

## **Optimization of photosynthesis, growth, and biochemical composition of the microalga *Rhodomonas salina***

an established diet for live feed copepods in aquaculture

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1 **Journal of Applied Phycology**

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3 **Optimization of photosynthesis, growth, and biochemical composition of the microalgae**  
4 ***Rhodomonas salina* – an established diet for live feed copepods in aquaculture**

5

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20

21

22 **Abstract**

23 The Cryptophyte *Rhodomonas salina* is widely used as feed for copepod cultures. However, the  
24 culturing conditions to obtain high quality algae have not yet been efficiently optimized. Therefore,  
25 we aimed to develop a cultivation protocol for *R. salina* to optimize its nutritional value and provide  
26 technical recommendations for later large scale production in algal photobioreactors. We studied  
27 photosynthesis, growth, pigments, fatty acids (FA) and free amino acids (FAA) composition of *R.*  
28 *salina* cultured at different irradiances (10-300  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) and nutrient availability  
29 (deficiency and excess). The optimal range of irradiance for photosynthesis and growth was 60-100  
30  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . The content of chlorophylls *a* and *c* decreased with increasing irradiance  
31 while phycoerythrin peaked at irradiances of 40-100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . The total FA content was  
32 maximal at optimal irradiances for growth, especially under nutrient deficiency. However, highly-  
33 unsaturated fatty acids, desired components for copepods, were higher under nutrient excess. The  
34 total FAA content was highest at limited irradiances (10-40  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) but a better  
35 composition with higher fraction of essential amino acids was obtained at saturated irradiances (60-  
36 140  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). These results demonstrate that quality and quantity of FA and FAA of *R.*  
37 *salina* can be optimized by manipulating the irradiance and nutrient conditions. We suggest that *R.*  
38 *salina* should be cultivated in a range of irradiance 60-100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  and nutrient excess  
39 to obtain the algae with high production and a balanced biochemical composition as feed for  
40 copepods.

41 Key words: algal production, amino acids, designer feed, fatty acids, phycoerythrin

42

## 43 **Introduction**

44 Microalgae are essential feeds for many cultured molluscs and larvae of marine fishes and  
45 crustaceans (Brown et al. 1997; Muller-Feuga et al. 2003). Microalgae are also used in aquaculture  
46 as food for other important live feeds such as for feeding or enriching rotifers, *Artemia* and  
47 copepods (Støttrup 2003; Dhert et al. 2001; Sorgeloos et al. 2001; Muller-Feuga et al. 2003). As  
48 live feeds in aquaculture, the optimization of their nutritional values, beside the biomass production,  
49 is of crucial importance and of special interest.

50 In microalgae, low irradiance may limit photosynthesis (Dunstan 1973), but high irradiances may  
51 cause photoinhibition (Neidhardt et al. 1998). Importantly, irradiance may influence the production  
52 and composition of the fatty acids in microalgae (Renaud et al. 1991; Mortillaro et al. 2009). Hence,  
53 it is possible to manipulate irradiance to optimize growth and the preferred biochemical quality of  
54 microalgae.

55 Another important factor regarding algal culture is inorganic nutrients that not only affect  
56 photosynthesis and productivity of cell biomass, but also influence the biochemical composition of  
57 microalgae (Hu 2004; Juneja et al. 2013). For example, under nutrient deficiency, microalgae  
58 exhibit low growth rates (Bi et al. 2014) and produce higher levels of total fatty acids (TFA), but  
59 levels of unsaturated fatty acids, the desired components for live feed in aquaculture, are low  
60 (Breteler et al. 2005).

61 Nutritional quality of microalgae species is associated with the level of highly unsaturated fatty  
62 acids (HUFA), especially Eicosapentaenoic acid (EPA; 20:5 n-3) and Docosahexaenoic acid (DHA;  
63 22:6 n-3) (Renaud et al. 1991). HUFAs (characterized by a carbon number  $\geq 20$  and double bonds  
64  $\geq 3$ ) are synthesized *de novo* only by photosynthetic organisms (Spector 1999), and are essential  
65 dietary nutrients for marine copepods (Fraser et al. 1989). HUFA, in particular, DHA and EPA  
66 appear to be very important in controlling reproduction, growth and metabolism in copepods  
67 (reviewed in Rasdi and Qin 2014). High dietary DHA/EPA ratios in feed improve survival, reduce  
68 time to maturity, increase maturation rate, female length of calanoid copepod species, egg  
69 production, and hatching success (Jónasdóttir 1994; Jónasdóttir and Kiørboe 1996; Payne and  
70 Rippingale 2000; Arendt et al. 2006; Rasdi and Qin 2014). Interestingly, copepods are carriers of  
71 high DHA/EPA ratios from microalgae into fish larvae (Parrish 2009). A DHA/EPA ratio  $\geq 2$  is  
72 regarded favorable for fish larval nutrition (Sargent et al. 1997). The enhance HUFA content in

73 algae prior to feeding to copepods is recommended as the nutrient content in copepods cannot be  
74 manipulated through enrichment techniques due to their avoidance behavior (Rasdi and Qin 2014).

75 Amino acids (AA) constitute another group of important biochemical constituents determining the  
76 nutritional quality of microalgae (Brown 1991). AAs are the building blocks for protein synthesis,  
77 and are involved in numerous specific physiological functions (Aragão et al. 2004). Some AAs are  
78 defined as essential amino acids (EAA) that either cannot be synthesized within the animal body or  
79 at an insufficient rate to meet the physiological needs for the growth of animals. EAAs must  
80 therefore be supplied from the diet. For copepods, microalgae are the only external source of the  
81 EAA (Wu 2009). AA composition of the algal prey also affects the egg production of the copepods  
82 (reviewed in Rasdi and Qin 2014).

83 Among the marine microalgae, species of the Cryptophyte genus *Rhodomonas*, such as *R. salina*, *R.*  
84 *baltica* and *R. reticulate*, are commonly cultivated for use as live feeds for scallop larvae (Malzahn  
85 and Boersma 2012), oyster larvae and spats (Brown et al. 1998; Muller-Feuga et al. 2003) and  
86 Queen conch veliger larvae (Aldana-Aranda and Patiño Suárez 1998). Especially, *Rhodomonas*  
87 species are excellent feeds for culturing copepods (Støttrup and Jensen 1990; Jónasdóttir 1994;  
88 Marinho da Costa and Fernández 2002; Zhang et al. 2013; Broglio et al. 2003).

89 The overall purpose of the present study is to develop a cultivation protocol for applying *R. salina*  
90 in large scale algal photobioreactors while optimizing their growth and nutritional value as algal  
91 feed for live feed calanoid copepods. We measured the photosynthesis of *R. salina* under  
92 irradiances from 10 to 300  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . In the next step, we conducted a full factorial  
93 growth experiment in which *R. salina* was cultivated in a series of irradiances ranging from 10 to  
94 140  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  under two inorganic nutrient levels: deficiency and excess. We quantified  
95 algal growth rates, three important pigments (chlorophyll *a*, chlorophyll *c* and phycoerythrin,  
96 Yaakob et al. 2014), fatty acids and free amino acids profiles for all irradiant levels and nutrient  
97 treatments. We have the ambition to develop a simple method where the copepod's biochemical  
98 profile is always optimal for the use as fish larvae feed by simply manipulating two algal growth  
99 factors (irradiance and nutrient). This will enable us to develop an intensive setup for a nutritious  
100 food chain delivering live feed on request for marine hatcheries.

