Optimizing the cultivation of the cryptophyte Rhodomonas salina for aquaculture

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Optimizing the cultivation of the cryptophyte *Rhodomonas salina* for aquaculture

Ph.D. Thesis

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Contents

Abstract (English)

In the aquaculture, marine fish species of commercial interest often require live feed at the first-feeding stage. Commonly used live feed organisms are rotifers and the brine shrimp *Artemia* although these are suboptimal due to inadequate size and nutritional value. Copepods are the natural prey of many marine species of fish larvae and decrease the mortality and abnormal development of fish larvae. A large-scale production of the *copepod Acartia tonsa* for aquaculture encompasses a large-scale production of a microalgal diet for *A. tonsa*. Several studies show that the cryptophyte *Rhodomonas* is an ideal microalgal diet as it has a superior nutritional content and increases the survival, growth and egg production of copepods. However, *Rhodomonas* is a rather sensitive genus and optimization of the cultivation is therefore required. The effects of various cultivation parameters were studied at small- and meso-scale and included: 1) modification of the growth medium, treatment of the water used for cultivation, and the growth rate of inocula at different initial densities, 2) evaluation of the application of commercial salts for cultivation, 3) evaluation of growth rate and production yield of densities of $5x10^5$, $1x10^6$, $1.5x10^6$ and $2x10^6$ cells ml^{-1} in a meso-scale tubular PBR, 4) evaluation of the biochemical content of five closely related strains of *Rhodomonas* at temperatures relevant for cultivation (15, 20 and 25 ˚C) to determine if there is an optimal temperature and strain more suitable for cultivation. Additional studies included an evaluation of using the heterotrophic dinoflagellate *Crypthecodinium cohnii* as a diet for *A. tonsa* compared to *R. salina*, and development of a simple and fast method for extraction and quantification of the pigment phycoerythrin from *Rhodomonas.*

Abstrakt (Dansk)

I akvakulturen kræver marine fiskearter af kommerciel interesse ofte levende foder, når de er fiskelarver. Typisk anvendte levende foderorganismer er hjuldyr og saltsøkrebs *Artemia*, selvom disse er suboptimale på grund af utilfredsstillende størrelse og næringsværdi. Vandlopper er det naturlige bytte for mange marine arter af fiskelarver, og mindsker dødeligheden og unormal udvikling af fiskelarver. En storskalaproduktion af vandloppen *Acartia tonsa* til akvakulturen omfatter en storskalaproduktion af en mikroalgediæt til *A. tonsa*. Flere undersøgelser viser, at cryptofytten *Rhodomonas* er en ideel mikroalgediæt, da den har et optimalt næringsindhold, og øger overlevelse, vækst og ægproduktion af vandlopper. *Rhodomonas* er dog en ret sensitiv slægt, og optimering of kultiveringen er derfor nødvendig. Effekten af forskellige kultiveringsparametre blev undersøgt på lille- og meso-skala og inkluderede: 1) modifikation af vækstmediet, behandling af det anvendte vand til kultivering og vækstraten af inokulum ved forskellige startkoncentrationer, 2) evaluering af anvendelse af kommercielle salte til kultivering, 3) evaluering af vækstrate og produktionsudbytte af densiteter på $5x10^5$, 1x106, $1,5x10^6$ og $2x10^6$ celler ml^{-1} i en meso-skala tubular PBR, 4) evaluering af det biokemiske indhold af fem nært beslægtede *Rhodomonas*-stammer ved temperaturer, der er relevante for dyrkning (15, 20 og 25 ˚C) for at bestemme, om der er en optimal temperatur og stamme, som er bedre egnet til kultivering. Yderligere studier omfattede en evaluering af anvendelsen af den heterotrofe dinoflagellat *Crypthecodinium cohnii* som diæt til *A. tonsa* sammenlignet med *R. salina* og udviklingen af en simpel og hurtig metode til ekstraktion og kvantificering af pigmentet fycoerytrin fra *Rhodomonas*.

Preface

The work described in this thesis was carried out primarily at the Department of Science and Environment, Environmental Dynamics at Roskilde University (DK) during the period January 2014 to March 2018. The main supervisor was Associate Professor Søren Laurentius Nielsen and the co-supervisor was Professor Benni Winding Hansen.

Meso-scale experiments were conducted at AgroTech, the Danish Technological Institute in Taastrup (DK). AgroTech was one of the project partners with facilities to conduct meso- and large-scale experiments with microalgae in photobioreactors.

The thesis includes 6 papers with 4 published and 4 as first author. Papers 1-4 are the main focus of the Ph.D. project while papers 5-6 are additional studies with relevance for the project.

Acknowledgements

First and foremost, I would like to express my gratitude to my supervisor Søren Laurentius Nielsen and co-supervisor Benni Wining Hansen for their motivation, enthusiasm, and immense scientific knowledge. Søren, your insight into the application of microalgae in various fields has been truly inspirational and encouraging. Benni, your eagerness for science is contagious.

I would like to thank everyone who is and has been in the Environmental Dynamics research group at RUC during the last 4 years.

I am grateful to AgroTech at The Danish Technological Institute for the opportunity to conduct research at their facility and for introducing me to their exciting innovative work with microalgae and plants. Also, it was exciting to follow the set-up and progress of the large-scale production of *Rhodomonas salina* and *Acartia tonsa* at AKVA group.

Finally, I would like to thank Per Juel Hansen for his fascinating and captivating stories about protists back in 2011 which convinced me to continue within the field of protists. And last, but by no means least, an enormous thank you to Hans Henrik Jakobsen for inviting me into his laboratory to work with the dancing cells of *Crypthecodinium cohnii* and for encouraging me to apply for this Ph.D. Thank you!

List of papers

Paper 1

Thoisen C, Vu MTT, Carron-Cabaret T, Jepsen PM, Nielsen SL and Hansen BW (2018). Small-scale experiments aimed at optimization of large-scale production of the microalga *Rhodomonas salina*. Published online 12 March in Journal of Applied Phycology. https://doi.org/10.1007/s10811-018-1434-1

Paper 2

Jepsen PM, **Thoisen C**, Carron-Cabaret T, Pinyol-Gallemí A, Nielsen SL and Hansen BW (2018). Effects of salinity, commercial salts and water types on cultivation of the cryptophyte microalgae *Rhodomonas salina* and the calanoid copepod *Acartia tonsa*. Published 8 February in Journal of the World Aquaculture Society. https://doi.org/10.1111/jwas.12508

Paper 3

Thoisen C, Pedersen JS, Jørgensen L, Kuehn A, Hansen BW and Nielsen SL. The effect of cell density on yield of biomass and fatty acids during meso-scale cultivation of the cryptophyte *Rhodomonas salina*. *In prep.*

Paper 4

Thoisen C, Hansen BW and Nielsen SL. A comparison of five strains of the cryptophyte *Rhodomonas*: Temperature effects on growth rate and biochemical profile. *In prep.*

Paper 5

Jakobsen HH, **Thoisen C** and Hansen BW (2018). *Crypthecodinium cohnii*: a promising prey toward large-scale intensive rearing of the live feed copepod *Acartia tonsa* (Dana). Aquaculture International 26(1):237-251. https://doi.org/10.1007/s10499-017-0207-1

Paper 6

Thoisen C, Hansen BW and Nielsen SL (2017). A simple and fast method for extraction and quantification of cryptophyte phycoerythrin. MethodsX 4:209-213. http://dx.doi.org/10.1016/j.mex.2017.06.002

Introduction

The COMA project

This thesis is part of the project COMA; COpepod egg Mass production for Aquaculture. The goal of the project is a commercial continuous large-scale production of high quality eggs from the calanoid copepod *Acartia tonsa* for the first-feeding fish larvae in the aquaculture. In our laboratory, females of our *A. tonsa* strain (DFH.AT1, Støttrup et al. (1986)) can produce 22 eggs d^{-1} (Jepsen et al. 2007) and the eggs are released into the water column where they sink to the bottom of the copepod tank. These eggs are harvested, rinsed and stored as resting eggs in 4 ˚C deoxygenated seawater. The eggs can be stored at 4 ˚C for 11 months while maintaining a hatching rate of 74 % (Drillet et al. 2006a). The stored eggs will be transported to fish hatcheries and added to the fish larval tanks, and after 24 hours the eggs hatch releasing copepod nauplii as first-feed for the fish larvae (Fig. 1). The goal of the COMA project is to produce 100×10^6 copepod eggs d^{-1}. Copepods feed on microalgae and a large-scale production of *A. tonsa* therefore encompasses a large-scale production of microalgae. This is my part in the COMA project; cultivation of the microalgal diet. The aim of my thesis is optimizing the large-scale production of the microalgal diet for *A. tonsa* by studying the effect of various parameters on the growth and biochemical profile of *Rhodomonas salina* in small- and meso-scale experiments. The findings from my work were then implemented to the large-scale production of *R. salina* by the team working with the large-scale setup. The chosen microalga for the COMA project is the cryptophyte *R. salina* as the genus *Rhodomonas* has shown to be superior to other microalgal diets used for copepod rearing (Arndt and Sommer 2014, Knuckey et al. 2005, McKinnon et al. 2003, Støttrup and Jensen 1990, Zhang et al. 2013).

Figure 1. Flow chart of the concept in the COMA project. From left to right: A large-scale production of the microalga *Rhodomonas salina* cultivated in photobioreactors is the diet for the copepod *Acartia tonsa* cultivated in a copepod tank. The eggs from *A. tonsa* are harvested and stored in containers. The eggs hatch when added to the fish tank releasing copepod nauplii as live feed for the fish larvae. (Illustration: AKVA group Denmark)

Live feed in aquaculture

The production of fish in aquaculture has been steadily increasing since the 1980s while the capture fishery production has been stagnant (Fig. 2). In 2014 the world per capita fish supply reached 20 kg with 50 % supplied from aquaculture (FAO 2016).
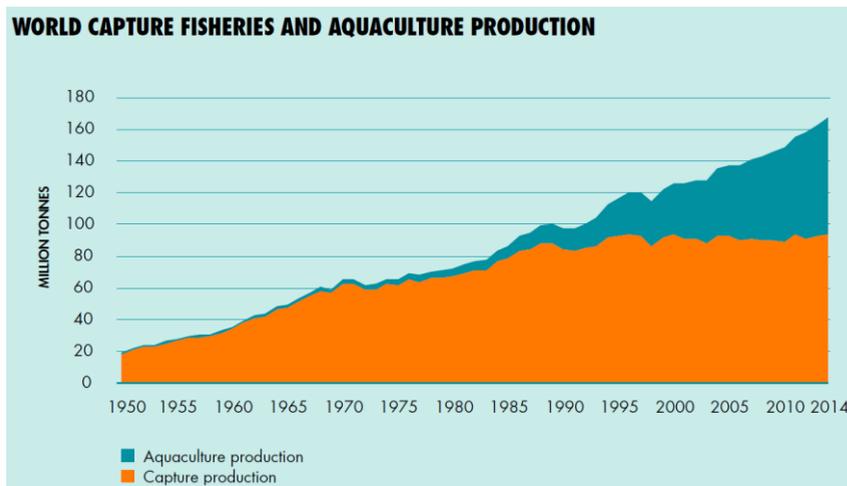
Figure 2. The world capture fisheries (orange) and aquaculture (blue) production in million tonnes from 1950 to 2014 (FAO 2016).

Many marine fish species of commercial interest require live feed at the first-feeding stage (Conceição et al. 2010). Commonly used live feed organisms in the marine larviculture are rotifers (50 μm-2 mm) and the brine shrimp *Artemia* (0.9-10 mm) as they are easy to cultivate at high density (Fig. 3). However, these organisms are not optimal as they are poor in the polyunsaturated fatty acids (PUFAs) docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) compared to copepods (Seixas et al. 2009, Støttrup et al. 1999). Furthermore, *Artemia* are too large sized for some species of fish larvae to consume and more difficult to digest compared to copepods (Dhont et al. 2013, Pedersen 1984).

