Utilization of centrate for the production of the marine microalgae *Nannochloropsis gaditana*

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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 depurate 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).

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

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

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).

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

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).

$$Iav = \frac{Io}{(Ka \cdot p \cdot Cb)}\left(1 - \exp\left(- Ka \cdot p \cdot Cb\right)\right)$$ 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·l^{-1}, much higher than
215 freshwater, which typically contains less than 1.0 g·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·l^{-1} for nitrogen and 1 mg·l^{-1}
293 for phosphorus. In all cases, the outlet nitrogen concentrations present were higher than 10
294 mg·l^{-1}, whatever the centrate percentage used in the culture medium; whereas the
295 phosphorus concentration was only lower than 1 mg·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$_N$·l^{-1}·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$_N$·l^{-1}·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$_P$·l^{-1}·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$_P$·l^{-1}·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 μE/m^2·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   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   ^{1} 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

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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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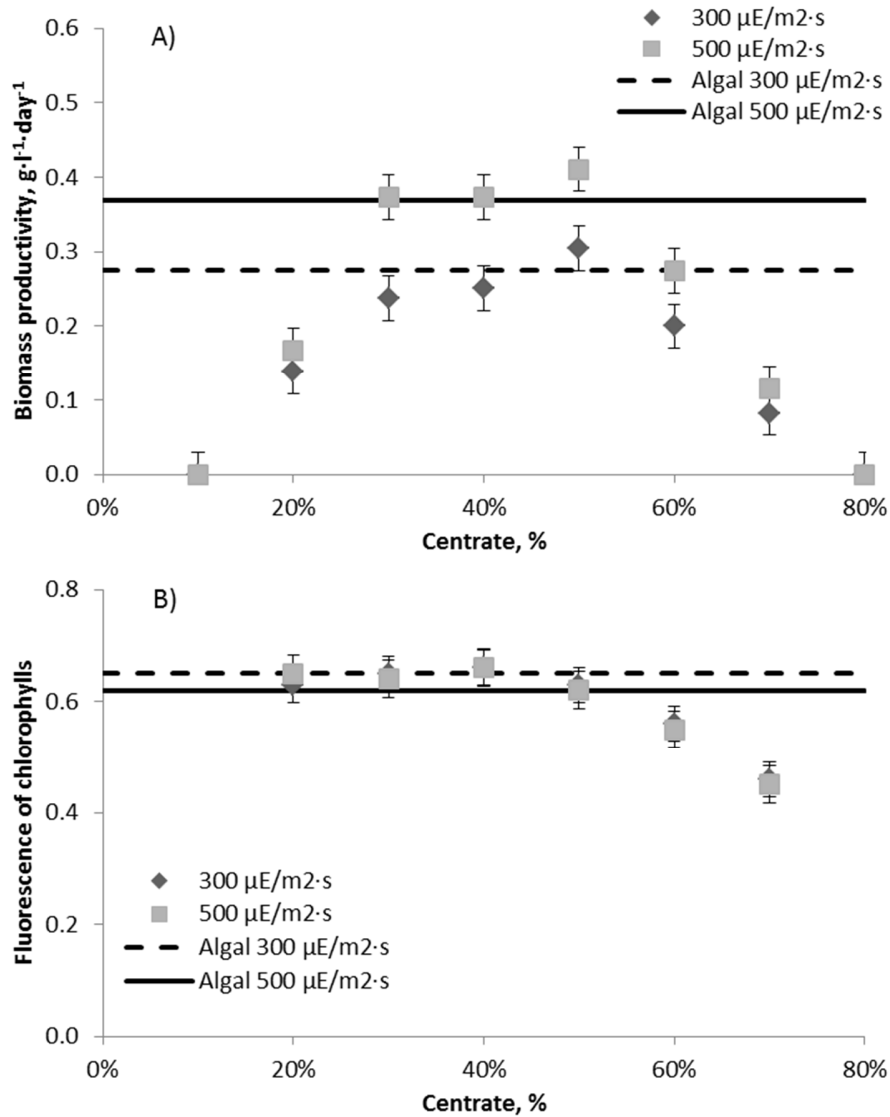
660