101 **Materials and methods**

102 *Microalgal material and culturing conditions*

103 The studied Cryptophyte *Rhodomonas salina* (equivalent spherical diameter 8 µm) was originally  
104 derived as SCCAP K-1487 of the Scandinavian Culture Collection of Algae and Protozoa  
105 (University of Copenhagen, Denmark). Cultures of *R. salina* was grown in acid washed 3-6 L round  
106 glass flasks containing autoclaved 0.2 µm filtered seawater (salinity 30 ‰) enriched with B1  
107 medium (1 mL L<sup>-1</sup> of seawater, Hansen 1989). The cultures were maintained under a continuous  
108 irradiance of 80 µmol photons m<sup>-2</sup> s<sup>-1</sup> Photosynthetic Active Radiation (PAR) in a climate room at  
109 20°C. The flasks were gently aerated with atmospheric air through 0.45 µm filters to mix the  
110 cultures to avoid temperature stratification, algal sedimentation, CO<sub>2</sub> depletion and O<sub>2</sub> build up.

111 *Photosynthesis measurements*

112 Measurements of photosynthesis-irradiance (P-I) curves were performed for 5 cell densities (0.1,  
113 0.5, 1, 2, 10 × 10<sup>6</sup> cells mL<sup>-1</sup>) at 16 increasing irradiances (0, 10, 20, 40, 60, 80, 100, 120, 140, 160,  
114 180, 200, 220, 240, 260, 300 µmol photons m<sup>-2</sup> s<sup>-1</sup> PAR), with n = 5 experimental replicates of each  
115 irradiances from 0 to 140 µmol photons m<sup>-2</sup> s<sup>-1</sup> and n = 2-3 experimental replicates for irradiances  
116 from 160 to 300 µmol photons m<sup>-2</sup> s<sup>-1</sup>. The photosynthesis of *R. salina* was measured when the  
117 algae was in the exponential phase for all treatments. A high concentration of algae was achieved  
118 by centrifuging at 1000 rpm for 5 minutes at 20°C. The algae suspension was diluted to the required  
119 cell concentration using fresh 0.2 µm filtered autoclaved seawater containing B1 medium. Net  
120 oxygen exchange rates were measured with a Clark-type oxygen electrode (S1 Oxygen Electrode  
121 Disc, Hansatech Instruments, Norfolk, UK) fitted in a stirred Hansatech DW3 chamber. Light was  
122 provided by a red LED-lamp (Hansatech LC1). The measurement at each irradiance was completed  
123 within 5 min when steady-state photosynthesis had been achieved.

124 Curves were fitted to the photosynthesis – irradiance data using the equation 1 (Platt et al. 1980):

125 
$$P^B = P_s^B \left( 1 - e^{\frac{-\alpha I}{P_s^B}} \right) e^{\frac{-\beta I}{P_s^B}} \quad (1)$$

126 Where  $P^B$ : photosynthetic rate at irradiance I

127  $P_s^B$ : Maximum theoretical (irradiance-saturated) photosynthetic rate in the absence of  
128 photoinhibition

129  $\alpha$ : Initial slope of the P-I curve (the quantum yield)

130  $\beta$ : Negative slope at high irradiance (photoinhibition)

131 In addition, the realized maximum photosynthetic rate attained ( $P_m^B$ ) and irradiance of maximum  
132 photosynthesis ( $I_m$ ) were calculated using the following equations 2 and 3 (Platt et al. 1980):

133 
$$P_m^B = P_s^B \left( \frac{\alpha}{\alpha + \beta} \right) \left( \frac{\alpha}{\alpha + \beta} \right)^{\frac{\beta}{\alpha}} \quad (2)$$

134 
$$I_m = \frac{P_s^B}{\alpha} \ln \left( \frac{\alpha + \beta}{\beta} \right) \quad (3)$$

### 135 *Experimental design for growth experiment*

136 In this experiment, microalgae were grown in a Multi-cultivator MC1000 OD (Photon Systems  
137 Instruments, Drasov, Czech Republic) with eight 100 mL test tubes. The test tubes were immersed  
138 in a 5L flat, rectangular glass container in which water was circulated by pump through an  
139 additional Cooling Unit AC-88 to maintain a stable temperature of 20°C in all test tubes. Between  
140 each slot, there was a plastic divider in the cultivation vessel to separate light regimes of individual  
141 tubes. Tubes were illuminated by white LEDs that were independently adjustable at up to 500  $\mu\text{mol}$   
142 photons  $\text{m}^{-2} \text{s}^{-1}$ . Each test tube was bubbled with atmospheric air.

143 To evaluate the effect of the irradiance and nutrients on growth and biochemical profile of *R. salina*,  
144 a factorial design of 8 irradiant levels (10, 20, 40, 60, 80, 100, 120 and 140  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ )  $\times$   
145 2 nutrient levels (deficiency and excess) was conducted (a total of 16 experimental treatments). All  
146 treatments were performed in duplicates (a total of 32 experimental units). The nutrient deficiency  
147 and excess treatments were not based on the initial nutrient concentrations but based on how many  
148 pulses nutrients were added to the culture. In nutrient deficiency, the nutrients were added only  
149 once at the start of the experiment (B1 medium, 1 mL  $\text{L}^{-1}$  of seawater, Hansen 1989), hence the  
150 nutrient level reduced over time and almost depleted at the end of the experiment. In the nutrient  
151 excess, the nutrients were added daily (v:v 1 mL B1 medium  $\text{L}^{-1} \text{day}^{-1}$  of algae culture), hence no  
152 depletion occurred. The initial density of *R. salina* was  $0.18 \pm 0.03 \times 10^6$  cells  $\text{mL}^{-1}$ . In the growth

153 experiment, all of algal cultures were grown for 5-6 days at 20°C, salinity 30 ‰ with the same flow  
154 of gas bubbling to ensure stirring and gas mass transfer.

#### 155 *Growth rate*

156 The cell density and cell biovolume of *R. salina* was determined every day by taking 1 mL of algae  
157 samples from each treatment and measuring by a Beckman Multisizer™3 Coulter Counter®  
158 (Beckman Coulter Inc., USA). All particles with a diameter in the range of 5-12 µm were  
159 considered as algal cells. The growth rate (day<sup>-1</sup>) of *R. salina* was calculated for the first three days  
160 of the experiment by fitting cell density increase during the exponential phase with an exponential  
161 growth equation 4:

$$162 \quad N_t = N_0 \times e^{(\mu \times t)} \quad (4)$$

163  $N_t$  is the cell density at time  $t$  (cells mL<sup>-1</sup>)

164  $N_0$  is the cell density at time zero (cells mL<sup>-1</sup>)

165  $\mu$  is the growth rate (day<sup>-1</sup>)

166  $t$  is the time (day)

167 The specific growth rate-irradiance curves was fitted using tangent hyperbola functions (Jassby and  
168 Platt 1976), equation 5:

$$169 \quad \mu = \mu_{\max} \tanh\left(\frac{\alpha I}{\mu_{\max}}\right) \quad (5)$$

170 Where,  $\mu$ : specific growth rate (day<sup>-1</sup>)

171  $\mu_{\max}$ : Maximum growth rate (day<sup>-1</sup>)

172  $I$ : Irradiance (µmol m<sup>-2</sup> day<sup>-1</sup>)

173  $\alpha$ : Initial slope of the curve (maximum quantum yield for growth, day<sup>-1</sup> [µmol photons m<sup>-2</sup> s<sup>-1</sup>]<sup>-1</sup>)

#### 174 *Cell biovolume*

175 The cell biovolume of the specific sample was determined as the mean of biovolume of all particles  
176 presented by the particle counter in the frequency diagram with a diameter in the range of 5-12 µm.



177 The presented cell biovolume of *R. salina* was the mean ( $\pm$  SDs) of cell biovolume of the algae in  
178 the stationary phase of the algal growth of all treatments when the biochemical composition of  
179 algae cell were analyzed.

