**The effect of cell density on biomass and fatty acid productivity during cultivation of *Rhodomonas salina* in a tubular photobioreactor**

Christina Thoisen1, Jakob Skov Pedersen2, Lars Jørgensen2, Anker Kuehn2, Benni Winding Hansen1, Søren Laurentius Nielsen1\*

1Roskilde University, Department of Science and Environment, Universitetsvej 1, 4000 Roskilde, Denmark.

2Danish Technological Institute, AgroTech, Gregersensvej 1, 2630 Taastrup, Denmark.

\*Corresponding author: nielsen@ruc.dk

Running title: Upscaling of *Rhodomonas salina* cultivation.

**Abstract**

The microalga *Rhodomonas salina* is widely used in aquaculture. There is a need for optimization of the growth of the microalgae and its content of essential fatty acids. Here, the fatty acid profile of *Rhodomonas* in relation to cell density during cultivation in a tubular PBR is investigated. It is expected that cell density is an important factor in controlling productivity and fatty acid content of the microalgae because cell density is important in determining light availability due to the self-shading of the algae. The carbon productivity as a function of cell density is described by a saturation curve. The carbon productivity and the productivity of total fatty acids is lowest at the lowest cell density, and independent of cell density at higher cell densities. The relative contribution of the two poly-unsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) increases with increasing cell density and saturates at 1 x 106 cells ml-1. We conclude that large-scale production of *Rhodomonas* in this tubular PBR should take place at cell densities of 1 x 106 cells ml-1, while there are indications for increasing difficulties in maintaining steady state production in this PBR at higher cell densities.

**Keywords**

Microalgae; Nutritional value; Photobioreactor; PUFA; Production conditions

**Introduction**

The marine cryptophyte *Rhodomonas salina* (Wislouch) D.R.A.Hill & R.Wetherbee is an important feed microalga in aquaculture. This applies to the production of shellfish ([Brown et al., 1998](#_ENREF_8); [Gagne et al., 2010](#_ENREF_17); [Fernandez-Reiriz et al., 2015](#_ENREF_16)), other types of invertebrates, such as sea urchins and sea cucumbers ([Wolcott & Messing, 2005](#_ENREF_40); [Yamamoto et al., 2015](#_ENREF_41); [Castilla-Gavilan et al., 2018](#_ENREF_9); [Sonnenholzner-Varas et al., 2018](#_ENREF_34)), as well as to the production of live feed such as *Artemia*, rotifers and copepods for the use in marine fish larviculture ([Seixas et al., 2009](#_ENREF_32); [Guevara et al., 2011](#_ENREF_20); [Jepsen et al., 2017](#_ENREF_23)). *Rhodomonas* is well suited for these purposes due to its high content of essential fatty acids and amino acids ([Huerlimann et al., 2010](#_ENREF_22); [Guevara et al., 2016](#_ENREF_19); [Vu et al., 2016](#_ENREF_36)), especially its content of the essential polyunsaturated fatty acids docosahexaenoic acid (DHA; C22:6 n-3), eicosapentaenoic acid (EPA; C20:5 n-3) and arachidonic acid (ARA; C20:4 n-6). The content of these fatty acids relative to each other in this microalgae is close to optimal for use in aquaculture ([Dalsgaard et al., 2003](#_ENREF_11); [Lund et al., 2008](#_ENREF_26); [Arndt & Sommer, 2014](#_ENREF_6)).

*Rhodomonas* is, however, considered relatively difficult to cultivate, especially in large-scale facilities ([Arndt & Sommer, 2014](#_ENREF_6); [Thoisen et al., 2018](#_ENREF_35); [Vu et al., 2019](#_ENREF_37)). In aquaculture, it is usually cultivated in batch culture in large plastic bags. There are very few examples in the literature of cultivation of *Rhodomonas* in large photobioreactors ([Vu et al., 2019](#_ENREF_37)), despite the advantages this form of cultivation would give in term of continuous cultivation, potentials for automation, high production rates and protection against contamination ([Eriksen et al., 1998](#_ENREF_15); [Mirón et al., 1999](#_ENREF_27); [Wang et al., 2012](#_ENREF_38)).

Because of this, there is a need for the development of production procedures for *Rhodomonas* in photobioreactors, including the need for establishment of cultivation conditions, optimizing not only growth of the microalgae, but also their content of essential fatty acids and amino acids. It is known from previous studies that the biochemical profile of *Rhodomonas* depends on its growth conditions. Nitrogen stress not only lowers the biomass productivity, but also yields a high lipid content, while decreasing the fraction of poly-unsaturated fatty acids, PUFAs ([Vu et al., 2016](#_ENREF_36)). High irradiances, on the other hand, increase both the biomass productivity and the content of PUFAs, but decrease the content of free amino acids ([Vu et al., 2016](#_ENREF_36)). Effects of cell density and dilution rate are much less well studied, not least because studies of the production of *Rhodomonas* in large-scale facilities are scarce. It can, however, be expected that cell density and dilution rate also will affect the biochemical profile of *Rhodomonas*, albeit in an indirect way through the effect of dilution rate on cell density and of cell density on light availability in the PBR. Available light during cultivation depends not only on external irradiance, but also on internal self-shading among the microalgal cells, which in turn depends on cell density in the photobioreactor. Previous work indicate that self-shading is more important in controlling light availability in a PBR than the external irradiance ([Jung et al., 2014](#_ENREF_24); [Vu et al., 2019](#_ENREF_37)). During steady-state growth, the dilution rate balances specific growth rate, which is an integrated expression of the growth conditions experienced by the microalgae. Generally, specific growth rates are higher at lower densities due to lower self-shading ([Chrismadha & Borowitzka, 1994](#_ENREF_10); [de Vicose et al., 2012](#_ENREF_12)), which could lead to a higher biomass productivity at low densities. However, given the low density of the culture, a number of trade-offs must be considered. One is that the productivity is the product of cell density and specific growth rate (dilution rate), another is that the harvested volume will contain a lower biomass in a larger volume of water, incurring higher costs in terms of wasted inorganic nutrients and higher harvesting costs ([Abdelaziz et al., 2013](#_ENREF_1); [Ahmad et al., 2014](#_ENREF_5); [Abdo et al., 2016](#_ENREF_2); [Abo et al., 2019](#_ENREF_3)).

