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

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Abstract

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

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

Introduction

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

In microalgae, low irradiance may limit photosynthesis (Dunstan 1973), but high irradiances may 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 microalgae.

Another important factor regarding algal culture is inorganic nutrients that not only affect photosynthesis and productivity of cell biomass, but also influence the biochemical composition of 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 levels of unsaturated fatty acids, the desired components for live feed in aquaculture, are low (Breteler et al. 2005).

Nutritional quality of microalgae species is associated with the level of highly unsaturated fatty acids (HUFA), especially Eicosapentaenoic acid (EPA; 20:5 n-3) and Docosahexaenoic acid (DHA; 22:6 n.-3) (Renaud et al. 1991). HUFAs (characterized by a carbon number ≥ 20 and double bonds ≥ 3) are synthesized *de novo* only by photosynthetic organisms (Spector 1999), and are essential dietary nutrients for marine copepods (Fraser et al. 1989). HUFA, in particular, DHA and EPA appear to be very important in controlling reproduction, growth and metabolism in copepods (reviewed in Rasdi and Qin 2014). High dietary DHA/EPA ratios in feed improve survival, reduce 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 Rippingale 2000; Arendt et al. 2006; Rasdi and Qin 2014). Interestingly, copepods are carriers of high DHA/EPA ratios from microalgae into fish larvae (Parrish 2009). A DHA/EPA ratio ≥ 2 is 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 nutritional quality of microalgae (Brown 1991). AAs are the building blocks for protein synthesis, and are involved in numerous specific physiological functions (Aragão et al. 2004). Some AAs are 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 therefore be supplied from the diet. For copepods, microalgae are the only external source of the EAA (Wu 2009). AA composition of the algal prey also affects the egg production of the copepods (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* in large scale algal photobioreactors while optimizing their growth and nutritional value as algal feed for live feed calanoid copepods. We measured the photosynthesis of *R. salina* under irradiances from 10 to 300 µmol photons m^{-2} s^{-1}. In the next step, we conducted a full factorial growth experiment in which *R. salina* was cultivated in a series of irradiances ranging from 10 to 140 µmol photons m^{-2} s^{-1} under two inorganic nutrient levels: deficiency and excess. We quantified algal growth rates, three important pigments (chlorophyll *a*, chlorophyll *c* and phycoerythrin, Yaakob et al. 2014), fatty acids and free amino acids profiles for all irradiant levels and nutrient 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 factors (irradiance and nutrient). This will enable us to develop an intensive setup for a nutritious food chain delivering live feed on request for marine hatcheries.

Materials and methods

Microalgal material and culturing conditions

The studied Cryptophyte *Rhodomonas salina* (equivalent spherical diameter 8 µm) was originally derived as SCCAP K-1487 of the Scandinavian Culture Collection of Algae and Protozoa (University of Copenhagen, Denmark). Cultures of *R. salina* was grown in acid washed 3-6 L round glass flasks containing autoclaved 0.2 µm filtered seawater (salinity 30 ‰) enriched with B1 medium (1 mL L^{-1} of seawater, Hansen 1989). The cultures were maintained under a continuous irradiance of 80 µmol photons m^{-2} s^{-1} Photosynthetic Active Radiation (PAR) in a climate room at 20°C. The flasks were gently aerated with atmospheric air through 0.45 µm filters to mix the cultures to avoid temperature stratification, algal sedimentation, CO_2 depletion and O_2 build up.

Photosynthesis measurements

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

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

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

Where P^B: photosynthetic rate at irradiance I

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

α: Initial slope of the P-I curve (the quantum yield)

β: Negative slope at high irradiance (photoinhibition)

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):

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

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

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 µmol 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*, a factorial design of 8 irradiant levels (10, 20, 40, 60, 80, 100, 120 and 140 µmol photons m^{-2} s^{-1}) × 2 nutrient levels (deficiency and excess) was conducted (a total of 16 experimental treatments). All treatments were performed in duplicates (a total of 32 experimental units). The nutrient deficiency 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 once at the start of the experiment (B1 medium, 1 mL L^{-1} of seawater, Hansen 1989), hence the nutrient level reduced over time and almost depleted at the end of the experiment. In the nutrient excess, the nutrients were added daily (v:v 1 mL B1 medium L^{-1} day^{-1} of algae culture), hence no depletion occurred. The initial density of *R. salina* was $0.18 \pm 0.03 \times 10^6$ cells mL^{-1}. In the growth 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.