#### 180 *Sample preparation for quantification of inorganic nutrients of algae cultures and pigments, fatty* 181 *acids and amino acids of algae cell*

182 To minimize the loss of culture volume due to the sampling, inorganic nutrient level analyses were  
183 carried out by measuring the samples taken from the first experimental replicate, whereas  
184 biochemical analyses of the algae cell *R. salina* were carried out by utilizing the samples taken from  
185 the second experimental replicate. During every second day, sample water was sampled from each  
186 test tube for analyzing nitrate (4 mL), ammonium (3 mL) and phosphate (6 mL). Culture water was  
187 filtered through 25 mm syringe filter (VWR International, USA) containing a Whatman GF/F glass  
188 fiber filter to remove algae, and the sample water was then stored in  $-20^{\circ}\text{C}$  for later analyses of  
189 nutrient compositions. In the stationary phase of the algal growth, samples of *R. salina* were taken  
190 on two different days from each treatment for analyzing chlorophyll *a* and chlorophyll *c* (chl *a* &  
191 chl *c*, 5 mL  $\times$  2 analytical replicates), phycoerythrin (PE, 5 mL  $\times$  2 analytical replicates), fatty acids  
192 (FA, 5 mL  $\times$  2-3 analytical replicates), and free amino acids (FAA, 5 mL  $\times$  2 analytical replicates).  
193 Then, samples were filtered onto three 12.8 mm diameter GF/C glass fiber filter (Whatman) and  
194 preserved in a biofreezer at  $-80^{\circ}\text{C}$  for later analyses of pigments, fatty acids and free amino acids  
195 compositions.

#### 196 *Inorganic nutrient analysis of R. salina culture*

197 Nitrate, ammonium and phosphate in the filtered water from the algae culture were quantified using  
198 colorimetric techniques. Nitrate concentration was determined by flow injection analysis using  
199 QuickChem Method 31-107-04-1-A (Diamond 1999). Ammonium concentration was analyzed by  
200 salicylate-hypochlorite method for determining ammonium in seawater described by Bower and  
201 Holm-Hansen (2011). Phosphate concentration was quantified by a Spectrachrom UV-1601 UV-  
202 Visible Spectrophotometer (Shimadzu, Kyoto, Japan) following the method described by  
203 S ndergaard and Riemann (1979). The final concentration of inorganic nutrients (nitrate,  
204 ammonium and phosphate) in the specific experimental treatment was defined as the average ( $\pm$ SD)  
205 concentration between the last two sampling days (day 4 and 6) of the experiment.

#### 206 *Analyses of algal pigments*

207 Chl *a* and chl *c* were extracted based on the methods described by Jeffrey and Humphrey (1975)  
208 and Ritchie (2006). Filter samples were lyophilized before extraction. Each of these filters was  
209 placed in a glass vial where 3.3 mL of 90% acetone was added. Samples were shaken in a whirly  
210 mixer. Then, samples were placed in the dark for 24 hours at 5°C. The extraction solvent in each  
211 vial was transferred into a quartz cuvette through a 0.2 µm pore size syringe filter in which the  
212 absorbance of each sample was measured at 664 nm and 630 nm on a GENESYS™ 6  
213 Spectrophotometer (ThermoSpectronic). The concentration of chl *a* and chl *c* is expressed as pg  
214 cell<sup>-1</sup>.

215 The PE was extracted based on the procedure described by Bennett and Bogorad (1973), Evans  
216 (1988) and Zimba (2012). After lyophilization, each of the filter samples for PE extraction was  
217 placed into a glass vial together with 3 mL of phosphate buffer (0.1 mol pH 7, 0.05 mol K<sub>2</sub>HPO<sub>4</sub>,  
218 0.05 mol KH<sub>2</sub>PO<sub>4</sub>). Samples were sonicated in ice-water bath for 15 minutes and then were left  
219 refrigerated for 12 hours. Extraction solvent was filtered through at 25 mm 0.2 µm pore size syringe  
220 filter and placed into a cuvette for measuring the absorbance spectrophotometrically at 455nm,  
221 564nm and 592nm. PE concentration was calculated as in Bennett and Bogorad (1973). The  
222 concentration of PE is expressed as pg cell<sup>-1</sup>.

### 223 *Analyses of fatty acids*

224 The FA composition of *R. salina* was determined by extraction of the lipids using a HPLC-grade  
225 chloroform: methanol mixture (Folch et al. 1957) followed by trans esterification process by acetyl  
226 chloride in methanol (see Drillet et al. 2006 for details). In brief, a chloroform: methanol mixture (3  
227 mL, v:v = 2:1) was added to each of the algal sample. A volume of 20 µL of internal standard (1000  
228 µg mL<sup>-1</sup> tricosanoic FA methyl ester [C23:0 FAME]) was also added for FA quantification.  
229 Thereafter, samples underwent ultrasound extraction for 15 minutes in an ice bath to break the algal  
230 cells. In the next step, samples were frozen at -20°C for 24 h for extraction. Subsequently, the  
231 extraction solvent from each sample was transferred to GC vials and was placed into an aluminum  
232 block at 60°C to evaporate the chloroform: methanol solvent by a flow of nitrogen. Thereafter,  
233 AcOMe/HCl reagent in Toluene (1 mL) was added into the GC vials. The GC vials were covered by  
234 aluminum caps and were placed in the aluminum block for 2 hours at 95°C. Next, the caps were  
235 removed to add 500 µL of 5% of NaHCO<sub>3</sub>, and two different phases appeared. The upper phase was  
236 transferred into a new GC vial. Subsequently, 500 µL of heptane was added and the upper phase  
237 was added to the new GC vial. The samples were evaporated at 65°C under a steady flow of

238 nitrogen. Thereafter, 0.5 ml of chloroform was added to each sample. Finally, all samples were  
239 analyzed by an Agilent GC6890N gas chromatograph while connected to an Agilent MS 5975 mass  
240 selective detector. The GC was equipped with a 60 m Agilent J&W DB23 column with 0.25 mm  
241 internal diameter and film thickness of 0.25  $\mu\text{m}$ . Splitless injection while running a positive electron  
242 ionization at 70 eV was selected. ChemStation software was used for MS peak integration. MS  
243 peaks were analyzed against a Supelco FAME standard mixture. Total fatty acid content (TFA) is  
244 expressed as  $\text{pg cell}^{-1}$ , while content of each FA is expressed as percentage of TFA (% of TFA).

#### 245 *Analyses of free amino acids*

246 The FAA of *R. salina* was analyzed based on the method also reported by Drillet et al. (2006). The  
247 filter samples were lyophilized 24h prior to extraction. FAA was extracted in 1 mL Milli-Q water  
248 by heating the filter samples to 95°C for 10 min. The extracts were filtered through 8 mm 0.2  $\mu\text{m}$   
249 pore size GHP polypropylene membrane filters. The FAA was derivatized (Yu et al. 1994) using a  
250 AccQFlour kit (Waters, MA, USA) and later separated on a Waters Alliance 2695 separation  
251 module with a 3.9  $\times$  150 mm AccQTag column. The separated AA derivatives were quantified by  
252 fluorescence (250 nm excitation and 395 nm emission) using an Alliance 2475 scanning  
253 fluorescence detector. Due to the limitation of the number of FAA samples (only one sample for  
254 each treatment), the FAA data was grouped into two categories according to the irradiance: i)  
255 limited irradiance (irradiance from 10 to 40  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ,  $n = 3$  for both nutrient deficiency  
256 and nutrient excess treatments) and ii) saturated irradiance (irradiance from 60 to 140  $\mu\text{mol photons}$   
257  $\text{m}^{-2} \text{ s}^{-1}$ ,  $n = 5$  for nutrient deficiency and  $n = 4$  for nutrient excess treatment). The saturated  
258 irradiances were from 60  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  where no further increase in the microalgal growth  
259 rate under the higher irradiance was observed (see the results). Total FAA is expressed as  $\text{pg cell}^{-1}$ ,  
260 while content of each amino acid is expressed as percentage of total FAA (% of total FAA).

