adv 25: 294-306.

Collos Y, Harrison PJ. 2014. Acclimation and toxicity of high ammonium concentrations to unicellular algae. Mar Pollut Bull 80:8-23.

Craggs RJ, McAuley PJ, Smith VJ. 1997. Wastewater nutrient removal by marine microalgae grown on a corrugated raceway. Water Res 31: 1701-1707.

Dong B, Ho N, Ogden KL, Arnold RG. 2014. Cultivation of Nannochloropsis salina in municipal wastewater or digester centrate. Ecotoxicol Environ Saf 103:45-53.

Fábregas J, Herrero C, Cabezas B, Abalde J. 1984. Growth of the marine microalga *Tetraselmis suecica* in batch cultures with different salinities. Aquaculture; 42: 207-215.

Godos Id, Vargas VA, Blanco S, González MCG, Soto R, García-Encina PA, Becares E, Muñoz R. 2010. A comparative evaluation of microalgae for the degradation of piggery wastewater under photosynthetic oxygenation. Bioresour Technol 101: 5150-5158.

Gómez C, Escudero R, Morales MM, Figueroa FL, Fernández-Sevilla JM, Acién FG. 2013. Use of secondary-treated wastewater for the production of *Muriellopsis* sp. Appl Microbiol Biotechnol 97:2239-2249.

González C, Marciniak J, Villaverde S, García-Encina PA, Muñoz R. 2008. Microalgae-based processes for the biodegradation of pretreated piggery wastewaters. Appl Microbiol Biotechnol 80:891-898.

Hernández E, Olguín EJ. 2002. Biosorption of heavy metals influenced by the chemical composition of *Spirulina* sp. (Arthrospira) biomass. Environ Technol 23: 1369-1377.

Klausmeler CA, Litchman E, Daufreshna T, Levin SA. 2004. Optimal nitrogen-to-phosphorus stoichiometry of phytoplankton. Nature 429: 171-174.

Kochert G. 1978. Handbook of phycological methods. London:Cambridge University Press.

Kumar A, Ergas S, Yuan X, Sahu A, Zhang Q, Dewulf J, Malcata FX, van Langenhove H. 2010. Enhanced CO_2 fixation and biofuel production via microalgae: Recent developments and future directions. Trends Biotechnol 28: 371-380.

Lardon L, Hélias A, Sialve B, Steyer JP, Bernard O. 2009. Life-cycle assessment of biodiesel production from microalgae. Environ Sci Technol 43: 6475-6481.

Li Y, Chen Y-, Chen P, Min M, Zhou W, Martinez B, Zhu J, Ruan R. 2011. Characterization of a microalga *Chlorella* sp. well adapted to highly concentrated municipal wastewater for nutrient removal and biodiesel production. Bioresour Technol 102: 5138-5144.

Marcilhac C, Sialve B, Pourcher A-, Ziebal C, Bernet N, Béline F. 2014. Digestate color and light intensity affect nutrient removal and competition phenomena in a microalgal-bacterial ecosystem. Water Res 64:278-287.

Mata TM, Martins AA, Caetano NS. 2010. Microalgae for biodiesel production and other applications: A review. Renew Sust Energ Rev 14:217-232.

Ministerio de Agricultura. 1982. Métodos oficiales de análisis: suelos y aguas. Ed. Ministerio de Agricultura, Madrid, Spain.

Molina-Grima, E. García Camacho, J.A. Sánchez Pérez, F.G. Acién Fernández, J.M. Fernández Sevilla. 1997. Evaluation of photosynthetic efficiency in microalgal cultures using averaged irradiance, Enz Microbial Technol, 21(5): 375-381

Molina-Grima, E., Acién, F.G., Medina, A.R. 2003. Downstream Processing of Cell-Mass and Products. In Handbook of Microalgal Culture, Blackwell Publishing Ltd, pp. 215-252.

Muñoz R, Jacinto M, Guieysse B, Mattiasson B. 2005. Combined carbon and nitrogen removal from acetonitrile using algal-bacterial bioreactors. Applied Microbiology and Biotechnology 67:699-707.

Muñoz R, Guieysse B. 2006. Algal-bacterial processes for the treatment of hazardous contaminants: A review. Water Res 40: 2799-2815.

Norsker NH, Barbosa MJ, Vermuë MH, Wijffels RH. 2011. Microalgal production: A close look at the economics. Biotechnol Adv 29(1): 24-27.

Olguín EJ, Galicia S, Mercado G, Pérez T. 2003. Annual productivity of *Spirulina* (*Arthrospira*) and nutrient removal in a pig wastewater recycling process under tropical conditions. J Appl Phycol 15: 249-257.

Olguín EJ. 2003. Phycoremediation: Key issues for cost-effective nutrient removal processes. Biotechnol Adv 22: 81-91.

Olguín EJ. 2012. Dual purpose microalgae-bacteria-based systems that treat wastewater and produce biodiesel and chemical products within a Biorefinery. Biotechnol Adv 30: 1031-1046.

Pittman JK, Dean AP, Osundeko O. 2011. The potential of sustainable algal biofuel production using wastewater resources. Bioresour Technol 102: 17-25.