Growth rate

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 Multisizer™3 Coulter Counter® (Beckman Coulter Inc., USA). All particles with a diameter in the range of 5-12 μm were 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 exponential growth equation 4:

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

N_t is the cell density at time t (cells mL^{-1})

N_0 is the cell density at time zero (cells mL^{-1})

μ is the growth rate (day^{-1})

t is the time (day)

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

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

Where, μ: specific growth rate (day^{-1})

μ_{max}: Maximum growth rate (day^{-1})

I: Irradiance (μmol m^{-2} day^{-1})

α: Initial slope of the curve (maximum quantum yield for growth, day^{-1} [μmol photons m^{-2} s^{-1}]$^{-1}$)

Cell biovolume

The cell biovolume of the specific sample was determined as the mean of biovolume of all particles 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 (± 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

To minimize the loss of culture volume due to the sampling, inorganic nutrient level analyses were 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 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 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 nutrient compositions. In the stationary phase of the algal growth, samples of *R. salina* were taken on two different days from each treatment for analyzing chlorophyll *a* and chlorophyll *c* (chl *a* & chl *c*, 5 mL × 2 analytical replicates), phycoerythrin (PE, 5 mL × 2 analytical replicates), fatty acids (FA, 5 mL × 2-3 analytical replicates), and free amino acids (FAA, 5 mL × 2 analytical replicates). Then, samples were filtered onto three 12.8 mm diameter GF/C glass fiber filter (Whatman) and preserved in a biofreezer at -80°C for later analyses of pigments, fatty acids and free amino acids compositions.

Inorganic nutrient analysis of R. salina culture

Nitrate, ammonium and phosphate in the filtered water from the algae culture were quantified using colorimetric techniques. Nitrate concentration was determined by flow injection analysis using 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-Visible Spectrophotometer (Shimadzu, Kyoto, Japan) following the method described by Søndergaard and Riemann (1979). The final concentration of inorganic nutrients (nitrate, ammonium and phosphate) in the specific experimental treatment was defined as the average (±SD) concentration between the last two sampling days (day 4 and 6) of the experiment.

Analyses of algal pigments

Chl *a* and chl *c* were extracted based on the methods described by Jeffrey and Humphrey (1975) 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 mixer. Then, samples were placed in the dark for 24 hours at 5°C. The extraction solvent in each vial was transferred into a quartz cuvette through a 0.2 µm pore size syringe filter in which the absorbance of each sample was measured at 664 nm and 630 nm on a GENESYS^{TM} 6 Spectrophotometer (ThermoSpectronic). The concentration of chl *a* and chl *c* is expressed as pg cell^{-1}.

The PE was extracted based on the procedure described by Bennett and Bogorad (1973), Evans (1988) and Zimba (2012). After lyophilization, each of the filter samples for PE extraction was placed into a glass vial together with 3 mL of phosphate buffer (0.1 mol pH 7, 0.05 mol K_2HPO_4, 0.05 mol KH_2PO_4). 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 concentration of PE is expressed as pg cell^{-1}.

Analyses of fatty acids

The FA composition of *R. salina* was determined by extraction of the lipids using a HPLC-grade 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 mL, v:v = 2:1) was added to each of the algal sample. A volume of 20 µL of internal standard (1000 µg mL^{-1} tricosanoic FA methyl ester [C23:0 FAME]) was also added for FA quantification. Thereafter, samples underwent ultrasound extraction for 15 minutes in an ice bath to break the algal cells. In the next step, samples were frozen at -20°C for 24 h for extraction. Subsequently, the 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, AcOMe/HCl reagent in Toluene (1 mL) was added into the GC vials. The GC vials were covered by aluminum caps and were placed in the aluminum block for 2 hours at 95°C. Next, the caps were removed to add 500 µL of 5% of $NaHCO_3$, and two different phases appeared. The upper phase was transferred into a new GC vial. Subsequently, 500 µL of heptane was added and the upper phase was added to the new GC vial. The samples were evaporated at 65°C under a steady flow of 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 µm. 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).

Analyses of free amino acids

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 by heating the filter samples to 95°C for 10 min. The extracts were filtered through 8 mm 0.2 µm pore size GHP polypropylene membrane filters. The FAA was derivatized (Yu et al. 1994) using a 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 fluorescence (250 nm excitation and 395 nm emission) using an Alliance 2475 scanning fluorescence detector. Due to the limitation of the number of FAA samples (only one sample for each treatment), the FAA data was grouped into two categories according to the irradiance: i) limited irradiance (irradiance from 10 to 40 µmol photons m^{-2} s^{-1}, n = 3 for both nutrient deficiency and nutrient excess treatments) and ii) saturated irradiance (irradiance from 60 to 140 µmol photons m^{-2} s^{-1}, n = 5 for nutrient deficiency and n = 4 for nutrient excess treatment). The saturated irradiances were from 60 µmol photons m^{-2} s^{-1} where no further increase in the microalgal growth rate under the higher irradiance was observed (see the results). Total FAA is expressed as pg cell^{-1}, while content of each amino acid is expressed as percentage of total FAA (% of total FAA).

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 composition results. Data were tested for homogeneity of variance (Cochran's test) and normal 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 $\alpha = 0.05$.

