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Published in:
Aquaculture Research

DOI:
10.1111/are.13868

Publication date:
2018

Document Version
Peer reviewed version

Citation for published version (APA):

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Download date: 22. Apr. 2021
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*

Keywords: Amino acids; Dilution rate; Fatty acids; Growth rate; Live feed; Upscaling
Abstract

A dual column photobioreactor (PBR) (2 × 47 L) with mixed CO₂/air bubbling was tested for cultivation of the microalga *Rhodomonas salina* as food for live feed copepods. In the continuous growth phase, the cell density was relatively stable at 2.40 ± 0.13 × 10⁶ cells mL⁻¹ at an average dilution rate of 0.46 ± 0.02 day⁻¹ throughout the 30-day experiment. The produced algae had a high content of both total fatty acids (TFA) and free amino acids (FAA). Especially, the harvested algae contained a high proportion of poly-unsaturated fatty acids (PUFA) that made up 80 % of the TFA and of essential amino acids (35% of all FAA), implicating desirable components as feed for copepods. The current PBR was sufficient to feed a culture of the calanoid copepod *Acartia tonsa* at a density of 2,500 adult L⁻¹ in ca. 500 L culture with a daily yield of approximately 17 × 10⁶ eggs. To be able to sustain the integrated copepods production, the suggested volume of the algae cultures should be ca. 20% of the copepod culture volume.
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 docosahexaenoic 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 (CO₂), 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 10⁷ 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.

57

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

The microalgae were kept in suspension by large bubbles from the air system, and atmospheric airflow was adjusted by a flow meter. Using another flow meter, a lower atmospheric flow was mixed with pure CO₂ from a pressure flask. The mixture of CO₂ and atmospheric airflow was regulated automatically using a feedback system, where opening or closing of solenoid valves were controlled by a pH probe in the column. The mixture of pure CO₂ and atmospheric air was passed through a membrane to increase formation of microbubbles to improve the mass transfer rate of CO₂ for algae growth.

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

Light was supplied by 4 pairs pr. PBR of LCD lamps (Aussmak ELT-S0335 T9/35W/2700K) from 4 sides and was adjusted to provide light intensities at 3 different levels: low, medium and high, corresponding to 393 ± 44, 608 ± 80 and 981 ± 133 µmol·m⁻²·s⁻¹ Photosynthetic Active Radiation (PAR), respectively.

The microalgae were harvested from an overflow at top of each culture column of the PBR. The harvested algae were stored in a tank and used for copepod cultures in 4 × 350 L tanks at Roskilde 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
in the PBR was brought into the continuous phase, where steady state conditions were obtained by adding UV-treated seawater by two pumps (ProMinent DULCOflex) at bottom of each of the two PBRs (Fig. 1) to dilute the algal density. The B1 medium was added at a rate of 1.2-4 mL L⁻¹ of newly added seawater.

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

The main atmospheric airflow was set at ca. 4 L min⁻¹ at the first 2-3 days of the experiment when the algal cell density was low and increased to 5-6 L min⁻¹ when the algal cell density was high from day 2-3 onward. The mixture of pure CO₂ (V/V = 2-5% of CO₂ in mixture) and atmospheric air (V= 2-2.5 L) was added to supply CO₂ and to control the pH at 8.1 ± 0.1.

Analytical procedures and measurements

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

The cell density and total volume of the harvested algae in each harvest tank (collected from the culture columns) were also measured daily. The cell density of *R. salina* was converted into carbon weight by assuming that one algal cell equals 47.4 pg C (Berggreen et al., 1988).
For analysis of fatty acids (FA) and free amino acids (FAA) in the microalgae, samples of about $10^6$ algal cells were collected during both the initial (14 samples; $n=14$) and the continuous growth phase (18 samples; $n=18$). The samples were filtered through a 25-mm diameter GF/C glass fiber filter (Whatman) and stored in Pyrex glass vials for FA analysis, or in 1.5 mL HPLC glass vials for FAA analysis, at -80 °C for later analysis FA and FAA compositions.