Figure 3. The brine shrimp *Artemia salina*, a rotifer and the copepod *Acartia tonsa*. (Artemia: http://amazinglife.bio/brine-shrimp-artemia-salina/, rotifer: https://mikesphyto.com/product/rotifers/, *A. tonsa*: B. Nilsson, RUC)

Copepods (≤ 1 mm crustaceans) are superior to rotifers and *Artemia* as copepod nauplii (65-120 μm) have a small size suitable as first-feed for fish larvae, a swimming pattern stimulation prey capture, and a favorable biochemical content (Conceição et al. 2010, Drillet et al. 2006b, Grageda et al. 2008, Shields et al. 1999). Furthermore, copepods are the natural prey of most fish larvae species and increase the larval performance and quality (Conceição et al. 2010 and references therein). Also, fish larvae have a low protein digestibility, but a high assimilation capacity for free amino acids (FAA), which are twice as abundant in copepods compared to *Artemia* (van der Meeren et al. 2008). Copepods are especially important for the rearing of species such as pink and red snapper, West Australian dhufish, grouper,

yellowtail, clownfish, halibut, cod and seabass (Dhont et al. 2013 and references therein).

The calanoid copepod *A. tonsa* has been cultivated and intensively studied as a live feed organism since 1981 and due to the extensive knowledge on *A. tonsa* and its resting eggs it is the chosen organism for the COMA project (e.g., Drillet et al. 2006a, Jepsen et al. 2007, Jepsen et al. 2016, Nilsson et al. 2017, Rayner et al. 2017, Støttrup et al. 1986, Støttrup and Jensen 1990, Støttrup et al. 1999). It is considered a challenge to cultivate copepods intensively at high densities and at large-scale due to stressors such as limited food resources, oxygen depletion, accumulation of metabolic products and physical interactions between individuals (Nilsson et al. 2017 and references therein). Cultivation of *A. tonsa* at densities > 2,500 individuals L^{-1} has shown to reduce egg harvest although no stress-related genes were found during cultivation at a fourfold density of 10,000 individuals L^{-1} after 12 h (Drillet et al. 2015, Nilsson et al. 2017).

Microalgal diet for copepods

The copepod *A. tonsa* can feed on various groups of microalgae such as cryptophytes (e.g., *Rhodomonas, Rhinomonas*), diatoms (e.g., *Chaetoceros*, *Thalassiosira*), and haptophytes (e.g., *Isochrysis*) either as monoalgal or mixed diet (Barroso et al. 2013, Drillet et al. 2011, Leandro et al. 2006, Øie et al. 2017, Støttrup and Jensen 1990, Teixeira et al. 2010, Zhang et al. 2013). Several studies, however, show that the cryptophyte *Rhodomonas* (Fig. 4) is an ideal microalgal diet as it increases the survival, growth and egg production of copepods (Arndt and Sommer 2014, Knuckey et al. 2005, McKinnon et al. 2003, Støttrup and Jensen 1990, Zhang et al. 2013). Furthermore, *Rhodomonas* contain the PUFAs DHA, EPA and arachidonic acid (ARA) in favorable amounts. These PUFAs are important for the

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survival, development, growth and pigmentation of fish larvae and the cell content of fatty acids in copepods is affected by the content of fatty acids in the microalgal diet (Arndt and Sommer 2014, Caramujo et al. 2008, Dalsgaard et al. 2003, de Lima et al. 2013, Karlsen et al. 2015, Støttrup et al. 1999). DHA and EPA are essential for copepod growth and reproduction but the role of ARA is not clear (Klein Breteler et al. 1999, Støttrup and Jensen 1990). Some copepods (the calanoid *Pseudodiaptomus annandelei*, the harpaticoids *Tisbe* sp. and *Tachidius discipes*, the cyclopoid *Apocyclops royi*) are most likely able to synthesize certain PUFAs and chain elongate fatty acids, but no indicia has yet been demonstrated for *A. tonsa* (Arndt and Sommer 2014, Kleppel et al. 1998, Rayner et al. 2015, Veloza et al. 2006). Also, a deficiency in amino acids in the diet affects the egg production of *A. tonsa* (Kleppel et al. 1998). Thus, not all microalgae are suitable as a monoalgal diet for *A. tonsa* as a deficiency in the nutritional value will affect *A. tonsa*. As an example, longer periods of cultivation revealed that the haptophyte *Isochrysis galbana* is not a suitable diet for *A. tonsa* leading to a reduction in egg hatching success and adult survival, which were likely due to a the low content of EPA and histidine found in this microalga (Kleppel et al. 1998, Zhang et al. 2013).

Figure 4. The cryptophyte *Rhodomonas* sp. (https://ncma.bigelow.org/ccmp768)

The size of the microalgal diet for *A. tonsa* is important as the upper size limit for particle capture is 10 to 15 µm for the youngest *A. tonsa* nauplii and the lower size limit for particle capture is 2 to 4 µm for all developmental stages (Berggreen et al. 1988). *Isochrysis* is about 4 µm which is in the lower limit for particle capture of all stages of *A. tonsa*. Contrary, *R. salina* is about 12 µm and more suitable in size for all stages of *A. tonsa* (Thoisen et al. *In prep.-a*).

Altering the biochemical composition of microalgae

Microalgae species are diverse and taxon-specific in their content of, e.g., protein, carbohydrate, fatty acids, amino acids, vitamins and pigments. Interestingly, the biochemical composition of microalgae can be manipulated to a certain degree by altering the parameters for cultivation. A well-known example is the accumulation of the pigment astaxanthin (a carotenoid) in the chlorophyte *Haematococcus pluvialis* when exposed to environmental and nutrient stress (Lorenz and Cysewski 2000). Another example is the high production of vitamin E and lutein (a carotenoid) in the haptophyte *Diacronema vlkianum* at 18 ˚C compared to when cultivated at 26 ˚C (Durmaz et al. 2009).

The lipid content can be increased considerably during conditions with unfavorable nutrient level, salinity, pH, temperature and light intensity (Hu et al. 2008). In particular, nitrogen limitation is a well-known method used to accumulate triacylglycerols (TAGs) which are storage lipids composed of saturated and monounsaturated fatty acids (Griffiths et al. 2012, Hu et al. 2008). Additionally, phosphate limitation can increase the lipid content, as well as silicate limitation in diatoms can increase the lipid content (reviewed in Hu et al. 2008). It is suggested that the accumulation of TAGs may serve as

depot for PUFAs for incorporation into membranes once conditions return to favorable (Cohen et al. 2000). However, the accumulation of TAGs is unfavorable when the microalgae are intended for aquaculture as the fraction of unsaturated fatty acids decreases (Qiao et al. 2016, Vu et al. 2016).

An effect of decreased temperature is generally a reduction in growth rate and increase in the content of unsaturated fatty acids to maintain cell membrane fluidity (reviewed in Hu et al. 2008 and Thompson 2005). Contrary, an increased temperature tend to decrease the content of PUFAs such as EPA and DHA while ARA increase (Durmaz et al. 2008, Durmaz et al. 2009, Guevara et al. 2016, Jiang and Chen 2000, Qiao et al. 2016, Renaud et al. 1995, Renaud et al. 2002, Thompson et al. 1992). In addition, saturating light intensities may yield a higher amount of total fatty acids (TFA) and PUFAs although the specific effect may be species-specific. In example, the production of EPA increased in the Eustigmatophyte *Monodus subterraneus* while DHA increase in *R. salina* (Lu et al. 2001, Vu et al. 2016).

No studies indicate that the biochemical composition of *Rhodomonas* can be manipulated in ways that, e.g., drastically increase the content of PUFAs relevant for aquaculture. The key parameters for cultivation of *Rhodomonas* are that the culture is not nutrient limited and is cultivated at a temperature and light intensity that does not negatively affect the growth rate or biochemical composition (Chaloub et al. 2015, Vu et al. 2016).

Large-scale cultivation of microalgae

Microalgae can be cultivated in open or closed cultivation systems. Open systems are raceway ponds and basins, and closed systems include plastic (polyethene) bags and photobioreactors (PBRs). Open and closed systems located outside are typically found at locations near equator where

the climate is more suitable for cultivation all year round, although precipitation and evaporation may negatively affect production in the open systems due to dilution of the culture as well as alteration of the salinity. Production in open and closed systems can also take place outdoors at other locations than equator but with limitations such as seasonal changes in temperature and light intensity. For example, the cyanobacteria *Arthrospira* is cultivated in California but only for 7 months of the year due to variations in temperature (Belay 2013). In Denmark, *R. salina* was successfully cultivated by the project partner AgroTech, the Danish Technological Institute, in a horizontal tubular PBR (750 L) in a greenhouse using natural light and no temperature regulation during winter. However, an increase in temperature to about 28 ˚C during March and April had detrimental effects on productivity (Jørgensen, pers. com.).

The open systems are easy to operate at a low cost but the level of control is low (Chang et al. 2017, Gupta et al. 2015). Due to a high risk of contamination, the open systems are restricted to species thriving at extreme conditions unfavorable for most other species such as high/low pH and salinity, e.g., the chlorophytes *Dunaliella*, *Chlorella, Haematococcus*, and the cyanobacteria *Spirulina* (Borowitzka 2005 and references therein). Contrary, closed systems in terms of PBRs allow a high degree of control of abiotic factors such as pH, salinity, nutrient level, mixing and density. If the PBRs are located inside also temperature and light intensity can be controlled allowing a higher degree of stability of the system (Chang et al. 2017). Furthermore, PBRs are available in numerous types of various volume, shape and design (e.g., flat panel, horizontal tubular, bubble column), and therefore cultivation of basically all types of microalgae is possible. However, the high level of controllability of PBRs is expensive and requires expertise, substantial

practical work, cleaning and monitoring to keep the system running at optimal conditions (Borowitzka and Vonshak 2017, Chang et al. 2017). Plastic bags are also an option and are considered easier and more affordable to use compared to PBRs as no cleaning is required. On the other hand, leakages are not uncommon and mounting and demounting of the bags is time consuming, especially for a continuous large-scale production running for several years (Huang et al. 2017). In addition, the typical outdoor lifespan of plastic bags is maximum 1 year while a tubular glass PBR may last > 50 years resulting in PBRs being the preferred system for long-term large-scale cultivation (Schott 2017).

The potential and limitations of the systems used for large-scale production of microalgae has been widely addressed (e.g., Borowitzka and Vonshak 2017, Chang et al. 2017, Gupta et al. 2015, Huang et al. 2017) and the primary factors hindering optimal productivity are light, pH, temperature, mixing and O_2 removal. At small-scale it is fairly easy to provide sufficient light and mixing of the system and reduce the settling of algal cells but during scale-up the change in dimension complicates this leading to a reduction in productivity (Borowitzka and Vonshak 2017). The light penetration into the culture depends on the material and diameter of the PBR and the density of the culture as a high density leads to self-shading (Chrismadha and Borowitzka 1994, Thoisen et al. *In prep.-b*). The light received by the algal cells is also affected by mixing and flow rate as these factors keep the algal cells in suspension and shift the cells between dark and light areas in the system (Gupta et al. 2015). Furthermore, mixing is essential for distributing nutrients in the system (Gupta et al. 2015). The hydrodynamic stress from flow rate and gas entrance velocity, however, may negatively affect the algal

cells by causing severe shear stress depending on the sensitivity of the species (Barbosa et al. 2003, Sung et al. 2014).