Results

Photosynthesis

The realized photosynthesis rate (P^B_m) and the initial slope of the P-I curves (α) decreased with the increase of cell density (Fig. 1 and Table 1). The P^B_m value was 22 times higher at the lowest cell density (0.1×10^6 cells mL^{-1} = 539.4×10^{-15} mol O_2 $cell^{-1}$ h^{-1}) compared to the highest cell density (10×10^6 cells mL^{-1} = 20.6×10^{-15} mol O_2 $cell^{-1}$ h^{-1}). In contrast, the irradiance of maximum photosynthesis (I_m) increased with increasing cell density. The lowest I_m values were 57.5-59.2 µmol photons m^{-2} s^{-1} occurring at the two lowest cell densities of 0.1-0.5 × 10^6 cells mL^{-1} and the highest I_m value was 103.0 µmol photons m^{-2} s^{-1} (ca. 1.5 times higher than the lowest I_m value) occurring at the highest cell density of 10×10^6 cells mL^{-1} (Table 1). The photoinhibition (β) 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 dark respiration was recorded at lowest cell density 0.1×10^6 cells mL^{-1} ($-319.1 \pm 19.8 \times 10^{-15}$ mol 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 density 10×10^6 cells mL^{-1} ($-17.9 \pm 5.7 \times 10^{-15}$ mol O_2 $cell^{-1}$ h^{-1} = 86.7% P^B_m). The ratio of relative dark respiration rate to maximum photosynthesis (R/P^B_m) increased slightly with increasing cell density (Table 1) indicating that the cell culture became less autotrophic with increasing cell density.

Microalgae growth

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

Maximum cell densities increased with increasing irradiance (Fig. 2b, Appendix 1). This pattern was consistently stronger in nutrient excess cultures than in nutrient deficient cultures (Appendix 1). The maximal cell density in nutrient excess (3.2-5.3 × 10^6 cells mL^{-1}) was about 2-3 times higher than in nutrient deficiency (1.7-1.9 × 10^6 cells mL^{-1}) at the irradiance from 80 µmol photons m^{-2} s^{-1} onwards, resulting in the overall higher maximal cell densities (Fig. 2b) in nutrient excess than in nutrient deficiency (Appendix 1).

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

Nutrient consumption

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

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

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

Algal pigments

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 µmol 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 µmol 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* 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 nutrients (Fig. 4c, Appendix 1). In nutrient deficiency, the PE rapidly increased at low irradiance and peaked at 40 µmol photons m^{-2} s^{-1} (ca. 18 pg cell^{-1}). Thereafter, the PE decreased rapidly to the lowest level at 140 µmol photons m^{-2} s^{-1} (ca. 3 pg cell^{-1}). In nutrient excess, the PE increased rapidly with increasing irradiance at low irradiances, reaching the highest concentration at the irradiance of 60 µmol photons m^{-2} s^{-1} (somewhat later compared to nutrient deficiency) and then remained at this high level until 100 µmol photons m^{-2} s^{-1} before slightly decreasing at 120-140 µmol photons m^{-2} s^{-1}. This difference generated a statistically significant Irradiance × Nutrient interaction (Appendix 1). In term of nutrients, the PE level was higher in nutrient excess (11.1 ± 5.5 pg cell^{-1}) than nutrient deficiency (8.9 ± 6.5 pg cell^{-1}) but this pattern was driven by the fact that PE levels were considerably higher in nutrient excess at the irradiance from 80 µmol photons m^{-2} s^{-1} onward.

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

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).

Fatty acids

Overall, the total fatty acids (TFA) content increased with increasing irradiance (Fig. 5a, Appendix1). The TFA was considerably higher (Appendix 1) in algae cultured in nutrient deficiency (34.5 ± 22.5 pg cell^{-1}) than in nutrient excess (11.4 ± 4.2 pg cell^{-1}). Most notably, TFA was ca. 3-4 times higher within the irradiance range of 60 to 140 µmol photons m^{-2} s^{-1} in nutrient deficient cultures in comparison to the nutrient excess, generating a statistically significant Irradiance × Nutrient interaction (Fig. 5a, Appendix 1).

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 µmol photons m^{-2} s^{-1}) had significant higher MUFA levels compared to other irradiances (Fig. 5b&c).

Irradiance also affected the content of short chain polyunsaturated fatty acids (SC-PUFA = 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 ± 3.1% 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 31.8 ± 2.5% and 15.1 ± 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).

Overall, the ratio between the two essential fatty acids Docosahexaenoic acid and Eicosapentaenoic acid (DHA/EPA) was lower at irradiance 10-20 µmol photons m^{-2} s^{-1} than at irradiance from 40-140 µmol 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 × Nutrient interaction (Appendix 1), but this interaction was driven by the fact that DHA was mostly 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 µmol photons m^{-2} s^{-1}) and saturated (irradiance from 60 to 140 µmol 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.

Free amino acids

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

The difference in the irradiance and nutrient showed no consistent effect on relative abundance of specific EAA (Table 2). The most abundant EAA in *R. salina*, argine was higher in saturated irradiance, especially under nutrient deficiency (Table 2). Seven out of ten EAA, including 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 at limited irradiance and in nutrient deficiency (Table 2). In contrast, histidine was higher in algae grown at limited irradiance and in nutrient deficiency (Table 2).

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*.

