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1 Journal of Applied Phycology

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3 4 5	Optimization of photosynthesis, growth, and biochemical composition of the microalgae <i>Rhodomonas salina</i> – an established diet for live feed copepods in aquaculture
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22 Abstract

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

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

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

and composition of the fatty acids in microalgae (Renaud et al. 1991; Mortillaro et al. 2009). Hence,

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

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).

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

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

Amino acids (AA) constitute another group of important biochemical constituents determining the 75 nutritional quality of microalgae (Brown 1991). AAs are the building blocks for protein synthesis, 76 and are involved in numerous specific physiological functions (Aragão et al. 2004). Some AAs are 77 78 defined as essential amino acids (EAA) that either cannot be synthesized within the animal body or at an insufficient rate to meet the physiological needs for the growth of animals. EAAs must 79 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).

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

The overall purpose of the present study is to develop a cultivation protocol for applying *R. salina* 89 in large scale algal photobioreactors while optimizing their growth and nutritional value as algal 90 feed for live feed calanoid copepods. We measured the photosynthesis of *R. salina* under 91 irradiances from 10 to 300 μ mol photons m⁻² s⁻¹. In the next step, we conducted a full factorial 92 growth experiment in which *R. salina* was cultivated in a series of irradiances ranging from 10 to 93 140 µmol photons m⁻² s⁻¹ under two inorganic nutrient levels: deficiency and excess. We quantified 94 algal growth rates, three important pigments (chlorophyll a, chlorophyll c and phycoerythrin, 95 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 profile is always optimal for the use as fish larvae feed by simply manipulating two algal growth 98 factors (irradiance and nutrient). This will enable us to develop an intensive setup for a nutritious 99 food chain delivering live feed on request for marine hatcheries. 100

101 Materials and methods

102 Microalgal material and culturing conditions

The studied Cryptophyte *Rhodomonas salina* (equivalent spherical diameter 8 µm) was originally 103 derived as SCCAP K-1487 of the Scandinavian Culture Collection of Algae and Protozoa 104 (University of Copenhagen, Denmark). Cultures of R. salina was grown in acid washed 3-6 L round 105 glass flasks containing autoclaved 0.2 µm filtered seawater (salinity 30 ‰) enriched with B1 106 medium (1 mL L⁻¹ of seawater, Hansen 1989). The cultures were maintained under a continuous 107 irradiance of 80 µmol photons m⁻² s⁻¹ Photosynthetic Active Radiation (PAR) in a climate room at 108 20°C. The flasks were gently aerated with atmospheric air through 0.45 µm filters to mix the 109 cultures to avoid temperature stratification, algal sedimentation, CO₂ depletion and O₂ build up. 110 111 Photosynthesis measurements

- Measurements of photosynthesis-irradiance (P-I) curves were performed for 5 cell densities (0.1, 112 $0.5, 1, 2, 10 \times 10^{6}$ cells mL⁻¹) at 16 increasing irradiances (0, 10, 20, 40, 60, 80, 100, 120, 140, 160, 113 180, 200, 220, 240, 260, 300 μ mol photons m⁻² s⁻¹ PAR), with n = 5 experimental replicates of each 114 irradiances from 0 to 140 μ mol photons m⁻² s⁻¹ and n = 2-3 experimental replicates for irradiances 115 from 160 to 300 μ mol photons m⁻² s⁻¹. The photosynthesis of *R. salina* was measured when the 116 algae was in the exponential phase for all treatments. A high concentration of algae was achieved 117 by centrifuging at 1000 rpm for 5 minutes at 20°C. The algae suspension was diluted to the required 118 cell concentration using fresh 0.2 µm filtered autoclaved seawater containing B1 medium. Net 119 oxygen exchange rates were measured with a Clark-type oxygen electrode (S1 Oxygen Electrode 120 Disc, Hansatech Instruments, Norfolk, UK) fitted in a stirred Hansatech DW3 chamber. Light was 121 provided by a red LED-lamp (Hansatech LC1). The measurement at each irradiance was completed 122 within 5 min when steady-state photosynthesis had been achieved. 123
- 124 Curves were fitted to the photosynthesis irradiance data using the equation 1 (Platt et al. 1980):