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

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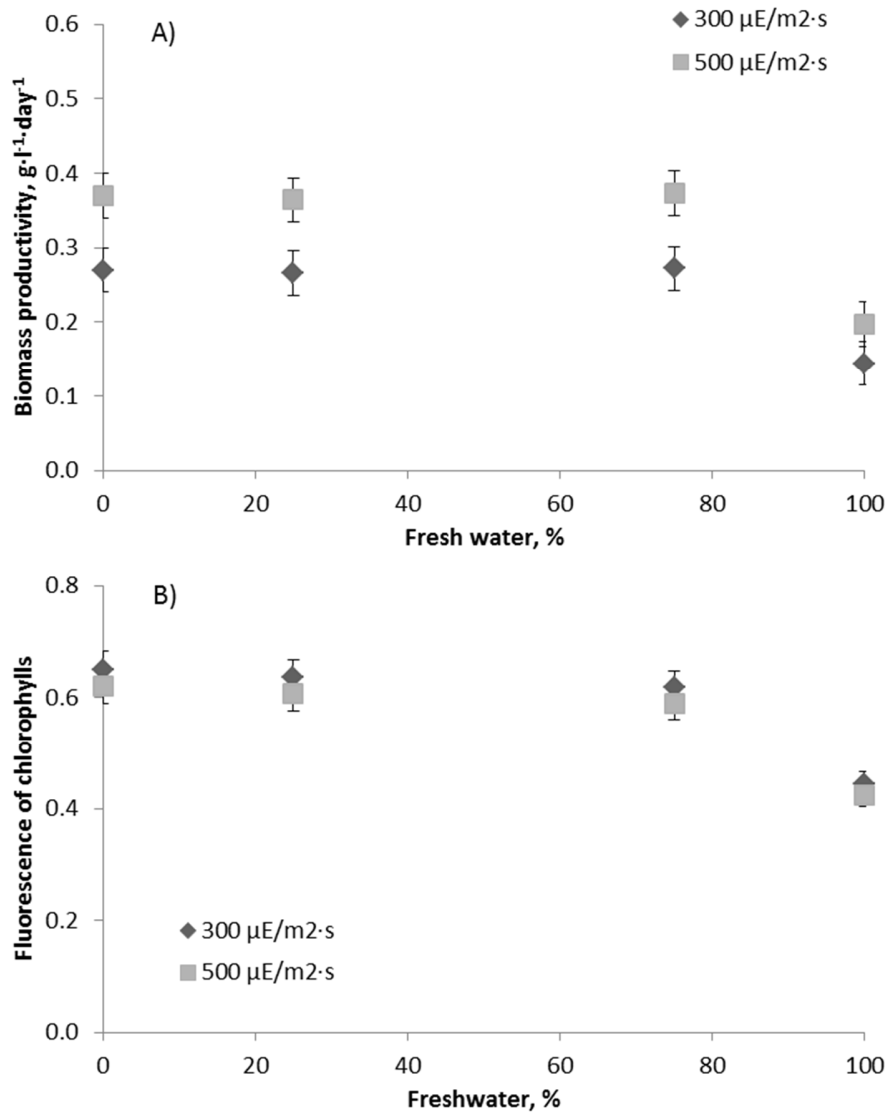
Figure 2: Variation of (A) biomass productivity and (B) fluorescence of chlorophylls of *N. gaditana* cultures as a function of the salinity in the culture medium. Experiments performed in semicontinuous mode at 0.25 1/day, at two irradiance levels, using Algal culture medium under the same culture conditions.

