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Testing the yield of a pilot-scale bubble column photobioreactor for cultivation of the microalga *Rhodomonas salina* as feed for intensive calanoid copepod cultures

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Running title: A PBR for cultivation of *Rhodomonas salina*

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15 **Abstract**

16 A dual column photobioreactor (PBR) (2 × 47 L) with mixed CO_2/air bubbling was tested for
17 cultivation of the microalga *Rhodomonas salina* as food for live feed copepods. In the continuous
18 growth phase, the cell density was relatively stable at 2.40 ± 0.13 $\times 10^6$ cells mL^{-1} at an average
19 dilution rate of 0.46 ± 0.02 day^{-1} throughout the 30-day experiment. The produced algae had a high
20 content of both total fatty acids (TFA) and free amino acids (FAA). Especially, the harvested algae
21 contained a high proportion of poly-unsaturated fatty acids (PUFA) that made up 80 % of the TFA
22 and of essential amino acids (35% of all FAA), implicating desirable components as feed for
23 copepods. The current PBR was sufficient to feed a culture of the calanoid copepod *Acartia tonsa* at
24 a density of 2,500 adult L^{-1} in ca. 500 L culture with a daily yield of approximately 17 × 10^6 eggs.
25 To be able to sustain the integrated copepods production, the suggested volume of the algae cultures
26 should be ca. 20% of the copepod culture volume.

27

Introduction

Microalgae are important live feeds for early life stages of many marine aquaculture species ranging from zooplankton to fish (e.g. Støttrup, 2003 ; Muller-Feuga et al., 2003). Microalgae are attractive feed organisms because of their size, rapid growth rate, potential for mass-cultivation, digestibility, and particularly their high nutritional value (Brown, 2002). The nutritional quality of microalgae is indicated by the abundance of highly unsaturated fatty acids (HUFA), particularly eicosapentaenoic acid (EPA), 20:5n-3 and decosahexaenoic acid (DHA), 22:6n-3 (Renaud et al., 1991) and the composition of amino acids (AAs) (Brown, 1991; Guisande et al., 2000). For example, HUFA are essential fatty acids for various aquaculture animals (Nichols, 2003), also sustaining growth and reproduction of copepods (Rasdi & Qin, 2016).

In aquaculture, microalgae are grown either in simple cultivation systems, such as open ponds, raceways or aerated open carboys using natural sun light or in more complex closed systems such as closed cylindrical tanks, vertical aerated column photobioreactors (PBRs) or tubular flat-plate PBRs (reviewed in Zmora & Richmond, 2004; Ugwu et al., 2008; Carvalho et al., 2006). The closed PBRs offer controllable culturing conditions such as temperature, light, pH, carbon dioxide (CO_2), but they require a high initial investment cost (Singh & Sharma, 2012; Ugwu et al., 2008).

The present study aimed to test the culturing capacity of a pilot-scale double bubble-column PBR (2 × 47 L) for cultivation of *R. salina* as food for intensive copepod production. Eriksen et al. (1998) succeeded in using a small-scale column PBR (1.7 L) to cultivate *Rhodomonas* sp. with a cell density reaching 10^7 cells mL^{-1} for up to 415 days continuously with a dilution rate of 0.6 day^{-1}. For large-scale cultivation of microalgae in the aquaculture industry, the production in experimental small-scale PBRs must be up-scaled and the efficiency maintained. Major challenges when upscaling microalgal production include increasing the total volume of the PBR, while at the same

time maintaining an optical path in the PBR that is short enough to still achieve maximal growth and output rates of the algae (Hu et al., 1996). Another challenge is to maintain a sufficient mixing in a large PBR without causing a shear stress high enough to break the algal cell membranes (Qiang & Richmond, 1996). In the present contribution, we tested capacity of a medium-sized PBR for production of microalgae with respect to both quantity and quality (fatty acids and amino acids) as feed for copepods.

Materials and methods

Algal strain

The cryptophyte *R. salina* was obtained as strain K-1487 from the Scandinavian Culture Collection of Algae and Protozoa (SCCAP). The stock culture of *R. salina* was grown in the laboratory at Roskilde University (RUC) at standard conditions as described by Vu et al. (2016). The cultures were maintained under a continuous irradiance of 80 μmol photons m−2 s−1 photosynthetically active radiation (PAR) in a climate room at 20 °C. The flasks were gently aerated with atmospheric air through 0.45-μm filters.

Design and description of bioreactors

R. salina was cultured in the dual column PBR shown in Fig. 1. Each culture column consists of a 150 cm high glass cylinder that for practical reasons is made of two 75 cm high glass cylinders on top of each other held together by a plastic ring. The inner diameter is 20 cm, giving a volume of 47 L for each of the two columns with a volume of 94 L for the entire PBR. The glass cylinders are placed on a polypropylene base and closed with silicon O-rings at the top and bottom.

73 Seawater was filtered through a 0.2 µm filter and purified with ultra-violet light (UV) (Ultra Violet
74 filter, Water.dk WP/75/60 at a fluence of 65 - 97 mW-s cm^{-2}) and nutrients were supplied trough a
75 peristaltic pump from the bottom of the culture column.

76 The microalgae were kept in suspension by large bubbles from the air system, and atmospheric
77 airflow was adjusted by a flow meter. Using another flow meter, a lower atmospheric flow was
78 mixed with pure CO_2 from a pressure flask. The mixture of CO_2 and atmospheric airflow was
79 regulated automatically using a feedback system, where opening or closing of solenoid valves were
80 controlled by a pH probe in the column. The mixture of pure CO_2 and atmospheric air was passed
81 through a membrane to increase formation of microbubbles to improve the mass transfer rate of
82 CO_2 for algae growth.