Rodríguez-Ruiz J, Belarbi E, García J, López D. 1998. Rapid simultaneous lipid extraction and transesterification for fatty acid analyses. Biotechnol Tech 12: 689-691.

Rosenberg JN, Oyler GA, Wilkinson L, Betenbaugh MJ. 2008. A green light for engineered algae: redirecting metabolism to fuel a biotechnology revolution. Curr Opin Biotechnol 19: 430-436.

San Pedro A, González-López CV, Acién FG, Molina-Grima E. 2014. Outdoor pilot-scale production of *Nannochloropsis gaditana*: influence of culture parameters and lipid production rates in tubular photobioreactors. Bioresour Technol http://dx.doi.org/10.1016/j.biortech.2014.07.052.

Sevrin-Reyssac J. 1998. Biotreatment of swine manure by production of aquatic valuable biomasses. Agric , Ecosyst Environ 68:177-186.

Schenk PM, Thomas-hall SR, Stephens E, Marx UC, Mussgnug JH, Posten C, Kruse O, Hankamer B. 2008. Second generation biofuels: high-efficiency microalgae for biodiesel production. Bioenergy Res 1: 20-43.

Sydney EB, da Silva TE, Tokarski A, Novak AC, de Carvalho JC, Woiciecohwski AL, Larroche C, Soccol CR. 2011. Screening of microalgae with potential for biodiesel

production and nutrient removal from treated domestic sewage. Appl Energy 88: 3291-3294.

Wang L, Li Y, Chen P, Min M, Chen Y, Zhu J, Ruan RR. 2010. Anaerobic digested dairy manure as a nutrient supplement for cultivation of oil-rich green microalgae *Chlorella* sp. Bioresour Technol 101: 2623-2628.

Yang J, Xu M, Zhang X, Hu Q, Sommerfeld M, Chen Y. 2011. Life-cycle analysis on biodiesel production from microalgae: Water footprint and nutrients balance. Bioresour Technol 102(1): 159–165.

652   Table 1: Composition of centrate obtained from a wastewater treatment plant used to
653   prepare culture medium by mixing with seawater at different proportions.

pH	8.31		
Conductivity	4.55 mmhos/cm 25ºC		
Compound	Concentration, mg/L	Compound	Concentration, mg/L
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

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656 Table 2: Salinity, nitrogen (nitrate-ammonium) and phosphorus content of the culture
657 medium used as a function of the centrate percentage added to seawater.

Centrate, %	Salinity, g/L	N-NO$_3$, mg/L	N-NH$_4$, mg/L	P-PO$_4$, mg/L
0% (Algal medium)	25.0	112.0	0.0	22.4
10%	22.9	0.7	47.9	3.6
20%	22.2	1.3	95.7	7.2
30%	19.5	2.0	143.6	10.8
40%	18.5	2.6	191.5	14.4
50%	16.9	4.6	334.0	25.1
60%	13.3	5.5	400.8	30.2
70%	11.0	6.4	467.5	35.2
80%	7.9	7.3	534.3	40.2

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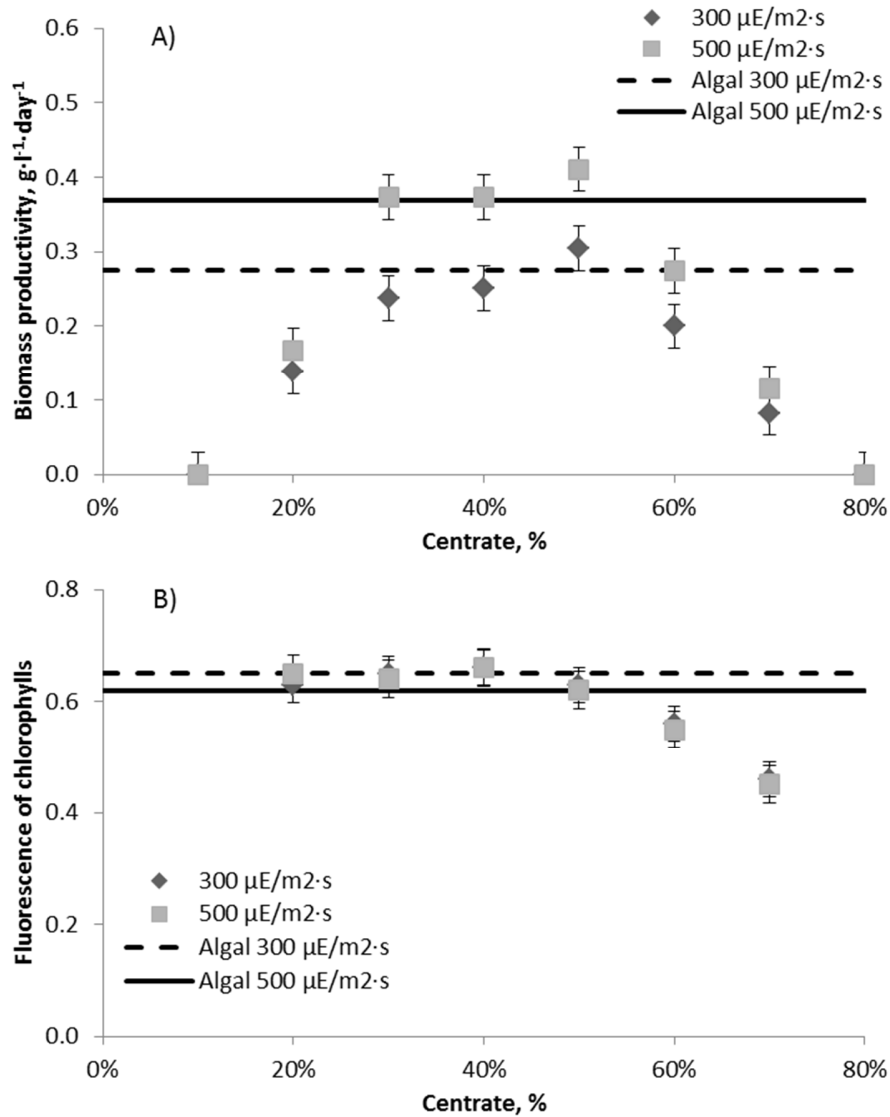
Figure 1: Variation of (A) biomass productivity and (B) fluorescence of chlorophylls of *N. gaditana* cultures as a function of the centrate percentage in the culture medium. Experiments performed in semicontinuous mode at 0.25 1/day, at two irradiance levels. Lines correspond to values obtained using Algal culture medium under the same culture conditions.