In tubular PBRs, the inorganic carbon incorporated during photosynthesis decrease with the length of the tubes while the accumulation of O_2 produced during photosynthesis increase and may inhibit photosynthesis and lead to photo-oxidation (Camacho Rubio et al. 1999 and references therein). An efficient way to add inorganic carbon and control pH is to add CO_2 controlled by feedback system continuously maintaining pH at the optimum for a given microalgal species or strain (Borowitzka and Vonshak 2017, Vu et al. *In prep.*). The accumulated O_2 in the culture can be removed by degassing in a collection tank but the concentration of O2 along the length of the tube may hinder photosynthesis depending on the tolerance of a given species resulting in decreased productivity (Camacho Rubio et al. 1999). As also pointed out by Borowitzka and Vonshak (2017), the optimal system for cultivation of microalgae depends on the species to be cultivated, and only by thorough studies with the respective system can the optimal conditions for cultivation of a given species be identified.

The chosen PBRs in the COMA project are horizontal tubular PBRs with a small tube diameter (6 cm) to increase the light penetration into the culture and thereby reduce light limitation. Initially, the cultivation of *R. salina* was intended to be conducted in a vertical bubble column PBR (2x 47 L) inspired by the 1.7 L PBR described by Eriksen et al. (1998). Although the upscaling of this PBR system provided successful cultivation of *R. salina* it was found to not be optimal due the large diameter (20 cm) of the column which resulted in light limitation in the inner part of the column (Vu et al. *In prep.*).

The aims of the studies

Rhodomonas has been shown to be an excellent diet for copepods in aquaculture and the benefit of using *Rhodomonas* is that there is no need to supply with another microalgal diet to fulfill the nutritional requirement of copepods. However, *Rhodomonas* is a rather delicate genus which is prone to crash due to, e.g., excessive light, nutrient limitation, fluctuations of pH and temperature, and contamination. The Ph.D. project was therefore based on using *Rhodomonas* as a mono-algal diet and to study how the cultivation of this microalga can be optimized in order to obtain a successful large-scale production (Fig. 5). My focus was:

Paper 1: Conduct small-scale experiments to determine the effects on *Rhodomonas* of parameters relevant for a large-scale production in PBRs. This included modification of the growth medium, sedimentation and PUFA content of five strains of *Rhodomonas*, treatment of the water used for cultivation, and the growth rate of inocula at different initial densities.

Paper 2: Evaluate the application of commercial salts for cultivation of *Rhodomonas* and the copepod *A. tonsa* in order to extend the production to locations without access to seawater.

Paper 3: Conduct meso-scale experiments with *Rhodomonas* in a horizontal tubular PBR to determine the growth rate and production yield of densities of 5×10^5, 1×10^6, 1.5×10^6 and 2×10^6 cells ml^{-1}.

Paper 4: Compare the biochemical content of five closely related strains of *Rhodomonas* at temperatures relevant for cultivation of the copepod *A. tonsa* (15, 20 and 25 ˚C) to determine if there is an optimal temperature for

cultivation of *Rhodomonas* which results in a high growth rate, and content of PUFAs and free amino acids.

Additional studies:

Paper 5: Copepods can feed on heterotrophic protists such as the dinoflagellates *Oxyrrhis marina* and *Gymnodinium dominans* (Broglio et al. 2003, Parrish et al. 2012). The aim of this study was to evaluate the potential for using the heterotrophic dinoflagellate *Crypthecodinium cohnii* as a diet for the copepod *A. tonsa* compared to *R. salina*.

Paper 6: Develop a simple and fast method for extraction and quantification of the pigment phycoerythrin from *Rhodomonas* as the methods currently available contain several procedures and equipment unnecessary for cryptophytes.
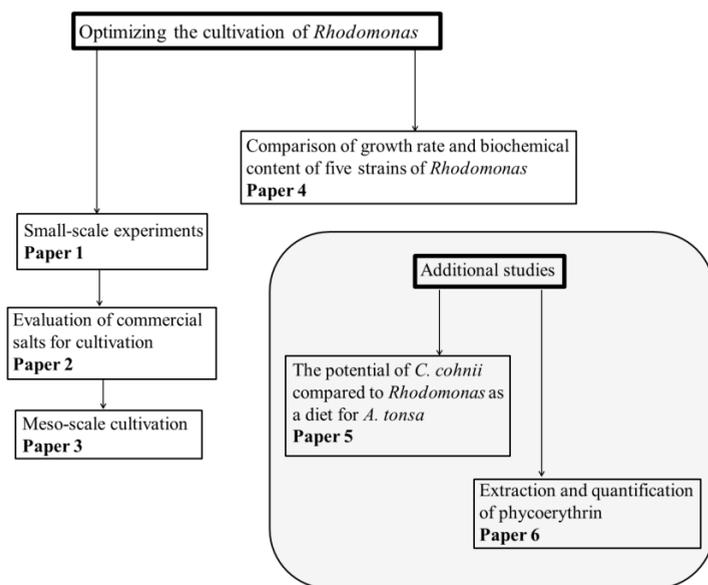
Figure 5. Overview and context of the studies included in the thesis on optimizing the cultivation of *Rhodomonas* for aquaculture.

General materials and methods

Maintenance of *Rhodomonas* stock cultures

A total of three species comprising five different strains were included in this thesis: *R. salina* (strains K-1487, K-0294 and LB 2763), *R. marina* (K-0435) and *R.* sp. (CCAP 995/5). The stock cultures were kept in conical flasks in a climate room at 17 ˚C at dim light intensity (15 µmol m^{-2} s^{-1}). The stock cultures were diluted regularly by transferring a few ml to new conical flasks and adding f/2 growth medium with seawater as the base. All materials, seawater and nutrient stock solutions were autoclaved prior to use to avoid contamination.

Small- and meso-scale experiments

For simplicity, the scales mentioned in this thesis are categorized as follows; small-scale <100 L, meso-scale: 100 to <500 L, and large-scale ≥500 L. The experiments in this thesis were primarily conducted at small-scale as this is a quick way to obtain an understanding of how various factors affect the growth rate and biochemical composition of microalgae. Meso- and large-scale experiments are extremely time consuming and unrealistic to use when the aim is to optimize the parameters for cultivation of microalgae within a short time frame. The results from small-scale experiments were implemented to the protocol for the commercial large-scale cultivation of *R. salina* in the COMA project. Results from small-scale experiments on the effect of parameters such as temperature, pH, salinity, modification of growth medium, and natural seawater (NSW) versus artificial seawater (ASW) mixed from commercial salts also apply to meso- and large-scale as this parameter is not affected by an upscaling of the systems. Contrary, the effect of parameters such as optimal cell density, light penetration and growth rate will differ between scales due to the dimensional changes during upscaling (e.g., tube

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diameter) resulting in different mixing and light penetration through glass flasks (small-scale) versus acrylic tubes (meso- and large-scale). Nonetheless, small-scale experiments studying these factors can provide guidance for the optimal cultivation at larger scales. Table 1 summarizes the scale, cultivation system and type of saltwater used in the experiments reported in the papers of this thesis.

For all experiments, *Rhodomonas* was studied during the exponential growth phase and cell concentration was determined either by enumeration on an electronic particle counter (Coulter Counter) or by optical density (OD) measured on a spectrophotometer. Analysis of fatty acids was conducted using gas chromatography-mass spectrometry (GC-MS) and analysis of free amino acids was conducted using high performance liquid chromatography (HPLC).

The small-scale experiments were conducted at Roskilde University. Cultivation flasks (balloons with aeration) or the table-sized PBR Multi-Cultivator MC1000 (Photon System Instruments, CZ) was used (Figs. 6 and 7). The table-sized PBR has eight aerated culture vessels (100 ml) each with a maximum culture volume of 85 ml and regulation of temperature and light intensity. This PBR is ideal for studying microalgae as the light can be adjusted for a variety of functions (e.g., continuous, blinking, different L:D cycles, increasing / decreasing intensity) while OD and temperature can be measured automatically and stored on an internal memory. The culture vessels are embedded in the same water tank and the temperature of the water is regulated via an external cooler (not show on figure 7). Due to the small volume of culture in the culture vessels and the high level of programming this system is ideal for quickly obtaining results.

Table 1. The scale of the experiments conducted including the type of cultivation system and type of seawater. NSW: natural seawater from Kattegat (DK) with a salinity of about 30-33. ASW: artificial seawater mixed from commercial salts with a salinity of about 27 to 33. The brands of the commercial salts are indicated by superscripts: Red Sea (RS), Red Sea - Coral Pro (CP) and Blue Treasure Salt (BT).

Paper no. and title	Scale	Cultivation (vol.)	Saltwater
1. Small-scale experiments aimed at optimization of large-scale production of the microalga *Rhodomonas salina*	Small	Table-sized PBR (8x 100 ml) Round bottom glass flasks (1 and 2 L) Glass beakers (250 ml)	SW and ASW[RS, CP, BT]
2. Effects of salinity, commercial salts and water types on cultivation of the cryptophyte microalgae *Rhodomonas salina* and the calanoid copepod *Acartia tonsa*	Small	Round bottom glass flasks (0.5 L)	ASW[RS, CP, BT]
3. The effect of cell density on yield of biomass and fatty acids during meso-scale cultivation of the cryptophyte *Rhodomonas salina*	Meso	Horizontal tubular PBR (250 L)	ASW[RS]
4. A comparison of five strains of the cryptophyte *Rhodomonas*: Temperature effects on growth rate and biochemical profile	Small	Table-sized PBR (8x 100 ml)	ASW[RS]
5. *Crypthecodinium cohnii*: a promising prey toward large-scale intensive rearing of the live feed copepod *Acartia tonsa* (Dana)	Small	Blue cap glass flasks (500 and 600 ml)	NSW
6. A simple and fast method for extraction and quantification of cryptophyte phycoerythrin	Small	Round bottom glass flasks (1 L)	NSW

Figure 6. 1 L balloon flasks used for cultivation for some of the small-scale experiments with *Rhodomonas salina*. The flasks are equipped with tubes for filtered aeration (0.2 µm).

Figure 7. The table-sized PBR (Multi-Cultivator MC1000) used for the majority of the small-scale experiments with *Rhodomonas*. The temperature in the water-filled chamber with the culture vessels (8x 100 ml) is adjustable, and the light behind each culture vessel can be individually programmed. (Photo: C. Thoisen)

The meso-scale experiments were conducted at AgroTech, the Danish Technological Institute (Taastrup, Denmark), in a horizontal tubular PBR of 250 L located in a walk-in climate chamber (Figs. 8 and 9). The total length of the tubes and bendings of the PBR was approximately 31 meters. Atmospheric air was pumped into the PBR from the lowest tube creating an upwards water flow through the tubes ensuring gas exchange and suspension of the algal cells. The pH of the culture was adjusted with CO_2 added through a valve connected to the air pump. If pH of the culture in the PBR increased above a given threshold, the CO_2 valve would open until the desired pH was reached. A more detailed description of the PBR is given in the materials and methods section in paper 3.