83 A cooling coil was installed inside each of the PBR culture columns and allowed adjustment of the
84 temperature in the PBR to 20ºC. A temperature sensor was used as an *in-situ* measuring probe to
85 regulate the feedback temperature system, by opening and closing solenoid valves.

86 Light was supplied by 4 pairs pr. PBR of LCD lamps (Aussmak ELT-S0335 T9/35W/2700K) from
87 4 sides and was adjusted to provide light intensities at 3 different levels: low, medium and high,
88 corresponding to 393 ± 44, 608 ± 80 and 981 ± 133 $\mu mol \cdot m^{-2} \cdot s^{-1}$ Photosynthetic Active Radiation
89 (PAR), respectively.

90 The microalgae were harvested from an overflow at top of each culture column of the PBR. The
91 harvested algae were stored in a tank and used for copepod cultures in 4 × 350 L tanks at Roskilde
92 University's copepod production facility (Fig. 1).

The PBR was connected and controlled by Programmable Logic Control (PLC). From the PLC, the light mode, temperature, pH, the pump flows (for input seawater, nutrients, copepod feeding) were set to required levels. Data such as pH and temperature were logged and stored every 30 minute.

Environmental parameters in the photobioreactor

Temperature and pH in the PBR in the three experiments were maintained at 20.01 ± 0.03 ºC and 8.11 ± 0.03, respectively. Nitrate concentrations were maintained at 2211 ± 374 µmol L^{-1} throughout the experiment, and the phosphate concentrations at 331 ± 33 µmol L^{-1} during the experiment. Nitrate concentrations were determined by flow injection analysis using QuickChem Method 31-107-04-1-A (detection limit is 0.02 µmol·L^{-1}, (Diamond, 1999)), while phosphate concentrations were quantified spectrophotometrically by the method described by Søndergaard and Riemann (1979).

Experimental design

The *R. salina* was cultured under conditions with nutrients in excess (B1-medium, added daily as 1 mL B1 L^{-1} seawater, (Hansen, 1989), and a high light intensity (60–140 µmol photons m^{-2} s^{-1}) to generate a relatively large algal biomass with desired fatty acids (FA) and free essential amino acids (FAA) profiles (*sensu* Vu et al., (2016)).

The growth of *R. salina* was investigated in the PBR as four replicates (2 times × 2 culture column). At start, each of the culture column (V = 47 L) was filled with UV-treated seawater and inoculated with fresh algae stock cultures to a total volume of ca. 42 L with the initial cell density of 125,229 ± 3,720 cells mL^{-1}. The PBR was run in the initial growth phase for five days. The B1 medium was added manually at the beginning of the experiment and every 1-2 days (rate at 1.2 mL L^{-1}, (Hansen, 1989) to ensure that nutrients were in excess in the culture. After the first five days, the algal culture

115 in the PBR was brought into the continuous phase, where steady state conditions were obtained by
116 adding UV-treated seawater by two pumps (ProMinent DULCOflex) at bottom of each of the two
117 PBRs (Fig. 1) to dilute the algal density. The B1 medium was added at a rate of 1.2-4 mL L^{-1} of
118 newly added seawater.

119 The light was set at the low intensity for the first 2 days when the algae density was low to avoid
120 photo-inhibition. When the algae density had increased after day 3, the light was changed to the
121 highest intensity to minimize a potential light limitation.

122 The main atmospheric airflow was set at ca. 4 L min^{-1} at the first 2-3 days of the experiment when
123 the algal cell density was low and increased to 5-6 L $min.^{-1}$ when the algal cell density was high
124 from day 2-3 onward. The mixture of pure CO_2 (V/V = 2-5% of CO_2 in mixture) and atmospheric
125 air (V= 2-2.5 L) was added to supply CO_2 and to control the pH at 8.1 ± 0.1.

126 *Analytical procedures and measurements*

127 Samples of *R. salina* (3-4 analytical replicates, each with 50-100 mL of culture medium per sample)
128 were taken daily from each culture column to determine growth and production of the algae and
129 biochemical composition of algal cells. The cell density of *R. salina* was determined daily by
130 measuring 3 to 4 replicates in a Beckman Multisizer™3 Coulter Counter® (Beckman Coulter Inc.,
131 USA). The specific growth rate (day^{-1}) of *R. salina* was calculated by fitting cell density increase in
132 each of the culture columns during the initial phase to an exponential growth function (Vu et al.,
133 2016).

134 The cell density and total volume of the harvested algae in each harvest tank (collected from the
135 culture columns) were also measured daily. The cell density of *R. salina* was converted into carbon
136 weight by assuming that one algal cell equals 47.4 pg C (Berggreen et al., 1988).

137

138 For analysis of fatty acids (FA) and free amino acids (FAA) in the microalgae, samples of about 10^6
139 algal cells were collected during both the initial (14 samples; n=14) and the continuous growth
140 phase (18 samples; n=18). The samples were filtered through a 25-mm diameter GF/C glass fiber
141 filter (Whatman) and stored in Pyrex glass vials for FA analysis, or in 1.5 mL HPLC glass vials for
142 FAA analysis, at -80 °C for later analysis FA and FAA compositions.

143 The FA composition was determined by extraction of the lipids using a HPLC-grade chloroform:
144 methanol mixture (Folch et al., 1957) followed by a trans-esterification process by acetyl chloride in
145 methanol. The detailed procedure of the FA analyses was described by Drillet et al. (2006) with
146 minor adjustments: addition of 20 µL internal standard (C-23-methylester, 1000 µg ml^{-1}) and no
147 sonication.