125
$$P^B = P_S^B \left(1 - e^{\frac{-\alpha I}{P_S^B}} \right) e^{\frac{-\beta I}{P_S^B}}$$
(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 α : Initial slope of the P-I curve (the quantum yield)
- 130 β : Negative slope at high irradiance (photoinhibition)

131 In addition, the realized maximum photosynthetic rate attained (P_m^B) and irradiance of maximum

photosynthesis (I_m) were calculated using the following equations 2 and 3 (Platt et al. 1980):

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

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

135 Experimental design for growth experiment

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

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

experiment, all of algal cultures were grown for 5-6 days at 20°C, salinity 30 ‰ with the same flow
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

samples from each treatment and measuring by a Beckman MultisizerTM3 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^{-1}) of *R*. salina was calculated for the first three days

of the experiment by fitting cell density increase during the exponential phase with an exponentialgrowth equation 4:

$$\mathbf{N}_{t} = \mathbf{N}_{0} \times \mathbf{e}^{(\mu \times t)} \left(4 \right)$$

164 N₀ is the cell density at time zero (cells mL⁻¹)

165
$$\mu$$
 is the growth rate (day⁻¹)

t is the time (day)

167 The specific growth rate-irradiance curves was fitted using tangent hyperbola functions (Jassby and168 Platt 1976), equation 5:

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

- 170 Where, μ : specific growth rate (day⁻¹)
- 171 μ_{max} : Maximum growth rate (day⁻¹)
- 172 I: Irradiance (μ mol m⁻² day⁻¹)

173 α : Initial slope of the curve (maximum quantum yield for growth, day⁻¹ [µmol photons m⁻² s⁻¹]⁻¹)

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

Sample preparation for quantification of inorganic nutrients of algae cultures and pigments, fatty 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 biochemical analyses of the algae cell R. salina were carried out by utilizing the samples taken from 184 185 the second experimental replicate. During every second day, sample water was sampled from each test tube for analyzing nitrate (4 mL), ammonium (3 mL) and phosphate (6 mL). Culture water was 186 187 filtered through 25 mm syringe filter (VWR International, USA) containing a Whatman GF/F glass fiber filter to remove algae, and the sample water was then stored in -20°C for later analyses of 188 nutrient compositions. In the stationary phase of the algal growth, samples of *R. salina* were taken 189 190 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 (FA, 5 mL \times 2-3 analytical replicates), and free amino acids (FAA, 5 mL \times 2 analytical replicates). 192 Then, samples were filtered onto three 12.8 mm diameter GF/C glass fiber filter (Whatman) and 193 194 preserved in a biofreezer at -80°C for later analyses of pigments, fatty acids and free amino acids compositions. 195

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

salicylate-hypochlorite method for determining ammonium in seawater described by Bower and

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 Søndergaard and Riemann (1979). The final concentration of inorganic nutrients (nitrate,
- ammonium and phosphate) in the specific experimental treatment was defined as the average $(\pm SD)$
- 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 placed in a glass vial where 3.3 mL of 90% acetone was added. Samples were shaken in a whirly 209 mixer. Then, samples were placed in the dark for 24 hours at 5°C. The extraction solvent in each 210 vial was transferred into a quartz cuvette through a 0.2 µm pore size syringe filter in which the 211 212 absorbance of each sample was measured at 664 nm and 630 nm on a GENESYSTM 6 Spectrophotometer (ThermoSpectronic). The concentration of chl a and chl c is expressed as pg 213 cell⁻¹. 214

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 K₂HPO₄,

218 0.05 mol KH₂PO₄). Samples were sonicated in ice-water bath for 15 minutes and then were left

refrigerated for 12 hours. Extraction solvent was filtered through at 25 mm 0.2 µm pore size syringe

filter and placed into a cuvette for measuring the absorbance spectrophotometrically at 455nm,

564nm and 592nm. PE concentration was calculated as in Bennett and Bogorad (1973). The

222 concentration of PE is expressed as $pg cell^{-1}$.