Photosynthesis

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

Algal growth

Regarding the specific growth rate versus irradiance curve, the saturating irradiance (60-140 µmol photons m^{-2} s^{-1}) for maximal growth and maximal growth rate (μ_{max}) obtained in our study are in agreement with previous studies on *R. salina* (Bartual et al. 2002; Hammer et al. 2002; Lafarga-De 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 µmol photons m^{-2} s^{-1} in 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-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 assimilation (Rückert and Giani 2004), hence a higher energetic cost is associated with nitrate use. Under low irradiance (< 20 µmol photons m^{-2} s^{-1}), the growth rate was very low, as also observed in other studies (Lewitus and Caron 1990; Bartual et al. 2002) that may be associated with the low photosynthetic rate at low irradiance.

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 algal growth, namely nitrate: 0.4 µmol L^{-1} (Falkowski 1975) and phosphate: 0.51 µmol L^{-1} (Smith and Kalff 1982). The nutrient deficiency only showed its effects on the algal growth from day 4 and 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 maximum cell density in nutrient deficient cultures was still higher than the reported value 1.53×10^6 cells mL^{-1} by Lafarga-De la Cruz et al. (2006), which may be a result of the difference in experimental set up. In Lafarga-De la Cruz et al. (2006), algae were cultivated in batch without aeration in (250 mL) Erlenmeyer flasks whereas in our study, the algae was cultivated in a Multi-cultivator with continuous bubbling of atmospheric air that may provide a better environment for growth by enhancing CO_2 addition, avoiding light/temperature stratification, algal sedimentation and O_2 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 period, especially under optimal range of irradiance and nutrient excess, where maximal cell densities were 2-3 times higher than those cultured in nutrient deficiency of the same light regimes, indicating a promising algal production.

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.

Algal pigments

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 carotenoids (Hu 2004). These results have been observed in Cryptophyte species (Faust and Gantt 1973; Lichtlé 1979), including *R. salina* (Bartual et al. 2002). In the present study, the chl *a* and chl *c* concentrations were higher at lower irradiance as a response to low irradiance (Hammer et al. 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 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* (PE/chl *a*) ratio of *R. salina* decreased with the increase of irradiance (> 40 μmol photons m^{-2} s^{-1}) in nutrient deficiency, which has been also observed in the Cyanophyceae *Anacystis nidulans* (Halldal 1958) or the Cryptophyceae *Chroomonas* sp. (Faust and Gantt 1973). Moreover, the PE/chl *a* ratio follow the pattern of total PE against irradiance, and can be explained by PE being more sensitive to variations in irradiance than chl *a*. (Brown and Richardson 1968).

A common pattern in algae is that cells respond to nutrient (nitrogen) deficiency by decreasing 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 *Pyrenomonas salina* (Lewitus and Caron 1990). In our study, while the PE and PE/chl *a* ratio were 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 concentration was 1.1 ± 1.0 µmol) was probably not too depletedto induce a reduction in chl *a* 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 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 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.

Fatty acids

In line with previous studies, the higher total fatty acids (TFA) was obtained at higher irradiance (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 increased sharply to very high level at irradiance 60-140 µmol photons m^{-2} s^{-1} under nutrient deficiency. The increase of TFA with increase of irradiance in nutrient deficiency could be 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-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 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 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 (Sharma et al. 2012). However, active biosynthesis of FA is maintained (Sharma et al. 2012). Consequently, the cells divert and deposit fatty acids into TAG (Sharma et al. 2012).

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 unsaturated fatty acids (SC-PUFA), but no correlation with SFA and highly unsaturated fatty acids (HUFA). We found that the irradiance of 20-140 µmol photons m^{-2} s^{-1} induced higher relative content of SC-PUFA (a sub-group of poly unsaturated fatty acids-PUFA) than at 10 µmol photons m^{-2} s^{-1} whereas the HUFA (also sub-group of PUFA) was highest at 40 µmol photons m^{-2} s^{-1} and 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-PUFA and HUFA as defined in our study). The self-shading of the high algae density ($1.1\text{-}4.1 \times 10^6$ cells mL^{-1}) recorded at high irradiance cultures may reduce the effect of the high irradiance on oxidative damage of the PUFA, indicating the potential to use density manipulation towards designer feed. In detail, irradiance had positive correlation with the relative abundance of DHA but not EPA. This result has also been observed in previous studies (e.g., Renaud et al. 1991; Harrison et al. 1990; Thompson et al. 1990). Harrison et al. (1990) found that Docosahexaenoic acid (DHA) 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 significantly for *Isochrysis*. The increase in DHA and decrease in EPA at high irradiance result in 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 µmol 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 deficiency comprised mainly of the higher relative contents of SFA 16:0, MUFA 18:1. On the other hand, under nutrient deficiency, the relative contents of HUFA, including EPA-20:5n-3 and DHA-22:6n-3, the most desired components for calanoid copepods (Arendt et al. 2006; Broglio et al. 2003), were lower. This phenomenon is common in many marine microalgae (Reitan et al. 1994). 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 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 generate a desired microalgae fatty acid profile.