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

For analysis of FAA, each vial containing a filter sample was added 1 mL Milli-Q water, heated to 95°C for 15 min and filtered through 0.2 µm cellulose nitrate syringe filters (VWR International, USA) after cooling. FAA concentrations were determined by HPLC and fluorescence detection after derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC). AQC was purchased from Waters as an AccQFlour kit (www.waters.com). The analysis was conducted as recommended by Waters, except for adjustment of the solvent gradient, and the AA derivatives were analyzed on a Waters HPLC system (Alliance 2695 solvent module, 2475 fluorescence detector and a 3.9 × 150 mm AccQTag column). Chromatogram peaks were identified from retention times and integrated against an external amino acid standard mixture (AA-S-18 amino acid mixture; www.sigma-aldrich.com).
The daily production of *R. salina* served as food for the calanoid copepod *Acartia tonsa* culture at Roskilde University, Denmark. The estimated production rate of the PBR for both culture columns (P, g C day⁻¹) was calculated as equation (Eq. 1):

\[ P = D \times V_A \times D_A \times W_A \]  
(Eq. 1)

In which

D: dilution rate (day⁻¹)

\[ V_A \]: filled volume (L) of the two culture columns of the photobioreactor

\[ D_A \]: density of the algae harvested, assuming that the density of algae harvested equals the density inside the culture columns (cells·L⁻¹)

\[ W_A \]: carbon weight of algae cells = 47.4×10⁻¹² g C cell⁻¹ (Berggreen et al. 1988)

The algae requirement (in carbon weight) for 1 L of copepods per day (\( C_A \), g C L⁻¹ day⁻¹) was calculated as equation (Eq. 2)

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

In which

\[ C_A \]: algae requirement (carbon units) for 1 L of copepods.

\[ D_C \]: density of copepods (ind L⁻¹), we used a density of 2,500 adult copepods·L⁻¹ which is reported to be the optimal density of *A. tonsa* for maximal egg yield in culture tanks (Drillet et al. 2015)
WF: The average carbon weight of female copepods = 3,193 ng C ind⁻¹ = 3,193×10⁻⁹ g C ind⁻¹ (Drillet et al. 2015)

WM: The average carbon weight of male copepods = 2,282 ng C ind⁻¹ = 2,282×10⁻⁹ g C ind⁻¹ (Drillet et al. 2015)

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

IRMax: 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 (VC, L) was estimated as equation (Eq. 3):

\[ V_C = \frac{P}{C_A} \quad \text{(Eq. 3)} \]

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)

\[ SEP = \frac{W_F \times IR_{Max}}{3 \times W_E} \quad \text{(Eq. 4)} \]

Whereas WE: 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):

\[ EP = SEP \times D_C \times F \times V_C \quad \text{(Eq. 5)} \]
Data on fatty acid and amino acid content were subjected to one-way ANOVA. 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 SYSTAT v. 13 with \( \alpha = 0.05 \). ANOVA was performed on data from the initial phase and the continuous phase separately, and as the ANOVA showed no significant differences in data within each phase (\( p > 0.05 \)), data on content of the various fatty acids and amino acids were pooled within each phase. Subsequently, differences between content in the two phases were tested for each compound by one-way ANOVA, using SYSTAT v. 13 with \( \alpha = 0.05 \).

**Results**

**Growth of *R. salina* in the photobioreactor**

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

**Fatty acids**

Data from each growth phase showed a similar content of fatty acids within the initial and continuous phase respectively (one-way ANOVA, \( p > 0.05 \)). The fatty acid data were therefore 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 \(\times 47\) 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 \(\frac{1}{4}\), 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 &
Suraishkumar, 2016) and in *Nannochloropsis oceanica* under nitrogen replete conditions as in the present study (Xiao et al., 2015). Another observation from the continuous phase in our cultures was an increased presence of desirable fatty acids, SC-PUFA (mainly C18:3 n-3) when the algae grew in the continuous phase. In contrast, algae harvested in the continuous phase contained less MUFA as compared to the algae in the initial phase. An enhanced content of SC-PUFA in microalgae has previously been observed under culture conditions utilizing nitrate as the nitrogen source, eliminating the pH fluctuations caused by ammonium use, and non-limiting concentrations of phosphate, as many of the SC-PUFAs are found as phospholipids (Yongmanitchai & Ward, 1991).

In aquaculture, a DHA/EPA ratio of 2 is proposed as an optimum criterion for live food organisms, leading to the preferred ratio in the next link in the aquaculture food chain, the fish larvae ((Reitan et al., 1994)). The DHA/EPA ratio obtained in the present study (DHA/EPA ratio of 0.7 to 0.8) was similar to previous studies on the same algal species (DHA/EPA ratio of 0.5 to 0.9; (Dunstan et al., 2005; Mansour et al., 2005; Vu et al., 2016; Guevara et al., 2016)), indicating that the preferred ratio of 2 was not reached. However, although both historical data and our data show that the DHA/EPA ratio in the cultured algae is below the optimum ratio, the DHA/EPA ratio will be closer to 2 by trophic upgrade when *Rhodomonas* is fed copepods that again are fed to the fish larvae.

Thus, Drillet et al. (2006) observed a DHA/EPA ratio of about 1.3 in fish larvae when fed *Acartia tonsa* based on *R. salina* from our laboratory strains.