223 Analyses of fatty acids

The FA composition of *R. salina* was determined by extraction of the lipids using a HPLC-grade 224 225 chloroform: methanol mixture (Folch et al. 1957) followed by trans esterification process by acetyl chloride in methanol (see Drillet et al. 2006 for details). In brief, a chloroform: methanol mixture (3 226 mL, v:v = 2:1) was added to each of the algal sample. A volume of 20 μ L of internal standard (1000 227 µg mL⁻¹ tricosanoic FA methyl ester [C23:0 FAME]) was also added for FA quantification. 228 229 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 block at 60°C to evaporate the chloroform: methanol solvent by a flow of nitrogen. Thereafter, 232 AcOMe/HCl reagent in Toluene (1 mL) was added into the GC vials. The GC vials were covered by 233 aluminum caps and were placed in the aluminum block for 2 hours at 95°C. Next, the caps were 234 removed to add 500 µL of 5% of NaHCO₃, and two different phases appeared. The upper phase was 235 transferred into a new GC vial. Subsequently, 500 µL of heptane was added and the upper phase 236 was added to the new GC vial. The samples were evaporated at 65°C under a steady flow of 237

nitrogen. Thereafter, 0.5 ml of chloroform was added to each sample. Finally, all samples were analyzed by an Agilent GC6890N gas chromatograph while connected to an Agilent MS 5975 mass selective detector. The GC was equipped with a 60 m Agilent J&W DB23 column with 0.25 mm internal diameter and film thickness of 0.25 μ m. Splitless injection while running a positive electron ionization at 70 eV was selected. ChemStation software was used for MS peak integration. MS peaks were analyzed against a Supelco FAME standard mixture. Total fatty acid content (TFA) is expressed as 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 filter samples were lyophilized 24h prior to extraction. FAA was extracted in 1 mL Milli-Q water 247 by heating the filter samples to 95°C for 10 min. The extracts were filtered through 8 mm 0.2 µm 248 pore size GHP polypropylene membrane filters. The FAA was derivatized (Yu et al. 1994) using a 249 250 AccQFlour kit (Waters, MA, USA) and later separated on a Waters Alliance 2695 separation module with a 3.9×150 mm AccQTag column. The separated AA derivatives were quantified by 251 fluorescence (250 nm excitation and 395 nm emission) using an Alliance 2475 scanning 252 fluorescence detector. Due to the limitation of the number of FAA samples (only one sample for 253 254 each treatment), the FAA data was grouped into two categories according to the irradiance: i) limited irradiance (irradiance from 10 to 40 μ mol photons m⁻² s⁻¹, n = 3 for both nutrient deficiency 255 and nutrient excess treatments) and ii) saturated irradiance (irradiance from 60 to 140 µmol photons 256 $m^{-2} s^{-1}$, n = 5 for nutrient deficiency and n = 4 for nutrient excess treatment). The saturated 257 irradiances were from 60 μ mol photons m⁻² s⁻¹ where no further increase in the microalgal growth 258 rate under the higher irradiance was observed (see the results). Total FAA is expressed as pg cell⁻¹, 259 260 while content of each amino acid is expressed as percentage of total FAA (% of total FAA).

261 *Statistical analyses*

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

- 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
- tests on data were carried out using SAS v. 9.3 with $\alpha = 0.05$.