Here, it is hypothesized that the biochemical profile of *Rhodomonas* during large-scale cultivation depends not only on nutrient availability, light and temperature, as previously described ([Huerlimann et al., 2010](#_ENREF_22); [Guevara et al., 2016](#_ENREF_19); [Vu et al., 2016](#_ENREF_36)), but also indirectly on cell density and hence on dilution rate during cultivation. Dilution rate and available light together control cell density, which in turn control light availability through microalgal self-shading. The purpose of this study is to establish relationships between cell density during production in a tubular photobioreactor and the productivity of *Rhodomonas*, aiming at identifying the optimal cultivation density both in relation to biomass production as well as to the productivity of the essential PUFAs, DHA, EPA and ARA.

**Materials and methods**

***Photobioreactor.***The cultivation system consisted of a horizontal tubular PBR (forming a closed system) with a total volume of 250 L, constructed from 2x12 transparent tubes (ø: 60 mm inner/64 mm outer, length 2000 mm) that were placed at a horizontal angle of 2,08 degrees (Fig. 1). The tubes were acrylic glass (polymethyl methacrylate, PMMA), connected by opaque bends of polyvinyl chloride (PVC-U). Filtered air (0.3 µm polypropylene DOE filter; van Borselen B.V.) with CO2 addition was supplied into the lowermost tube by an air pump (ALITA, model AL-60SB). This provided an upwards flow of water through the connected tubes into a 77 L dark collection tank (included in the total volume of 250 L), from where the culture entered the lowermost tube again. The airflow was set at 60 L min-1, controlled by the pump, provide at a pressure of 15 kPa. This airflow generated a flow rate of approximately 10 L min-1 of liquid culture, but it must be emphasized that this flow rate is far from constant in a PBR operating with a gas-filled headspace, where the flow is inherently turbulent. The CO2 concentration in the air was continuously adjusted to maintain a constant pH in the growth medium of 8.2 ± 0.3 (see below). The CO2 addition to the air was controlled by feedback from the inserted pH-electrode to maintain this pH. The PBR was operated with a gas-filled headspace in the tubes, taking up approximately 80 – 100 L of the total volume of 250 L. The duration for obtaining a homogenous culture in the PBR after addition of a culture was 2 h and 13 min. The PBR was located in a controlled climate room at 19 °C and sensors were inserted directly into the PBR in the lowermost tube for measurements of pH, temperature and O2 saturation. The pH and O2 sensors (Mettler Toledo; Easy Sense pH 32 sensor and Easy Sense O2 21 sensor) were connected to a transmitter (Mettler Toledo M 200Easy), connected to a data logger (CR1000, Campbell Scientific). The data logger stored one measurement per minute, which was used to calculate the average pH, temperature, dilution rate and light intensity. The light sources (6x SENMATIC, FL300 grow white LED Fixture, SENMATIC, Søndersø, Denmark) were placed 1 meter in front of the PBR and the intensity was adjusted via the Fionia Lighting Interface Software (SENMATIC, Søndersø, Denmark). A light measuring device (Li-Cor LI-193 Spherical Quantum Sensor), measuring the photosynthetic photon flux fluence rate, was positioned centrally on the sixth lowest tube. Nutrients (f/2 growth medium) and harvested culture were kept in two separate 250 L tanks. Two separate dosing pumps (Grundfos ALLDOS, DDC) controlled inflow of nutrients to the PBR and outflow of culture from the PBR. Nutrients and artificial seawater (ASW) were filtered (1 µm followed by 0.3 µm, Borospun filters) prior to entering the PBR tank.

***Artificial seawater.***
The commercial salt Red Sea Salt (Red Sea Ltd, Houston, USA) was mixed with ion-exchanged water in a 600 L tank to a salinity of approximately 30 and pumped through the filters of the PBR with a water pump (Gardena). When filling the PBR with ASW, a flow meter (Gardena) was connected to the inlet to the filters to measure the total volume of ASW entering the PBR (150 to 170 L).

***Microalga and inoculation of the PBR.***The cryptophyte *Rhodomonas salina* (strain K-1487, originally obtained from SCCAP; Scandinavian Culture Collection of Algae & Protozoa) was cultivated in f/2 growth medium ([Guillard & Ryther, 1962](#_ENREF_21)) without cobalt as described in [Thoisen et al. (2018)](#_ENREF_35). Cultivation was carried out with addition of growth medium in triple concentration to avoid nutrient limitation. Inoculum cultures for the PBR were cultivated in 5 L glass flasks with aeration (0.2 µm filtered) at a light intensity of 100 µmol m-2 s-1 and a temperature of 19 ˚C in the same climate room as the PBR.