Regarding the general composition of FA in *R. salina* cultured in the PBR, the composition agreed with previous results of especially PUFA (made up ca. 80% of TFA in this study), including SC-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. 17.11×10⁶ eggs per culture tank. Therefore, to be able to feed the copepods optimally, the required volume of algal culture at the cell density
achieved in this study is ca. 20% of the copepod culture. It should be noted that the estimation of algae feeding to the copepods was based on the grazing rate of adult copepods. For younger life stages, e.g., nauplii and copepodite stages, the demand for algae is substantially smaller.

Recommendations

The microalga *R. salina* was successfully cultivated in the current bubble-column PBR and produced algal biomass with a high biochemical quality and that can immediately be fed out to copepod cultures. Based on this, it is recommended that *R. salina* should be cultivated with the addition of pure CO2 in a mixture with atmospheric air (V/V=2-5% of CO2 in the mixture) to maintain the pH at 8.1. The harvested algae in the continuous growth phase obtained a high TFA content with a relative high abundance of PUFA (including SC-PUFA and HUFA) as well as a high content of FAA, which is of crucial importance for enhancing the egg production, the egg hatching success, and the somatic growth of the cultured copepods. For copepod production in aquaculture facilities, the estimated volume of algae cultures should be about 20% v/v of copepod culture.

Acknowledgments

This work was funded by the Danish National Strategic Research Council IMPAQ-IMProvement of AQuaculture high quality fish fry production grant (Grant no. 10-093522) to Benni Winding Hansen and the Danish National Advanced Technology Foundation COMA-COpepod egg Mass production in Aquaculture grant (Grant no. 67-2013-1) to Benni Winding Hansen and Søren Laurentius Nielsen. We would like to thank Anne B. Faarborg and Rikke Guttesen (Roskilde University, Denmark) for laboratory assistance.


Jose S, Suraishkumar GK (2016) High carbon (CO2) supply leads to elevated intracellular acetyl CoA levels and increased lipid accumulation in *Chlorella vulgaris*. *Algal Res.*, 19, 307-315.


Captions for figures

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

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

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

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

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

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

Table 3 Estimated ability to support the copepod *Acartia tonsa* production (2500 ind L$^{-1}$) by *Rhodomonas salina* algae produced in the current bubble column photobioreactors
<table>
<thead>
<tr>
<th>Variables</th>
<th>Initial phase</th>
<th>Continuous phase</th>
<th>df1</th>
<th>df2</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0</td>
<td>1.74±0.33a</td>
<td>1.65±0.21a</td>
<td>1</td>
<td>31</td>
<td>0.065</td>
<td>0.065</td>
<td>0.0585</td>
<td>0.81</td>
</tr>
<tr>
<td>C16:0</td>
<td>9.56±0.91a</td>
<td>11.42±0.57b</td>
<td>1</td>
<td>31</td>
<td>3.011</td>
<td>3.011</td>
<td>10.655</td>
<td>0.003</td>
</tr>
<tr>
<td>C18:0</td>
<td>1.18±0.19a</td>
<td>0.56±0.07b</td>
<td>1</td>
<td>31</td>
<td>3.011</td>
<td>3.011</td>
<td>10.655</td>
<td>0.003</td>
</tr>
<tr>
<td>SFA</td>
<td>12.68±1.02a</td>
<td>13.78±0.65a</td>
<td>1</td>
<td>31</td>
<td>9.456</td>
<td>9.456</td>
<td>0.891</td>
<td>0.353</td>
</tr>
<tr>
<td>C16:1</td>
<td>0.88±0.23a</td>
<td>1.23±0.24a</td>
<td>1</td>
<td>31</td>
<td>0.967</td>
<td>0.967</td>
<td>1.032</td>
<td>0.318</td>
</tr>
<tr>
<td>C18:1</td>
<td>3.45±0.57a</td>
<td>3.59±0.37a</td>
<td>1</td>
<td>31</td>
<td>0.159</td>
<td>0.159</td>
<td>0.0473</td>
<td>0.829</td>
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<tr>
<td>C22:1</td>
<td>6.71±2.48a</td>
<td>1.26±3.03b</td>
<td>1</td>
<td>31</td>
<td>234.318</td>
<td>234.318</td>
<td>6.137</td>
<td>0.019</td>
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<tr>
<td>MUFA</td>
<td>11.37±1.97a</td>
<td>6.49±0.54b</td>
<td>1</td>
<td>31</td>
<td>187.522</td>
<td>187.522</td>
<td>7.056</td>
<td>0.013</td>
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<tr>
<td>C18:2</td>
<td>10.48±1.55a</td>
<td>15.25±1.39b</td>
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<td>31</td>
<td>179.275</td>
<td>179.275</td>
<td>5.244</td>
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<tr>
<td>C18:3 n-6</td>
<td>1.80±0.26a</td>
<td>2.15±0.08b</td>
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<td>31</td>
<td>0.964</td>
<td>0.964</td>
<td>2.042</td>
<td>0.163</td>
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<tr>
<td>C18:3 n-3</td>
<td>18.26±0.93a</td>
<td>22.46±0.84b</td>
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<td>139.059</td>
<td>11.203</td>
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<td>C18:4</td>
<td>21.43±1.07a</td>
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<td>106.342</td>
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<td>SC-PUFA</td>
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<tr>
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<td>1.06±0.18a</td>
<td>2.00±0.14b</td>
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<td>6.905</td>
<td>17.639</td>
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<tr>
<td>C20:5 n-3 (EPA)</td>
<td>12.47±0.57a</td>
<td>11.86±0.73a</td>
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<td>2.936</td>
<td>2.936</td>
<td>0.394</td>
<td>0.535</td>
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<tr>
<td>C22:6 n-3 (DHA)</td>
<td>10.41±0.70a</td>
<td>8.23±0.39b</td>
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<td>37.347</td>
<td>37.347</td>
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<tr>
<td>HUFA</td>
<td>23.95±1.06a</td>
<td>22.11±1.08a</td>
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<td>26.684</td>
<td>26.684</td>
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<td>DHA/EPA</td>
<td>0.84±0.04a</td>
<td>0.71±0.02b</td>
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<td>0.138</td>
<td>10.136</td>
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<td>TFA</td>
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<td>41.37±2.46b</td>
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<td>1328.75</td>
<td>1328.75</td>
<td>12.118</td>
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</table>