Figure 8. The tubular PBR (250 L) during a meso-scale experiment with *Rhodomonas salina*. (Photo: C. Thoisen)
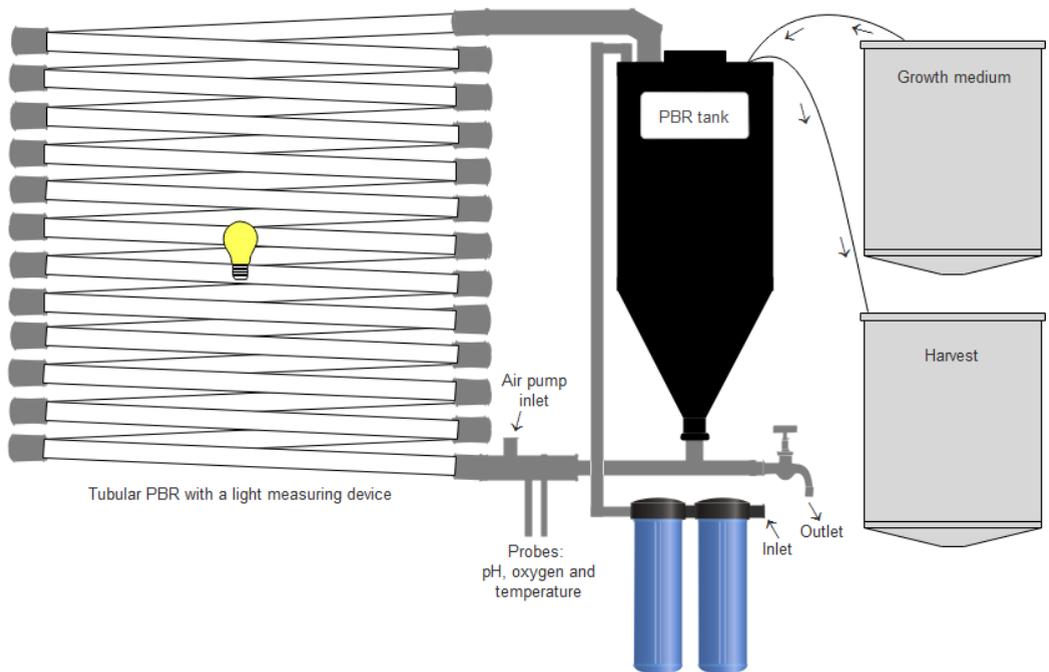
Figure 9. Illustration of the 250 L tubular PBR system used for meso-scale experiments with *Rhodomonas salina*. Data from the probes (pH, O_2, temperature), light measuring device, growth medium and harvest tanks (hence dilution rate) was continuously logged and saved on a computer. (Illustration: C. Thoisen)

Main findings

Paper 1

The cryptophyte *Rhodomonas* is an excellent diet for copepods which are used as live feed for fish larvae in the aquaculture. However, *Rhodomonas* is a delicate microalga and can therefore be difficult to cultivate if the expertise is not provided. The findings from this study are intended as tools to optimize the large-scale production of *R. salina* which will hopefully lead to an increased use of this microalga as a microalgal diet for copepods in aquaculture. The main findings are:

- The f/2 growth medium can be optimized by excluding $CoCl_2$ and adding NH_4^+ as the nitrogen source. $CoCl_2$ is problematic for a large-scale production as the European Union requires elaborated and detailed documentation for the use of this toxic compound. Our study shows that exclusion of $CoCl_2$ from the growth medium does not affect the growth rate or cell content of the PUFAs EPA, DHA and ARA. The growth rate of *R. salina* can be increased when adding NH_4^+ as the nitrogen source compared to NO_3^-.
- The temporal sedimentation of five strains of *Rhodomonas* showed that there is a difference and that strains K-1487 and K-0435 has the highest motility. It is proposed that perhaps biofouling of the PBR can be reduced by cultivating a strain with a low temporal sedimentation.

- A large-scale production of microalgae involves time consuming practical work which includes maintenance of inoculum to start production in the PBR. By addressing the growth rates of inocula with various initial densities, we highlighted the importance of adjusting the volume and density of the inoculum according to the desired production in order to reduce the work load associated with this step.

Paper 2

Jepsen P M, **Thoisen C**, Carron-Cabaret T, Pinyol-Gallemí A, Nielsen SL and Hansen BW (2018). Effects of salinity, commercial salts and water types on cultivation of the cryptophyte microalgae *Rhodomonas salina* and the calanoid copepod *Acartia tonsa*. Published February 8th 2018 in Journal of the World Aquaculture Society.

Land based aquaculture located far from the sea necessitates the use of commercial salt. Thus, it is highly relevant to study the effect of commercial salts on the cultivation of *R. salina* as well as the copepod *A. tonsa*. Commercial salts vary in composition as they are produced for specific aquarium types but the specific compositions of the salts are not available for the consumer. Thus, we studied three commercial salts widely available on the market to evaluate their potential for a large-scale cultivation of *R. salina* and *A. tonsa*. The commercial salts were mixed in either *d*H_2O or tap water and NSW was the control. In addition, we studied the effect of salinity on *R. salina* and *A. tonsa*. The main findings for *R. salina* were:

- Cultivation in NSW had an optimum salinity at 29.1 obtaining a growth rate of 1.2 d$^{-1.}$

- The highest growth rates were obtained at salinities of 20 and 30 in NSW (1.21 and 1.11 d^{-1}) and the commercial salt Red Sea (RS) (1.22 and 1.19 d^{-1}) mixed with dH$_2$O. However, all the commercial salts mixed with dH$_2$O yielded promising growth rates in the range 1.02 to 1.22 d^{-1}.

- The growth rate was lower when the commercial salts were mixed with tap water compared to dH$_2$O. Although no differences were observed in the ion composition analyzes, we speculate that unknown impurities of the tap water may have contributed to the lower growth rates observed.

Paper 3

Thoisen C, Pedersen JS, Jørgensen L, Kuehn A, Hansen BW and Nielsen SL. The effect of cell density on yield of biomass and fatty acids during meso-scale cultivation of the cryptophyte *Rhodomonas salina*. *In prep.*

Studies on cultivation of *Rhodomonas* at meso- or large-scale is not available in the literature. We therefore described the cultivation of *R. salina* in a 250 L PBR at densities of 1x10^6, 1.5x10^6 and 2x10^6 cells ml^{-1}. Results from paper 1 and 2 on the use of strain K-1487 with a high motility, exclusion of CoCl$_2$ from the f/2 growth medium, and application of commercial salts for cultivation were implemented. Data on cultivation parameters (light intensity, pH, and temperature) and the resulting dilution rates, yields and cell content of PUFAs are given. A description of this meso-scale cultivation of *R. salina* can potentially lead to an increased use of this microalga in aquaculture. The main findings were:

- The studied densities of 1×10^6, 1.5×10^6 and 2×10^6 cells ml^{-1} are suitable for a durable yield of biomass of *R. salina*. The highest dilution rate (hence growth rate) obtained was 0.52 d^{-1} at 1×10^6 cells ml^{-1}

- The light intensity at 1×10^6, 1.5×10^6 and 2×10^6 cells ml^{-1} was approximately 200 µmol m^{-2} s^{-1} and the amount of light received by cells in the culture was reduced with increasing density due to self-shading. It is therefore likely that the density of *R. salina* can be increased at higher light intensities.

- When transferring the microalgal diet to a tank with copepods it is desirable to have a high biomass of the microalga in a low volume of culture. Thus, densities of 1.5×10^6 and 2×10^6 cells ml^{-1} were most suitable as the volume and biomass was approximately 50 L d^{-1} with 8×10^{10} cells^{-1}.

Paper 4

Thoisen C, Hansen BW and Nielsen SL. A comparison of five strains of the cryptophyte *Rhodomonas*: Temperature effects on growth rate and biochemical profile. *In prep.*

The growth rate and biochemical profile of closely related strains of *Rhodomonas* were studied at temperatures of 15, 20 and 25 ˚C to evaluate their suitability for use as a microalgal diet in aquaculture. Although the strains are closely related they may have distinct biochemical profiles (Lang et al. 2011) and respond differently to an increase in temperature. The experimental temperatures of 15, 20 and 25 ˚C were chosen as they are within

the acceptable range for cultivation of *Rhodomonas* as well as the copepod *A. tonsa* (Peck et al. 2014). The main findings were:

- The five strains of *Rhodomonas* were similar with regards to CN content and cell length. Only strain CCAP 995/5 had a significantly longer cell length.

- The FA profiles of the strains revealed a high separation of strains CCAP 995/5 and K-0294 from the other strains.

- There was no consistent effect of temperature on the growth rate or content of FAs. Contrary to what was expected, the growth rate did not increase with temperature for the majority of the strains. Three of the five strains had a decreased content of EPA at the highest temperature (25 ˚C). The effect of temperature on the content of DHA in the strains was less noticeable. Interestingly, the only strain to not obtain a significantly higher content of ARA at 25 ˚C was K-0435.

- The effect of temperature on the total content of free amino acids (FAAs) was significant for strains LB 2763 and CCAP 995/5 but the effect was not similar with the highest content of FAA at 25 and 15 ˚C, respectively.

- The effect of temperature on the content of EAA (essential amino acids) was significant for three strains (K-0435, LB 2763 and CCAP 995/5) but the effect was not similar as the highest content in the stains was found at different temperatures. Interestingly, histidine (HIS) was found at very low concentrations or was absent or below detection limit at 15 ˚C for all the strains, except CCAP 995/5.

Paper 5

Jakobsen HH, **Thoisen C** and Hansen BW (2018). *Crypthecodinium cohnii*: a promising prey toward large-scale intensive rearing of the live feed copepod *Acartia tonsa* (Dana). Aquaculture International 26(1):237-251.

Although *Rhodomonas* is known to be an excellent diet for copepods other feed items are also suitable. This includes the heterotrophic dinoflagellate *Crypthecodinium cohnii*. The beneficial aspect of *C. cohnii* is cultivation without light at a densities as high as 100 g/L (de Swaaf et al. 2003) and high growth rates up to 3.4 d^{-1} (Tuttle and Loeblich 1975).

- Gross growth efficiency of the copepod *A. tonsa* when fed *C. cohnii* was about 22 % compared to > 36 % when feed *R. salina*.

- Egg hatching success of *A. tonsa* when fed *C. Cohnii* was 60 % compared to 89 % when fed *R. salina*.

- The cell content of C and N in *C. cohnii* and *R. salina* was not very different on a cell volume (μm^3) basis.

- There were major differences in the FA content as 64 % of the FAs were DHA in *C. cohnii* compared to 12 % in *R. salina*. The content of EPA was very low in *C. cohnii* (0.2 %) compared to 18 % in *R. salina*. Furthermore, the content of ARA was not abundant in *C. cohnii* (0.01 %) or *R. salina* (0.19 %), and seems in this short-term study to be less important for copepods compared to fish larvae. However, fish may be able to retain or *de novo* produce ARA.

- *R. salina* is superior to *C. cohnii* as a microalgal diet for *A. tonsa* but nonetheless *C. cohnii* is still an interesting alternative.

Paper 6

Thoisen C, Hansen BW and Nielsen SL (2017). A simple and fast method for extraction and quantification of cryptophyte phycoerythrin. MethodsX 4:209-213.

Rhodomonas contain the red pigment phycobiliprotein and numerous methods for the extraction of phycoerythrin are available. However, the methods in the literature are based on various species, e.g., species with a rigid cell wall, and often involve chemicals and equipment which are too comprehensive and time consuming for more simple purposes such as comparing the content of phycoerythrin between cultivation conditions or strains without a cell wall. Thus, the purpose of this study was to compare different methods of extraction using *R. salina* to find a method that is simple and fast for extraction and quantification of cryptophyte phycoerythrin. The main findings were:

- The best simple method was to filter *R. salina* onto a filter and store at -80 ˚C (24 h) until extraction with phosphate buffer at 4 ˚C (24 h). This method (treatment 5) had duration of two days and yielded the highest content of phycoerythrin (8.0 pg cell^{-1}).

- A more simple method (treatment 1) consisting of merely extraction (24 h) also resulted in good results, although the measured content of phycoerythrin was slightly lower (6.5 pg cell^{-1}) compared to treatment 5.

- Methods where storage at -80 ˚C is followed by lyophilization (24 h) (treatment 7 and 8) are not recommended, as the measured content of phycoerythrin was lowest in these treatments (5.1 and 4.3 pg cell^{-1}).

Status on the cultivation of *Rhodomonas salina* for aquaculture and future perspectives

At present, there are no available studies on the cultivation of *Rhodomonas* at meso- or large-scale. Hopefully, publication of the papers in this thesis will contribute to the expansion of the use of *Rhodomonas* as well as copepods in aquaculture as cultivation of these valuable organisms can be successful under the right conditions. The most important findings for the optimization of the cultivation of *Rhodomonas* for aquaculture were:

- exclusion of $CoCl_2$ from the f/2 growth medium (paper 1)
- application of commercial salts for cultivation (paper 2)
- a description of parameters resulting in successful cultivation and biomass yield in a meso-scale tubular PBR (250 L) (paper 3)
- the suitability of various strains of *Rhodomonas* as a microalgal diet for *A. tonsa* and their relatively steady growth rate in an interval from 15 to 25 ˚C (paper 4)

The COMA project has been successful and demonstrated that 8x 500 L PBRs with a concentration of *R. salina* at $1x10^6$ to $1.5x10^6$ cells ml^{-1} can provide sufficient diet for four copepod tanks (each 4,200 L) yielding a total production of $136x10^6$ copepod eggs d^{-1} (Højgaard, pers. com.). This production could provide sufficient live feed for approximately $1x10^6$ fish larvae (Jepsen, pers. com.).