 Equipment and artificial seawater (ASW) for the inoculum cultures were autoclaved prior to use. Likewise, stock solutions and vitamins for the f/2 growth medium for the inoculum cultures and PBR culture were autoclaved prior to use.

To start production, the PBR was filled with filtered ASW and amounts of nutrients corresponding to the total volume of ASW were added directly into the PBR tank. Following this, 25 to 35 L of dense inoculum culture was poured into the PBR tank. Immediately after inoculation, the irradiance was set to 100 µmol m-2 s-1 to avoid light inhibition of the microalgal cells. After 2 - 3 days, varying between runs, the irradiance was set to the desired value when the culture in the PBR reached a density of approximately 5 x 105 cells ml-1. Dilution of the PBR (growth medium input and harvest output) was activated 2 - 3 days after inoculation to avoid nutrient limitation and was regulated as needed to maintain the culture at a steady density.

***Experimental conditions and sampling.***Nominal cell densities, the target cell densities we attempted to achieve during the PBR runs, of 0.5 x 106, 1 x 106 , 1.5 x 106 and 2 x 106 cells ml-1 were cultivated in the PBR at a temperature of 19.0 ± 0.1 ˚C, and pH 8.2 ± 0.3. Two experimental runs were performed at each nominal cell density. Actually achieved average cell densities during the cultivation runs are shown in Table 1. These were measured as described below. Irradiance was set to 100, 200 and 175 µmol m-2 s-1 for nominal cell densities of 0.5 x 106, 1 x 106 and 1.5 x 106 cells ml-1, respectively. For a nominal cell density of 2 x 106 cells ml-1, the irradiance for the first and second run was 200 and 250 µmol m-2 s-1, respectively. The average irradiance inside the PBR was calculated according to



where Iavg is the average irradiance in the PBR, I0 is the incident irradiance at the PBR surface, α is an empirically determined proportionality coefficient (found here to be 3.62 x 10-7 ml cell-1 cm-1), ρ is the culture density (cells ml-1) and L is the length of the optical path (6 cm) ([Grima et al., 1996](#_ENREF_18); [Molina Grima et al., 1996](#_ENREF_28); [Acién Fernández et al., 1997](#_ENREF_4)).

The two PBR runs with nominal cell densities of 1.5 x 106 cells ml-1 were used as a basis for the remaining experiments. During these runs, the cell density was kept constant for 8 – 11 days to ensure the stable operation of the PBR. After the conclusion of these runs, the cell density in the PBR was either up- or down-regulated to the desired density, nominally 0.5, 1 or 2 x 106 cells ml-1. The PBR was run for 3 – 5 days during these experiments.

The cell density was determined from replicate samples (n = 3) taken from the PBR tank. Prior to sampling, approximately 500 ml of the PBR culture was allowed to pass through the tap at the bottom of the PBR tank to remove sedimented cells. The optical density (OD) was measured and the density calculated using a constant obtained from a linear regression between OD750 nm (Eppendorf BioSpectrometer) and cell enumeration on a Coulter Counter model 4E (Beckman) using the same methodology as described in [Nielsen and Hansen (2019)](#_ENREF_29), from where the proportionality factor is also derived:



Carbon content of the cells and carbon productivity is 47.4 pg cell-1, based on data in [Berggreen et al. (1988)](#_ENREF_7).

Fatty acid analyses were performed on the same samples. The volume removed each day was noted in order to calculate the remaining total volume in the PBR throughout the experimental run.

The average dilution rate (d-1) for an experimental run was calculated from the total water volume of the PBR and the exchange rate (nutrient input / harvest output, L d-1):



***Fatty acids*.**Samples for comparing fatty acid composition between experimental conditions were filtered through 0.2 µm filters (WhatmanTM GF/C), which were placed in pre-combusted Pyrex glass vials and stored at -80 ˚C until further analysis. Extraction was done by a chloroform : methanol mixture followed by a trans esterification of the lipids by acetyl chloride in methanol, and the fatty acid methyl esters were analyzed by gas chromatography mass spectrometry (GCMS) as described in [Drillet et al. (2006)](#_ENREF_13) but with minor adjustments. Each sample was added 2 ml chloroform, 1 ml methanol and 20 µL of the internal standard C23-methylester at a concentration of 1000 µg ml-1, and extracted at -20 ˚C for 24 h. The liquid from each sample was then transferred to a clean GC vial and the chloroform: methanol solvent was evaporated by placing the GC vials in an aluminum block at 60°C and applying a flow of nitrogen into the opening of the GC vials. Then, 1 ml of a reagent solution composed of toluene, methanol and acetyl chloride (66 ml : 85 ml : 15 ml) was added to each GC vial. The vials were capped and heated for 2 h at 95°C in an aluminum block. Hereafter, the GC vials were uncapped and added 500 µL of 5% (w) NaHCO3 that had been deoxygenated by bubbling with nitrogen for 2 h. The two phases present in the GC vial were mixed and separated using a glass Pasteur pipette (250 mm). The upper phase was transferred to a new GC vial. The original GC vial was added 500 µL heptane, the two phases were mixed and separated, and the upper phase was transferred to the new GC vial containing the previous upper phase. The step with heptane was then repeated. The content in the new GC vials was evaporated by placing the GC vials in an aluminum block at 60°C and applying a flow of nitrogen into the opening of the GC vials. The GC vials were removed from the aluminum block and added 0.5 ml chloroform. The GC vials were capped and stored at -80°C until analysis on GCMS. The content of fatty acids was integrated and calculated in the program MSD ChemStation (E.02.02.1431, Agilent Technologies, Inc.).