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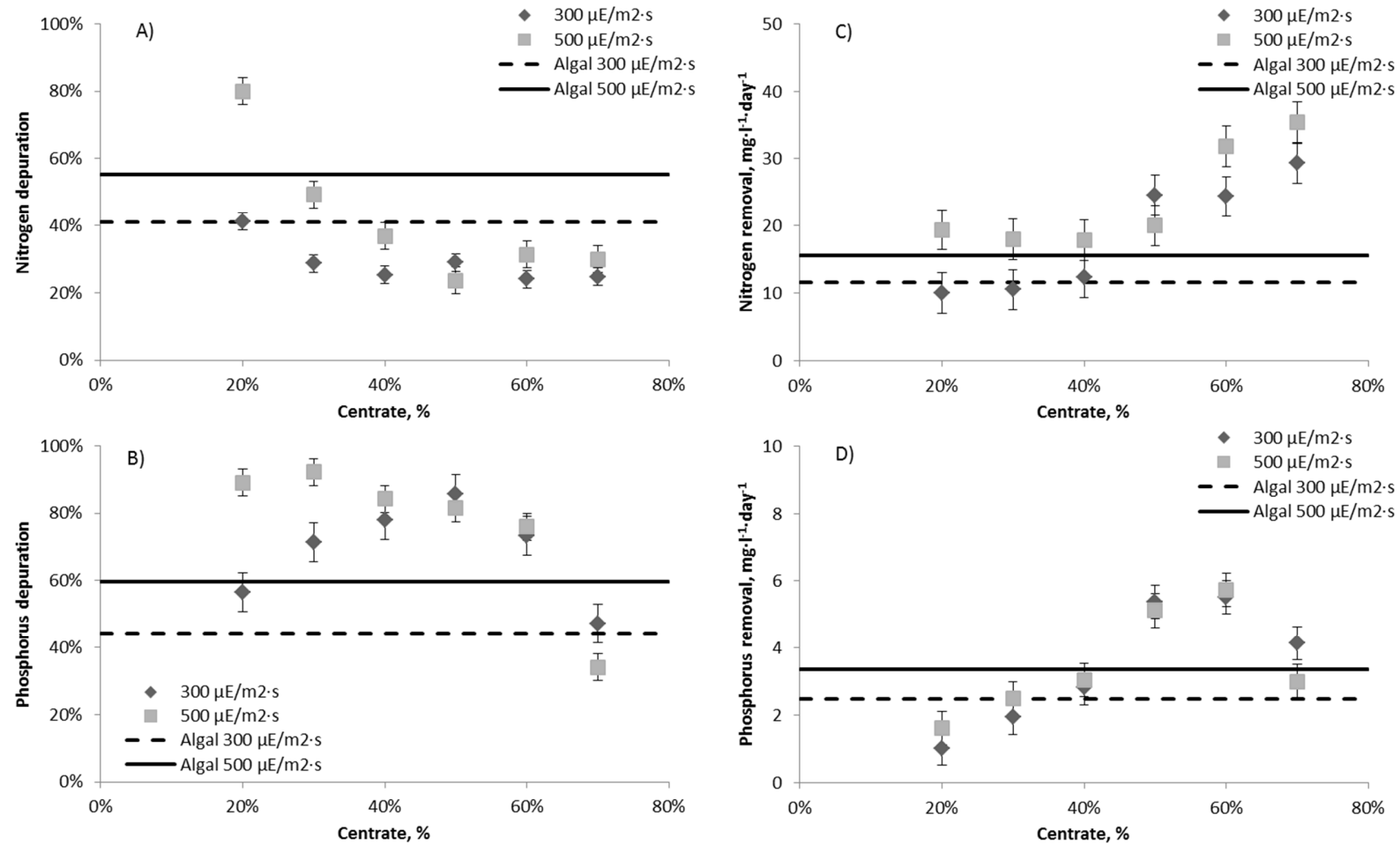
Figure 3: Variation of nitrogen (A) and phosphorus (B) depuration in addition removal capacity of nitrogen (C) and phosphorus (D) of *N. gaditana* cultures as a function of the centrate percentage in the culture medium. Experiments performed in semicontinuous mode at 0.25 1/day, at two irradiance levels.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