Future research should focus on identifying the optimal light intensity for cultivation of different densities of *Rhodomonas* in the tubular PBRs as the density and growth rate most likely can be increased compared to that obtained in paper 3. Also factors such as nutrient level and smaller tube diameter will be relevant to evaluate in order to increase productivity of *R.*

salina. Another concern is to provide a microalgal diet for the newly hatched *A. tonsa* nauplii in the fish tank as they can only survive a few days without food. A solution could be to provide the microalgal diet as an algae paste although studies show that live microalgae are the most optimal diet for copepods (Milione and Zeng 2007, Rayner and Hansen *Submitted*). A recent study on *I. galbana* paste as feed resulted in low growth of *A. tonsa* due to high mortality of the nauplii compared to when offering live *I. galbana* (Rayner and Hansen *Submitted*).

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Paper 1

<u>Title</u>

Small-scale experiments aimed at optimization of large-scale production of

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Small-scale experiments aimed at optimization of large-scale production of the microalga Rhodomonas salina

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Abstract
The cryptophyte Rhodomonas is an important feed item for live feed organisms in aquaculture and although large-scale culti-vation of Rhodomonas in photobioreactors (PBRs) is feasible, the production needs to be optimized through further studies of specific factors. Through small-scale experiments, several factors relevant for an on-going large-scale production of Rhodomonas were studied and the results presented here provide a useful insight on factors that can help future large-scale production. The content of polyunsaturated fatty acids (PUFAs) and the temporal sedimentation was compared in five strains of Rhodomonas. Strain K-1487 (R. salina) was chosen as the most suitable for cultivation in PBRs due to a good biochemical content of PUFAs and low cell sedimentation. The f/2 growth medium used for cultivation was modified by excluding $CoCl_2$ which did not affect either growth rate or cell content of the PUFAs, DHA, EPA, and ARA. Furthermore, the growth medium was modified by adding the nitrogen source as ammonium (NH_4^+), nitrate (NO_3^-), urea, or combinations of these, with NH_4^+ yielding a significantly higher growth rate of 1.30 ± 0.07 day^{-1}. The seawater used for cultivation was exposed to three types of treatments which gave no significant difference in the growth rate: (1) filtration (0.2 μm) + autoclaving, (2) filtration (0.2 μm) + UV-radiation, and (3) filtration (0.2 μm). Finally, the results for growth rates of inocula at initial densities ranging from 2000 to 200,000 cells mL^{-1} showed that growth rate decreased with increasing density but a final density of 10^6 cells mL^{-1} was obtained fastest with the highest initial density. With the present findings, several barriers for effective cultivation of Rhodomonas are solved and future large-scale production has become a great step closer.

Keywords Seawater · Fatty acids · Growth medium modification · Cell density · Seawater treatment · Sedimentation · Initial density

Introduction

In marine aquaculture, microalgae are used as feed for larvae and benthic stages of filter feeders (Tremblay et al. 2007; Fernández-Reiriz et al. 2015) as well as for pelagic live feed organisms such as copepods, rotifers, and brine shrimp (McKinnon et al. 2003; Srivastava et al. 2006; Seixas et al. 2009). The microalgal cryptophyte Rhodomonas improves the survival, growth, lipid

content, and reproduction of brine shrimp, copepods, and scallop larvae (McKinnon et al. 2003; Knuckey et al. 2005; Tremblay et al. 2007; Seixas et al. 2009; Ohs et al. 2010; Zhang et al. 2013), and contain the essential polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (EPA, 20:5ω 3), docosahexaenoic acid (DHA, 22:6ω 3), and arachidonic acid (ARA, 20:4ω 6) in ratios optimal for aquaculture organisms (Guevara et al. 2016; Vu et al. 2016; Jakobsen et al. 2018). These PUFAs are essential for the survival and development of fish larvae (Bell and Sargent 2003; Sargent et al. 1997; Sargent et al. 1999) and are transferred to the fish larvae through the live feed.

The existing literature on Rhodomonas primarily discuss the nutritional value of the microalga as a diet for live feed organisms in aquaculture based on its biochemical composition with the majority focusing on copepods (e.g., Støttrup et al. 1999; McKinnon et al. 2003; Knuckey et al. 2005; Drillet et al. 2006; Seixas et al. 2009; Ohs et al. 2010; de Lima et al. 2013; Zhang

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et al. 2013, Arndt and Summer 2014; Jakobsen et al. 2018). The biochemical composition of *Rhodomonas* has also been studied at different temperatures (Renaud et al. 2002), irradiances, and nutrient levels (Guevara et al. 2016; Vu et al. 2016), at different growth phases (Boelen et al. 2017), and when cultivated in various growth media (Valenzuela-Espinoza et al. 2005; Huerlimann et al. 2010). In addition, the content of the pigment phycoerythrin has been studied at different temperatures (Chaloub et al. 2015), irradiances (Bartual et al. 2002; Chaloub et al. 2015; Vu et al. 2016), and nutrient levels (Eriksen and Iversen 1995; Chaloub et al. 2015; Vu et al. 2016). A recent small-scale study by Jepsen et al. (2018) evaluated the effect of salinity and different commercial salts on *Rhodomonas salina* and the copepod *Acartia tonsa* with positive outcomes for large-scale cultivation located without access to seawater. Aside from Jepsen et al. (2018), studies specifically regarding a meso- or large-scale production of *R. salina*, or optimization hereof, are not found in the literature. The aim was therefore to study factors acting as barriers for large-scale production of *Rhodomonas* as a microalgal diet for live feed organisms in aquaculture. This motivated us to focus on (1) the necessity of $CoCl_2$ (cobalt(II) chloride) in the f/2 growth medium, (2) the content of PUFAs in five strains of *Rhodomonas* to identify the most suitable strain, (3) the temporal sedimentation of the five strains of *Rhodomonas* to identify the one with the lowest sedimentation rate which could potentially reduce biofouling of the PBR, (4) the effect on growth rate by adding nitrogen as different sources to the growth medium, (5) the effect of different types of seawater treatment on the growth rate, and finally (6) the growth rate of different initial inoculum densities.

Large-scale cultivation of microalgae in PBRs is extremely time and labor consuming to conduct and therefore small-scale experiments were conducted to study the various factors and obtain useful results within a short period. It can be problematic to transfer certain results from small- to large-scale systems as there is a dimensional factor hindering an exact scale-up. Nonetheless, factors such as nutrient requirement, commercial salts, and treatment of seawater are restricted to the organism and results regarding these factors can therefore be transferred directly from small to large scale. Contrary, the specific growth rate of initial cell densities will most likely be affected between scales but it still provides a guidance to estimate the size and density of the inoculum for a desired production. In the following sections, each studied factor is introduced to clarify why these factors are important to study when pursuing an ambition of optimizing the large-scale production of *Rhodomonas*.

Successful meso- and large-scale cultivation of *R. salina* in tubular and vertical PBRs has taken place the last 4 years at Roskilde University and its project partners. One of the limiting factors for cultivation is the need for cleaning of the PBR at a regular frequency due to biofouling. During cleaning, the

PBR is shut down which is an economic loss for the production. The period for cultivation could potentially be prolonged by substituting with a *Rhodomonas* strain with a lower rate of cell sedimentation (i.e., high motility) and thereby reduce the tendency of biofouling. Furthermore, since closely related species and strains of a given microalga are known to have deviating biochemical compositions, it is important to compare the PUFA content between strains of *Rhodomonas* to identify the most suitable strain as a microalgal diet for the aquaculture (Lang et al. 2011; Guevara et al. 2016).

The cultivation of microalgae necessitates growth media, and numerous recipes are available and generally target a broad range of species (Harrison et al. 1980; Keller et al. 1987). The growth medium is therefore likely to contain unnecessary or excessive amounts of certain components for cultivation of specific species. To our knowledge, there is no growth medium specifically defined according to the nutrient requirements for *Rhodomonas*. The nitrogen source can be added as ammonium (NH_4^+), nitrate (NO_3^-), or urea and the preferred source is species-specific (e.g., Giordano 1997; Lourenço et al. 2002). The increased growth rate of some microalgal species obtained when cultivated on NH_4^+ (Giordano 1997) is assumed to be coupled to the lower demand of reductants for assimilation (Dortch 1990). Growth media also contain different trace metals but the requirement of various trace metals is species-specific and some microalgal species can substitute a given trace metal with another (e.g., Timmermans et al. 2001; Xu et al. 2007). The compound $CoCl_2$ in growth media is problematic for a large-scale production as it is widely recognized as a toxic substance. Exposure limits, as well as limits for tolerated daily intake (TDI), have been established by both the European Chemicals Agency (ECHA) in the European Union as well as by the National Institute of Occupational Safety and Health (NIOSH) in the United States of America. In particular, the European Union guidelines involve producing elaborated and detailed documentation for the use of $CoCl_2$. Producing this necessary documentation is both manpower requiring and time consuming and since some microalgal species are able to substitute cobalt (Co) with another trace metal, or simply does not require Co, it is relevant to study if $CoCl_2$ can be excluded from the large-scale production of *Rhodomonas* with no consequences for the yield.

During cultivation of microalgae, it is essential that unwanted organisms are not introduced to the culture. The treatments applied to eliminate unwanted organisms at small-scale (≤ 20 L) are filtration and autoclaving (e.g., Lourenço et al. 2002; Knuckey et al. 2005; de Lima et al. 2013; Arndt and Sommer 2014; Vu et al. 2016) while larger volumes generally are treated by filtration and UV radiation (e.g., Summerfelt 2003; Bamba et al. 2014). Common for these types of treatments is no addition of chemicals or production of toxic residues that may negatively affect the microalgae (Rhodes et al.

2008). However, filtration does not sterilize as small bacteria and certainly viruses can pass through depending on the pore size of the filter material. Autoclaving is an effective sterilization method although it can raise pH of seawater and cause precipitation of nutrients (Jones 1967; Filip and Middlebrooks 1975). This, however, can easily be overcome by controlling pH during cultivation and adding sterilized nutrients post autoclaving. Nevertheless, autoclavation is unrealistic in large-scale productions and UV radiation is widely used in, e.g., the aquaculture, where pre-filtration is crucial for optimal effectiveness (Summerfelt 2003). The small-scale experiments in the present study use autoclaved seawater to define the optimal cultivation conditions of *Rhodomonas* and a comparison of the seawater treatments used at the different scales is therefore necessary for detecting possible effects of a given treatment of the seawater used for cultivation of *Rhodomonas*.

The size and density of inocula used to initiate microalgae cultures are important, especially for a large-scale production. A common rule of thumb is that the inoculum for a new culture should be minimum 10% (*v/v*) of the original culture. However, there are, to our knowledge, no studies explaining or confirming the validity of this rule, and it most likely depends on the species and the purpose of the cultivation. Contrary, the cell density of a culture affects the growth rate as, e.g., self-shading may reduce growth at higher densities. For a large-scale cultivation, it is relevant to study the growth rate of inocula at various initial cell densities to estimate when a given biomass for production is reached. Furthermore, it is time consuming to maintain a large volume of inocula cultures for a large-scale production and this can be reduced by merely maintaining the specific volume of inoculum necessary for the production.