#### 261 *Statistical analyses*

262 Response variables were subjected to two-way ANOVA with nutrient and irradiance as fixed  
263 factors. Tukey tests were subsequently used to compare individual means across significantly  
264 different treatment levels where relevant. For the results of Tukey tests for maximum cell density,  
265 nutrients, algal pigments, total fatty acids content and the DHA/EPA ratio, it was so difficult to see  
266 the different letters in bars as there are many bars in each figure that for clarity we prefer not adding  
267 the letters above the bars to indicate the statistically difference. We therefore only provide the

268 results of the Tukey test in a letter code in the tables for fatty acids composition and free amino acid  
269 composition results. Data were tested for homogeneity of variance (Cochran's test) and normal  
270 distribution (Kolmogorov-Smirnoff goodness of fit test) before being analyzed by ANOVA. All  
271 tests on data were carried out using SAS v. 9.3 with  $\alpha = 0.05$ .

## 272 Results

### 273 *Photosynthesis*

274 The realized photosynthesis rate ( $P^B_m$ ) and the initial slope of the P-I curves ( $\alpha$ ) decreased with the  
275 increase of cell density (Fig. 1 and Table 1). The  $P^B_m$  value was 22 times higher at the lowest cell  
276 density ( $0.1 \times 10^6$  cells mL<sup>-1</sup> =  $539.4 \times 10^{-15}$  mol O<sub>2</sub> cell<sup>-1</sup> h<sup>-1</sup>) compared to the highest cell density  
277 ( $10 \times 10^6$  cells mL<sup>-1</sup> =  $20.6 \times 10^{-15}$  mol O<sub>2</sub> cell<sup>-1</sup> h<sup>-1</sup>). In contrast, the irradiance of maximum  
278 photosynthesis ( $I_m$ ) increased with increasing cell density. The lowest  $I_m$  values were 57.5-59.2  
279  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  occurring at the two lowest cell densities of  $0.1\text{-}0.5 \times 10^6$  cells mL<sup>-1</sup> and the  
280 highest  $I_m$  value was 103.0  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  (ca. 1.5 times higher than the lowest  $I_m$  value)  
281 occurring at the highest cell density of  $10 \times 10^6$  cells mL<sup>-1</sup> (Table 1). The photoinhibition ( $\beta$ )  
282 decreased with increasing cell density (Fig. 1, Table 1). Dark respiration (R) decreased with  
283 increasing cell density, indicated by the higher oxygen consumption per cell (Table 1). The highest  
284 dark respiration was recorded at lowest cell density  $0.1 \times 10^6$  cells mL<sup>-1</sup> ( $-319.1 \pm 19.8 \times 10^{-15}$  mol  
285 O<sub>2</sub> cell<sup>-1</sup> h<sup>-1</sup> = 59.1%  $P^B_m$ ) which was ca. 18 times higher than the dark respiration of cells at highest  
286 density  $10 \times 10^6$  cells mL<sup>-1</sup> ( $-17.9 \pm 5.7 \times 10^{-15}$  mol O<sub>2</sub> cell<sup>-1</sup> h<sup>-1</sup> = 86.7%  $P^B_m$ ). The ratio of relative  
287 dark respiration rate to maximum photosynthesis ( $R/P^B_m$ ) increased slightly with increasing cell  
288 density (Table 1) indicating that the cell culture became less autotrophic with increasing cell  
289 density.

### 290 *Microalgae growth*

291 As the specific growth rate was measured during the exponential growth phase, where no nutrient  
292 deficiency had yet set in, there is no significant difference in the specific growth rate between  
293 nutrient deficiency and nutrient excess treatments. These were therefore pooled for fitting the same  
294 specific growth rate-irradiance curve (Fig. 2a). The specific growth rate increased with the increase  
295 of irradiance and reached a plateau from the irradiance of 60  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  onward (Fig. 2a).  
296 The maximal growth rate ( $\mu_{\text{max}}$ ) was 0.752 day<sup>-1</sup> with the maximal quantum yield for growth of  
297 0.014 cells day<sup>-1</sup> [ $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ]<sup>-1</sup>. Algae did not grow ( $\mu \approx 0$ ) at the lowest irradiance (10  
298  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ).

299 Maximum cell densities increased with increasing irradiance (Fig. 2b, Appendix 1). This pattern  
300 was consistently stronger in nutrient excess cultures than in nutrient deficient cultures (Appendix 1).  
301 The maximal cell density in nutrient excess ( $3.2\text{-}5.3 \times 10^6$  cells mL<sup>-1</sup>) was about 2-3 times higher

302 than in nutrient deficiency ( $1.7-1.9 \times 10^6$  cells mL<sup>-1</sup>) at the irradiance from 80  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$   
303 onwards, resulting in the overall higher maximal cell densities (Fig. 2b) in nutrient excess than in  
304 nutrient deficiency (Appendix 1).

305 The cell biovolume of *R. salina* was found to be 200-300  $\mu\text{m}^3$  (Fig. 2c). Linear regression revealed  
306 no significant relationship between specific growth rate and cell biovolume,  $p = 0.633$  and  $p =$   
307  $0.923$ , for nutrient excess and deficiency, respectively, indicating that cell biovolume is independent  
308 of growth rate in this study, regardless of treatment.

### 309 *Nutrient consumption*

310 The initial concentration of nitrate was  $1092.9 \pm 108.3 \mu\text{mol L}^{-1}$  in all treatments. The final nitrate  
311 concentrations (Fig. 3a) remaining in the algae culture decreased with increasing irradiance  
312 (Appendix 1) and were higher in nutrient excess than in nutrient deficiency (Appendix 1). The  
313 lowest final nitrate concentrations were recorded at the irradiances of 140  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  ( $1.1$   
314  $\pm 1.0 \mu\text{mol L}^{-1}$ ) and 120  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  ( $418.3 \pm 384.9 \mu\text{mol L}^{-1}$ ) in nutrient deficiency and  
315 nutrient excess, respectively. In addition, the variation in the final nitrate concentrations was wider  
316 under nutrient deficiency than under nutrient excess (Appendix 1).

317 At the start of the experiment, the ammonium concentration was under the detection limit ( $0.05$   
318  $\mu\text{mol L}^{-1}$ ) in all treatments. At the end of the experiment, the ammonium concentration was only  
319 detectable in the treatments at irradiances of higher than 20  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  and of higher than  
320 40  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  for nutrient deficiency and nutrient excess (Fig. 3b), respectively. The final  
321 ammonium concentration increased with increasing irradiance (Appendix 1) and was considerably  
322 higher in nutrient excess than nutrient deficiency (Appendix 1).

323 The initial concentration of phosphate was  $160.9 \pm 29.0 \mu\text{mol L}^{-1}$  in all treatments. The final  
324 phosphate concentration (Fig. 3a) remaining in the algae culture decreased with increasing  
325 irradiance (Appendix 1) and was higher at nutrient excess than at nutrient deficiency (Appendix 1).  
326 At the end of the experiment, the lowest phosphate concentration was obtained at the highest  
327 irradiance 140  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  in both nutrient deficiency ( $86.7 \pm 35.0 \mu\text{mol L}^{-1}$ ) and nutrient  
328 excess ( $112 \pm 10.2 \mu\text{mol L}^{-1}$ ). The decrease in final phosphate concentration with irradiance was  
329 more pronounced in nutrient deficiency than nutrient excess (Appendix 1).