272 **Results**

273 *Photosynthesis*

The realized photosynthesis rate (P^{B}_{m}) and the initial slope of the P-I curves (α) decreased with the 274 increase of cell density (Fig. 1 and Table 1). The P^{B}_{m} value was 22 times higher at the lowest cell 275 density $(0.1 \times 10^6 \text{ cells mL}^{-1} = 539.4 \times 10^{-15} \text{ mol } O_2 \text{ cell}^{-1} \text{ h}^{-1})$ compared to the highest cell density 276 $(10 \times 10^6 \text{ cells mL}^{-1} = 20.6 \times 10^{-15} \text{ mol } O_2 \text{ cell}^{-1} \text{ h}^{-1})$. In contrast, the irradiance of maximum 277 photosynthesis (I_m) increased with increasing cell density. The lowest I_m values were 57.5-59.2 278 μ mol photons m⁻² s⁻¹ occurring at the two lowest cell densities of 0.1-0.5 × 10⁶ cells mL⁻¹ and the 279 highest I_m value was 103.0 μ mol photons m⁻² s⁻¹ (ca. 1.5 times higher than the lowest I_m value) 280 occurring at the highest cell density of 10×10^6 cells mL⁻¹ (Table 1). The photoinhibition (β) 281 282 decreased with increasing cell density (Fig. 1, Table 1). Dark respiration (R) decreased with increasing cell density, indicated by the higher oxygen consumption per cell (Table 1). The highest 283 dark respiration was recorded at lowest cell density 0.1×10^6 cells mL⁻¹ (-319.1 ± 19.8 × 10⁻¹⁵ mol 284 O_2 cell⁻¹ h⁻¹ = 59.1% P^B_m) which was ca. 18 times higher than the dark respiration of cells at highest 285 density 10×10^6 cells mL⁻¹ (-17.9 ± 5.7 ×10⁻¹⁵ mol O₂ cell⁻¹ h⁻¹ = 86.7% P^B_m). The ratio of relative 286 dark respiration rate to maximum photosynthesis (R/P^{B}_{m}) increased slightly with increasing cell 287 density (Table 1) indicating that the cell culture became less autotrophic with increasing cell 288 289 density.

290 *Microalgae growth*

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

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 $(3.2-5.3 \times 10^6 \text{ cells mL}^{-1})$ was about 2-3 times higher

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

The cell biovolume of *R. salina* was found to be 200-300 µm³ (Fig. 2c). Linear regression revealed 305 no significant relationship between specific growth rate and cell biovolume, p = 0.633 and p =306 307 0.923, for nutrient excess and deficiency, respectively, indicating that cell biovolume is independent of growth rate in this study, regardless of treatment.

309 Nutrient consumption

308

The initial concentration of nitrate was $1092.9 \pm 108.3 \mu mol L^{-1}$ in all treatments. The final nitrate 310

concentrations (Fig. 3a) remaining in the algae culture decreased with increasing irradiance 311

(Appendix 1) and were higher in nutrient excess than in nutrient deficiency (Appendix 1). The 312

lowest final nitrate concentrations were recorded at the irradiances of 140 μ mol photons m⁻² s⁻¹ (1.1 313

 \pm 1.0 µmol L⁻¹) and 120 µmol photons m⁻² s⁻¹ (418.3 \pm 384.9 µmol L⁻¹) in nutrient deficiency and 314

nutrient excess, respectively. In addition, the variation in the final nitrate concentrations was wider 315 under nutrient deficiency than under nutrient excess (Appendix 1). 316

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

The initial concentration of phosphate was $160.9 \pm 29.0 \,\mu$ mol L⁻¹ in all treatments. The final 323

phosphate concentration (Fig. 3a) remaining in the algae culture decreased with increasing 324

irradiance (Appendix 1) and was higher at nutrient excess than at nutrient deficiency (Appendix 1). 325

At the end of the experiment, the lowest phosphate concentration was obtained at the highest 326

irradiance 140 μ mol photons m⁻² s⁻¹ in both nutrient deficiency (86.7 \pm 35.0 μ mol L⁻¹) and nutrient 327

- excess ($112 \pm 10.2 \mu$ mol L⁻¹). The decrease in final phosphate concentration with irradiance was 328
- 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,

Appendix 1). The highest chl *a* (ca. 6.7 pg cell⁻¹) recorded at the irradiance of 20 μ mol m⁻² s⁻¹ was

ca. 3 times higher than the lowest chl *a* (ca. 2.4 pg cell⁻¹) recorded at the highest irradiance (140

 μ mol m⁻² s⁻¹). Chl *a* levels did not differ between nutrient deficiency and nutrient excess (Appendix

1). There was a statistically significant interaction between the irradiance and nutrient on chl a

336 concentrations (Appendix 1).