***Statistical methods.***

 Changes in cell density over time during steady state growth and the relationship between irradiance and carbon productivity were analyzed using least squares regression analysis ([Sokal & Rohlf, 1995](#_ENREF_33)), including evaluation of whether the slope of the resulting line was significantly different from zero ([Sokal & Rohlf, 1995](#_ENREF_33)).

 The relationships between cell density (x) and the productivity of carbon and fatty acids (y) were analyzed by fitting a hyperbolic function and evaluating the resulting coefficients of determination (R2 – values). The modified hyperbola III from the software package SIGMAPLOT 14.0 was used, where *a* = ymax; *b* = ymax – ymin, ymax and ymin being the maximum and minimum values of the dependent variable, y, whether carbon productivity, productivity of total fatty acids or productivity of individual fatty acids, respectively. The two parameters *c* and *d* are dimensionless parameters determining the shape of the saturation curve:



 Fatty acid contents were subjected to one-way ANOVA with cell density as the fixed factor. Tukey tests were subsequently used to compare individual means across significantly different levels of cell density where relevant. Data were tested for homogeneity of variance (Cochran’s test) and normal distribution (Kolmogorov-Smirnoff goodness-of-fit test) before being analyzed by ANOVA ([Sokal & Rohlf, 1995](#_ENREF_33)). All tests were carried out using the software package SYSTAT v. 13 with α = 0.05.

**Results**

The nominal cell densities we attempted to achieve during the total of eight runs of the PBR in this study were 0.5 x 106, 1.0 x 106 1.5 x 106 and 2.0 x 106 cells ml-1 respectively, but obviously obtaining exactly these numbers of cells were not possible. The actual average cell densities achieved during steady-state growth were 467,000 – 500,000 cells ml-1, 990,000 – 1,044,000 cells ml-1, 1,044,000 – 1,455,000 cells ml-1 and 1,840,000 – 2,385,000 cells ml-1, respectively (Table 1). Possible trends in changes in cell densities over time were analyzed using least squares linear regression (Fig. 2). None of these showed any significant changes in cell densities over time during steady state (slopes of lines not statistically different from zero, p > 0.05, Fig. 1). During the runs, it was attempted to maintain similar average irradiances within the PBR by varying the incident irradiance from 100 – 250 µmol m-2 s-1 (Table 1). This resulted in average irradiances within the PBR of 48 – 82 µmol m-2 s-1 (Table 1). Despite this variation in average irradiances within the PBR, no effects on productivity, measured as carbon productivity, as a consequence of available light within the range in irradiances, used here, could be seen (Fig. 3). This was analyzed using least squares linear regression. The slope of the resulting regression line was not statistically different from zero (p > 0.05, Fig. 3).

 The dilution rates achieved in this study varied from 0.17 – 0.52 d-1. Carbon productivities varied from 0.950 – 5.200 g C d-1 (Table 1), with the lowest values achieved at the two lowest cell densities (Fig. 3), while carbon productivities saturated at approximately 1.0 x 106 cells ml-1 and showed no further increase at higher cell densities (Fig. 4).

 The content of total fatty acids (TFA) varied from 10.7 pg cell-1 at the highest cell density to 13.1 pg cell-1 at the lowest cell density (Table 2). ANOVA followed by a Tukey test showed that the cell TFA content was significantly higher at the lowest cell density than at the two higher cell densities (ANOVA, p < 0.0001, Table 3) where the TFA contents did not differ significantly from each other (ANOVA, p > 0.05). The TFA productivity showed a clear saturating pattern (Fig. 5, R2 = 0.802) with the lowest productivities at the lowest cell densities and productivities saturating at approximately 1 x 106 cells ml-1 (Fig. 5).

 The cell content of EPA varied from 9.9 % of TFA at the lowest cell density to 12.9 % of TFA at the highest (Table 2). The ANOVA and Tukey tests showed a statistically significant increase in the proportion of EPA in TFA with cell density (ANOVA, p < 0.0001, Table 3) across the three density levels. As for TFA, EPA content showed a statistically significant saturation relationship to cell density (Fig. 5, R2 = 0.778) with the lowest productivities at the lowest cell density and saturating at approximately 1.0 x 106 cells ml-1 (Fig. 5).

 The cell content of DHA varied from 8.1 % of TFA at the lowest cell density to 9.1 % of TFA at the highest (Table 2). ANOVA followed by a Tukey test showed that the proportion of DHA in TFA was significantly lower at the lowest cell density than at the two higher cell densities (ANOVA, p = 0.001, Table 3) where the proportions of DHA in TFA did not differ significantly from each other (ANOVA, p > 0.05). As for TFA, DHA productivities showed a statistically significant saturation relationship to cell density (Fig. 5, R2 = 0.788) with the lowest productivities at the lowest cell density and saturating at approximately 1.0 x 106 cells ml-1 (Fig. 5).

 The cell content of ARA evinced a non-significant variation between 0.61 % of TFA to 0.70 % of TFA (ANOVA p > 0.05, Table 2, Table 3). When considering the productivities of ARA, this still showed a statistically significant saturating pattern (Fig. 5, R2 = 0.736) with the lowest productivities at the lowest cell densities, and saturating at approximately 1.0 x 106 cells ml-1 (Fig. 5).