Materials and methods

Algal strains and general culture conditions

Five species/strains of *Rhodomonas* were obtained from culture collections and are referred to their respective strain identity (Table 1). The strains were cultivated in natural seawater (NSW) with a salinity of 30–35 collected from > 30 m depth in the Kattegat (DK) and filtered through a series of filters (terminal pore size of 0.2 μm). Equipment, NSW, and growth medium stock solutions were autoclaved (15 min at 125 °C) prior to use. Irradiance was continuous (24:0 light/dark cycle) and measured with a Hansatech Instruments LTD Quantitherm light meter QRT1 (see below for specific irradiance in the separate experiments). The f/2 growth medium (without addition of silicate) was used for cultivation (Guillard and Ryther 1962; Guillard 1975), except in experiment 5 "Seawater treatment." Cell concentration was enumerated on a Coulter Counter (Beckman) using the

computer program Multisizer 3, except experiment 4 "Nitrogen source" and experiment 6 "Initial density" (see the respective experimental sections below). Growth rates were calculated by fit of exponential growth functions on either cell concentration or optical density (OD) over time.

Experiment 1: CoCl$_2$

Strain K-1487 was cultivated in two versions of f/2 growth medium: a regular version and a version without addition of CoCl$_2$ (this strain is referred to as K-1487*). Cultivation took place in a small-scale PBR (Multi-Cultivator MC1000, Photon System Instruments, Czech Republic) with eight test tubes (each 85 mL) and aeration at 20 °C and irradiance of 85 μmol photons m^{-2} s^{-1} ($n = 4$). The experimental period was 5 days with a start concentration of $64,460 \pm 3234$ cells mL^{-1}. Samples for fatty acids were taken on day 6 and analyzed as described in "Experiment 2: Fatty acids" section. Nutrients were added daily.

Experiment 2: fatty acids

Samples for comparing the fatty acid composition between the *Rhodomonas* strains were taken from exponentially growing semi-batch cultures in 1 L round-bottom flasks with aeration at 17 °C and irradiance of approximately 13 μmol photons m^{-2} s^{-1} ($n = 3$). The low irradiance was chosen to maintain reduced, but still exponential, growth rates in these cultures. The cells were filtered onto 0.2 μm glass microfiber filters (Whatman GF/C) and stored at − 80 °C until analyzed according to Drillet et al. (2006) with minor adjustments: addition of 20 μL internal standard (C23-methylester, 1000 μg mL^{-1}) and no sonication.

Experiment 3: temporal sedimentation

Each *Rhodomonas* strain was transferred to individual 250 mL beakers ($n = 5$) at a cell concentration of $418,423 \pm 28,400$ cells mL^{-1}, except CCAP 995/5 at $99,500 \pm 3536$ cells mL^{-1}. Replicates were left undisturbed at room temperature in the stagnant water and samples for cell enumeration were withdrawn 1 cm below the water surface after intervals of 1 and 6 h.

Experiment 4: nitrogen source

The nitrogen source in the f/2 growth medium was changed from NO$_3^-$ (nitrate) to NH$_4^+$ (ammonium) and CO(NH$_2$)$_2$ (urea), and combinations of these with an equimolar amount of N in all treatments (Table 2). Cultivation of strain K-1487* took place in the small-scale PBR (described in "Experiment 1: CoCl$_2$" section) at 20 °C and irradiance of 100 μmol photons m^{-2} s^{-1} ($n = 4$). Start concentration was $141,500 \pm 18,742$

Table 1 The studied strains of *Rhodomonas* obtained from various culture collections

Species	Strain	Culture collection
R. salina	K-1487	Scandinavian Culture Collection of Algae & Protozoa (SCCAP)
R. salina	K-0294	Scandinavian Culture Collection of Algae & Protozoa (SCCAP)
R. salina	LB 2763	The University of Texas at Austin (UTEX)
R. marina	K-0435	Scandinavian Culture Collection of Algae & Protozoa (SCCAP)
R. sp.	CCAP 995/5	Culture Collection of Algae and Protozoa (CCAP)

cells mL^{-1} and determined by a build-in OD measuring device measuring automatically every 30 min for 30 h. An equation between cell concentration (Coulter Counter - Beckman) and absorbance (Spectrophotometer - Genesys 6, Thermo Scientific) was obtained by linear regression to estimate the cell concentration by OD:

$$Cell\ concentration\ \left(mL^{-1}\right) = \left(\frac{abs_{550\ nm} - 0.0026}{0.0002}\right) *100 \quad (1)$$

where $abs_{550\ nm}$ is the absorbance of the sample at 550 nm.

Experiment 5: seawater treatment

Strain K-1487 was cultivated in filtered (0.2 μm) NSW exposed to autoclavation or UV radiation. For comparison, the growth rate of K-1487 in filtered NSW without further treatment was included. Cultivation took place in aerated 2 L round-bottom flasks with B1 growth medium added at experimental start and irradiance of 67 ± 7 μmol photons m^{-2} s^{-1} at 20 °C ($n = 4$). The experimental period was 4 days with a start concentration of $169,500 \pm 2500$ cells mL^{-1}. Cell concentrations were determined by OD as described in "Experiment 4: Nitrogen source" section.

Experiment 6: initial density

Strain K-1487 was inoculated at increasing initial densities from 2000 to 200,000 cells mL^{-1} in aerated 1 L round-bottom flasks at 18.5 ± 0.7 °C and irradiance of 103 μmol photons m^{-2} s^{-1} ($n = 3$). The specific initial densities were 2000 ± 0, 7000 ± 0, 9500 ± 4183, $43,667 \pm 2582$, $104,500 \pm 7583$ and $196,167 \pm 4916$ cells mL^{-1}. Nutrients were added at experimental start. Cell concentration during the exponential

growth phase was determined daily by OD as described in "Experiment 4: Nitrogen source" section. The cell concentration (N) over time was plotted as $\ln(N/N_0)$ where N_0 is the cell concentration at the experimental start. Data were fitted to the modified Gompertz equation described in Zwietering et al. (1990):

$$y = A exp\left(-exp\left(\frac{\mu_m e}{A}(\lambda - t) + 1\right)\right) \quad (2)$$

The maximum specific growth rate (μ_m) and lag time (λ) can be calculated from the parameters (a, b, c) obtained from the fit:

$$\mu_m = \frac{a*c}{e} \quad (3)$$

$$\lambda = \frac{b-1}{c} \quad (4)$$

Statistical analysis

Data on the cell content of fatty acids, growth rate on nitrogen sources, different seawater treatments, and different initial cell densities were subjected to one-way ANOVAs. Significant results were followed by a Holm-Sidak post hoc test to compare individual means across significantly different levels.

Data on temporal sedimentation was recorded as percentage of cells remaining in the upper 1 cm of the water column after 1 and 6 h and logit-transformed (Sokal and Rohlf 1995) prior to analysis with one-way ANOVA followed by a Holm-Sidak post hoc test to compare individual means across significantly different levels.

Table 2 The concentration (g L^{-1}) of the specific nitrogen sources in the modified f/2 stock solutions and the volume (mL L^{-1}) of each stock solution added to 1 L seawater for cultivation of K-1487. All N-sources had an equimolar amount of N

Nitrogen source	Stock solution (g L^{-1})	Growth medium (mL L^{-1})	N (mole)
NO_3^- (NaNO)	12.4	1	0.8826
NH_4^+ (NH$_4$Cl)	47.2	1	0.8826
Urea (CO(NH$_2$)$_2$)	26.5	1	0.8826
NH_4^+ + NO_3^-		0.5 + 0.5	0.8826
Urea + NO_3^-		0.5 + 0.5	0.8826
Urea + NH_4^+		0.5 + 0.5	0.8826

Data on the growth rate in f/2 growth medium with and without $CoCl_2$ was subjected to a two-tailed t test.

Prior to ANOVAs and t tests, data were tested for constant variance (Spearman's rank correlation) and normality (Shapiro-Wilk test). All tests were carried out using SigmaPlot 12.0 (Systat Software) with $\alpha = 0.05$.

Results

$CoCl_2$

The growth rate of strain K-1487 in the two treatments (with and without $CoCl_2$ added to the f/2 growth medium) was not statistically significant at an average of 0.69 ± 0.04 day^{-1} ($p = 0.765$) (Fig. 1). Likewise, the cell content of the PUFAs DHA, EPA, and ARA was not statistically significant different between the two treatments with averages of 2.6 ± 0.1, 3.9 ± 0.4, and 0.2 ± 0.1 pg cell^{-1}, respectively (one-way ANOVA, $p \leq 0.453$).

Fatty acids

The cell content of the PUFAs DHA, EPA, and ARA was compared in the five strains of *Rhodomonas* (Fig. 2). The content of EPA and ARA was not statistically different between strains ranging from 1.9 ± 0.3 to 3.1 ± 0.1 pg EPA cell^{-1} and 0.07 ± 0.05 to 0.22 ± 0.05 pg ARA cell^{-1} (one-way ANOVA, EPA; $p = 0.267$ ARA; $p = 0.156$). However, ARA was either not present or below detection limit in CCAP 995/5. The content of DHA was statistically significant higher in CCAP 995/5 with 4.0 ± 0.1 pg cell^{-1} (one-way ANOVA, $p \leq 0.018$). The cell content of EPA was higher than DHA in all strains, except CCAP 995/5 where the opposite was observed.

The highest ratios of DHA/EPA were 1.3 and 1.0 for CCAP 995/5 and K-1487*, respectively (Table 3). The remaining strains had similar DHA/EPA ratios of 0.7 and 0.8. For EPA/ARA, the highest ratios were 5.1 and 4.4 for K-1487 and K-0435, respectively.

Temporal sedimentation

The cell sedimentation of the *Rhodomonas* strains was measured after 1 and 6 h to identify the strain with the lowest sedimentation. The cell density (%) in the upper 1 cm water column of undisturbed seawater was significantly different between the *Rhodomonas* strains at the given time intervals (Fig. 3). After 1 h, the cell density of K-1487, K-1487*, and K-0435 was statistically highest with > 80% of the cells remaining in the water column. After 6 h, the cell density was still highest for K-1487 and K-1487* with 54 ± 2 and $63 \pm 3\%$, respectively (one-way ANOVA, 1 h; $p < 0.001$, 6 h; $p < 0.001$). Merely the two significantly highest groups (A, a and B, b) at each given time interval are considered here.

Nitrogen source

The effect of the nitrogen source on the growth rate of strain K-1487* was studied by adding NO_3^-, NH_4^+, urea, or combinations of these (Fig. 4). Cultivation with NH_4^+ as the nitrogen source yielded a significantly higher growth rate of 1.3 ± 0.07 day^{-1} compared to the growth rate for NO_3^- of 1.0 ± 0.08 day^{-1} (one-way ANOVA, $p = 0.046$). Contrary, urea and combinations of the nitrogen sources gave no statistically significant difference in growth rate compared to both NO_3^- and NH_4^+.