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 obtained when the *R. salina* algae are cultured at saturated irradiances and in nutrient excess 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 2014). Therefore, the relatively higher abundance of PUFA of *R. salina* cultured in saturated irradiances and in nutrient excess will benefit the performance of copepods. Please note that the nutrient content in copepods, unlike rotifer and *Artemia*, cannot be manipulated through enrichment 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 2014; Rasdi et al. 2015).

Free amino acids

The irradiance had significant effect on both the quantity and the composition of free amino acids (FAA), whereas nutrient levels only affected the composition of the FAA. In particular, the limited irradiance induced higher total FAA whereas relative abundance of essential amino acid was higher 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, 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 been reported before, higher total FAA at lower cultured irradiance was observed in a seaweed *Caulerpa prolifera* (Khaleafa et al. 1982). The effects of irradiance on FAA composition in our 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 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. 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 changes in FAA composition of *R. salina* in different cultured irradiance and nutrient condition in our study may not entirely reflect the protein bound AAs.

Recommendations

The microalgae *Rhodomonas salina* is a preferred feed item for e.g. copepods used as live feed in hatcheries (Zhang et al. 2013). With our purpose to formulate designer feed for these promising zooplankton live feed organisms, several scenarios are possible when cultivating *R. salina*. i) One can either prioritize high microalga productivity where saturated irradiance and excess inorganic nutrients are implicit. This condition will generate a relatively larger algal biomass with a better fatty acids and free essential amino acids profiles. ii) Another strategy involves saturated irradiance and nutrient deficiency, which generates algal biomass with high total fatty acids content and remain an appropriate DHA/EPA ratio. Moreover, a relatively high content of highly unsaturated 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 purposes is to cultivate the microalgae at 60-100 μmol photons m^{-2} s^{-1} irradiance and in nutrient excess.

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References

Aldana-Aranda D, Patiño Suárez V (1998) Overview of diets used in larviculture of three caribbean conchs: Queen conch *Strombus gigas*, Milk conch *Strombus costatus* and Fighting conch *Strombus pugilis*. Aquaculture 167 (3–4):163-178.

Aragão C, Conceição LEC, Dinis MT, Fyhn H-J (2004) Amino acid pools of rotifers and Artemia under different conditions: nutritional implications for fish larvae. Aquaculture 234 (1–4):429-445.

Arendt KE, Jónasdóttir SH, Hansen PJ, Gärtner S (2006) Effects of dietary fatty acids on the reproductive success of the calanoid copepod *Temora longicornis*. Mar Biol 148 (6):1415-1415.

Bartual A, Lubián LM, Gálvez JA, Niell FX (2002) Effect of irradiance on growth, photosynthesis, pigment content and nutrient consumption in dense cultures of *Rhodomonas salina* (Wislouch) (Cryptophyceae). Cienc Mar 28:381-392.

Bennett A, Bogorad L (1973) Complementary chromatic adaptation in a filamentous blue-green alga. J Cell Biol 2:419-435.

Bi R, Arndt C, Sommer U (2014) Linking elements to biochemicals: Effects of nutrient supply ratios and growth rates on fatty acid composition of phytoplankton species. J Phycol 50 (1):117-130.

Bower CE, Holm-Hansen T (2011) A salicylate–hypochlorite method for determining ammonia in seawater. Can J Fish Aquat Sci 37 (5):794-798.

Breteler WCMK, Schogt N, Rampen S (2005) Effect of diatom nutrient limitation on copepod development: Role of essential lipids. Mar Ecol Prog Ser 291:125-133.

Broglio E, Jónasdóttir SH, Calbet A, Jakobsen HH, Saiz E (2003) Effect of heterotrophic versus autotrophic food on feeding and reproduction of the calanoid copepod *Acartia tonsa*: Relationship with prey fatty acid composition. Aquat Microb Ecol 31:267-278.

Brown MR (1991) The amino-acid and sugar composition of 16 species of microalgae used in mariculture. J Exp Mar Biol Ecol 145 (1):79-99.

Brown MR, Dunstan GA, Jeffrey SW, Volkman JK, Barrett SM, LeRoi J-M (1993a) The influence of irradiance on the biochemical composition of the Prymnesiophyte *Isochrysis* sp. (Clone T-ISO). J Phycol 29 (5):601-612.

Brown MR, Dunstan GA, Norwood SJ, Miller KA (1996) Effects of harvest stage and light on the biochemical composition of the diatom *Thalassiosira pseudonana*. J Phycol 32 (1):64-73.

Brown MR, Garland CD, Jeffrey SW, Jameson ID, Leroi JM (1993b) The gross and amino acid compositions of batch and semi-continuous cultures of I*sochrysis* sp. (clone T.ISO), *Pavlova lutheri* and *Nannochloropsis oculata*. J Appl Phycol 5 (3):285-296.

Brown MR, Jeffrey SW, Volkman JK, Dunstan GA (1997) Nutritional properties of microalgae for mariculture. Aquaculture 151:315-331.

Brown MR, McCausland MA, Kowalski K (1998) The nutritional value of four Australian microalgal strains fed to Pacific oyster *Crassostrea gigas* spat. Aquaculture 165 (3-4):281-293.