148 For analysis of FAA, each vial containing a filter sample was added 1 mL Milli-Q water, heated to
149 95°C for 15 min and filtered through 0.2 µm cellulose nitrate syringe filters (VWR International,
150 USA) after cooling. FAA concentrations were determined by HPLC and fluorescence detection
151 after derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC). AQC was
152 purchased from Waters as an AccQFlour kit (www.waters.com). The analysis was conducted as
153 recommended by Waters, except for adjustment of the solvent gradient, and the AA derivatives
154 were analyzed on a Waters HPLC system (Alliance 2695 solvent module, 2475 fluorescence
155 detector and a 3.9 × 150 mm AccQTag column). Chromatogram peaks were identified from
156 retention times and integrated against an external amino acid standard mixture (AA-S-18 amino
157 acid mixture; www.sigma-aldrich.com).

158

159 *Calculated capacity of the photobioreactor to produce algal biomass for copepod cultures*

160 The daily production of *R. salina* served as food for the calanoid copepod *Acartia tonsa* culture at
161 Roskilde University, Denmark. The estimated production rate of the PBR for both culture columns
162 (P, g C day^{-1}) was calculated as equation (Eq. 1):

163 $$P = D \times V_A \times D_A \times W_A \quad \text{(Eq. 1)}$$

164 In which

165 D: dilution rate (day^{-1})

166 V_A: filled volume (L) of the two culture columns of the photobioreactor

167 D_A: density of the algae harvested, assuming that the density of algae harvested equals the density
168 inside the culture columns (cells·L^{-1})

169 W_A: carbon weight of algae cells = 47.4×10^{-12} g C $cell^{-1}$ (Berggreen et al. 1988)

170 The algae requirement (in carbon weight) for 1 L of copepods per day (C_A, g C L^{-1} day^{-1}) was
171 calculated as equation (Eq. 2)

172 $$C_A = D_C \times [W_F \times F + W_M(1 - F)] \times IR_{Max} \quad \text{(Eq. 2)}$$

173 In which

174 C_A: algae requirement (carbon units) for 1 L of copepods.

175 D_C: density of copepods (ind L^{-1}), we used a density of 2,500 adult copepods·L^{-1} which is reported
176 to be the optimal density of *A. tonsa* for maximal egg yield in culture tanks (Drillet et al. 2015)

W_F: The average carbon weight of female copepods = 3,193 ng C ind^{-1} = $3{,}193\times10^{-9}$ g C ind^{-1} (Drillet et al. 2015)

W_M: The average carbon weight of male copepods =2,282 ng C ind^{-1} = $2{,}282\times10^{-9}$ g C ind^{-1} (Drillet et al. 2015)

F: the female: male ratio = 0.45 (Drillet et al. 2015)

IR_{Max}: maximum specific carbon ingestion rate of *R. salina* K-1487 by the same strain of *A. tonsa* = 1.32 day^{-1} (Berggreen et al. 1988)

The volume of copepod culture that can be sustained by feeding on the current algae production (V_C, L) was estimated as equation (Eq. 3):

$$V_C = \frac{P}{C_A} \qquad \text{(Eq.3)}$$

The egg production of *A. tonsa* was estimated assuming that 1/3 of carbon ingested by female copepods is allocated to egg production (Kiørboe et al. 1985). Theoretical specific egg production per individual (SEP, eggs $female^{-1}$ day^{-1}) was calculated as equation (Eq. 4)

$$SEP = \frac{W_F \times IR_{Max}}{3 \times W_E} \qquad \text{(Eq. 4)}$$

Whereas W_E: the carbon weight of the egg: 45.7×10^{-9} g C egg^{-1} (Kiørboe et al. 1985)

Potential number of eggs produced with the current algal production (EP, eggs day^{-1}) was estimated as equation (Eq. 5):

$$EP = SEP \times D_C \times F \times V_c \quad \text{(Eq. 5)}$$

195 *Statistical analysis*

196 Data on fatty acid and amino acid content were subjected to one-way ANOVA. Data were tested for
197 homogeneity of variance (Cochran's test) and normal distribution (Kolmogorov-Smirnoff goodness
198 of fit test) before being analyzed by ANOVA. All tests on data were carried out using SYSTAT v.
199 13 with $\alpha = 0.05$. ANOVA was performed on data from the initial phase and the continuous phase
200 separately, and as the ANOVA showed no significant differences in data within each phase ($p >$
201 0.05), data on content of the various fatty acids and amino acids were pooled within each phase.
202 Subsequently, differences between content in the two phases were tested for each compound by
203 one-way ANOVA, using SYSTAT v. 13 with $\alpha = 0.05$.

204 **Results**

205 *Growth of R. salina in the photobioreactor*

206 In the initial phase (day 1 to 5), the specific growth rate of *R. salina* was 0.81 ± 0.07 day^{-1} (mean ±
207 SE). The average cell density increased from Day 0 and peaked at $4.17 \pm 1.43 \times 10^6$ cells mL^{-1}
208 (mean ± SE) at Day 5 (Fig. 2). From Day 6, the cell density remained relative stable at 2.40 ± 0.13
209 $\times 10^6$ cells mL^{-1} (mean ± SE). The dilution rate varied over time with an average of 0.46 day^{-1}
210 throughout the experiment (Fig. 2). The average production rate for one 47 L PBR column was
211 determined to $44.60 \pm 5.25 \times 10^9$ cells day^{-1} (mean ± SE), equivalent to 2.11 ± 0.25 g C day^{-1}.