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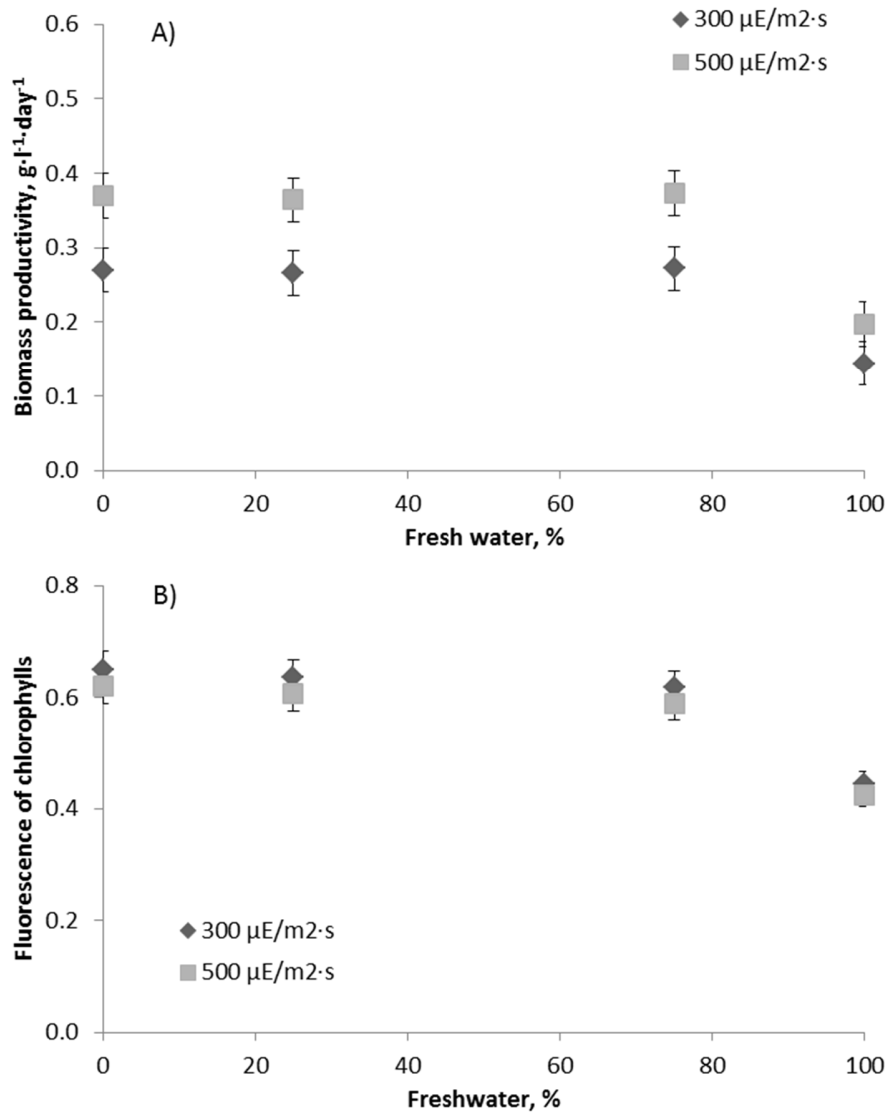
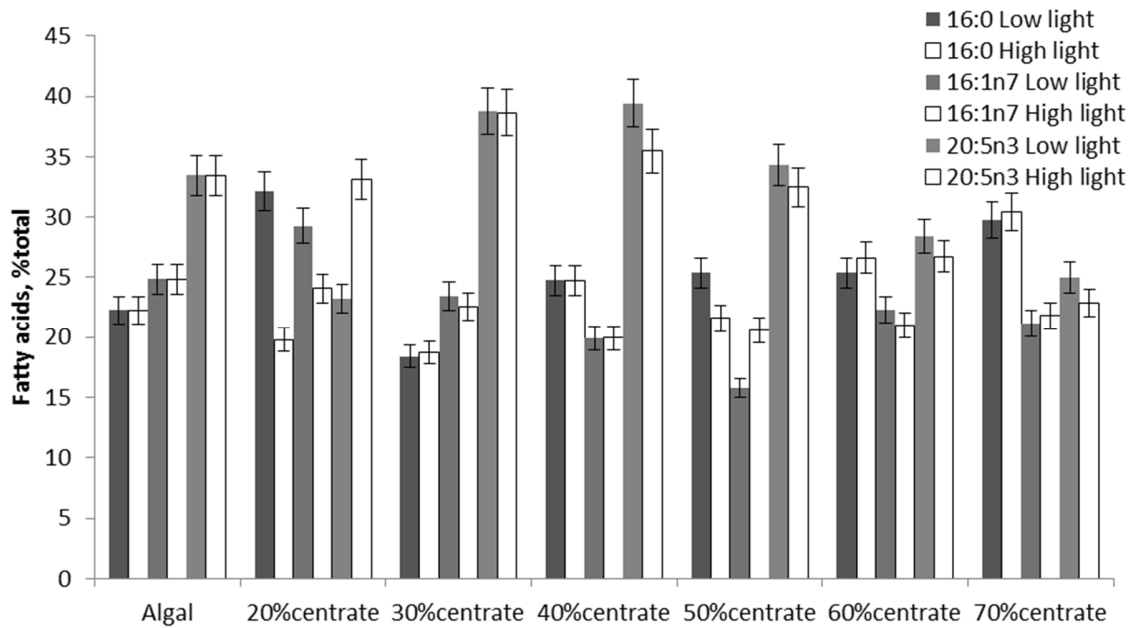Figure 2: Variation of (A) biomass productivity and (B) fluorescence of chlorophylls of *N. gaditana* cultures as a function of the salinity in the culture medium. Experiments performed in semicontinuous mode at 0.25 1/day, at two irradiance levels, using Algal culture medium under the same culture conditions.