Brown TE, Richardson FL (1968) The effect of growth environment on the physiology of algae: light intensity. J Phycol 4 (1):38-54.

Claybrook DL (1983) Nitrogen metabolism. In: Mantel LH (ed) The biology of Crustacea: 5. Internal anatomy and physiological regulation. The biology of Crustacea. Academic Press, New York, pp 162-213.

Dhert P, Rombaut G, Suantika G, Sorgeloos P (2001) Advancement of rotifer culture and manipulation techniques in Europe. Aquaculture 200 (1–2):129-146.

Diamond D (1999) Determination of nitrate in brackish or seawater by flow injection analysis. QuikChem Method 31-107-04-1-A. Millwaukee, WI 53218-1239 USA.

Dongre SK, Manglawat S, Singh P, Yadav M, Tiwari A (2014) Effect of environmental parameters enhancing the micro algal lipid as a sustainable energy source for biodiesel production-A review. Int J Biol Pharm Res 5 (4):327-335.

Drillet G, Jørgensen NOG, Sørensen TF, Ramløv H, Hansen BW (2006) Biochemical and technical observations supporting the use of copepods as live feed organisms in marine larviculture. Aquacult Res 37:756-772.

Dunstan GA, Brown MR, Volkman JK (2005) Cryptophyceae and rhodophyceae; chemotaxonomy, phylogeny, and application. Phytochemistry 66 (21):2557-2570.

Dunstan WM (1973) A comparison of the photosynthesis - light intensity relationship in phylogenetically different marine microalgae. J Exp Mar Biol Ecol 13:181-187.

Eriksen NT, Iversen JJL (1995) Photosynthetic pigments as nitroen stores in the cryptophyte alga *Rhodomonas* sp. J Mar Biotechnol 3:193-195.

Evans LV (1988) The effect of spectral composition and irradiance level on pigment levels in seaweed. Experimental phycology: A laboratory manual. Cambridge University Press, Cambridge.

Falkowski PG (1975) Nitrate uptake by marine phytoplankton: Comparison of half- saturation constants for seven species. Limnol Oceanogr 20:412-417.

Faust MA, Gantt E (1973) Effect of light intensity and glycerol on the growth, pigment composition, and ultrastructure of *Chroomonas* sp. J Phycol 9 (4):489-495.

Folch J, Lees M, Stanley GHS (1957) A simple method for isolation and purification of total lipids from animal tissues. J Biol Chem 226:497-509.

Fraser AJ, Sargent JR, Gamble JC (1989) Lipid class and fatty acid composition of *Calanus finmarchicus* (Gunnerus), *Pseudocalanus* sp. and *Temora longicornis* Muller from a nutrient-enriched seawater enclosure. J Exp Mar Biol Ecol 130 (1):81-92.

Halldal P (1958) Pigment formation and growth in blue-green algae in crossed gradients of light intensity and temperature. Physiol Plant 11:401-420.

Hammer A, Schumann R, Schubert H (2002) Light and temperature acclimation of *Rhodomonas salina* (Cryptophyceae): Photosynthetic performance. Aquat Microb Ecol 29:287-286.

Hansen PJ (1989) The red tide dinoflagellate *Alexandrium tamarense*: Effects on behaviour and growth of a tintinnid ciliate. Mar Ecol Prog Ser 53:105-116.

Harrison PJ, Thompson PA, Calderwood GS (1990) Effects of nutrient and light limitation on the biochemical composition of phytoplankton. J Appl Phycol 2 (1):45-56.

Harwood J (1998) Involvement of Chloroplast Lipids in the Reaction of Plants Submitted to Stress. In: Paul-André S, Norio M (eds) Lipids in Photosynthesis: Structure, Function and Genetics, vol 6. Advances in Photosynthesis and Respiration. Springer Netherlands, pp 287-302.

Hu Q (2004) Environmental effects on cell composition. In: Richmond A (ed) Handbook of microalgal culture: Biotechnology and applied phycology, vol XVIII, 566 s. Blackwell Science, Oxford, pp 83-93.

Huntley M, Sykes P, Rohan S, Marin V (1986) Chemically-mediated rejection of dinoflagellate prey by the copepods *Calanus pacificus* and *Paracalanus parvus*: mechanism, occurrence and significance. Mar Ecol Prog Ser 28:105-120.

Jassby AD, Platt T (1976) Mathematical formulation of the relationship between photosynthesis and light for phytoplankton. Limnol Oceanogr 21 (4):540-547.

Jeffrey SW, Humphrey GF (1975) New spectrophotometric equations for determining chlorophylls a1, b1, c1 and c2 in higher plants, algae and natural phytoplankton. Biochem Physiol Pflanzen 167:191-194.

Jónasdóttir SH (1994) Effects of food quality on the reproductive success of *Acartia tonsa* and *Acartia hudsonica*: laboratory observations. Mar Biol 121 (1):67-81.

Jónasdóttir SH, Kiørboe T (1996) Copepod recruitment and food composition: do diatoms affect hatching success? Mar Biol 125:743-750.