**Discussion**

During large-scale production of microalgae in a PBR, the productivities of e.g. PUFAs, is a function of cell density, specific growth rate, which equals dilution rate under steady state conditions, as well as the concentration of PUFAs in the microalgal biomass. The relationship between cell density and specific growth rate or dilution rate is inverse, so that the specific growth rate declines with increasing cell density, especially due to light limitation caused by self-shading in the microalgal culture ([Richmond, 2004](#_ENREF_31)).

In this study, we operated a tubular PBR at constant cell density and dilution rate for 3 – 11 days at four different cell densities, from 0.5 x 106 to 2.0 x 106 cells ml-1. Under these conditions, it is not possible to maintain the irradiance within the PBR constant. We maintained an irradiance within the PBR at approximately 50 – 80 µmol m-2 s-1 and found that *Rhodomonas* showed no signs of its carbon productivity being related to irradiance within this range despite that it has previously been found that in small-scale experiments *Rhodomonas* is light limited at irradiances below some 60 µmol m-2 s-1 ([Vu et al., 2016](#_ENREF_36)). The apparent discrepancy may be because our calculated estimates of available light in the PBR are minimum estimates as the PBR was operating with a gas-filled headspace and the calculation assumes a completely filled PBR. Calculating the available light in a partially gas-filled PBR is, however, not straightforward as the liquid is moving turbulently.

The carbon productivity as a function of cell density could in this study be described by a saturation curve. At the highest cell densities, a lower specific growth rate was compensated for by the higher cell densities, thus keeping carbon productivity constant. The concentration of total fatty acids, TFA, in the microalgal biomass declined with increasing cell density in this study. The relative contribution of the two poly-unsaturated fatty acids EPA and DHA increased with increasing cell density, while the relative contribution of the third poly-unsaturated fatty acid, ARA, did not vary as a consequence of increasing cell density. The variation in the biomass content of both TFA and the individual PUFAs is, however, relatively small, so TFA as well as the three individuals PUFAs evince the same type of saturation curve as a function of increasing cell density as seen for the carbon biomass productivity. The productivity of fatty acids is lowest at the lowest cell densities, where the higher specific growth rate does not compensate for the low cell density, and higher, and constant, at the three highest cell densities, where the product of cell density and specific growth rate is more or less constant. In the present study, cultivation was carried out with addition of growth medium in triple dose to avoid nutrient limitation as [Vu et al. (2016)](#_ENREF_36) demonstrated that *Rhodomonas* has a more suitable biochemical profile for aquaculture when cultivated in excess of nutrients. The ratio of DHA/EPA in *Rhodomonas* in the present study of 0.6 to 0.7 is within the range of what has previously been reported for *Rhodomonas* with ratios from 0.5 to 1.5 ([Dunstan et al., 2005](#_ENREF_14); [Pleissner et al., 2012](#_ENREF_30); [Guevara et al., 2016](#_ENREF_19); [Vu et al., 2016](#_ENREF_36)). Contrary, the ratio of EPA/ARA is high compared to other studies (4 to 13.2 versus the present values of 14.1 to 21.5) ([Dunstan et al., 2005](#_ENREF_14); [Guevara et al., 2016](#_ENREF_19)). Literature on the effect of density on fatty acids is limited and ambiguous. A study by [Lu et al. (2001)](#_ENREF_25) reported a density effect on TFA at 88 µmol m-2 s-1 but not at a lower irradiance (44 µmol m-2 s-1) for the eustigmatophyte *Monodus subterraneus*, while [Chrismadha and Borowitzka (1994)](#_ENREF_10) reported small and unclear effects for the diatom *Phaeodactylum tricornutum*.

The results of the present study indicate that large-scale production of *Rhodomonas* in our PBR should take place at cell densities of 1.0 x 106 cells ml-1. At lower cell densities, the biomass productivity as well as the productivity of PUFAs, crucial for the nutritional value of this microalga in aquaculture, will be lower, while there are indications for increasing difficulties in maintaining steady state production in the PBR at the higher cell density of 2 million cells ml-1 (Fig. 2). The results achieved here can be expected to be valid for PBRs of a design similar to ours, e.g. operating with a gas-filled headspace. These PBRs are becoming more and more common, especially due to the advantages of not having to consider de-gassing of the culture ([Waycott, 2020](#_ENREF_39)). During these production conditions, the biomass productivity of *Rhodomonas* as well as its contents of essential PUFAs are lower than what has been reported in the literature when *Rhodomonas* is grown under optimal small-scale conditions in a 1.5 L bubble column PBR and in bottle experiments in the laboratory ([Eriksen et al., 1998](#_ENREF_15); [Vu et al., 2016](#_ENREF_36)). Under these conditions, maximum specific growth rates of about 0.75 d-1 have been found. This is to be expected when scaling up production of microalgae due to the larger volumes and greater significance of limiting factors such as self-shading ([Richmond, 2004](#_ENREF_31)).

**Acknowledgements**The authors would like to thank lab technician Hanne Hasselager (Danish Technological Institute, Agro Tech) for help maintaining the microalgal cultures and lab technician Rikke Guttesen (Roskilde University, Department of Science and Environment) for assisting with the fatty acids analysis. The study was funded by Innovation Fund Denmark COMA – Copepod egg Mass production in Aquaculture grant (Grant. no. 67-2013-1).

**Data availability statement**

All data in presented in this paper are original for this study. The dataset is available as: Thoisen, Christina, Pedersen, Jakob Skov, Jørgensen, Lars, Kuehn, Anker, Hansen, Benni Winding, & Nielsen, Søren Laurentius (2019). The effect of cell density on biomass and fatty acid during upscaling of Rhodomonas salina cultivation - the dataset. [Data set]. Zenodo. <http://doi.org/10.5281/zenodo.3470791>.