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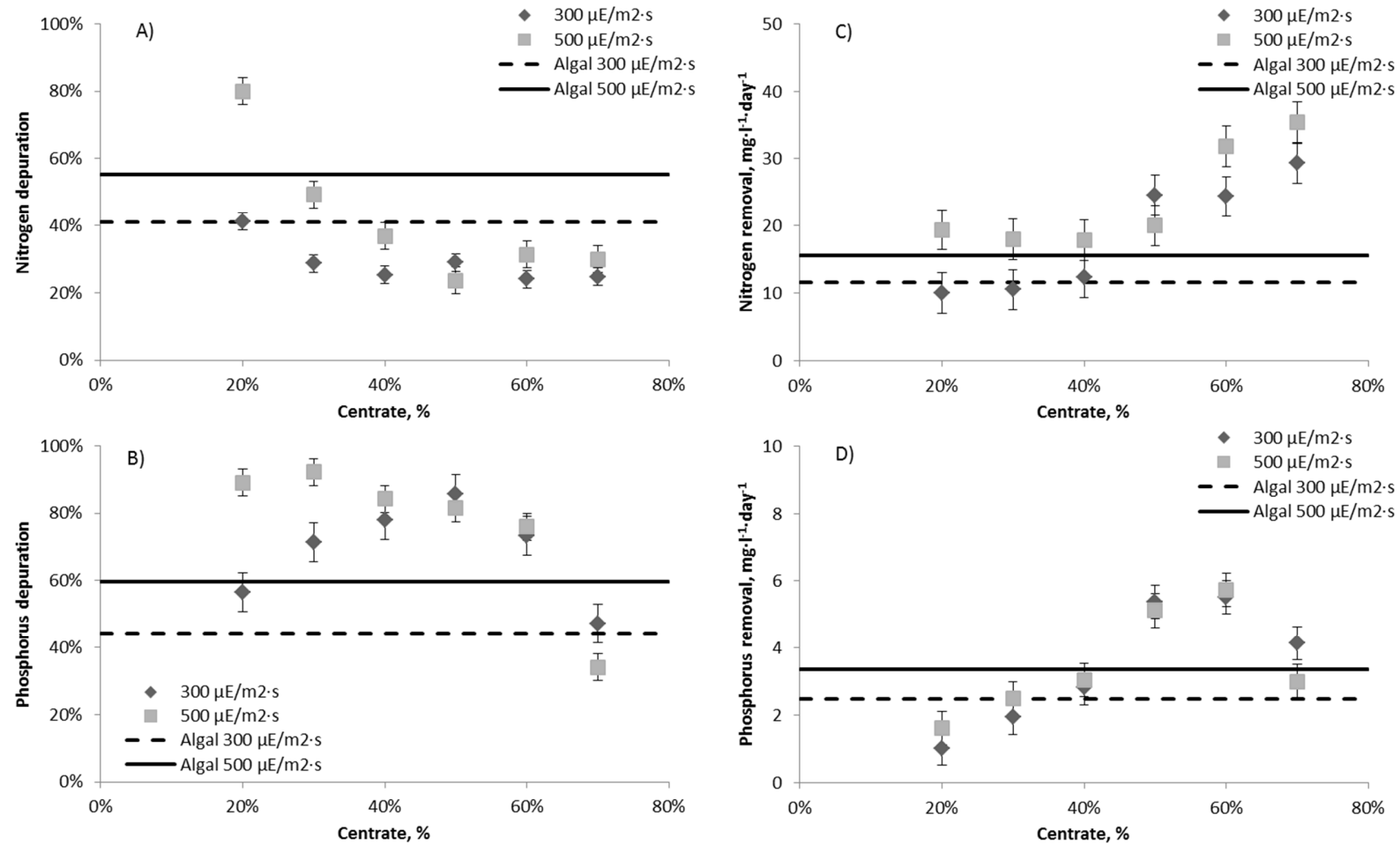
Figure 3: Variation of nitrogen (A) and phosphorus (B) depuration in addition removal capacity of nitrogen (C) and phosphorus (D) of *N. gaditana* cultures as a function of the centrate percentage in the culture medium. Experiments performed in semicontinuous mode at 0.25 1/day, at two irradiance levels. Lines correspond to values obtained using Algal culture medium under the same culture conditions.