Juneja A, Ceballos RM, Murthy GS (2013) Effects of environmental factors and nutrient availability on the biochemical composition of algae for biofuels production: A review. Energies 6:4607-4638.

Khaleafa AF, Mohsen AF, Shaalan SH (1982) Effect of different light intensities on growth, amino-acid, fat and sugar concentrations in *Caulerpa prolifera* (Foerskal) Lamouroux. Hydrobiol Bull 16 (2-3):207-212.

Kromkamp JC, Peen J (2001) Oxygen comsumption in the light by unicellular algae. In: PS2001 Proceedings 12th International Congress on Photosynthesis. CSIRO Publishing, Brisbane, Australia, pp 1-6.

Lafarga-De la Cruz F, Valenzuela-Espinoza E, Millán-Núñez R, Trees CC, Santamaría-del-Ángel E, Núñez-Cebrero F (2006) Nutrient uptake, chlorophyll a and carbon fixation by *Rhodomonas sp.* (Cryptophyceae) cultured at different irradiance and nutrient concentrations. Aquacult Eng 35:51-60.

Lewitus AJ, Caron DA (1990) Relative effects of nitrogen or phosphorus depletion and light intensity on the pigmentation, chemical composition, and volume of *Pyrenomonas salina* (Cryptophyceae) Mar Ecol Prog Ser 61:171-181.

Lichtlé C (1979) Effects of nitrogen deficiency and light of high intensity on *Cryptomonas rufescens* (Cryptophyceae). Protoplasma 101 (4):283-299.

Malzahn AM, Boersma M (2012) Effects of poor food quality on copepod growth are dose dependent and non-reversible. Oikos 121 (9):1408-1416.

Mansour M, Frampton DF, Nichols P, Volkman J, Blackburn S (2005) Lipid and fatty acid yield of nine stationary-phase microalgae: Applications and unusual C24–C28 polyunsaturated fatty acids. J Appl Phycol 17 (4):287-300.

Marinho da Costa Rr, Fernández F (2002) Feeding and survival rates of the copepods *Euterpina acutifrons* Dana and *Acartia grani* Sars on the dinoflagellates *Alexandrium minutum* Balech and *Gyrodinium corsicum* Paulmier and the Chryptophyta *Rhodomonas baltica* Karsten. J Exp Mar Biol Ecol 273 (2):131-142.

Mortillaro JM, Pitt KA, Lee SY, Meziane T (2009) Light intensity influences the production and translocation of fatty acids by zooxanthellae in the jellyfish *Cassiopea* sp. J Exp Mar Biol Ecol 378 (1–2):22-30.

Muller-Feuga A, Moal J, Kaas R (2003) The microalgae of aquaculture. In: Støttrup JG, McEvoy LA (eds) Live feeds in Marine Aquaculture. Blackwell Publising, Oxford, pp 206-299.

Neidhardt J, Benemann J, Zhang L, Melis A (1998) Photosystem-II repair and chloroplast recovery from irradiance stress: Relationship between chronic photoinhibition, light-harvesting chlorophyll antenna size and photosynthetic productivity in *Dunaliella salina* (green algae). Photosynthesis Res 56 (2):175-184.

Parrish CC (2009) Essential fatty acids in aquatic food webs. In: Arts MT, Brett MT, Kainz MJ (eds) Lipids in Aquatic Ecosystems. Springer, New York, pp 309-326.

Payne MF, Rippingale RJ (2000) Evaluation of diets for culture of the calanoid copepod *Gladioferens imparipes*. Aquaculture 187 (1-2):85-96.

Pereira DC, Trigueiro TG, Colepicolo P, Marinho-Soriano E (2012) Seasonal changes in the pigment composition of natural population of *Gracilaria domingensis* (Gracilariales, Rhodophyta). Rev Bras Farmacogn 22:874-880.

Piorreck M, Baasch K-H, Pohl P (1984) Biomass production, total protein, chlorophylls, lipids and fatty acids of freshwater green and blue-green algae under different nitrogen regimes. Phytochemistry 23 (2):207-216.

Platt T, Gallegos C, Harrison W (1980) Photoinhibition of photosynthesis in natural assemblages of marine phytoplankton. J Mar Res 38:687-701.

Rasdi NW, Qin JG (2014) Improvement of copepod nutritional quality as live food for aquaculture: a review. Aquacult Res n/a-n/a: DOI: 10.1111/are.12471.

Rasdi NW, Qin JG, Li Y (2015) Effects of dietary microalgae on fatty acids and digestive enzymes in copepod *Cyclopina kasignete*, a potential live food for fish larvae. Aquacult Res n/a-n/a:DOI: 10.1111/are.12778.

Reitan KI, Rainuzzo JR, Olsen Y (1994) Effect of nutrient limitation on fatty acid and lipid content of marine microalgae. J Phycol 30 (6):972-979.

Renaud SM, Parry DL, Thinh L-V, Kuo C, Padovan A, Sammy N (1991) Effect of light intensity on the proximate biochemical and fatty acid composition of *Isochrysis* sp. and *Nannochloropsis oculata* for use in tropical aquaculture. J Appl Phycol 3 (1):43-53.