Fig. 1 The growth rate (day^{-1}, striped bars) and cell content (pg) of the PUFAs DHA (black bars), EPA (light gray bars), and ARA (dark gray bars) of K-1487 cultivated with and without $CoCl_2$ added to the f/2 growth medium. Mean values ± S.D. ($n = 4$)
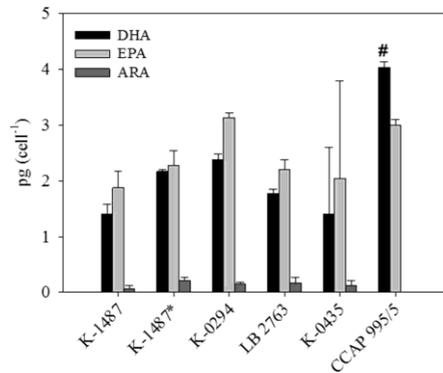
Fig. 2 The cell content (pg) of DHA (black bars), EPA (light gray bars), and ARA (dark gray bars) in the *Rhodomonas* strains. Symbol (#) indicates a statistical difference. ARA was not present or below detection limit in CCAP 995/5. Mean values ± S.D. ($n = 3$)

Paper 1

Table 3 The cell content of DHA, EPA and ARA in the *Rhodomonas* strains expressed as % of TFA, and the ratios of DHA/EPA and EPA/ARA. ARA was not present or below detection limit in CCAP 995/5. Mean values ± S.D. ($n = 3$)

Strain	DHA	EPA	ARA	DHA/EPA	EPA/ARA
K-1487	8.0 ± 0.8	10.7 ± 1.6	2.1 ± 1.8	0.7	5.1
K-1487*	8.3 ± 1.1	8.6 ± 0.6	4.2 ± 0.5	1.0	2.0
K-0294	7.4 ± 0.2	9.8 ± 0.4	2.7 ± 0.4	0.7	3.6
LB 2763	10.1 ± 1.4	12.4 ± 1.1	4.6 ± 2.1	0.8	2.7
K-0435	7.3 ± 0.4	10.6 ± 0.1	2.4 ± 1.3	0.7	4.4
CCAP 995/5	13.9 ± 0.6	10.3 ± 0.3	−	1.3	−

Seawater treatment

The growth rate of K-1487 cultivated in NSW treated with (1) filtration (0.2 μm) + autoclaving, (2) filtration (0.2 μm) + UV radiation, and (3) filtration (0.2 μm) was compared. The results show that there were no significant differences on growth rates in the treatments with an average of 0.7 ± 0.1 day^{-1} (one-way ANOVA, $p = 0.833$). However, cultivation in simply filtered NSW became contaminated after ~ 1 week of cultivation with an unidentified nanoflagellate (personal observations) limiting the period of cultivation.

Initial density

The temporal cell concentration of initial densities of K-1487* in the range of 2000 to 200,000 cells mL^{-1} was fitted to the modified Gompertz equation (Zwietering et al. 1990) to calculate the exponential growth rate (Figs. 5 and 6). The initial density of 2000 and 7000 cells mL^{-1} obtained the highest growth rates at 1.4 day^{-1}. A trend was observed with the growth rate gradually decreasing with increasing initial density to 0.7 day^{-1} at 200,000 cells mL^{-1}. The lag time was calculated to be shorter than the sampling interval for cell enumeration and was therefore not considered further.

The time required for the initial densities to reach a biomass of 10^6 cells mL^{-1} successively decreased with increasing initial density; 6.8 days for 2000 cells mL^{-1} and 2.8 days for 200,000 cells mL^{-1} (Table 4). However, initial densities of 40,000 and 100,000 cells mL^{-1} both reached 10^6 cells mL^{-1} after just 3.8 days.

Discussion

The findings in this study represent a step toward a broader implementation of *Rhodomonas* as a microalgal diet for live feed organisms in aquaculture as relevant practicalities for large-scale production are sought clarified.

Various growth media recipes are available but, e.g., the specific trace metal requirement of phytoplankton varies and it depends on the species if a specific trace metal can be substituted with another trace metal. Examples on Co from the literature showing this species specificity are the coccolithophore *Emiliania huxleyi* substitute Co and Zn (zinc) with each other (Xu et al. 2007), the diatoms *Thalassiosira pseudonana* and *T. ocenica* largely substitute
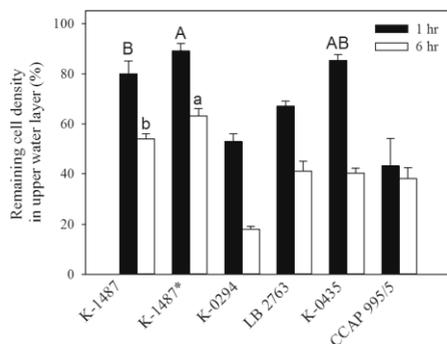
Fig. 3 The cell density (%) of the *Rhodomonas* strains in the upper 1 cm water column after 1 (black bars) and 6 h (white bars) in undisturbed water. Letters A and B indicate the two statistically significant groups at each given time interval with the highest percentage of cells remaining (1 h, uppercase; 6 h, lowercase). Statistically significant differences at lower densities are not indicated. Mean values ± S.D. ($n = 5$)
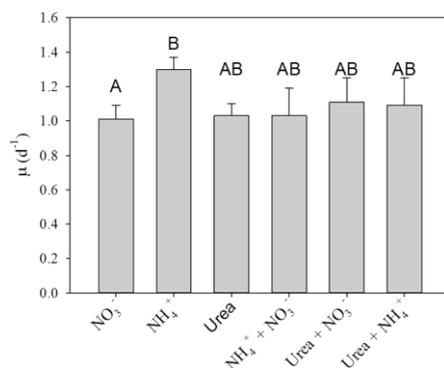
Fig. 4 The growth rate (day^{-1}) of K-1487* cultivated with the nitrogen sources NO$_3^-$, NH$_4^+$, urea, and combinations of these (1:1). Letters (A, B) indicate statistically significant groups. Mean values ± S.D. ($n = 4$)
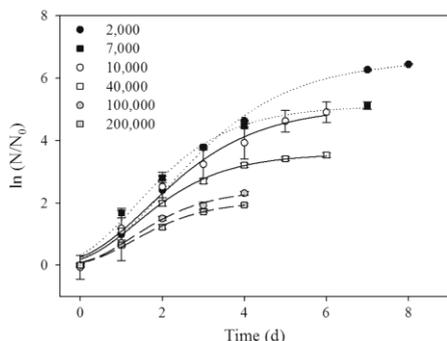
Fig. 5 The temporal cell concentration ($\ln(N/N_0)$) of different initial densities (cells mL^{-1}) of K-1487* fitted to the modified Gompertz equation. Mean values ± S.D. ($n = 3$)

Zn with Co (Sunda and Huntsman 1995; Yee and Morel 1996), the prymnesiophyte *Phaeocystis antarctica* substitute Zn with Co although Zn is preferred (Saito and Goepfert 2008), the diatom *Chaetoceros calcitrans* lack a substitution of Zn with Co (Timmermans et al. 2001), and the cyanobacteria *Synechococcus bacillaris* and *Prochlorococcus* require Co for growth (Sunda and Huntsman 1995; Saito et al. 2002). Studies on the trace metal requirements of *Rhodomonas* are lacking and our study did not seek to clarify the requirement of all the trace metals in the f/2 growth medium. Nevertheless, it is a key finding for a large-scale production of *Rhodomonas* that exclusion of CoCl$_2$ from the growth medium does not affect neither the growth rate nor the cell content of DHA, EPA, and ARA as these parameters are essential for aquaculture. Large quantities
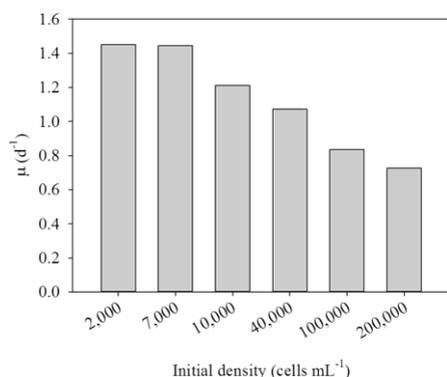
Fig. 6 The growth rates (day^{-1}, ●) of different initial densities of K-1487* calculated with parameters from the fit to the modified Gompertz equation

of growth medium are prepared during a large-scale production of microalgae and exclusion of CoCl$_2$ will ease production by bypassing the required elaborated and detailed documentation required by, e.g., ECHA and NIOSH. However, it must be highlighted that NSW contain small amounts of Co (0.00005 μm kg^{-1} according to Atkinson and Bingman 1997). Thus, either strain K-1487* can substitute Co with another trace metal, or there is an adequate amount of Co present in NSW.

A further modification of the f/2 growth medium was the addition of various nitrogen sources. The highest growth rate of strain K-1487* was obtained with NH$_4^+$ as the nitrogen source and similar to the results reported by Lewitus and Caron (1990) for *Pyrenomonas* (now *Rhodomonas*) *salina* with a growth rate at 1.2 day^{-1} (135 μmol, 21 °C). However, Lourenço et al. (1997) reported that the cryptophyte *Hillea* sp. could not grow on NH$_4^+$ unless reduced to a concentration equal to half of that used in the present study. The lower demand of reductants for assimilation of NH$_4^+$ is a plausible explanation for the increase in growth rate observed in the present experiment (Dortch 1990). However, studies have shown that the biochemical content of microalgae may be altered when supplied with different nitrogen sources and future studies must clarify if NH$_4^+$ alters the biochemical profile (in particular the PUFAs) of *Rhodomonas* (Fidalgo et al. 1998; Lourenço et al. 2002). Providing the nitrogen source in the form of NH$_4^+$ may cause an acidification of the culture as NH$_4^+$ is taken up by the microalgae in the form of NH$_3$, leaving a proton in the medium. However, when the cell concentration of microalgae increases during cultivation, the photosynthetic activity raises pH. In this experiment, pH was not controlled or adjusted but the effect of acidification is assumed to be minor, as seawater is generally well buffered due to its high content of carbonates (Goldman et al. 1982), and the cells in our experiment grew exponentially during the experimental period indicating no negative effect of pH. It must be stressed that the positive effect on growth rate of providing NH$_4^+$ as the nitrogen source obviously is larger than any negative effects of growth medium acidification on growth rate. In many plant and algal growth media (but not in the f/2 growth medium), the nitrogen source is provided as both NH$_4^+$ and NO$_3^-$ because the acidifying effect of NH$_4^+$ uptake counters the alkalizing effect of NO$_3^-$ uptake (Asher and Edwards 1983). In the present study, however, providing NH$_4^+$ as the only nitrogen source evidently gave the highest growth rate despite any effects of NH$_4^+$-uptake on pH or of pH on the NH$_3$/NH$_4^+$ equilibrium. When producing microalgae at large scale, pH is usually controlled in a feedback system by CO$_2$ addition as pH in the photobioreactor will increase during microalgal growth due to the photosynthetic uptake of CO$_2$ and HCO$_3^-$.

The microalgal diet has been shown to affect the composition of fatty acids in copepods (Støttrup et al. 1999; Caramujo et al. 2008; de Lima et al. 2013) and particularly *Rhodomonas*

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Table 4 The time (day) for the different initial densities of K-1487* to reach a density of 10^6 cells mL^{-1}

Initial density (cells mL^{-1})	2000	7000	10,000	40,000	100,000	200,000
Time (day)	6.8	6.0	5.3	3.8	3.8	2.8

is praised as an excellent diet for the copepod *Acartia* by improving the nauplii survival, development rate, and reproduction (Knuckey et al. 2005; Zhang et al. 2013; Arndt and Sommer 2014). While a DHA/EPA/ARA ratio of 10:5:1 is considered optimal for some marine fish larvae (Sargent et al. 1999), studies on the specific nutritional requirement of PUFAs in copepods are limited (see references in Camus and Zeng 2010), and some species, e.g., *Pseudodiaptomus annandalei*, *Tisbe furcata*, and *Nitokra lacustris*, may de novo synthesize certain fatty acids (Parrish et al. 2012; Raynar et al. 2015). A short-term study (96 h) by Jakobsen et al. (2018) indicates that ARA is less important for *Acartia tonsa* (Dana) as similar reproductive rates were obtained on a diet of *R. salina* (K-1487) compared to a diet of the heterotrophic dinoflagellate *Crypthecodinium cohnii* with cell contents of ARA at 0.19 and 0.01% TFA, respectively. However, it is most likely not the case in long-term growth studies with the copepod. All of our studied *Rhodomonas* strains, except CCAP 995/5, are suitable as a microalgal diet for *A. tonsa* but to supply fish larvae with the essential ARA through the live feed (i.e., *A. tonsa*) a *Rhodomonas* strain with high ARA content must be offered as the microalgal diet. This excludes strain CCAP 995/5 unless ARA is supplied from another source. However, this would include another factor in the production line which is undesirable. The cell content of PUFAs in the strains in the present study can likely be increased and result in an improved nutritional value of *Rhodomonas* as a microalgal diet for live feed organisms as studies have reported an effect on the content of PUFAs in *Rhodomonas* when changing the temperature, light intensity, and nutrient level (Renaud et al. 2002; Guevara et al. 2016; Vu et al. 2016).