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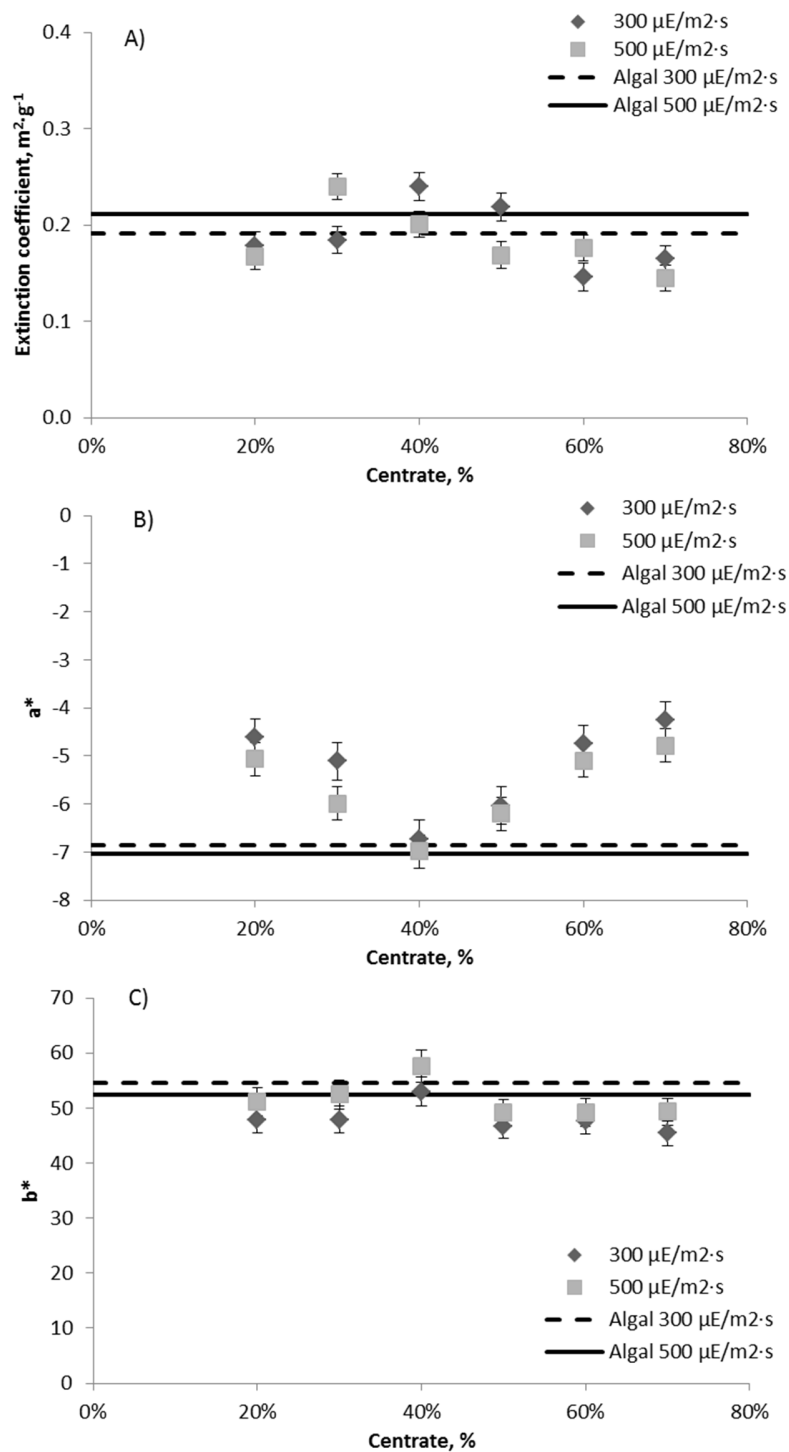
Figure 4: Variation of optical properties of *N. gaditana* cultures as a function of the centrate percentage in the culture medium. A) Extinction coefficient, B) colour coordinate a*, C) Colour coordinate b*. Experiments performed in semicontinuous mode at 0.25 1/day, at two irradiance levels. Lines correspond to values obtained using Algal culture medium under the same culture conditions.