Rhiel E, Mörschel E, Wehrmeyer W (1985) Correlation of pigment deprivation and ultrastructural organization of thylakoid membranes in *Cryptomonas maculata* following nutrient deficiency. Protoplasma 129 (1):62-73.

Ritchie R (2006) Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents. Photosynthesis Res 89 (1):27-41.

Rückert GV, Giani A (2004) Effect of nitrate and ammonium on the growth and protein concentration of *Microcystis viridis* Lemmermann (Cyanobacteria). Rev Bras Bot 27:325-331.

Sargent JR, McEvoy LA, Bell JG (1997) Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. Aquaculture 155 (1-4):117-127.

Sharma KK, Schuhmann H, Schenk PM (2012) High lipid induction in microalgae for biodiesel production. Energies 5 (5):1996-1073.

Shifrin NS, Chisholm SW (1981) Phytoplankton lipids: Interspecific differences and effects of nitrate, silicate and light-dark cycles. J Phycol 17 (4):374-384.

Smith REH, Kalff J (1982) Size-dependent phosphorus uptake kinetics and cell quota in phytoplankton. J Phycol 18 (2):275-284.

Søndergaard M, Riemann B (1979) Ferskvandsbiologiske analysemetoder. Akademisk Forlag, Copenhagen.

Sorgeloos P, Dhert P, Candreva P (2001) Use of brine shrimp, *Artemia* spp., in larval crustacean nutrition: a review. Aquaculture 200:147-159.

Spector A (1999) Essentiality of fatty acids. Lipids 34 (1):S1-S3.

Sriharan S, Bagga D, Nawaz M (1991) The effects of nutrients and temperature on biomass, growth, lipid production, and fatty acid composition of *Cyclotella cryptica* Reimann, Lewin, and Guillard. Appl Biochem Biotechnol 28-29 (1):317-326.

Støttrup JG (2003) Production and nutrional value of copepods. In: Støttrup JG, McEvoy LA (eds) Live feeds in Marine Aquaculture. Blackwell Publising, Oxford, pp 145-205.

Støttrup JG, Jensen J (1990) Influence of algal diet on feeding and egg-production of the calanoid copepod *Acartia tonsa* Dana. J Exp Mar Biol Ecol 141 (2-3):87-105.

Thompson PA, Harrison PJ, Whyte JNC (1990) Influence of irradiance on the fatty acid composition of phytoplankton. J Phycol 26 (2):278-288.

Wilson RP (1985) Amino acid and protein requirements of fish. In: Cowey CB, Mackie AM, Bell JG (eds) Nutrition and Feeding in Fish. Academic Press, London, pp 1-16.

Wu G (2009) Amino acids: metabolism, functions, and nutrition. Amino Acids 37 (1):1-17.

Yaakob Z, Ali E, Zainal A, Mohamad M, Takriff MS (2014) An overview: Biomolecules from microalgae for animal feed and aquaculture. J Biol Res-Thessalon 21:6 (6).

Yu J, Li G, Krull I, Cohen S (1994) Polymeric 6-aminoquinone, an activated carbamate reagent for derivatization of amines and amino acids by high performance liquid chromatography. J Chromatogr B Biomed Appl 658 (2):249-260.

Zhang J, Wu C, Pellegrini D, Romano G, Esposito F, Ianora A, Buttino I (2013) Effects of different monoalgal diets on egg production, hatching success and apoptosis induction in a Mediterranean population of the calanoid copepod *Acartia tonsa* (Dana). Aquaculture 400-401 (0):65-72.

Zimba PV (2012) An improved phycobilin extraction method. Harmful Algae 17:35-39.

Caption for figures

Fig. 1 Net photosynthesis per cell of *R. salina* (× 10^{-15} mol O_2 $cell^{-1}$ h^{-1}) incubated at different cell densities and irradiance levels. Note: the number of experimental replicates, n = 5 for irradiances from 0-140 µmol photons m^{-2} s^{-1} and n = 2-3 for irradiances from 160-300 µmol 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. Data are indicated by means (± 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 (± 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 *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 (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, 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

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 µmol 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 = 2-3 for irradiances from 160-300 µmol photons m^{-2} s^{-1}

Note: α = Initial slope of the photosynthesis-irradiance curve; β = Negative slope at high irradiance; I_m = irradiance of maximum photosynthesis, R = Dark respiration = mean ± SDs (plus/minus standard deviation) of dark respiration at 0 µmol photons m^{-2} s^{-1} of 5 experimental replicates at a specific cell density. Units: α, β = 10^{-15} mol O_2 $cell^{-1}$ h^{-1} [µmol photons m^{-2} s^{-1}]$^{-1}$; P_m^B, R = 10^{-15} mol O_2 $cell^{-1}$ h^{-1} and I_m = µmol photons m^{-2} 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 limited irradiance = mean ± SDs (plus/minus standard deviation) of FAA/EAA at irradiance from 10-40 µmol photons m^{-2} s^{-1}; values for saturated irradiance = mean ± SDs of FAA/EAA at irradiance from 60-140 µmol 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 different treatments