212 *Fatty acids*

213 Data from each growth phase showed a similar content of fatty acids within the initial and
214 continuous phase respectively (one-way ANOVA, $p > 0.05$). The fatty acid data were therefore
215 pooled within each phase for further analysis. The total fatty acid (TFA) content of *R. salina*

increased from 28.38 ± 2.81 pg cell^{-1} (mean ± SE, n = 18) in the initial phase, to 41.37 ± 2.46 pg cell^{-1} (mean ± SE, n = 18) during the continuous phase (Fig. 3A, Table 1). This increase was statistically significant (one-way ANOVA, $p < 0.001$). The DHA content was significantly lower (2.74 ± 0.19 pg cell^{-1}, mean ± SE) in the initial phase (one-way ANOVA, $p< 0.01$) than in the continuous phase (3.27 ± 0.16 pg cell^{-1}, mean ± SE, Fig. 3A), but the relative abundance of DHA as percentage of TFA was higher in the initial phase (10.41 ± 0.70 % of TFA, mean ± SE) than that in the continuous phase (8.23 ± 0.39 % (mean ± SE) of TFA, Table 1). Similarly, the EPA content increased significantly (one-way ANOVA, $p < 0.01$) from 3.45 ± 0.35 pg cell^{-1} (mean ± SE) in the initial phase to 4.71 ± 0.26 pg cell^{-1} (mean ± SE) in the continuous phase (Fig. 3A), but the relative abundance remained at about 12 % of TFA, independent of the growth phases (Table 1). However, the DHA/EPA ratio decreased from 0.84 ± 0.04 (mean ± SE) in the initial phase to 0.71 ± 0.02 % (mean ± SE) of TFA in the continuous phase (Table 1).

The fatty acids of *R. salina* were dominated by short chain polyunsaturated fatty acids (SC-PUFA). These fatty acids evinced a statistically significant lower content (one-way ANOVA, $p < 0.01$) in the initial phase (51.99 ± 1.81 % of TFA, mean ± SE) than in the continuous phase (57.62 ± 1.72 % of TFA, mean ± SE, Fig. 3B, Table 1). In contrast, the relative abundance of the monounsaturated fatty acids (MUFA) was significantly lower in the continuous phase (one-way ANOVA, $p < 0.01$) (6.49 ± 0.54 % of TFA), as compared to the initial phase (11.37 ± 1.97 % of TFA, Fig. 3B, Table 1). The relative abundance of highly saturated fatty acids (HUFA) and the saturated fatty acids (SFA) were not significantly different in the two growth phases (one-way ANOVA, $p > 0.05$, Fig. 3B, Table 1).

Free amino acids

As was the case for the fatty acids, data from each growth phase showed a similar content of FAA within the initial and continuous phase respectively (one-way ANOVA, $p > 0.05$). The FAA data were thus also pooled within each phase for further analysis. The total free amino acid (TFAA) content was relative stable at 3 pg cell^{-1}, regardless the growth phase (one-way ANOVA, $p > 0.05$, Fig. 4A, Table 2). The essential amino acids made up around 1 pg cell^{-1}, corresponding to ca. 35% of TFAA, irrespective of the growth phase (one-way ANOVA, $p > 0.05$, Fig. 4A, Table 2). No significant differences in the relative abundance of the various EAA were observed between different growth phases either (one-way ANOVA, $p > 0.05$, Fig. 4B, Table 2). The most abundant EAA was arginine (ca. 18% of TFAA), followed by lysine (ca. 4% of TFAA, Fig. 4B).

Capacity of the photobioreactor to produce algal biomass for a copepod culture

Our estimation shows that the current production of the two column PBR can be 92.74 $\times 10^9$ cells day^{-1} which can sustain ca. 500 L of copepod *A. tonsa* culture at the density of 2500 ind. L^{-1} (Table 3). The estimated number of eggs produced from such *A. tonsa* cultures were 17.11 $\times 10^6$ eggs day^{-1} (Table 3).

Discussion

The purpose of this study was testing of a dual bubble-column PBR (2 × 47 L) for cultivation of the microalga R. salina to examine if the methodology can be scaled up for commercial production in the aquaculture industry. For large-scale production, a stable, continuous, and high-quality production of algae is needed if the algal should serve as feed for cultures of sustain calanoid copepods that later can be used as live feed in marine fish hatcheries. Thus, the overall aim was to integrate PBR and copepod culture facilities at a future industrial scale.

Cell density and daily production of PBR

The specific growth rate obtained in this study (0.81 ± 0.07 day^{-1}) was similar to the previously reported growth rates of *Rhodomonas* species (0.3-0.8 day^{-1}, (Guevara et al., 2016; Vu et al., 2016; Lafarga-De la Cruz et al., 2006; Eriksen et al., 1998). In contrast to these previous studies, we successfully up-scaled the *Rhodomonas* cultures from low-volume cultures of 0.1-1.7 L to volumes closer to a future commercial scale. In a substantially smaller PBR system (1.5 L cultures), *Rhodomonas* sp. was produced and harvested continuously for 415 d at a cell density of 10^7 cells mL^{-1} and with a dilution rate of 0.6 day^{-1} (Eriksen et al., 1998). In our 30 times larger PBRs, the cell density was about one quarter of this during the continuous phase, but the dilution rates of algae obtained were almost the same as in the study of Eriksen et al. (1998). The lower cell density obtained in our PBR reflects its much larger inner diameter (20 cm vs. 7.5 cm (Eriksen et al., 1998; Zhang & Richmond, 2003)), but the larger volume in our PBR more than compensates for the lower volumetric productivity. This means that although the upscaling lowers the production efficiency (PE) with a factor ¼, the total volumetric productivity is about 7.5 higher than in small-scale facilities.