**Ethics statement**

This work only involved microalgae. No experiments on animals were performed, so no ethical approval of this work applies.

**Conflicts of interest**

The authors declare no conflicts of interest.

**Author contribution**

Christina Thoisen did most of the experimental work and compiled data. Jakob Skov Pedersen, Anker Kuehn and Lars Jørgensen contributed to the experimental work and commented on the manuscript. Benni Winding Hansen and Søren Laurentius Nielsen conceived the rationale behind the study and analyzed the data; Søren Laurentius Nielsen wrote the manuscript and Benni Winding Hansen commented on it.

**References**

Abdelaziz AEM, Leite GB, Hallenbeck PC (2013) Addressing the challenges for sustainable production of algal biofuels: II. Harvesting and conversion to biofuels. *Environmental Technology,* **34**, 1807-1836.

Abdo SM, Wahba SZ, Ali GH, El-Enin SAA, El-Khatib KM, El-Galad MI, El Diwani G (2016) Preliminary economic assessment of biofuel production from microalgae. *Renewable & Sustainable Energy Reviews,* **55**, 1147-1153.

Abo BO, Odey EA, Bakayoko M, Kalakodio L (2019) Microalgae to biofuels production: a review on cultivation, application and renewable energy. *Reviews on Environmental Health,* **34**, 91-99.

Acién Fernández FG, García Camacho F, Sánchez Pérez JA, Fernández Sevilla JM, Molina Grima E (1997) A model for light distribution and average solar irradiance inside outdoor tubular photobioreactors for the microalgal mass culture. *Biotechnology and Bioengineering,* **55**, 701-714.

Ahmad AL, Yasin NHM, Derek CJC, Lim JK (2014) Comparison of harvesting methods for microalgae *Chlorella* sp. and its potential use as a biodiesel feedstock. *Environmental Technology,* **35**, 2244-2253.

Arndt C, Sommer U (2014) Effect of algal species and concentration on development and fatty acid composition of two harpacticoid copepods, *Tisbe* sp. and *Tachidius discipes,* and a discussion about their suitability for marine fish larvae. *Aquaculture Nutrition,* **20**, 44-59.

Berggreen U, Hansen B, Kiorboe T (1988) Food size spectra, ingestion and growth of the copepod *Acartia tonsa* during development - implications for determination of copepod production. *Marine Biology,* **99**, 341-352.

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

Castilla-Gavilan M, Buzin F, Cognie B, Dumay J, Turpin V, Decottignies P (2018) Optimising microalgae diets in sea urchin *Paracentrotus lividus* larviculture to promote aquaculture diversification. *Aquaculture,* **490**, 251-259.

Chrismadha T, Borowitzka MA (1994) Effect of cell density and irradiance on growth, proximate composition and eicosapentaenoic acid production of *Phaeodactylum tricornutum* grown in a tubular photobioreactor. *Journal of Applied Phycology,* **6**, 67-74.

Dalsgaard J, St John M, Kattner G, Muller-Navarra D, Hagen W (2003) Fatty acid trophic markers in the pelagic marine environment. In: *Advances in Marine Biology, Vol 46* (ed. by Southwards AJ, Tyler PA, Young CM, Fuiman LA)*,* pp. 225-340.

de Vicose GC, Porta A, Viera MP, Fernandez-Palacios H, Izquierdo MS (2012) Effects of density on growth rates of four benthic diatoms and variations in biochemical composition associated with growth phase. *Journal of Applied Phycology,* **24**, 1427-1437.

Drillet G, Iversen MH, Sorensen TF, Ramlov H, Lund T, Hansen BW (2006) Effect of cold storage upon eggs of a calanoid copepod, *Acartia tonsa* (Dana) and their offspring. *Aquaculture,* **254**, 714-729.

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

Eriksen NT, Poulsen BR, Iversen JJL (1998) Dual sparging laboratory-scale photobioreactor for continuous production of microalgae. *Journal of Applied Phycology,* **10**, 377-382.

Fernandez-Reiriz MJ, Irisarri J, Labarta U (2015) Feeding behaviour and differential absorption of nutrients in mussel *Mytilus galloprovincialis*: Responses to three microalgae diets. *Aquaculture,* **446**, 42-47.

Gagne R, Tremblay R, Pernet F, Miner P, Samain JF, Olivier F (2010) Lipid requirements of the scallop *Pecten maximus* (L.) during larval and post-larval development in relation to addition of *Rhodomonas sauna* in diet. *Aquaculture,* **309**, 212-221.

Grima EM, Sevilla JMF, Pérez JAS, Camacho FG (1996) A study on simultaneous photolimitation and photoinhibition in dense microalgal cultures taking into account incident and averaged irradiances. *Journal of Biotechnology,* **45**, 59-69.

Guevara M, Arredondo-Vega BO, Palacios Y, Saez K, Gomez PI (2016) Comparison of growth and biochemical parameters of two strains of *Rhodomonas salina* (Cryptophyceae) cultivated under different combinations of irradiance, temperature, and nutrients. *Journal of Applied Phycology,* **28**, 2651-2660.

Guevara M, Bastardo L, Cortez R, Arredondo-Vega B, Romero L, Gomez P (2011) *Rhodomonas salina* (Cryptophyta) pastes as feed for *Brachionus plicatilis* (Rotifera). *Revista De Biologia Tropical,* **59**, 1503-1515.