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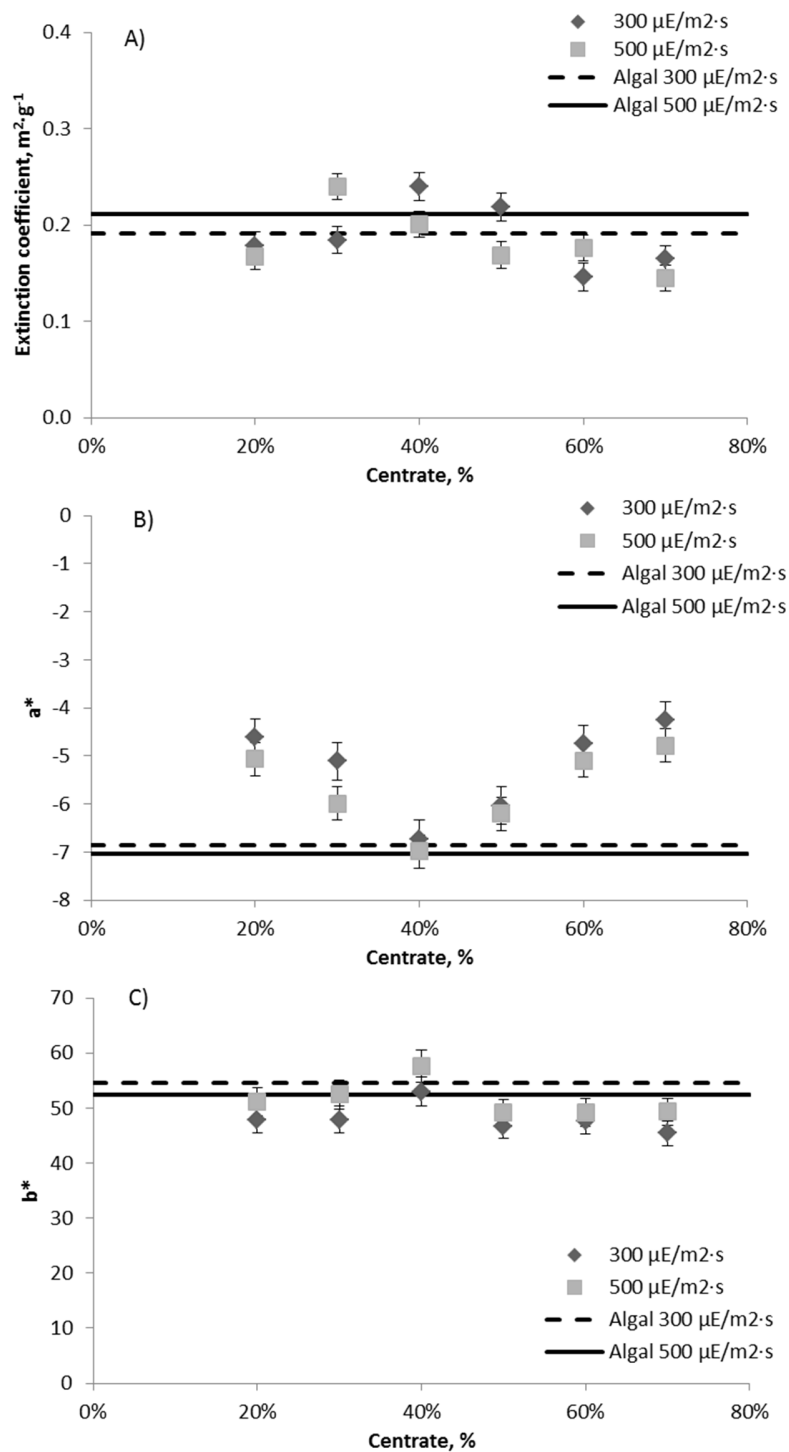
Figure 4: Variation of optical properties of *N. gaditana* cultures as a function of the centrate percentage in the culture medium. A) Extinction coefficient, B) colour coordinate a*, C) Colour coordinate b*. Experiments performed in semicontinuous mode at 0.25 1/day, at two irradiance levels. Lines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