Nutritional quality of algae

Compared to the initial exponential phase, a higher TFA content was found in *R. salina* during the continuous phase. Since microalgae for copepod feed typically are harvested in the continuous phase, this observation is important. This increase in TFA is often associated with nitrogen limitation (Gladue & Maxey, 1994), but this is not the case in the present study, as the nitrogen concentration in the PBR is kept at a high, non-limiting, level. Our results indicate, however, that *R. salina* accumulate more FA in steady state growth than under the initial phase. A similar pattern has been observed for *Chlorella* vulgaris when growing under a high supply of CO_2 (Jose &

283 Suraishkumar, 2016) and in *Nannochloropsis oceanica* under nitrogen replete conditions as in the
284 present study (Xiao et al., 2015). Another observation from the continuous phase in our cultures
285 was an increased presence of desirable fatty acids, SC-PUFA (mainly C18:3 n-3) when the algae
286 grew in the continuous phase. In contrast, algae harvested in the continuous phase contained less
287 MUFA as compared to the algae in the initial phase. An enhanced content of SC-PUFA in
288 microalgae has previously been observed under culture conditions utilizing nitrate as the nitrogen
289 source, eliminating the pH fluctuations caused by ammonium use, and non-limiting concentrations
290 of phosphate, as many of the SC-PUFAs are found as phospholipids (Yongmanitchai & Ward,
291 1991).

292 In aquaculture, a DHA/EPA ratio of 2 is proposed as an optimum criterion for live food organisms,
293 leading to the preferred ratio in the next link in the aquaculture food chain, the fish larvae ((Reitan
294 et al., 1994)). The DHA/EPA ratio obtained in the present study (DHA/EPA ratio of 0.7 to 0.8) was
295 similar to previous studies on the same algal species (DHA/EPA ratio of 0.5 to 0.9; (Dunstan et al.,
296 2005; Mansour et al., 2005; Vu et al., 2016; Guevara et al., 2016)), indicating that the preferred
297 ratio of 2 was not reached. However, although both historical data and our data show that the
298 DHA/EPA ratio in the cultured algae is below the optimum ratio, the DHA/EPA ratio will be closer
299 to 2 by trophic upgrade when *Rhodomonas* is fed copepods that again are fed to the fish larvae.
300 Thus, Drillet et al. (2006) observed a DHA/EPA ratio of about 1.3 in fish larvae when fed *Acartia*
301 *tonsa* based on *R. salina* from our laboratory strains.

302 Regarding the general composition of FA in *R. salina* cultured in the PBR, the composition agreed
303 with previous results of especially PUFA (made up ca. 80% of TFA in this study), including SC-
304 PUFA and HUFA ((Vu et al., 2016)). PUFA are also well known to be crucial for the production

and hatching success of copepod eggs (Arendt et al., 2005; Broglio et al., 2003; Jónasdóttir et al., 2009).

The increase in cell-specific content of FA in the continuous phase was not observed in TFAA that remained unchanged during the entire study period. This uncoupling between FA and FAA was also observed in another marine alga in which precursors for FA increased, while most of the FAA declined during N starvation (Zhang et al., 2016). These authors suggested that the changes in intracellular FAA pool reflected synthesis and catabolism of AA, as well as proteolytic activity, e.g. degradation of Rubisco. Thus, in *R. salina*, synthesis and catabolism of FAA may have been balanced with proteolytic activity throughout the study.

The FAA content in zooplankton is reported to be closely dependent on content and composition in algae used as feed for the zooplankton (e.g., Laabir et al., 1999) and in this respect, *Rhodomonas* is considered a very promising food alga for various zooplankton species, including our target species (Drillet et al. 2006). For the next trophic level, the fish larvae, Rayner et al. (2017) has recently published an overview of fish larval needs. Essential FAs, especially leucine and valine, as well as threonine, arginine and methionine are considered most important. These amino acids were all present in significant amounts in the present *R. salina* strain.

Availability as food for copepod productions

Based on our estimation, the current daily production of *R. salina* in our two PBRs, about 4.40 g C day^{-1}, can sustain an intensive copepod production tank at a volume of ca. 500 L at an optimum density of ca. 2,500 adult copepods L^{-1}, as proposed in Drillet et al. (2015). This copepod production can deliver a daily egg production of ca. 17.11×10^6 eggs per culture tank. Therefore, to be able to feed the copepods optimally, the required volume of algal culture at the cell density

327 achieved in this study is ca. 20% of the copepod culture. It should be noted that the estimation of
328 algae feeding to the copepods was based on the grazing rate of adult copepods. For younger life
329 stages, e.g., nauplii and copepodite stages, the demand for algae is substantially smaller.

330 *Recommendations*

331 The microalga *R. salina* was successfully cultivated in the current bubble-column PBR and
332 produced algal biomass with a high biochemical quality and that can immediately be fed out to
333 copepod cultures. Based on this, it is recommended that *R. salina* should be cultivated with the
334 addition of pure CO_2 in a mixture with atmospheric air (V/V=2-5% of CO_2 in the mixture) to
335 maintain the pH at 8.1. The harvested algae in the continuous growth phase obtained a high TFA
336 content with a relative high abundance of PUFA (including SC-PUFA and HUFA) as well as a high
337 content of FAA, which is of crucial importance for enhancing the egg production, the egg hatching
338 success, and the somatic growth of the cultured copepods. For copepod production in aquaculture
339 facilities, the estimated volume of algae cultures should be about 20% v/v of copepod culture.