- Cellular chlorophyll c (chl c) levels decreased with increasing irradiance (Fig. 4b, Appendix 1). The chl c levels were not affected by nutrient treatment (Appendix 1). However, there was an interaction between irradiance and nutrient on chl c (Appendix 1).
- The cellular phycoerythrin (PE) levels were influenced by both irradiance (Fig. 4c, Appendix 1) and 340 nutrients (Fig. 4c, Appendix 1). In nutrient deficiency, the PE rapidly increased at low irradiance 341 and peaked at 40 μ mol photons m⁻² s⁻¹ (ca. 18 pg cell⁻¹). Thereafter, the PE decreased rapidly to the 342 lowest level at 140 µmol photons m⁻² s⁻¹ (ca. 3 pg cell⁻¹). In nutrient excess, the PE increased 343 rapidly with increasing irradiance at low irradiances, reaching the highest concentration at the 344 irradiance of 60 μ mol photons m⁻² s⁻¹ (somewhat later compared to nutrient deficiency) and then 345 remained at this high level until 100 µmol photons m⁻² s⁻¹ before slightly decreasing at 120-140 346 347 μ mol photons m⁻² s⁻¹. This difference generated a statistically significant Irradiance \times Nutrient interaction (Appendix 1). In term of nutrients, the PE level was higher in nutrient excess (11.1 ± 5.5 348 pg cell⁻¹) than nutrient deficiency (8.9 ± 6.5 pg cell⁻¹) but this pattern was driven by the fact that PE 349 levels were considerably higher in nutrient excess at the irradiance from 80 µmol photons m⁻² s⁻¹ 350 onward. 351
- The PE/chl a ratio (Fig. 4d) resembled the patterns of the PE. The PE/chl a ratio was affected by 352 both irradiance (Appendix 1) and nutrients (Appendix 1). Specifically in nutrient deficiency, the 353 PE/chl a ratio rapidly increased at low irradiances, peaking at an irradiance of 40 µmol photons m⁻² 354 s^{-1} (4.2 pg cell⁻¹), then decreased steadily with increasing irradiances, attaining the lowest level at 355 140 μ mol photons m⁻² s⁻¹ (1.2 pg cell⁻¹). In nutrient excess, the PE/chl *a* ratio increased with 356 increasing irradiance and reached a plateau (2.9-3.5 pg cell⁻¹) from the irradiance of 40 µmol 357 photons m⁻² s⁻¹ onwards (Fig. 4d). This difference also generated a statistically significant 358 interaction (Appendix 1) and resulted in an overall higher PE/chl a ratio in nutrient excess than in 359 nutrient deficiency. 360

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

Appendix 1). The TFA was considerably higher (Appendix 1) in algae cultured in nutrient

deficiency $(34.5 \pm 22.5 \text{ pg cell}^{-1})$ than in nutrient excess $(11.4 \pm 4.2 \text{ pg cell}^{-1})$. Most notably, TFA

368 was ca. 3-4 times higher within the irradiance range of 60 to 140 μ mol photons m⁻² s⁻¹ in nutrient

369 deficient cultures in comparison to the nutrient excess, generating a statistically significant

370 Irradiance × Nutrient interaction (Fig. 5a, Appendix 1).

371 Irradiance had statistically significant effects on the relative abundance of the mono-unsaturated

fatty acids (MUFA) (Fig. 5b&c, Appendix 1), especially in nutrient excess, generating an

interaction of Irradiance × Nutrient on MUFA (Appendix 1). Algae cultured at lowest and highest

irradiance (10 and 140 μ mol photons m⁻² s⁻¹) had significant higher MUFA levels compared to other

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

pattern was independent of nutrient levels (Appendix 1). SC-PUFA increased with increase in

irradiance. On the other hand, the changing in irradiance levels did not affect the concentration of

the saturated fatty acids (SFA) and highly unsaturated fatty acids (HUFA) (Fig. 5b&c, Appendix 1).