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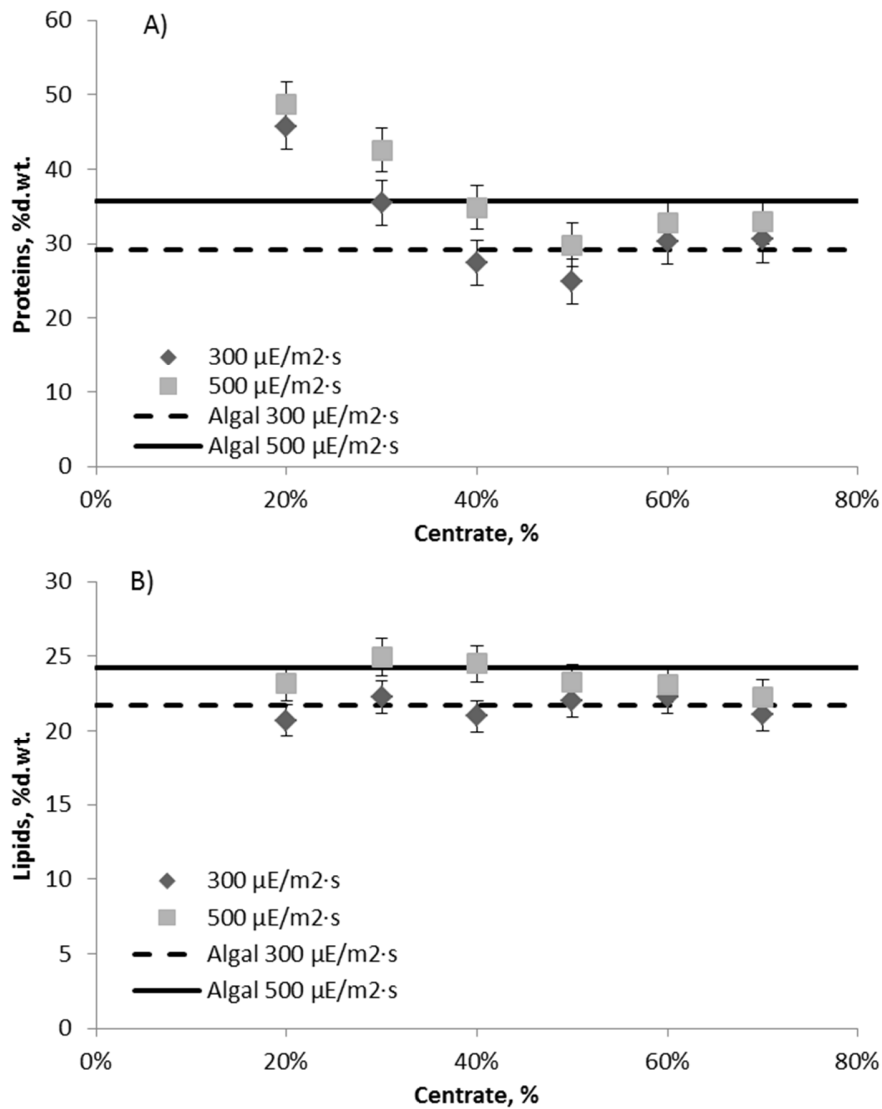
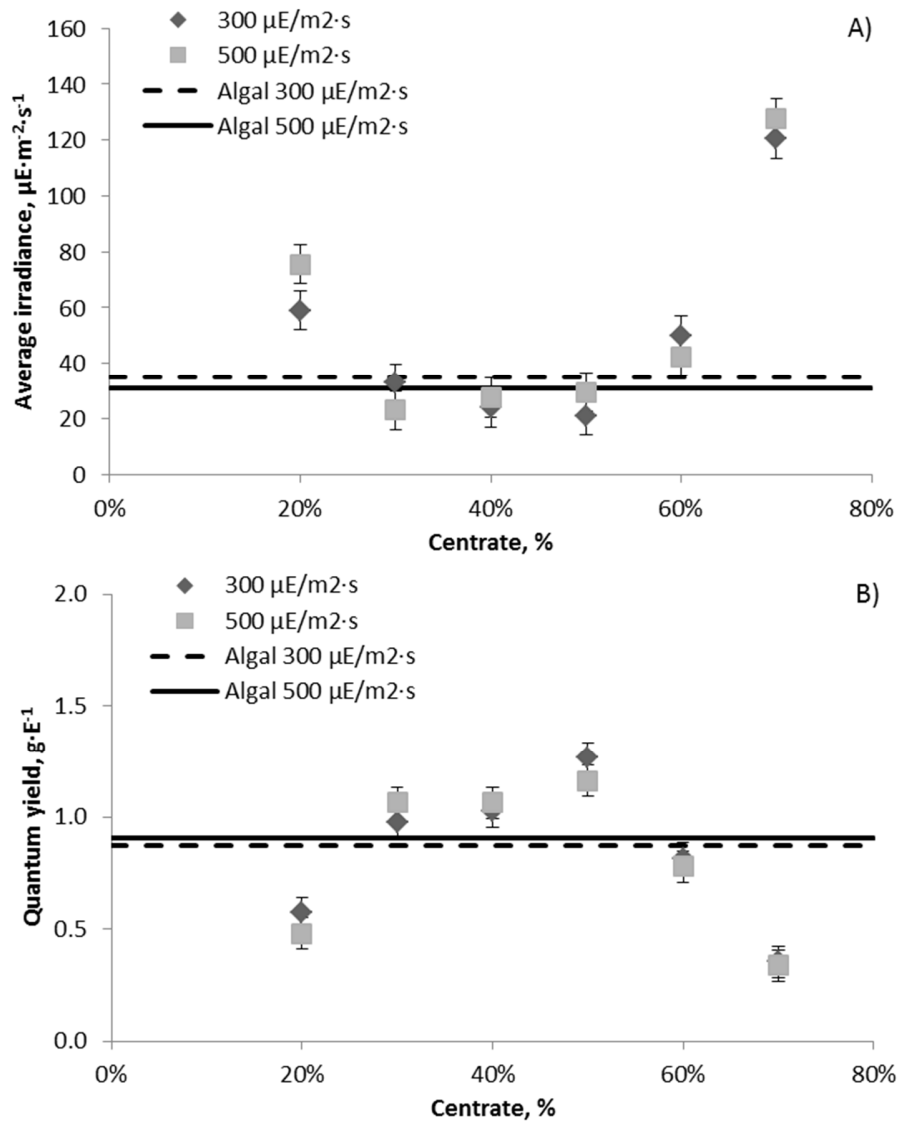Figure 5: Variation of (A) protein and (B) lipid content of *N. gaditana* cultures as a function of the centrate percentage in the culture medium. Experiments performed in semicontinuous mode at 0.25 1/day, at two irradiance levels. Lines correspond to values obtained using Algal culture medium under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