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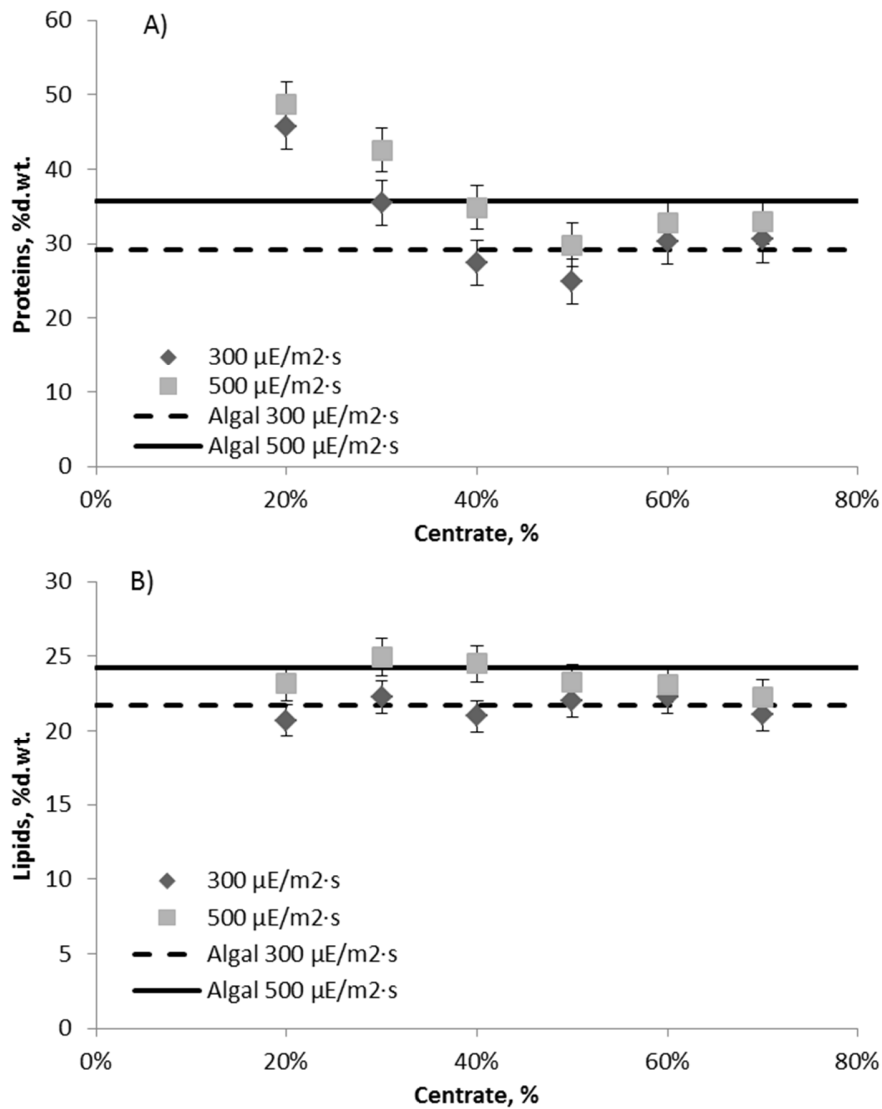
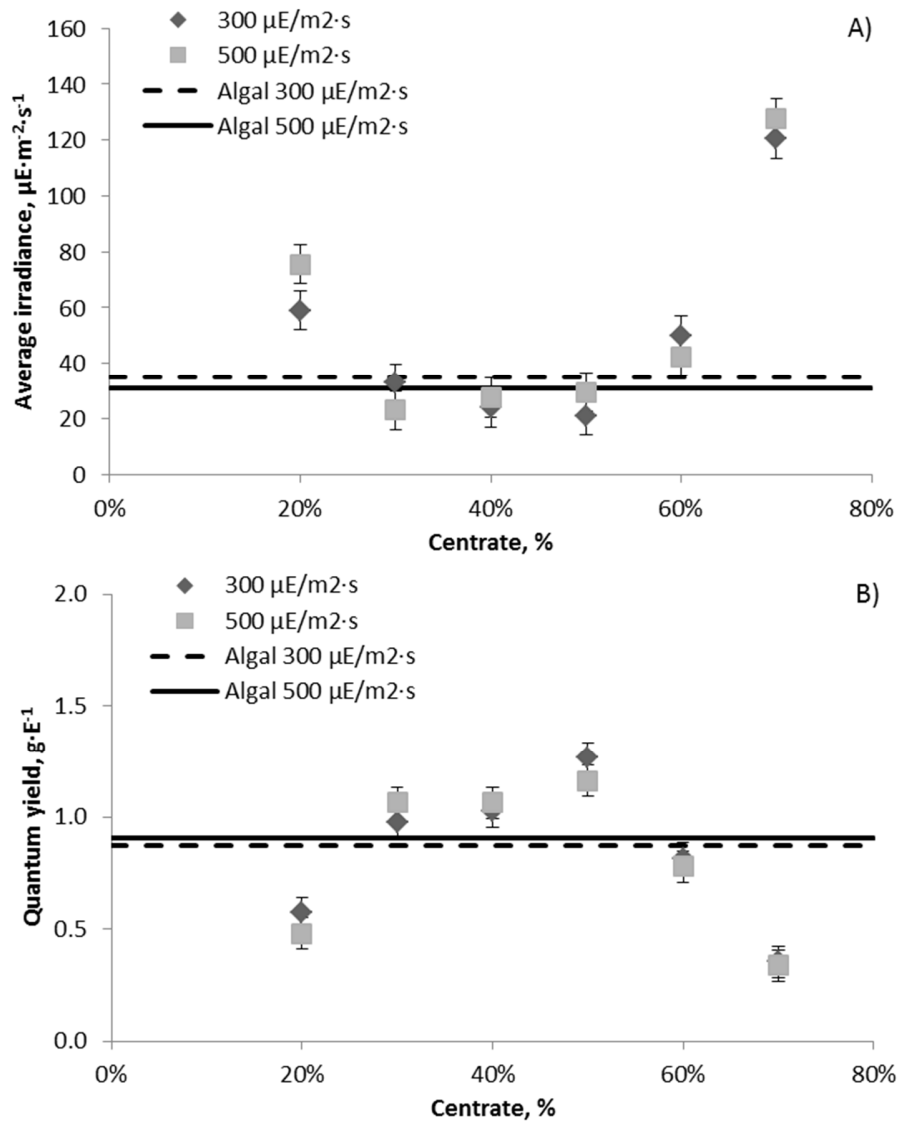Figure 5: Variation of (A) protein and (B) lipid content of *N. gaditana* cultures as a function of the centrate percentage in the culture medium. Experiments performed in semicontinuous mode at 0.25 1/day, at two irradiance levels. Lines correspond to values obtained using Algal culture medium under the same culture conditions.