Guillard RR, Ryther JH (1962) Studies of marine planktonic diatoms. 1. *Cyclotella nana* hustedt, and *Detonula confervacea* (Cleve) Gran. *Canadian Journal of Microbiology,* **8**, 229-&.

Huerlimann R, de Nys R, Heimann K (2010) Growth, Lipid Content, Productivity, and Fatty Acid Composition of Tropical Microalgae for Scale-Up Production. *Biotechnology and Bioengineering,* **107**, 245-257.

Jepsen PM, Bjorbaek NS, Rayner TA, Vu MTT, Hansen BW (2017) Recommended feeding regime and light climate in live feed cultures of the calanoid copepod *Acartia tonsa* Dana. *Aquaculture International,* **25**, 635-654.

Jung EE, Jain A, Voulis N, Doud DFR, Angenent LT, Erickson D (2014) Stacked optical waveguide photobioreactor for high density algal cultures. *Bioresource Technology,* **171**, 495-499.

Lu CM, Rao K, Hall D, Vonshak A (2001) Production of eicosapentaenoic acid (EPA) in *Monodus subterraneus* grown in a helical tubular photobioreactor as affected by cell density and light intensity. *Journal of Applied Phycology,* **13**, 517-522.

Lund I, Steenfeldt SJ, Suhr KI, Hansen BW (2008) A comparison of fatty acid composition and quality aspects of eggs and larvae from cultured and wild broodstock of common sole (*Solea solea* L.). *Aquaculture Nutrition,* **14**.

Mirón AS, Gómez AC, Camacho FG, Grima EM, Chisti Y (1999) Comparative evaluation of compact photobioreactors for large-scale monoculture of microalgae. *Journal of Biotechnology,* **70**, 249-270.

Molina Grima E, García Camacho F, Sánchez Pérez JA, Acién Fernández FG, Fernández Sevilla JM (1996) Growth yield determination in a chemostat culture of the marine microalga *Isochrysis galbana*. *Journal of Applied Phycology,* **8**, 529-534.

Nielsen SL, Hansen BW (2019) Evaluation of the robustness of optical density as a tool for estimation of biomass in microalgal cultivation: The effects of growth conditions and physiological state. *Aquaculture Research,* **50**, 2698-2706.

Pleissner D, Eriksen NT, Lundgreen K, Riisgaard HU (2012) Biomass composition of blue mussels, *Mytilus edulis*, is affected by living site and species of ingested microalgae. *ISRN Zoology,* **2012**.

Richmond A (2004) Biological principles of mass cultivation. In: *Handbook of Microalgal Culture: Biotechnology and Applied Phycology* (ed by Richmond A). Blackwell Publishing Ltd*,* pp. 125-177.

Seixas P, Coutinho P, Ferreira M, Otero A (2009) Nutritional value of the cryptophyte *Rhodomonas lens* for *Artemia* sp. *Journal of Experimental Marine Biology and Ecology,* **381**, 1-9.

Sokal RR, Rohlf FJ (1995) *Biometry,* W.H. Freeman and Company, New York.

Sonnenholzner-Varas JI, Touron N, Orrala MMP (2018) Breeding, larval development, and growth of juveniles of the edible sea urchin *Tripneustes depressus*: A new target species for aquaculture in Ecuador. *Aquaculture,* **496**, 134-145.

Thoisen C, Vu MTT, Carron-Cabaret T, Jepsen PM, Nielsen SL, Hansen BW (2018) Small-scale experiments aimed at optimization of large-scale production of the microalga *Rhodomonas salina*. *Journal of Applied Phycology,* **30**, 2193-2202.

Vu MTT, Douette C, Rayner TA, Thoisen C, Nielsen SL, Hansen BW (2016) Optimization of photosynthesis, growth, and biochemical composition of the microalga *Rhodomonas salina*-an established diet for live feed copepods in aquaculture. *Journal of Applied Phycology,* **28**, 1485-1500.

Vu MTT, Jepsen PM, Jorgensen NOG, Hansen BW, Nielsen SL (2019) Testing the yield of a pilot-scale bubble column photobioreactor for cultivation of the microalga *Rhodomonas salina a*s feed for intensive calanoid copepod cultures. *Aquaculture Research,* **50**, 63-71.

Wang B, Lan CQ, Horsman M (2012) Closed photobioreactors for production of microalgal biomasses. *Biotechnol Adv,* **30**, 904-912.

Waycott B (2020) Making algae can get expensive. Innovations aim to bring costs down. In: *Global Aquaculture Advocate*. Global Aquaculture Alliance.

Wolcott R, Messing CG (2005) A comparison of diets and water agitation methods for larval culture of the edible sea urchin, *Tripneustes ventricosus* (Echinodermata : Echinoidea). *Bulletin of Marine Science,* **77**, 177-190.

Yamamoto S, Okauchi M, Yoshimatsu T (2015) Dietary value of microalga Rhodomonas sp as a live food for sea cucumber *Apostichopus japonicus* larvae. *Nippon Suisan Gakkaishi,* **81**, 973-978.