### 330 *Algal pigments*

331 Overall, cellular chlorophyll *a* (chl *a*) levels decreased with increasing irradiance (Fig. 4a,  
332 Appendix 1). The highest chl *a* (ca. 6.7 pg cell<sup>-1</sup>) recorded at the irradiance of 20 μmol m<sup>-2</sup> s<sup>-1</sup> was  
333 ca. 3 times higher than the lowest chl *a* (ca. 2.4 pg cell<sup>-1</sup>) recorded at the highest irradiance (140  
334 μmol m<sup>-2</sup> s<sup>-1</sup>). Chl *a* levels did not differ between nutrient deficiency and nutrient excess (Appendix  
335 1). There was a statistically significant interaction between the irradiance and nutrient on chl *a*  
336 concentrations (Appendix 1).

337 Cellular chlorophyll *c* (chl *c*) levels decreased with increasing irradiance (Fig. 4b, Appendix 1). The  
338 chl *c* levels were not affected by nutrient treatment (Appendix 1). However, there was an interaction  
339 between irradiance and nutrient on chl *c* (Appendix 1).

340 The cellular phycoerythrin (PE) levels were influenced by both irradiance (Fig. 4c, Appendix 1) and  
341 nutrients (Fig. 4c, Appendix 1). In nutrient deficiency, the PE rapidly increased at low irradiance  
342 and peaked at 40 μmol photons m<sup>-2</sup> s<sup>-1</sup> (ca. 18 pg cell<sup>-1</sup>). Thereafter, the PE decreased rapidly to the  
343 lowest level at 140 μmol photons m<sup>-2</sup> s<sup>-1</sup> (ca. 3 pg cell<sup>-1</sup>). In nutrient excess, the PE increased  
344 rapidly with increasing irradiance at low irradiances, reaching the highest concentration at the  
345 irradiance of 60 μmol photons m<sup>-2</sup> s<sup>-1</sup> (somewhat later compared to nutrient deficiency) and then  
346 remained at this high level until 100 μmol photons m<sup>-2</sup> s<sup>-1</sup> before slightly decreasing at 120-140  
347 μmol photons m<sup>-2</sup> s<sup>-1</sup>. This difference generated a statistically significant Irradiance × Nutrient  
348 interaction (Appendix 1). In term of nutrients, the PE level was higher in nutrient excess (11.1 ± 5.5  
349 pg cell<sup>-1</sup>) than nutrient deficiency (8.9 ± 6.5 pg cell<sup>-1</sup>) but this pattern was driven by the fact that PE  
350 levels were considerably higher in nutrient excess at the irradiance from 80 μmol photons m<sup>-2</sup> s<sup>-1</sup>  
351 onward.

352 The PE/chl *a* ratio (Fig. 4d) resembled the patterns of the PE. The PE/chl *a* ratio was affected by  
353 both irradiance (Appendix 1) and nutrients (Appendix 1). Specifically in nutrient deficiency, the  
354 PE/chl *a* ratio rapidly increased at low irradiances, peaking at an irradiance of 40 μmol photons m<sup>-2</sup>  
355 s<sup>-1</sup> (4.2 pg cell<sup>-1</sup>), then decreased steadily with increasing irradiances, attaining the lowest level at  
356 140 μmol photons m<sup>-2</sup> s<sup>-1</sup> (1.2 pg cell<sup>-1</sup>). In nutrient excess, the PE/chl *a* ratio increased with  
357 increasing irradiance and reached a plateau (2.9-3.5 pg cell<sup>-1</sup>) from the irradiance of 40 μmol  
358 photons m<sup>-2</sup> s<sup>-1</sup> onwards (Fig. 4d). This difference also generated a statistically significant  
359 interaction (Appendix 1) and resulted in an overall higher PE/chl *a* ratio in nutrient excess than in  
360 nutrient deficiency.

361 The PE content tended to be negatively correlated to the cell density when cultivated in nutrient  
362 deficiency ( $p = 0.0579$ , Fig. 4e). In nutrient excess, the PE content was not significantly related to  
363 the cell density ( $p = 0.3434$ , Fig. 4f).

#### 364 *Fatty acids*

365 Overall, the total fatty acids (TFA) content increased with increasing irradiance (Fig. 5a,  
366 Appendix 1). The TFA was considerably higher (Appendix 1) in algae cultured in nutrient  
367 deficiency ( $34.5 \pm 22.5$  pg cell<sup>-1</sup>) than in nutrient excess ( $11.4 \pm 4.2$  pg cell<sup>-1</sup>). Most notably, TFA  
368 was ca. 3-4 times higher within the irradiance range of 60 to 140  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  in nutrient  
369 deficient cultures in comparison to the nutrient excess, generating a statistically significant  
370 Irradiance  $\times$  Nutrient interaction (Fig. 5a, Appendix 1).

371 Irradiance had statistically significant effects on the relative abundance of the mono-unsaturated  
372 fatty acids (MUFA) (Fig. 5b&c, Appendix 1), especially in nutrient excess, generating an  
373 interaction of Irradiance  $\times$  Nutrient on MUFA (Appendix 1). Algae cultured at lowest and highest  
374 irradiance (10 and 140  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) had significant higher MUFA levels compared to other  
375 irradiances (Fig. 5b&c).

376 Irradiance also affected the content of short chain polyunsaturated fatty acids (SC-PUFA =  
377 characterizing by a carbon number  $< 20$  and double bonds  $> 1$ ) (Fig. 5b&c, Appendix 1). This  
378 pattern was independent of nutrient levels (Appendix 1). SC-PUFA increased with increase in  
379 irradiance. On the other hand, the changing in irradiance levels did not affect the concentration of  
380 the saturated fatty acids (SFA) and highly unsaturated fatty acids (HUFA) (Fig. 5b&c, Appendix 1).

381 Nutrient had a statistically significant effect on the SFA (Appendix 1) and HUFA (Appendix 1) of  
382 the algae (Fig. 5b&c). Relative abundance of SFA was considerably higher when algae were  
383 cultured during nutrient deficiency ( $26.8 \pm 8.3$  % of TFA) compared to those in nutrient excess  
384 ( $11.0 \pm 3.1$ % of TFA). In contrast, the relative abundance of HUFA was ca. 2 times higher in algae  
385 cultured in nutrient excess than those cultured in nutrient deficiency (Appendix 1), accounting for  
386  $31.8 \pm 2.5$ % and  $15.1 \pm 5.7$  % of TFA, respectively. Especially Eicosapentaenoic acid (EPA), the  
387 most abundant HUFA in *R. salina*, was 22.1 % of TFA in nutrient excess, which was ca. 2 times  
388 higher than in nutrient deficiency (10.3 % of TFA).



389 Overall, the ratio between the two essential fatty acids Docosahexaenoic acid and Eicosapentaenoic  
390 acid (DHA/EPA) was lower at irradiance 10-20  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  than at irradiance from 40-140  
391  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  (Appendix 1). The nutrient had no effect on DHA/EPA ratio which varied  
392 from 0-0.86 in nutrient deficiency and from 0.28-0.57 in nutrient excess. There was an Irradiance  $\times$   
393 Nutrient interaction (Appendix 1), but this interaction was driven by the fact that DHA was mostly  
394 not synthesized at the two lowest irradiance level (Fig. 5d).

395 More details in fatty acids composition of *R. salina* cultured in nutrient deficiency and nutrient  
396 excess under limited (irradiance from 10 to 40  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) and saturated (irradiance from  
397 60 to 140  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) irradiances was summarized in Appendix 2. Noticeably, relative  
398 abundance of SFA 18:0 was highest at saturated irradiance and under nutrient deficiency, whereas,  
399 relative abundance of SC-PUFA 18:4, DHA and EPA was highest at saturated irradiance and in  
400 nutrient excess.