Nutrient had a statistically significant effect on the SFA (Appendix 1) and HUFA (Appendix 1) of

the algae (Fig. 5b&c). Relative abundance of SFA was considerably higher when algae were

cultured during nutrient deficiency (26.8 ± 8.3 % of TFA) compared to those in nutrient excess

 $(11.0 \pm 3.1\% \text{ of TFA})$. In contrast, the relative abundance of HUFA was ca. 2 times higher in algae

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

most abundant HUFA in *R. salina*, was 22.1 % of TFA in nutrient excess, which was ca. 2 times

higher than in nutrient deficiency (10.3 % of TFA).

- 389 Overall, the ratio between the two essential fatty acids Docosahexaenoic acid and Eicosapentaenoic
- acid (DHA/EPA) was lower at irradiance 10-20 μ mol photons m⁻² s⁻¹ than at irradiance from 40-140
- μ mol photons m⁻² s⁻¹ (Appendix 1). The nutrient had no effect on DHA/EPA ratio which varied
- 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).
- More details in fatty acids composition of *R. salina* cultured in nutrient deficiency and nutrient excess under limited (irradiance from 10 to 40 μ mol photons m⁻² s⁻¹) and saturated (irradiance from 60 to 140 μ mol photons m⁻² s⁻¹) irradiances was summarized in Appendix 2. Noticeably, relative abundance of SFA 18:0 was highest at saturated irradiance and under nutrient deficiency, whereas, relative abundance of SC-PUFA 18:4, DHA and EPA was highest at saturated irradiance and in nutrient excess.

401 Free amino acids

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

- The relative abundance of sub-total EAA (% of total FAA) was affected by the irradiance and the
 interaction Irradiance × Nutrient, but not the nutrient levels (Table 2). In contrast with total FAA,
 the relative abundance of sub-total EAA was significantly higher at saturated irradiance, especially
 in nutrient in excess (31.89±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*, argine 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
- 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 algae420 grown at limited irradiance and in nutrient deficiency (Table 2).

422 **Discussion**

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

425 *Photosynthesis*

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

In addition, the respiration rate in darkness was also higher at low cell density than at high cell
density, probably due to the higher cellular photosynthetic activity at low cell densities, leading to
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 μ mol 448 photons m⁻² s⁻¹) for maximal growth and maximal growth rate (μ_{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 *Pyrenemonas salina* (a taxonomic synonym of *R*.

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

460 Until the end of the exponential phase (day 3), the specific growth rate of *R. salina* did not differ between nutrient levels as nutrients in all treatments was still above the half saturation constants for 461 algal growth, namely nitrate: 0.4 μ mol L⁻¹ (Falkowski 1975) and phosphate: 0.51 μ mol L⁻¹ (Smith 462 and Kalff 1982). The nutrient deficiency only showed its effects on the algal growth from day 4 and 463 464 onwards where the nitrate concentration was almost depleted. Not surprisingly, the cell densities in nutrient deficient cultures did not increase beyond this point. However, in the current study, the 465 466 maximum cell density in nutrient deficient cultures was still higher than the reported value $1.53 \times$ 10⁶ cells mL⁻¹ by Lafarga-De la Cruz et al. (2006), which may be a result of the difference in 467 experimental set up. In Lafarga-De la Cruz et al. (2006), algae were cultivated in batch without 468 aeration in (250 mL) Erlenmeyer flasks whereas in our study, the algae was cultivated in a Multi-469 cultivator with continuous bubbling of atmospheric air that may provide a better environment for 470 growth by enhancing CO₂ addition, avoiding light/temperature stratification, algal sedimentation 471 472 and O₂ build up. In contrast, the nitrate concentration in nutrient excess remained very high during all 6 days of the experiment. As a result, cell densities increased continuously throughout this 473 period, especially under optimal range of irradiance and nutrient excess, where maximal cell 474 densities were 2-3 times higher than those cultured in nutrient deficiency of the same light regimes, 475 indicating a promising algal production. 476