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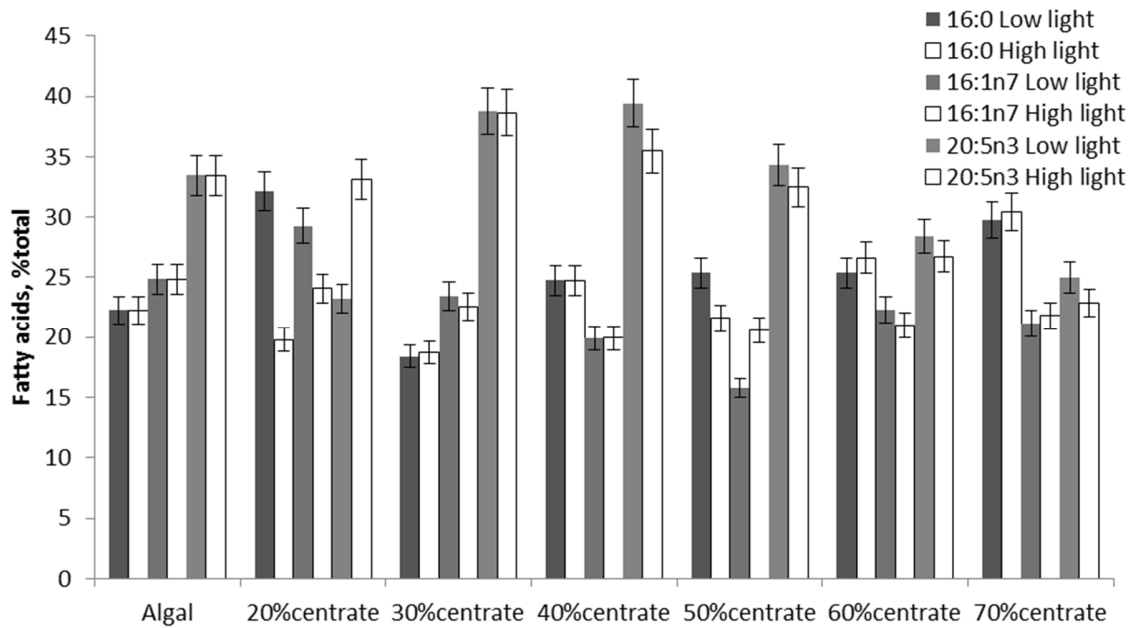
Figure 6: Variation of fatty acid profile of *N. gaditana* cultures as a function of the centrate percentage in the culture medium. Experiments performed in semicontinuous mode at 0.25 1/day, at two irradiance levels: Low light=300 $\mu E/m^2 s$ (filled symbols), High light=500 $\mu E/m^2 s$ (empty symbols).