#### 401 *Free amino acids*

402 The total free amino acids (FAA) and specific essential amino acids (EAA, mean  $\pm$  SDs of total  
403 FAA) for copepods (Claybrook 1983) and fish growth (Wilson 1985) that were present in *R. salina*  
404 cells cultured at different irradiance and nutrient conditions are summarized in table 2. Total FAA  
405 was affected by the irradiance (Table 2), but not by the nutrient levels (Table 2). The total FAA was  
406 significantly higher when the algal cells were cultivated at limited irradiance ( $8.3 \pm 2.5 \text{ pg cell}^{-1}$  at  
407 10-40  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) than in the saturated irradiance ( $4.3 \pm 1.3 \text{ pg cell}^{-1}$  at 60-140  $\mu\text{mol}$   
408  $\text{photons m}^{-2} \text{s}^{-1}$ ). There was no interaction between irradiance and nutrient levels on total FAA of  
409 algae (Table 2).

410 The relative abundance of sub-total EAA (% of total FAA) was affected by the irradiance and the  
411 interaction Irradiance  $\times$  Nutrient, but not the nutrient levels (Table 2). In contrast with total FAA,  
412 the relative abundance of sub-total EAA was significantly higher at saturated irradiance, especially  
413 in nutrient in excess ( $31.89 \pm 4.24$  % of total FAA, Table 2).

414 The difference in the irradiance and nutrient showed no consistent effect on relative abundance of  
415 specific EAA (Table 2). The most abundant EAA in *R. salina*, arginine was higher in saturated  
416 irradiance, especially under nutrient deficiency (Table 2). Seven out of ten EAA, including  
417 isoleucine, leucine, lysine, methionine, phenylalanine, threonine, valine, were generally higher in  
418 the algae cells cultured under saturated irradiance and in nutrient excess compared to those cultured

419 at limited irradiance and in nutrient deficiency (Table 2). In contrast, histidine was higher in algae  
420 grown at limited irradiance and in nutrient deficiency (Table 2).

421

## 422 **Discussion**

423 In this study, we found strong effects of irradiance and/or nutrient levels on all measured variables  
424 such as photosynthesis, growth rate, and biochemical composition of *Rhodomonas salina*.

### 425 *Photosynthesis*

426 The photosynthesis rate of *R. salina* at all five cell densities increased with increasing irradiance  
427 until reaching a saturating irradiance ( $I_m$ ) of 60-100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . The range of  $I_m$  found in  
428 this study confirmed what has been documented in previous studies ( $I_m \geq 60 \mu\text{mol photons m}^{-2} \text{s}^{-1}$   
429 Hammer et al. 2002; and  $I_m \geq 200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  Bartual et al. 2002). Photoinhibition,  
430 indicated by  $\beta > 0$  (Platt et al. 1980), occurred at all cell densities, but higher cell densities reduced  
431 the photoinhibition as indicated by the rapid decrease of  $\beta$  with increasing cell densities. This  
432 density-mediated decrease in photoinhibition is probably due to an increase in self-shading at higher  
433 cell densities. This finding was in contrast with previous studies (e.g., Hammer et al. 2002; Bartual  
434 et al. 2002) where *R. salina* showed no photoinhibition when being exposed to irradiances from 0-  
435 1200  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . This difference could be a result of methodical differences. In our study,  
436 photosynthesis measurements were carried out using the culture maintained at 80  $\mu\text{mol photons m}^{-2}$   
437  $\text{s}^{-1}$  at a cell density of ca  $1.5\text{-}2 \times 10^6 \text{ cells mL}^{-1}$ , which was then diluted to different cell densities.  
438 Therefore, the algae may already be acclimated to relatively low irradiances before the  
439 measurements took place while in other studies the photosynthesis was measured at low algal cell  
440 densities (although it was unclear which algal densities that these studies used) at different  
441 irradiances, from 11-320  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  (Bartual et al. 2002) or 10-150  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$   
442 (Hammer et al. 2002).

443 In addition, the respiration rate in darkness was also higher at low cell density than at high cell  
444 density, probably due to the higher cellular photosynthetic activity at low cell densities, leading to  
445 higher metabolic maintenance cost (Kromkamp and Peen 2001).

### 446 *Algal growth*

447 Regarding the specific growth rate versus irradiance curve, the saturating irradiance (60-140  $\mu\text{mol}$   
448  $\text{photons m}^{-2} \text{s}^{-1}$ ) for maximal growth and maximal growth rate ( $\mu_{\text{max}}$ ) obtained in our study are in  
449 agreement with previous studies on *R. salina* (Bartual et al. 2002; Hammer et al. 2002; Lafarga-De  
450 la Cruz et al. 2006). The maximal growth rate of *Pyrenomonas salina* (a taxonomic synonym of *R.*

451 *salina*) was somewhat higher ( $1.2 \text{ day}^{-1}$ ) at a saturating irradiance of ca.  $100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  in  
452 the study of Lewitus and Caron (1990). The higher specific growth rate in the previous study in  
453 comparison to our study is most likely due to the use of a more preferable nitrogen source-  
454 ammonium for algal growth instead of nitrate in our study. It is well known that algae can take up  
455 and assimilate ammonium directly while they have to reduce nitrate to ammonium before  
456 assimilation (Rückert and Giani 2004), hence a higher energetic cost is associated with nitrate use.  
457 Under low irradiance ( $< 20 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ), the growth rate was very low, as also observed in  
458 other studies (Lewitus and Caron 1990; Bartual et al. 2002) that may be associated with the low  
459 photosynthetic rate at low irradiance.

460 Until the end of the exponential phase (day 3), the specific growth rate of *R. salina* did not differ  
461 between nutrient levels as nutrients in all treatments was still above the half saturation constants for  
462 algal growth, namely nitrate:  $0.4 \mu\text{mol L}^{-1}$  (Falkowski 1975) and phosphate:  $0.51 \mu\text{mol L}^{-1}$  (Smith  
463 and Kalff 1982). The nutrient deficiency only showed its effects on the algal growth from day 4 and  
464 onwards where the nitrate concentration was almost depleted. Not surprisingly, the cell densities in  
465 nutrient deficient cultures did not increase beyond this point. However, in the current study, the  
466 maximum cell density in nutrient deficient cultures was still higher than the reported value  $1.53 \times$   
467  $10^6 \text{ cells mL}^{-1}$  by Lafarga-De la Cruz et al. (2006), which may be a result of the difference in  
468 experimental set up. In Lafarga-De la Cruz et al. (2006), algae were cultivated in batch without  
469 aeration in (250 mL) Erlenmeyer flasks whereas in our study, the algae was cultivated in a Multi-  
470 cultivator with continuous bubbling of atmospheric air that may provide a better environment for  
471 growth by enhancing  $\text{CO}_2$  addition, avoiding light/temperature stratification, algal sedimentation  
472 and  $\text{O}_2$  build up. In contrast, the nitrate concentration in nutrient excess remained very high during  
473 all 6 days of the experiment. As a result, cell densities increased continuously throughout this  
474 period, especially under optimal range of irradiance and nutrient excess, where maximal cell  
475 densities were 2-3 times higher than those cultured in nutrient deficiency of the same light regimes,  
476 indicating a promising algal production.

477 As cell biovolume was not significantly affected by growth rate, and has been found to be constant  
478 across experimental conditions and treatment, we have chosen to express all data on biochemical  
479 composition on a per cell basis.