Note: SFA: saturated fatty acids, MUFA: mono unsaturated fatty acids, SC-PUFA: short chain-poly unsaturated fatty acids, HUFA: highly unsaturated fatty acids. Units of TFA: pg cell⁻¹, specific FA/FA group: % of TFA. Values for limited irradiance = mean ± SEs of TFA, specific FA or FA groups at different growth phases (with n = 18 replicates for initial phase and n = 14 replicates for the continuous phase). Different letters in the same row denote the significant differences (Tukey test) in the TFA, or the same specific FA or FA groups between the different growth phases.
<table>
<thead>
<tr>
<th>Variables</th>
<th>df1</th>
<th>df2</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>p</th>
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<td>0.0043</td>
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<td>Thr</td>
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<td>1.92</td>
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<td>0.283</td>
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<td>Arg</td>
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<td>31</td>
<td>34.195</td>
<td>34.195</td>
<td>1.017</td>
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<td>Val</td>
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<td>2.257</td>
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<td>Met</td>
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<td>0.0421</td>
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<td>Leu</td>
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<td>Estimated parameters</td>
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<tr>
<td>Dilution rate, D (day$^{-1}$)</td>
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<tr>
<td>Total volume of the two culture columns (L)</td>
<td>94.0</td>
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<td>Actual filled volume of the two culture columns, $V_A$ (L)</td>
<td>84.0</td>
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<td>Density of the produced algae (DA, 10$^9$ cells L$^{-1}$)</td>
<td>2.40</td>
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<td>Algae production (P, 10$^9$ cells day$^{-1}$)</td>
<td>92.74</td>
<td></td>
<td></td>
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<tr>
<td>Algae production (P, g C day$^{-1}$)</td>
<td>4.40</td>
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<td>The algae requirement for 1 L of copepods (2500 ind L$^{-1}$) per day (CA, g C L$^{-1}$ day$^{-1}$)</td>
<td>0.0089</td>
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<tr>
<td>The volume of copepod culture that can be sustained by feeding on the current algae production ($V_C$, L)</td>
<td>495</td>
<td></td>
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</tr>
<tr>
<td>Specific egg production (SEP, egg female$^{-1}$·day$^{-1}$)</td>
<td>30.70</td>
<td></td>
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</tr>
<tr>
<td>Potential number of eggs produced with the current algal production (EP, $\times 10^6$ eggs day$^{-1}$)</td>
<td>17.11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1
Figure 2

[Graph showing cell density and dilution rate over experiment days]
Figure 3
Figure 4

(A) Free amino acids (μg cell⁻¹) for Total FAA and Total EAA.

(B) EAA (% of TFAA) for individual amino acids: His, Thr, Arg, Val, Met, Ile, Leu, Lys, Phe.

- Initial phase represented by black bars.
- Continuous phase represented by gray bars.