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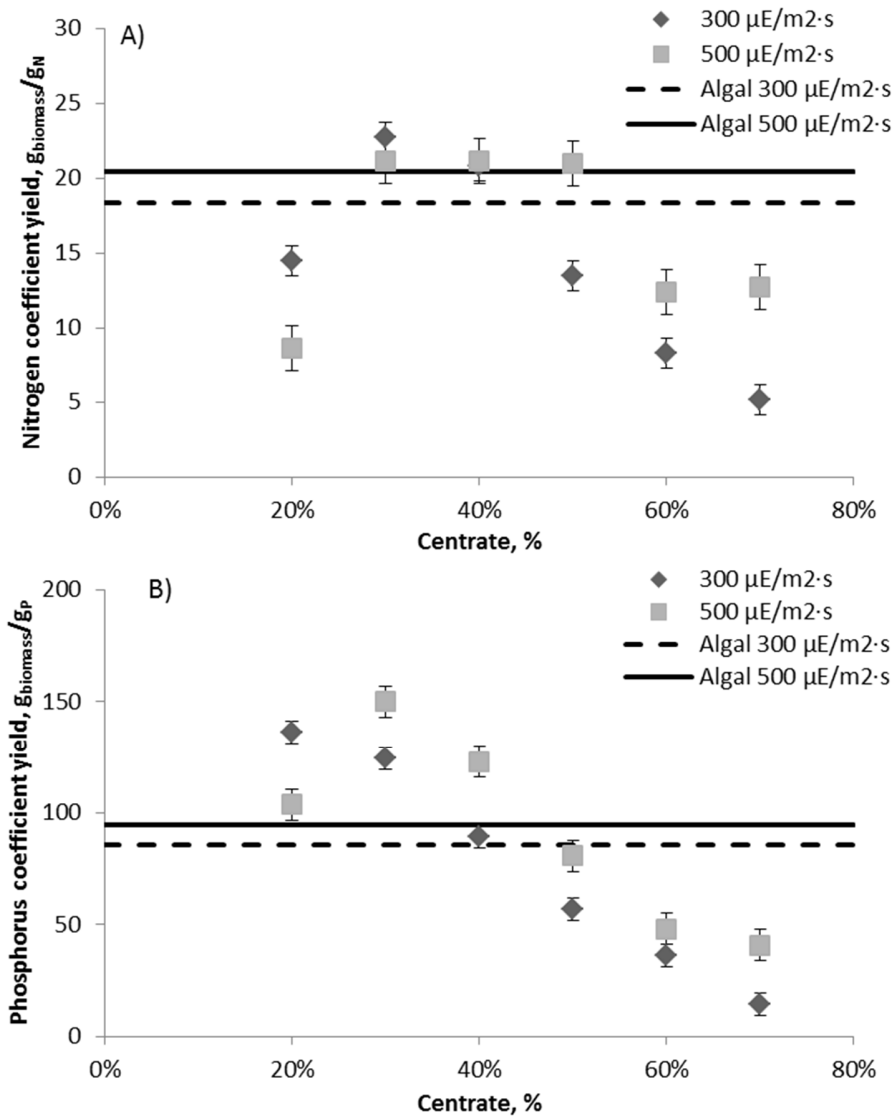
Figure 7: Variation of (A) nitrogen and (B) phosphorus coefficient yields of *N. gaditana*
cultures as a function of the centrate percentage in the culture medium. Experiments
performed in semicontinuous mode at 0.25 1/day, at two irradiance levels. Lines