As cell biovolume was not significantly affected by growth rate, and has been found to be constant
across experimental conditions and treatment, we have chosen to express all data on biochemical
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) and other light harvesting pigments, such as chlorophyll b (chl b), chlorophyll c (chl c), and primary 482 carotenoids (Hu 2004). These results have been observed in Cryptophyte species (Faust and Gantt 483 1973; Lichtlé 1979), including R. salina (Bartual et al. 2002). In the present study, the chl a and chl 484 c concentrations were higher at lower irradiance as a response to low irradiance (Hammer et al. 485 486 2002; Hu 2004). When the algae are exposed to high irradiance, phycobiliproteins and carotenoids act as protection mechanisms again the excess light (Pereira et al. 2012). Therefore, it was expected 487 488 that PE content would increase at high irradiance to increase the photoprotection, which indeed was observed in our study under nutrient excess. In our experiment, the phycoerythrin (PE) and chl a 489 490 (PE/chl *a*) ratio of *R*. salina decreased with the increase of irradiance (> 40 μ mol photons m⁻² s⁻¹) in nutrient deficiency, which has been also observed in the Cyanophyceae Anacystis nidulans (Halldal 491 1958) or the Cryptophyceae Chroomonas sp. (Faust and Gantt 1973). Moreover, the PE/chl a ratio 492 follow the pattern of total PE against irradiance, and can be explained by PE being more sensitive to 493 variations in irradiance than chl a. (Brown and Richardson 1968). 494

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 Cryptomonas rufescens (Lichtlé 1979), Cryptomonas maculata (Rhiel et al. 1985) and 497 498 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 content. For the chl *a* content, the nitrate concentration in our experiment (the lowest nitrate 500 501 concentration was $1.1 \pm 1.0 \,\mu$ 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 concentrations below 0.5 µmol to total exhausted from day 4 of the experiment. The drop of PE and 503 504 PE/chl a ratio under nutrient deficiency has been observed before in R. salina (Bartual et al. 2002). This nutrient-induced reduction in PE and PE/chl *a* ratio is explained by mobilization of nitrogen 505 506 from PE (Bartual et al. 2002).

In our study, PE decreases with increasing cell density under nutrient deficiency, indicating that
this phycobillipigment is scavenged as a nitrogen source under nutrient deficiency (Bartual et al.
2002; Lewitus and Caron 1990; Eriksen and Iversen 1995) as photoprotection is decreasing with
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. 1984; Hu 2004; Shifrin and Chisholm 1981, but see Harrison et al. 1990). Strikingly, TFA levels 514 increased sharply to very high level at irradiance 60-140 μ mol photons m⁻² s⁻¹ under nutrient 515 deficiency. The increase of TFA with increase of irradiance in nutrient deficiency could be 516 517 correlated to the increase of triacylglycerols-TAG (Sharma et al. 2012; Dongre et al. 2014). This is likely associated with the synthesis of TAG (generally contain saturated fatty acids-SFA and mono-518 519 unsaturated fatty acids-MUFA) that mostly occur under adequate light conditions, hence this synthesis can be maximized when cultivated at light saturation (Dongre et al. 2014). Besides, in 520 521 nutrient deficiency condition (nitrogen starvation), many algal species accumulate lipids due to that these constituents do not contain N (mostly TAG including SFA and MUFA, Shifrin and Chisholm 522 523 1981; Sharma et al. 2012). When nutrients are limited, the cell division rate decreases steadily, hence the requirement for membrane compounds reduce or almost reach no requirement any more 524 (Sharma et al. 2012). However, active biosynthesis of FA is maintained (Sharma et al. 2012). 525 526 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. Specifically, irradiance had a positive correlation with the relative abundance of short chain-poly 528 unsaturated fatty acids (SC-PUFA), but no correlation with SFA and highly unsaturated fatty acids 529 (HUFA). We found that the irradiance of 20-140 µmol photons m⁻² s⁻¹ induced higher relative 530 content of SC-PUFA (a sub-group of poly unsaturated fatty acids-PUFA) than at 10 µmol photons 531 $m^{-2} s^{-1}$ whereas the HUFA (also sub-group of PUFA) was highest at 40 µmol photons $m^{-2} s^{-1}$ and 532 533 relative constant for all other irradiances. This result did not reflect the general rule reported by Harwood (1998) when high irradiance usually leads to oxidative damage of PUFA (including SC-534 535 PUFA and HUFA as defined in our study). The self-shading of the high algae density $(1.1-4.1 \times 10^6)$ cells mL⁻¹) recorded at high irradiance cultures may reduce the effect of the high irradiance on 536 oxidative damage of the PUFA, indicating the potential to use density manipulation towards 537 538 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) was relatively constant over a range of irradiance for Chaetoceros and Thalassiosira but increased 542 significantly for *Isochrysis*. The increase in DHA and decrease in EPA at high irradiance result in 543