**Table 1**

Data from the eight runs of the PBR. Nominal cell density: The cell density we attempted to maintain in the PBR (cells ml-1). Average cell density: Actual average cell density during the run (cells ml-1). Incident irradiance: Irradiance (PAR) measured at the surface of the PBR (µmol m-2 s-1). Average irradiance in PBR: Average irradiance (PAR) in the PBR calculated as described in the Materials and Methods – section (µmol m-2 s-1). It must be noted that these values are minimum estimates of available irradiance as the calculation assumes a full PBR, while it was in effect operated with an air-filled headspace. Dilution rate: Number of times per day the volume of the PBR was replenished with fresh growth medium (d-1). Productivity: Average productivity of carbon ( g C d-1), total fatty acids (mg TFA d-1), arachidonic acid (mg ARA d-1), eicosapentaenoic acid (mg EPA d-1), and docosahexaenoic acid (mg DHA d-1) during the runs.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Nominal cell density** | **Average cell density** | **Incident irradiance** | **Average irradiance in PBR** | **Dilution rate** | **Productivity** |
|  |  |  |  |  | C | TFA | ARA | EPA | DHA |
| 500,000 | 466,735 | 100 | 63 | 0.30 | 0.954 | 264.2 | 1.85 | 26.29 | 21.26 |
| 500,000 | 499,543 | 100 | 61 | 0.42 | 1.511 | 418.4 | 2.93 | 41.63 | 33.66 |
| 1,000,000 | 990,344 | 200 | 82 | 0.52 | 3.897 | 935.3 | 5.67 | 108.93 | 80.24 |
| 1,000,000 | 1,044,315 | 200 | 79 | 0.52 | 4.119 | 988.5 | 6.00 | 115.13 | 84.80 |
| 1,500,000 | 1,454,980 | 175 | 53 | 0.33 | 3.882 | n/a | n/a | n/a | n/a |
| 1,500,000 | 1,527,799 | 175 | 51 | 0.37 | 4.546 | n/a | n/a | n/a | n/a |
| 2,000,000 | 2,384,809 | 250 | 48 | 0.17 | 2.847 | 641.4 | 3.96 | 83.00 | 58.32 |
| 2,000,000 | 1,839,823 | 200 | 49 | 0.40 | 5.204 | 1,172.6 | 7.25 | 151.73 | 106.61 |

**Table 2.** The cell content of total fatty acids (TFA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid (ARA) in *Rhodomonas salina* as well as the ratio of DHA/EPA and EPA/ARA. Nominal cell density is given as cells ml-1, TFA as pg cell-1. EPA, DHA and ARA are % of TFA. Data are given as mean ± 1 SD.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Nominal cell density** | **TFA** | **EPA**  | **DHA**  | **ARA** | **DHA/EPA** | **EPA/ARA** |
| 500,000 | 13.1 ± 0.9 | 9.9 ± 0.2 | 8.1 ± 0.3 | 0.70 ± 0.07 | 0.8 | 14.1 |
| 1,000,000 | 11.4 ± 0.7 | 11.7 ± 0.4 | 8.6 ± 0.3 | 0.61 ± 0.08 | 0.7 | 19.2 |
| 2,000,000 | 10.7 ± 0.4 | 12.9 ± 0.3 | 9.1 ± 0.3 | 0.61 ± 0.03 | 0.7 | 21.1 |

**Table 3**

Results of one-way ANOVA testing the effect of cell density on total fatty acids (TFA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid (ARA) in *Rhodomonas salina* in this study.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Variable | Source of variation | *SS* | *df* | *MS* | *F* ratio | *p* |
| TFA | Cell density | 24.383 | 2 | 12.192 | 21.590 | < 0.0001 |
|  | Error | 11.294 | 20 | 0.565 |  |  |
| EPA | Cell density | 34.635 | 2 | 17.317 | 58.683 | < 0.0001 |
|  | Error | 5.902 | 20 | 0.295 |  |  |
| DHA | Cell density | 4.229 | 2 | 2.115 | 9.715 | 0.001 |
|  | Error | 4.354 | 20 | 0.218 |  |  |
| ARA | Cell density | 0.046 | 2 | 0.023 | 3.469 | 0.051 |
|  | Error | 0.132 | 20 | 0.007 |  |  |

**Figure legends**

**Figure 1.** Schematic overview of the construction of the PBR used in this study. Two different views of the PBR are included: From the side, including a schematic overview of air inlet, growth medium dosing and harvest pump and tank; from the top, including the placement of the LED light panel.

**Figure 2.** Development in cell density during the eight experiments (runs of the PBR). Circles: nominal cell density 0.5 x 106 cells ml-1, squares: nominal cell density 1.0 x 106 cells ml-1, triangles: nominal cell density 1.5 x 106 cells ml-1, diamonds: nominal cell density 2.0 x 106 cells ml-1. The straight lines are least squares regression lines, added to illustrate the trend in cell density during the PBR run. None of the regression lines has slopes that are statistically significantly different from 0, so no trend in cell density can be detected during any of the PBR runs.

**Figure 3.** The relationship between carbon productivity of the PBR and calculated average irradiance inside the PBR. The dotted line represent the least squares linear regression of this relationship. The slope of the line is not statistically significantly different from 0.

**Figure 4.** The relationship between cell density and carbon productivity from the eight PBR runs. The line represent the fitted hyperbolic function. The coefficient of determination was R2 = 0.802.

**Figure 5.** The relationship between cell density and total fatty acids (TFA, panel A), as well as the three fatty acids arachidonic acid (ARA, circles), eicosapentaenoic acid (EPA, triangles) and docosahexaenoic acid (DHA, squares) (panel B). The lines represent the fitted hyperbolic function. The coefficients of determination are as follows: TFA: R2 = 0.767, ARA: R2 = 0.736, EPA: R2 = 0.778, DHA: R2 = 0.788.