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Figure 6: Variation of fatty acid profile of *N. gaditana* cultures as a function of the centrate percentage in the culture medium. Experiments performed in semicontinuous mode at 0.25 1/day, at two irradiance levels: Low light=300 $\mu E/m^2 s$ (filled symbols), High light=500 $\mu E/m^2 s$ (empty symbols).

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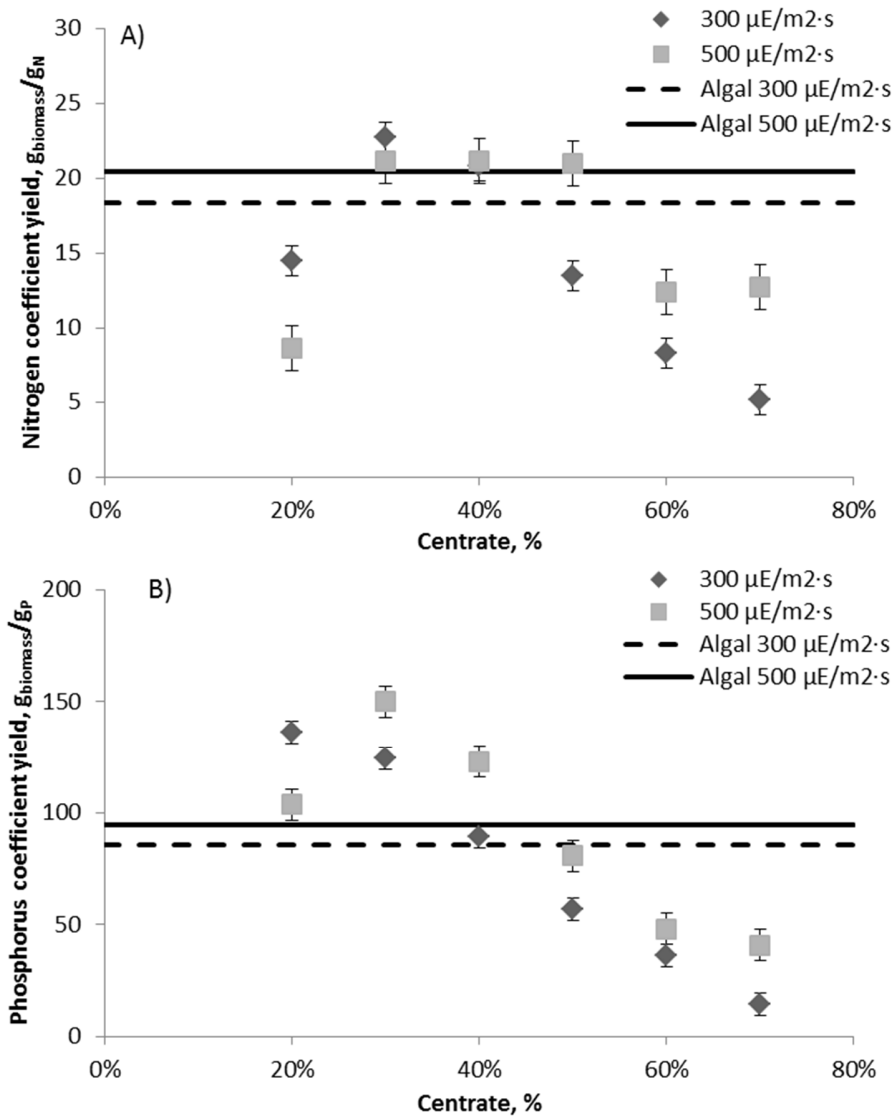
Figure 7: Variation of (A) nitrogen and (B) phosphorus coefficient yields of *N. gaditana*
cultures as a function of the centrate percentage in the culture medium. Experiments
performed in semicontinuous mode at 0.25 1/day, at two irradiance levels. Lines
correspond to values obtained using Algal culture medium under the same culture
conditions.