correspond to values obtained using Algal culture 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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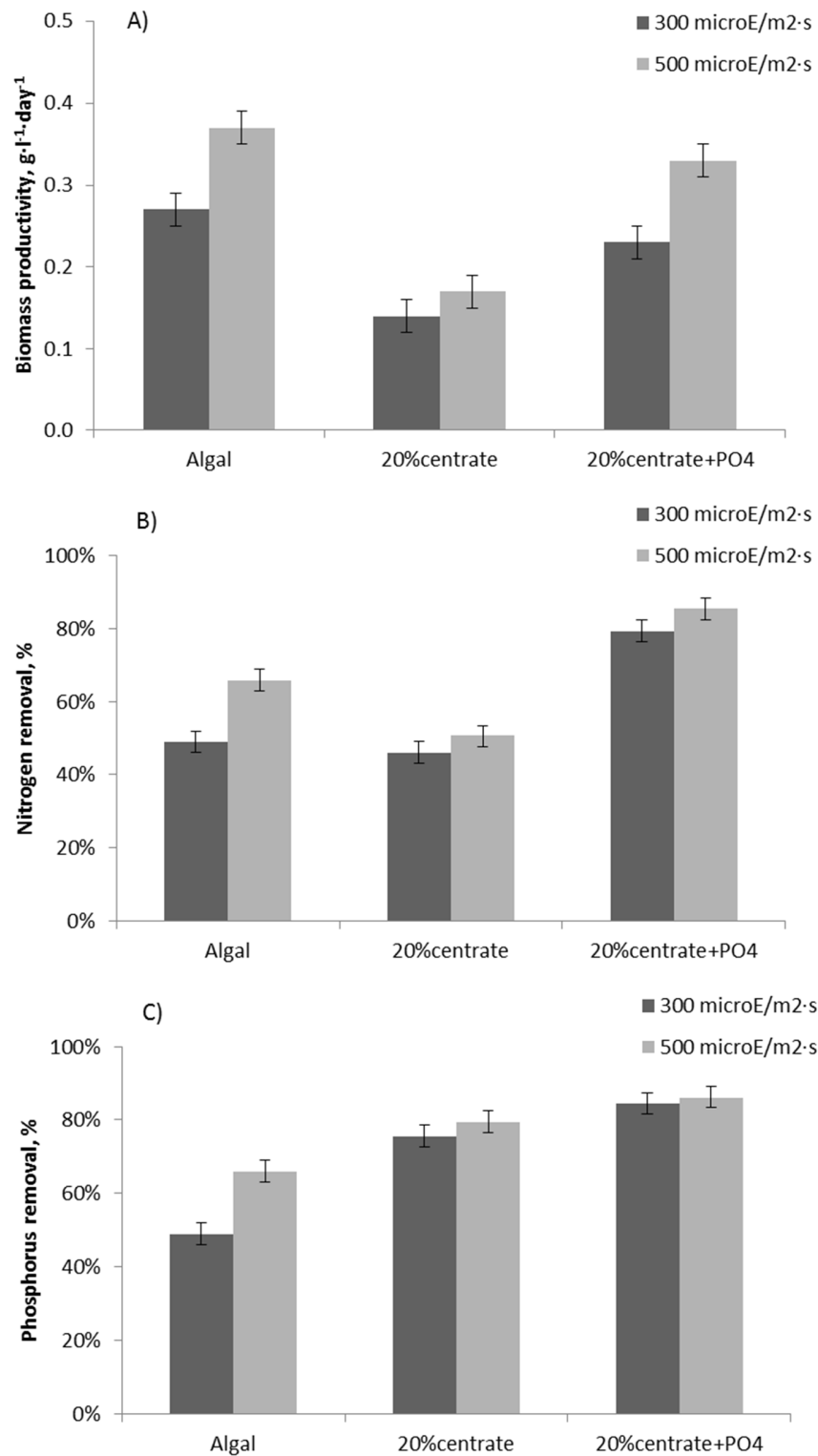
Figure 9: Variation of (A) biomass productivity, (B) nitrogen and (C) phosphorus removal
with average irradiance as a function of the composition of the culture medium used.
Experiments performed in semicontinuous mode at 0.25 1/day.