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

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

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

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

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

588 **Recommendations**

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

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- 811
- 812

814 **Caption for figures**

- **Fig. 1** Net photosynthesis per cell of *R*. *salina* ($\times 10^{-15}$ mol O₂ cell⁻¹ h⁻¹) incubated at different cell
- 816 densities and irradiance levels. Note: the number of experimental replicates, n = 5 for irradiances
- from 0-140 μ mol photons m⁻² s⁻¹ and n = 2-3 for irradiances from 160-300 μ mol photons m⁻² s⁻¹
- Fig. 2 The specific growth rate (SGR) (a), maximum cell density (b) and the plot between SGR and
- cell biovolume (c) of *Rhodomonas salina* in response to different irradiance and nutrient levels.
- B20 Data are indicated by means (\pm SDs) of specific growth rate of algae cultured in nutrient deficiency
- and nutrient excess at the same levels of irradiance. Note: the number of experimental replicates, n
- = 4 (2 replicates from nutrient deficiency and 2 replicates from nutrient excess treatments)
- Fig. 3 Nitrate, ammonium and phosphate concentrations in the culture media of *Rhodomonas salina*
- with different irradiance and nutrient levels. Data are means $(\pm SDs)$ of nutrient at day 4 and day 6,
- the number of analytical replicates, n = 2
- **Fig. 4** The chlorophyll a (a), chlorophyll c (b), phycoerythrin (PE) (c) and the
- 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
- irradiance levels. The number of analytical replicates, n = 2 for all of presented parameters. In
- figure 4 e and f, the solid lines are the regression lines between PE and cell density, the dashed lines
- are the 95% confident interval of these regression lines
- **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:
- 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
- nutrient deficiency and n = 2 for nutrient excess treatment
- 837

838 Caption for tables

- **Table 1** Summary of photosynthetic-irradiance parameters for five algae densities of *Rhodomonas*
- salina exposed to 16 increasing irradiances from 0 to 300 μ mol photons m⁻² s⁻¹ PAR, with the
- number of experimental replicates, n = 5 for irradiances from 0-140 µmol photons $m^{-2} s^{-1}$ and n = 2-
- 842 3 for irradiances from 160-300 μ mol photons m⁻² s⁻¹
- 843 Note: α = Initial slope of the photosynthesis-irradiance curve; β = Negative slope at high irradiance;
- 844 I_m = irradiance of maximum photosynthesis, R = Dark respiration = mean ± SDs (plus/minus
- standard deviation) of dark respiration at 0 μ mol photons m⁻² s⁻¹ of 5 experimental replicates at a
- specific cell density. Units: α , $\beta = 10^{-15}$ mol O₂ cell⁻¹ h⁻¹ [µmol photons m⁻² s⁻¹]⁻¹; P_m^B , R = 10⁻¹⁵ mol
- 847 $O_2 \text{ cell}^{-1} \text{ h}^{-1} \text{ and } I_m = \mu \text{mol photons } m^{-2} \text{ s}^{-1}$
- Table 2 Total free amino acids (FAA) and essential amino acids (EAA) in *Rhodomonas salina*cultured in different irradiance and nutrient levels
- Note: Units of total FAA = $pg cell^{-1}$; 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 μ mol photons m⁻² s⁻¹; values for saturated irradiance = mean \pm SDs of FAA/EAA at
- irradiance from 60-140 μ mol photons m⁻² s⁻¹; n is the number of experimental replicates. Different
- letters in the same row denote the significant differences in the same specific EAA between the
- 855 different treatments