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Figure 8: Variation of (A) average irradiance and (B) light-use efficiency of *N. gaditana* cultures as a function of the centrate percentage in the culture medium. Experiments performed in semicontinuous mode at 0.25 1/day, at two irradiance levels. Lines correspond to values obtained using Algal culture medium under the same culture conditions.

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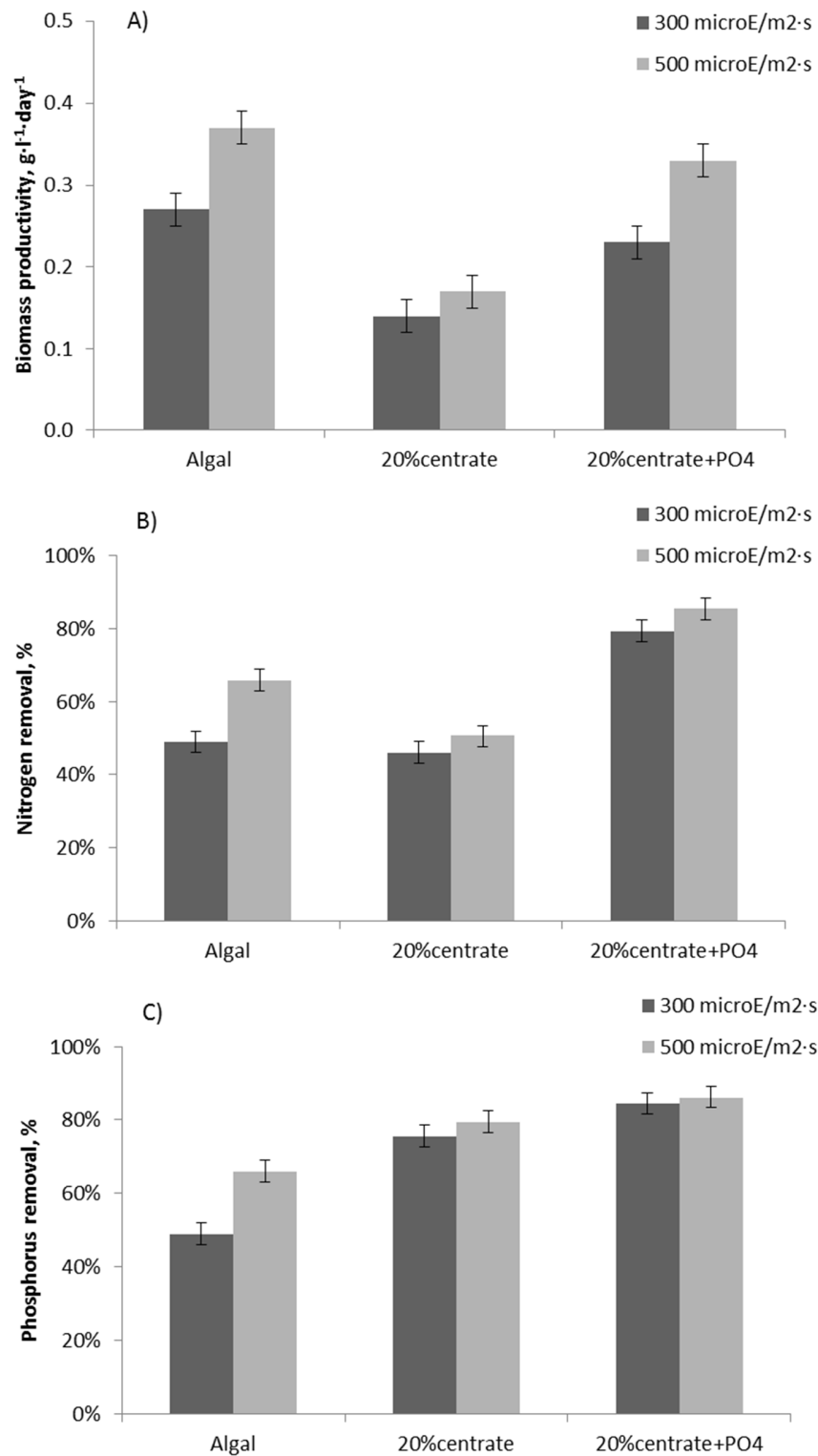
Figure 9: Variation of (A) biomass productivity, (B) nitrogen and (C) phosphorus removal
with average irradiance as a function of the composition of the culture medium used.
Experiments performed in semicontinuous mode at 0.25 1/day.