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454 **Captions for figures**

455 **Fig. 1** The pilot-scale bubble column photobioreactor for cultivation of *Rhodomonas salina*
456 connecting with the copepod cultures. Note: figure not drawn to scale

457 **Fig. 2** Cell density of *Rhodomonas salina* cultured in the PBR. The data indicated by means (±SE) of
458 the cell density (●) and dilution rate (Δ) of algae cultured in the PBR with 4 replicates from day 0 to
459 day 16 and only 1 replicate from day 17 onwards.

460 **Fig. 3** The total fatty acids, absolute DHA and EPA content (A) and the fatty acids main group
461 composition (B) of *Rhodomonas salina* during initial (black bar) and continuous phase (grey bar).
462 Data presented by means ± SE. Different letters represent significant differences of a specific fatty
463 acid or main group of fatty acids at $p = 0.05$ between the two growth phases.

464 **Fig. 4** The total free amino acids (TFAA), absolute total essential amino acids (TEAA) content (A)
465 and the relative essential amino acids composition (B) of *Rhodomonas salina* during the initial
466 (black bar) and continuous phase (grey bar) growth phase. Data presented by means ± SE.

467

468

469 **Captions for tables**

470 **Table 1** Total fatty acids (TFA) and FA compositions of *Rhodomonas salina* cultured in the
471 photobioreactors at different growth phases (initial vs continuous phase)

472 **Table 2** Results of one-way ANOVA testing the effects of different growth phases (initial vs
473 continuous phase) on total free amino acids, total essential amino acids content and specific amino
474 acids of *Rhodomonas salina* cultured in the photobioreactors

475 **Table 3** Estimated ability to support the copepod *Acartia tonsa* production (2500 ind L^{-1}) by
476 *Rhodomonas salina* algae produced in the current bubble column photobioreactors

477

478 **Table 1**

Variables	Initial phase	Continuous phase	df1	df2	SS	MS	F	p
C14:0	1.74±0.33[a]	1.65±0.21[a]	1	31	0.065	0.065	0.0585	0.81
C16:0	9.56±0.91[a]	11.42±0.57[b]	1	31	3.011	3.011	10.655	0.003
C18:0	1.18±0.19[a]	0.56±0.07[b]	1	31	3.011	3.011	10.655	0.003
SFA	12.68±1.02[a]	13.78±0.65[a]	1	31	9.456	9.456	0.891	0.353
C16:1	0.88±0.23[a]	1.23±0.24[a]	1	31	0.967	0.967	1.032	0.318
C18:1	3.45±0.57[a]	3.59±0.37[a]	1	31	0.159	0.159	0.0473	0.829
C22:1	6.71±2.48[a]	1.26±0.30[b]	1	31	234.318	234.318	6.137	0.019
MUFA	11.37±1.97[a]	6.49±0.54[b]	1	31	187.522	187.522	7.056	0.013
C18:2	10.48±1.55[a]	15.25±1.39[b]	1	31	179.275	179.275	5.244	0.029
C18:3 n-6	1.80±0.26[a]	2.15±0.08[b]	1	31	0.964	0.964	2.042	0.163
C18:3 n-3	18.26±0.93[a]	22.46±0.84[b]	1	31	139.059	139.059	11.203	0.002
C18:4	21.43±1.07[a]	17.74±1.72[a]	1	31	106.342	106.342	2.863	0.101
SC-PUFA	51.99±1.81[a]	57.62±1.72[b]	1	31	249.151	249.151	7.982	0.008
C20:4 n-6	1.06±0.18[a]	2.00±0.14[b]	1	31	6.905	6.905	17.639	<0.001
C20:5 n-3 (EPA)	12.47±0.57[a]	11.86±0.73[a]	1	31	2.936	2.936	0.394	0.535
C22:6 n-3 (DHA)	10.41±0.70[a]	8.23±0.39[b]	1	31	37.347	37.347	8.224	0.007
HUFA	23.95±1.06[a]	22.11±1.08[a]	1	31	26.684	26.684	1.428	0.241
DHA/EPA	0.84±0.04[a]	0.71±0.02[b]	1	31	0.138	0.138	10.136	0.003
TFA	28.38±2.81[a]	41.37±2.46[b]	1	31	1328.75	1328.75	12.118	0.002

479 Note: SFA: saturated fatty acids, MUFA: mono unsaturated fatty acids, SC-PUFA: short chain-poly
480 unsaturated fatty acids, HUFA: highly unsaturated fatty acids. Units of TFA: pg $cell^{-1}$, specific
481 FA/FA group: % of TFA. Values for limited irradiance = mean ± SEs of TFA, specific FA or FA
482 groups at different growth phases (with n = 18 replicates for initial phase and n = 14 replicates for
483 the continuous phase). Different letters in the same row denote the significant differences (Tukey
484 test) in the TFA, or the same specific FA or FA groups between the different growth phases.