480 *Algal pigments*

481 In response to the decrease in irradiance, the phytoplankton typically increases chlorophyll *a* (chl *a*)  
482 and other light harvesting pigments, such as chlorophyll *b* (chl *b*), chlorophyll *c* (chl *c*), and primary  
483 carotenoids (Hu 2004). These results have been observed in Cryptophyte species (Faust and Gantt  
484 1973; Lichtlé 1979), including *R. salina* (Bartual et al. 2002). In the present study, the chl *a* and chl  
485 *c* concentrations were higher at lower irradiance as a response to low irradiance (Hammer et al.  
486 2002; Hu 2004). When the algae are exposed to high irradiance, phycobiliproteins and carotenoids  
487 act as protection mechanisms against the excess light (Pereira et al. 2012). Therefore, it was expected  
488 that PE content would increase at high irradiance to increase the photoprotection, which indeed was  
489 observed in our study under nutrient excess. In our experiment, the phycoerythrin (PE) and chl *a*  
490 (PE/chl *a*) ratio of *R. salina* decreased with the increase of irradiance ( $> 40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) in  
491 nutrient deficiency, which has been also observed in the Cyanophyceae *Anacystis nidulans* (Halldal  
492 1958) or the Cryptophyceae *Chroomonas* sp. (Faust and Gantt 1973). Moreover, the PE/chl *a* ratio  
493 follows the pattern of total PE against irradiance, and can be explained by PE being more sensitive to  
494 variations in irradiance than chl *a*. (Brown and Richardson 1968).

495 A common pattern in algae is that cells respond to nutrient (nitrogen) deficiency by decreasing  
496 pigment content (Hu 2004). This pattern has been reported in several Cryptophyte species such as  
497 *Cryptomonas rufescens* (Lichtlé 1979), *Cryptomonas maculata* (Rhiel et al. 1985) and  
498 *Pyrenomonas salina* (Lewitus and Caron 1990). In our study, while the PE and PE/chl *a* ratio were  
499 indeed lower in nutrient deficiency, we observed no effect of nutrients on the chl *a* and chl *c*  
500 content. For the chl *a* content, the nitrate concentration in our experiment (the lowest nitrate  
501 concentration was  $1.1 \pm 1.0 \mu\text{mol}$ ) was probably not too depleted to induce a reduction in chl *a*  
502 content like in the study of Bartual et al. (2002) in which *R. salina* suffered under nitrogen  
503 concentrations below  $0.5 \mu\text{mol}$  to total exhaustion from day 4 of the experiment. The drop of PE and  
504 PE/chl *a* ratio under nutrient deficiency has been observed before in *R. salina* (Bartual et al. 2002).  
505 This nutrient-induced reduction in PE and PE/chl *a* ratio is explained by mobilization of nitrogen  
506 from PE (Bartual et al. 2002).

507 In our study, PE decreases with increasing cell density under nutrient deficiency, indicating that  
508 this phycobillipigment is scavenged as a nitrogen source under nutrient deficiency (Bartual et al.  
509 2002; Lewitus and Caron 1990; Eriksen and Iversen 1995) as photoprotection is decreasing with  
510 increasing cell density and thus increasing self-shading.

511 *Fatty acids*

512 In line with previous studies, the higher total fatty acids (TFA) was obtained at higher irradiance  
513 (Sharma et al. 2012; Dongre et al. 2014) and nutrient deficiency (Sriharan et al. 1991; Piorreck et al.  
514 1984; Hu 2004; Shifrin and Chisholm 1981, but see Harrison et al. 1990). Strikingly, TFA levels  
515 increased sharply to very high level at irradiance 60-140  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  under nutrient  
516 deficiency. The increase of TFA with increase of irradiance in nutrient deficiency could be  
517 correlated to the increase of triacylglycerols-TAG (Sharma et al. 2012; Dongre et al. 2014). This is  
518 likely associated with the synthesis of TAG (generally contain saturated fatty acids-SFA and mono-  
519 unsaturated fatty acids-MUFA) that mostly occur under adequate light conditions, hence this  
520 synthesis can be maximized when cultivated at light saturation (Dongre et al. 2014). Besides, in  
521 nutrient deficiency condition (nitrogen starvation), many algal species accumulate lipids due to that  
522 these constituents do not contain N (mostly TAG including SFA and MUFA, Shifrin and Chisholm  
523 1981; Sharma et al. 2012). When nutrients are limited, the cell division rate decreases steadily,  
524 hence the requirement for membrane compounds reduce or almost reach no requirement any more  
525 (Sharma et al. 2012). However, active biosynthesis of FA is maintained (Sharma et al. 2012).  
526 Consequently, the cells divert and deposit fatty acids into TAG (Sharma et al. 2012).

527 In our study, irradiance did not have a consistent effect on specific groups of fatty acids.  
528 Specifically, irradiance had a positive correlation with the relative abundance of short chain-poly  
529 unsaturated fatty acids (SC-PUFA), but no correlation with SFA and highly unsaturated fatty acids  
530 (HUFA). We found that the irradiance of 20-140  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  induced higher relative  
531 content of SC-PUFA (a sub-group of poly unsaturated fatty acids-PUFA) than at 10  $\mu\text{mol photons}$   
532  $\text{m}^{-2} \text{s}^{-1}$  whereas the HUFA (also sub-group of PUFA) was highest at 40  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  and  
533 relative constant for all other irradiances. This result did not reflect the general rule reported by  
534 Harwood (1998) when high irradiance usually leads to oxidative damage of PUFA (including SC-  
535 PUFA and HUFA as defined in our study). The self-shading of the high algae density ( $1.1-4.1 \times 10^6$   
536  $\text{cells mL}^{-1}$ ) recorded at high irradiance cultures may reduce the effect of the high irradiance on  
537 oxidative damage of the PUFA, indicating the potential to use density manipulation towards  
538 designer feed. In detail, irradiance had positive correlation with the relative abundance of DHA but  
539 not EPA. This result has also been observed in previous studies (e.g., Renaud et al. 1991; Harrison  
540 et al. 1990; Thompson et al. 1990). Harrison et al. (1990) found that Docosahexaenoic acid (DHA)  
541 increased as a function of irradiance for all three microalgae whereas Eicosapentaenoic acid (EPA)  
542 was relatively constant over a range of irradiance for *Chaetoceros* and *Thalassiosira* but increased  
543 significantly for *Isochrysis*. The increase in DHA and decrease in EPA at high irradiance result in

544 an increase of DHA/EPA ratio. In our study, the DHA/EPA of *R. salina* ratio was in the range of  
545 0.51-0.70, except for the low irradiance (10-20  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) where algae did not produce  
546 DHA or at least below detection limit of our method. The similar range of DHA/EPA has been  
547 reported before in this algal species (Mansour et al. 2005; Dunstan et al. 2005; Drillet et al. 2006).

548 In term of fatty acids composition, it is important to note that the higher TFA level under nutrient  
549 deficiency comprised mainly of the higher relative contents of SFA 16:0, MUFA 18:1. On the other  
550 hand, under nutrient deficiency, the relative contents of HUFA, including EPA-20:5n-3 and DHA-  
551 22:6n-3, the most desired components for calanoid copepods (Arendt et al. 2006; Broglio et al.  
552 2003), were lower. This phenomenon is common in many marine microalgae (Reitan et al. 1994).  
553 The nutrient limitation probably reduced the synthesis of n-3 PUFA (Reitan et al. 1994). As we can  
554 see in our study, the relative abundance of HUFA, especially DHA and EPA of *R. salina*, was  
555 considerably higher in nutrient excess than in nutrient deficiency. Depending on the purpose of  
556 aquaculture, with different desires of fatty acids, the high or low nutrient medium can be chosen to  
557 generate a desired microalgae fatty acid profile.