485

Table 2

Variables	df1	df2	SS	MS	F	*p*
TFAA	1	31	0.0043	0.0043	0.003	0.958
TEAA	1	31	0.149	0.149	0.325	0.573
His	1	31	0.0059	0.0059	0.0176	0.895
Thr	1	31	1.92	1.92	1.193	0.283
Arg	1	31	34.195	34.195	1.017	0.321
Val	1	31	2.257	2.257	0.319	0.576
Met	1	31	1.699	1.699	0.876	0.357
Ile	1	31	0.0421	0.0421	0.0317	0.86
Leu	1	31	1.878	1.878	2.084	0.159
Lys	1	31	0.0205	0.0205	0.00494	0.944
Phe	1	31	3.502	3.502	2.258	0.143

489 **Table 3**

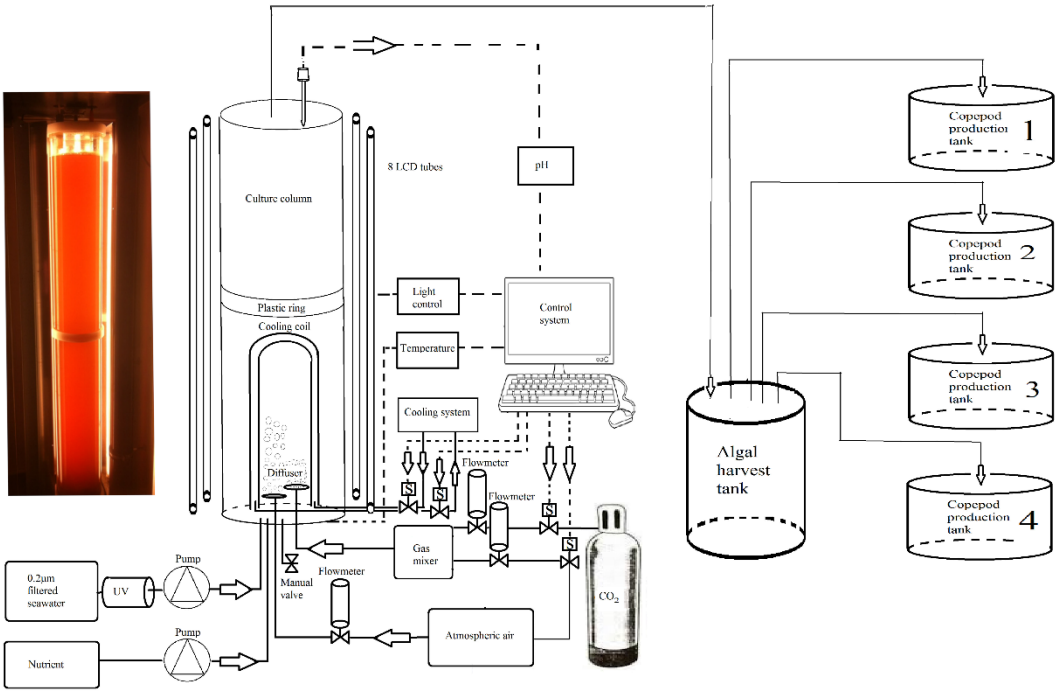
Estimated parameters	
Dilution rate, D (day^{-1})	0.46
Total volume of the two culture columns (L)	94.0
Actual filled volume of the two culture columns, V_A (L)	84.0
Density of the produced algae (DA, 10^9 cells L^{-1})	2.40
Algae production (P, 10^9 cells day^{-1})	92.74
Algae production (P, g C day^{-1})	4.40
The algae requirement for 1 L of copepods (2500 ind L^{-1}) per day (CA, g C L^{-1} day^{-1})	0.0089
The volume of copepod culture that can be sustained by feeding on the current algae production (V_C, L)	495
Specific egg production (SEP, egg $female^{-1}$ ·day^{-1})	30.70
Potential number of eggs produced with the current algal production (EP, $\times 10^6$eggs day^{-1})	17.11