558 In general the higher the level of relative abundance of PUFA (including SC-PUFA and HUFA)  
559 content in the *R. salina*, the better feed for copepods (reviewed in Rasdi and Qin 2014). This is  
560 obtained when the *R. salina* algae are cultured at saturated irradiances and in nutrient excess  
561 condition. Previous studies showed that the increase in PUFA (including SC-PUFA and HUFA) in  
562 algae diet would enhance the egg production and somatic growth of copepods (Rasdi and Qin  
563 2014). Therefore, the relatively higher abundance of PUFA of *R. salina* cultured in saturated  
564 irradiances and in nutrient excess will benefit the performance of copepods. Please note that the  
565 nutrient content in copepods, unlike rotifer and *Artemia*, cannot be manipulated through enrichment  
566 techniques due to their avoidance (Huntley et al. 1986; Rasdi and Qin 2014; Rasdi et al. 2015) and  
567 nutrient content in copepods can only be enhanced by feeding on high quality algae (Rasdi and Qin  
568 2014; Rasdi et al. 2015).

#### 569 *Free amino acids*

570 The irradiance had significant effect on both the quantity and the composition of free amino acids  
571 (FAA), whereas nutrient levels only affected the composition of the FAA. In particular, the limited  
572 irradiance induced higher total FAA whereas relative abundance of essential amino acid was higher  
573 at saturated irradiance. A more favorable essential amino acids (EAA) profile was obtained at

574 higher irradiance with higher contents of most of the specific EAAs, such as arginine, isoleucine,  
575 leucine, lysine, methionine, phenylalanine, threonine and valine. This indicates a potential for  
576 designer feed at high irradiance. While the changes in total FAA under different irradiances has not  
577 been reported before, higher total FAA at lower cultured irradiance was observed in a seaweed  
578 *Caulerpa prolifera* (Khaleafa et al. 1982). The effects of irradiance on FAA composition in our  
579 study were in contrast with previous studies on different algae species *Isochrysis* sp., *Pavlova*  
580 *lutheri* and *Nannochloropsis oculata* where the amino acid composition of the proteins of  
581 microalgae has been shown relatively unaffected by the growth phase (normally nutrient is depleted  
582 in the stationary phase) and light conditions (Brown et al. 1993a; Brown et al. 1993b; Brown et al.  
583 1996). The difference in the effect of irradiance on FAA composition of our study and previous  
584 studies could be a result of the species and/or strain specific responses. However, it is noted that the  
585 changes in FAA composition of *R. salina* in different cultured irradiance and nutrient condition in  
586 our study may not entirely reflect the protein bound AAs.

587



588 **Recommendations**

589 The microalgae *Rhodomonas salina* is a preferred feed item for e.g. copepods used as live feed in  
590 hatcheries (Zhang et al. 2013). With our purpose to formulate designer feed for these promising  
591 zooplankton live feed organisms, several scenarios are possible when cultivating *R. salina*. i) One  
592 can either prioritize high microalga productivity where saturated irradiance and excess inorganic  
593 nutrients are implicit. This condition will generate a relatively larger algal biomass with a better  
594 fatty acids and free essential amino acids profiles. ii) Another strategy involves saturated irradiance  
595 and nutrient deficiency, which generates algal biomass with high total fatty acids content and  
596 remain an appropriate DHA/EPA ratio. Moreover, a relatively high content of highly unsaturated  
597 fatty acids will occur as a result of nutrient excess, invariant of irradiance. Overall, the most durable  
598 and recommended compromise for large scale production in algal photobioreactors for most  
599 purposes is to cultivate the microalgae at 60-100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  irradiance and in nutrient  
600 excess.

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609

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814 **Caption for figures**

815 **Fig. 1** Net photosynthesis per cell of *R. salina* ( $\times 10^{-15}$  mol O<sub>2</sub> cell<sup>-1</sup> h<sup>-1</sup>) incubated at different cell  
816 densities and irradiance levels. Note: the number of experimental replicates, n = 5 for irradiances  
817 from 0-140  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  and n = 2-3 for irradiances from 160-300  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$

818 **Fig. 2** The specific growth rate (SGR) (a), maximum cell density (b) and the plot between SGR and  
819 cell biovolume (c) of *Rhodomonas salina* in response to different irradiance and nutrient levels.  
820 Data are indicated by means ( $\pm$  SDs) of specific growth rate of algae cultured in nutrient deficiency  
821 and nutrient excess at the same levels of irradiance. Note: the number of experimental replicates, n  
822 = 4 (2 replicates from nutrient deficiency and 2 replicates from nutrient excess treatments)

823 **Fig. 3** Nitrate, ammonium and phosphate concentrations in the culture media of *Rhodomonas salina*  
824 with different irradiance and nutrient levels. Data are means ( $\pm$  SDs) of nutrient at day 4 and day 6,  
825 the number of analytical replicates, n = 2

826 **Fig. 4** The chlorophyll *a* (a), chlorophyll *c* (b), phycoerythrin (PE) (c) and the  
827 phycoerythrin/chlorophyll *a* (PE/chl *a*) ratio (d) and the relationship between PE and cell density of  
828 *Rhodomonas salina* cultured in nutrient deficiency (e) and nutrient excess (f) under different  
829 irradiance levels. The number of analytical replicates, n = 2 for all of presented parameters. In  
830 figure 4 e and f, the solid lines are the regression lines between PE and cell density, the dashed lines  
831 are the 95% confident interval of these regression lines

832 **Fig. 5** Total fatty acid (TFA) (a), the FA composition in nutrient deficiency (b) and nutrient excess  
833 (c), and the DHA/EPA ratio (d) of *Rhodomonas salina* under different irradiance levels. SFA:  
834 saturated fatty acids; MUFA: mono unsaturated fatty acids; HUFA: highly unsaturated fatty acids,  
835 SC-PUFA: short chain-poly unsaturated fatty acids. The number of analytical replicates, n = 3 for  
836 nutrient deficiency and n = 2 for nutrient excess treatment

837

838 **Caption for tables**

839 **Table 1** Summary of photosynthetic-irradiance parameters for five algae densities of *Rhodomonas*  
840 *salina* exposed to 16 increasing irradiances from 0 to 300  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  PAR, with the  
841 number of experimental replicates,  $n = 5$  for irradiances from 0-140  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  and  $n = 2$ -  
842 3 for irradiances from 160-300  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$

843 Note:  $\alpha$  = Initial slope of the photosynthesis-irradiance curve;  $\beta$  = Negative slope at high irradiance;  
844  $I_m$  = irradiance of maximum photosynthesis,  $R$  = Dark respiration = mean  $\pm$  SDs (plus/minus  
845 standard deviation) of dark respiration at 0  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  of 5 experimental replicates at a  
846 specific cell density. Units:  $\alpha, \beta = 10^{-15} \text{ mol O}_2 \text{ cell}^{-1} \text{ h}^{-1} [\mu\text{mol photons m}^{-2} \text{s}^{-1}]^{-1}$ ;  $P_m^B, R = 10^{-15} \text{ mol}$   
847  $\text{O}_2 \text{ cell}^{-1} \text{ h}^{-1}$  and  $I_m = \mu\text{mol photons m}^{-2} \text{s}^{-1}$

848 **Table 2** Total free amino acids (FAA) and essential amino acids (EAA) in *Rhodomonas salina*  
849 cultured in different irradiance and nutrient levels

850 Note: Units of total FAA =  $\text{pg cell}^{-1}$ ; sub-total EAA, specific EAA = % of total FAA. Values for  
851 limited irradiance = mean  $\pm$  SDs (plus/minus standard deviation) of FAA/EAA at irradiance from  
852 10-40  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ; values for saturated irradiance = mean  $\pm$  SDs of FAA/EAA at  
853 irradiance from 60-140  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ;  $n$  is the number of experimental replicates. Different  
854 letters in the same row denote the significant differences in the same specific EAA between the  
855 different treatments