490

491

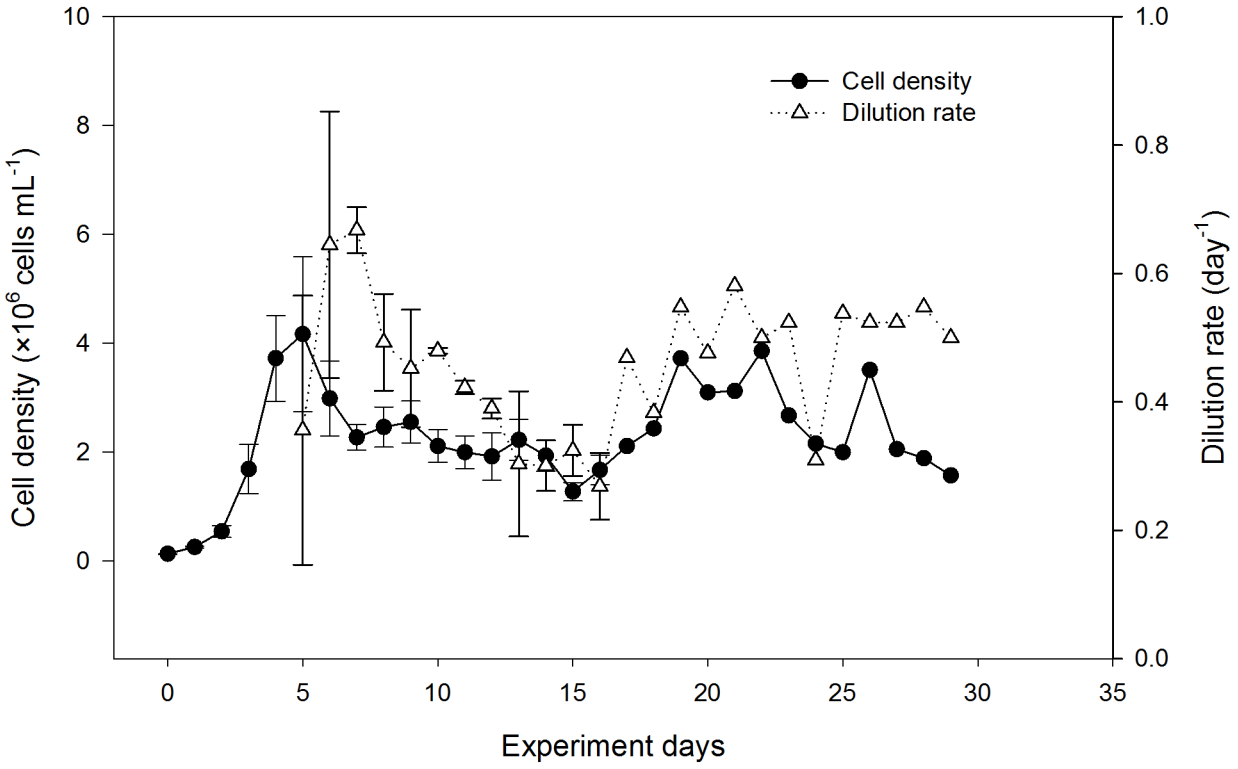
492

493 **Figure 1**



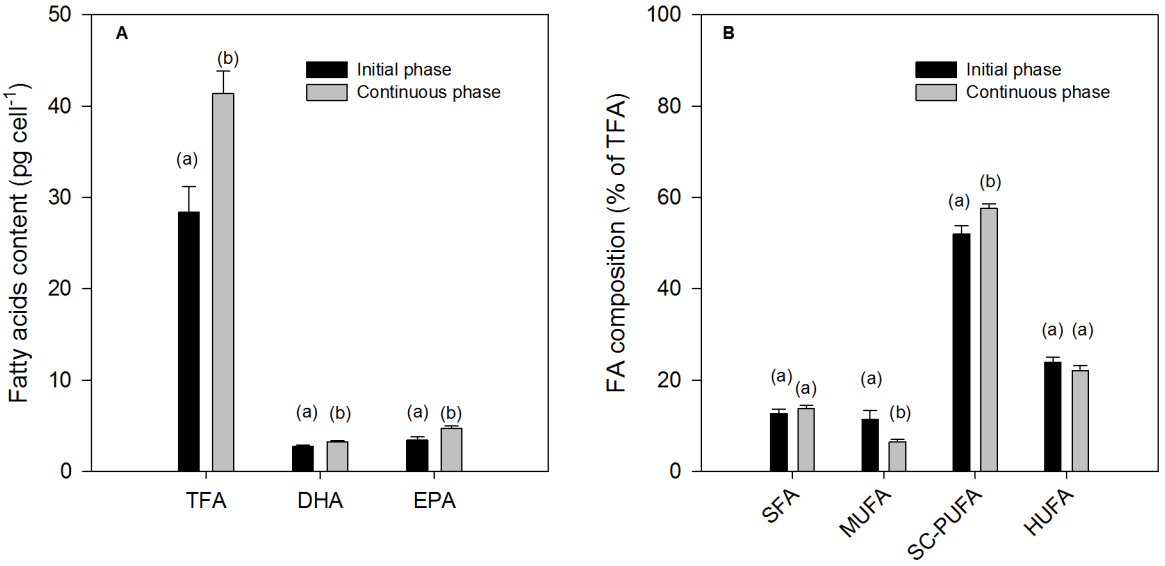
494

Figure 2


Cell density
Dilution rate
Cell density (×10^6 cells mL^-1)
Dilution rate (day^-1)
Experiment days
0
2
4
6
8
10
0.0
0.2
0.4
0.6
0.8
1.0
0
5
10
15
20
25
30
35

498 **Figure 3**

499



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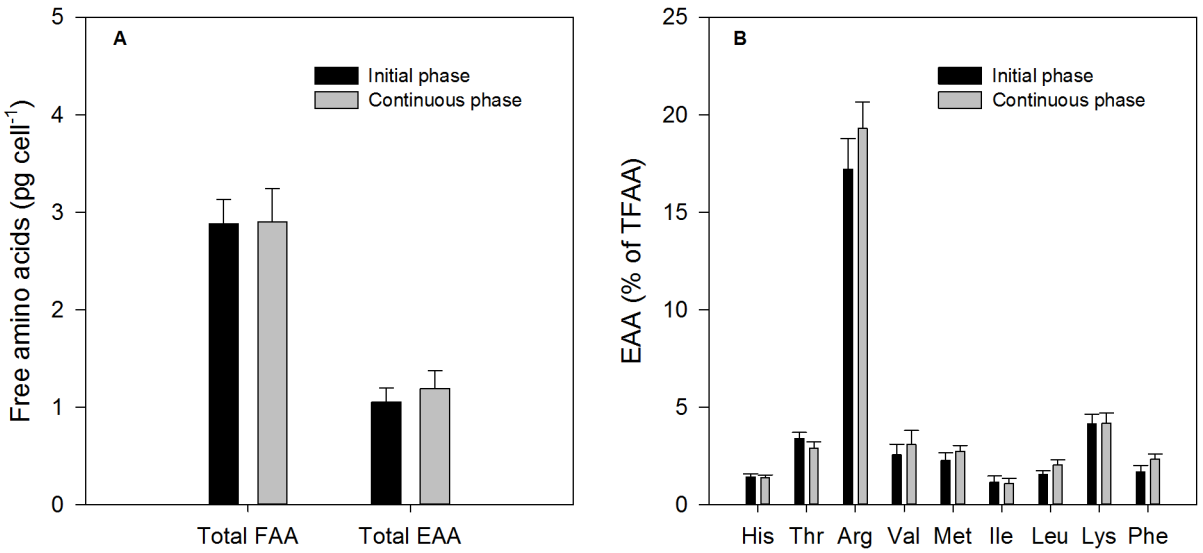
502 **Figure 4**



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