The strains exhibited different temporal sedimentation and strains K-1487 and K-1487* were identified as most suitable for cultivation in PBRs due to a low sedimentation. However, this study did not find an explanation for the low sedimentation in these two strains compared to the other strains. Data analysis on cell length (data not presented), total fatty acid content (data not presented), and bio volume (data not presented) showed no correlation with temporal sedimentation. The cultivation of a strain with a low sedimentation is expected to reduce the inevitable biofouling and necessary cleaning frequency of large-scale PBRs resulting in an increase of production.

The seawater for large-scale cultivation of microalgae used as a diet for live feed organisms in aquaculture requires a treatment without addition of chemicals and antibiotics as some organisms may otherwise be negatively affected

(Rhodes et al. 2008). Large volumes of water in PBRs should be provided easy, cheap, and effective to meet all practical requirements. Furthermore, the end-product of the food chain, the fish, is intended for human consumption and must live up to high production standards. Studies comparing the growth rate of microalgae in seawater treated by autoclaving, filtration, and UV radiation are few. In the present study, the growth rate of *Rhodomonas* was not affected by any of these treatments. This indicates that our results obtained from small-scale experiments with *Rhodomonas* (using autoclaved seawater) can be directly implemented to a large-scale production (typically using UV-radiated seawater). Contrary to our results, Jorquera et al. (2002) obtained a lower growth rate of the prymnesiophyte *Isochrysis galbana* in UV-radiated seawater compared to autoclaved seawater which may be due to differences in the sensitivity of microalgal species to the toxic residues that can be produced during UV radiation. It is therefore optimal to combine filtration with UV radiation as filtration improves the efficiency of UV by removing particles shading the radiation (Liltved and Cripps 1999). Other examples from the literature on treatments used in aquaculture include, e.g., electrolytic treatment (Jorquera et al. 2002) and ozone (Summerfelt 2003).

To start the cultivation of microalgae in a PBR, an inoculum of a given volume and density is required to obtain the desired production within a given time frame. The initial density was shown to negatively affect the growth rate of strain K-1487* with increasing density. The growth rate was measured during the exponential phase; thus, limitation of nutrients is unlikely the cause for the observed decreased growth rate with increasing initial density. Also, a density of 200,000 cells mL^{-1} is by far a dense *Rhodomonas* culture and improbable to cause significant self-shading.

Generally, it is required to reach a desired cell density as fast as possible to produce sufficient microalgal feed for the live feed organisms. An example based on the present findings; a cell density of 10^6 cells mL^{-1} is desired after approximately 3 days in a 500-L PBR. The PBR must then be inoculated with an inoculum of 200 L with a density of 500,000 cells mL^{-1} which will result in an initial density of 200,000 cell mL^{-1} in the PBR. Contrary, if the PBR is inoculated with 10 L of the same inoculum as above, the initial density in PBR is 10,000 cells mL^{-1} and the desired cell density is not reached until approximately 5 days after inoculation. Thus, the production efficiency must be adjusted depending on the facility's capacity for maintaining inoculum cultures of a given volume and the time allowed before cultivation at a desired cell density is reached. Knowledge on these parameters is valuable

tools when planning a large-scale production of any given microalgae.

Recommendations

All of the studied strains of *Rhodomonas* are suitable for use in aquaculture when considering their content of PUFAs, except CCAP 995/5 which did not contain a traceable amount of ARA. However, we recommend the strain K-1487 for a large-scale production in PBRs due to its low sedimentation which potentially could decrease excessive biofouling of the PBR system. We also recommend that the f/2 growth medium is optimized by modifying the components according to the nutritional demand of K-1487. Our results clearly show that $CoCl_2$ can be excluded without affecting the growth rate and content of PUFAs, and that the nitrogen source can be added as NH_4^+ in order to increase the growth rate. However, future studies must clarify if the cell content of PUFAs is altered compared to when adding the nitrogen source as NO_3^-. The water should be UV-radiated to avoid contamination and prolong the period of cultivation. A time-consuming step in large-scale production is the maintenance of inoculum. Our results on growth rates for different initial densities of the inoculum are a guideline and should be measured for the specific PBR system used for cultivation. By adjusting the volume and density of the inoculum, the labor cost used for maintenance hereof can be minimized. With the present findings, several barriers for effective cultivation are solved and future large-scale production has become a great step closer.

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Paper 2

<u>Title</u>

Effects of salinity, commercial salts and water types on cultivation of the cryptophyte microalgae *Rhodomonas salina* and the calanoid copepod *Acartia tonsa*.

<u>Status</u>

Effects of Salinity, Commercial Salts, and Water Type on Cultivation of the Cryptophyte Microalgae *Rhodomonas salina* and the Calanoid Copepod *Acartia tonsa*

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Abstract

Marine aquaculture facilities positioned far from the sea need access to seawater (SW); hence, commercial salts are often the chosen solution. In marine hatcheries, most fish larvae require live feed (zooplankton) that are in turn fed with microalgae. The objective of this research was to investigate the applicability of commercial salts and clarify the potential effects on the cultivation of the microalga *Rhodomonas salina* and the copepod *Acartia tonsa*. Three commercial salts were tested, Red Sea Salt (RS), Red Sea – Coral Pro Salt (CP), and Blue Treasure Salt. *R. salina* was cultured at salinities of 10, 20, and 30 psu resulting in equal growth rates at salinities 20 and 30 in SW and RS mixed with deionized (DI) water. The optimum salinity for *R. salina* was 29 psu. For *A. tonsa* eggs, we observed highest hatching success in 30 psu with CP or RS mixed with DI water. The egg hatching success was not affected by salinities 15−40 and optimal hatching was obtained at 27 psu. Results confirm it was possible to use commercial salts for rearing of both *R. salina* and *A. tonsa*, widening the application of these species for aquaculture facilities without access to SW.

KEYWORDS

commercial salts, development stage progression, optimal algal growth, optimal copepod egg hatching success, salinity

The success of marine larval rearing is highly dependent on high-quality food for the first feeding of fish larvae (Dhert et al. 2001; Turingan et al. 2005). Traditionally, rotifers and subsequently *Artemia* are used as live feed, although copepods are required for larvae of some fish species (Shields et al. 1999; Mahjoub et al. 2013; Abate et al. 2015).

Most copepods depend on microalgae for food, preferably those with high amounts of long-chain fatty acids together with contents of essential fatty and amino acids. The cryptophyte *Rhodomonas* has been shown to be a promising candidate as food algae within aquaculture,

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TABLE 1. *Elemental composition of salts in the water types used in the experiment.*

	FW	SW	RS	CP	BT	MW	RS[a]	CP	BT
PSU	0	35	35	35	35		35.5	35	35
Major cations (mmol/kg)									
Na$^+$	13	445.3	314.9	417.9	400.3	23.0	N/A	N/A	413.0
K$^+$	0.1	10.1	31.4	10.1	10.6	39.1	10.0	10.2	
Mg^{2+}	0.6	51.7	168.6	51.7	55.9	24.3	52.7	57.2	58.6
Ca^{2+}	2.4	10.6	2.9	12.0	11.5	40.1	10.7	11.6	11.0
Sum	5.0	517.7	517.9	491.7	478.4		N/A	N/A	492.5
Major anions (mmol/kg)									
Cl$^-$	0.76	573.4	567.4	528.8	521.6	35.5	N/A	N/A	493.0
SO$_4{}^{2-}$	0.97	28.1	75.4	69.9	69.9	32.1	N/A	N/A	74.8
Br$^-$	N/A	Trace	N/A	0.8	N/A	79.9	N/A	N/A	0.4
Sum	1.7	601.4	642.7	599.6	591.4		N/A	N/A	568.1
HCO$_3{}^-$ (at pH 6.0)	2.95	N/A	N/A	N/A	N/A	61	N/A	N/A	N/A
Nutrients (µmol/kg)									
PO$_4$:P	Trace	96.8	0.1	N/A	N/A	31.0	N/A	N/A	N/A
NO$_3$:N	0.2	7.1	0.2	N/A	N/A	14.0	N/A	N/A	N/A
NH$_4$:N	Trace	71.4	Trace	36.5	33.8	14.0	N/A	N/A	N/A
Trace (µmol/kg)									
Mo	Trace	Trace	Trace	N/A	N/A	95.9	N/A	N/A	N/A
Cu	Trace	Trace	Trace	N/A	N/A	63.5	N/A	N/A	N/A
Zn	Trace	2,4	0.1	N/A	N/A	65.4	N/A	N/A	N/A
Mn	Trace	0.7	Trace	N/A	N/A	54.9	N/A	N/A	N/A
Fe	Trace	2,9	Trace	N/A	N/A	55.8	N/A	N/A	Trace
B	Trace	3.7	0.6	N/A	N/A	10.8	N/A	N/A	0.5

BT = Blue Treasure Salt; CP = Red Sea − Coral Pro Salt; FW = tap water; MW = molecular weight, g/mol; RS = Red Sea Salt; SW = seawater.

[a]Levels according to the manufacturer, note RS is 35.5 psu.

Paper 2

for oysters (González-Araya et al. 2012), blue mussels (Riisgård et al. 2011), and copepods (Støttrup and Jensen 1990; Knuckey et al. 2005; Ohs et al. 2010; Zhang et al. 2013; Arndt and Sommer 2014). *Rhodomonas* has also been used in paste form for rotifers (Guevara et al. 2011) and copepods (T. A. Rayner, Roskilde University, Roskilde, unpublished data), and more importantly, it has a suitable cell size for all developmental stages of *Acartia tonsa* (Berggreen et al. 1988).

Acartia tonsa is a euryhaline cosmopolitan copepod often associated with the neritic zone, tolerating salinities from 5 to 72 psu (Paffenhöfer and Stearns 1988; Cervetto et al. 1999; Chinnery and Williams 2004; Højgaard et al. 2008; Ohs et al. 2009). *A. tonsa* has been grown in laboratory cultures in seawater (SW) since the 1980s (Støttrup et al. 1986). Already in 1999, Kusk and Wollenberger (1999) suggested a synthetic saltwater medium for toxicity tests and cultures of

A. tonsa. They cultured both *Rhodomonas salina* and *A. tonsa* for 8 mo in a home-blended synthetic medium. Although this synthetic saltwater is well proven to work with *R. salina* and *A. tonsa*, the recipe is rather complex to mix. Therefore, a ready-to-mix, off-the-shelf, commercially available salt is more relevant to test on cultures of *R. salina* and *A. tonsa*. In the present study, we tested the effects of three different commercial salts with different origin and composition (Table 1), and natural SW as a control, on *R. salina* growth rate and *A. tonsa* egg hatching success and subsequent development stage progression. Extensive ranges of salinities (from 0 to 70) were used to evaluate the hatching success of *A. tonsa* eggs to further test the phenotypic plasticity of the embryos in the eggs. Thus, we evaluated commercial sea salt mixes on all stages of cultures of *R. salina* and *A. tonsa* to reduce dependency on natural SW.

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Materials and Methods

All experiments were conducted in a walk-in temperature-controlled room at 17 ± 2 C at Roskilde University, Denmark.

Water Quality

In the copepod development experiment, dissolved oxygen and pH were measured each morning on Days 0, 4, 7, and 10 in each bottle using a handheld Oxyguard Polaris 2 for oxygen, and pH with an Oxyguard handy pH portable pH/Redox meter (Oxyguard International A/S, Farum, Denmark). The temperature was measured in the temperature-controlled room using HOBO Data Loggers (UA-002-64 HOBO Pendant Temp/Light, 64 K, Bourne, MA), set to log every hour during the entire experiment.

Samples for determination of inorganic nutrients, total ammonia nitrogen (TAN), nitrate (NO_3^-), and nitrite (NO_2^-) were sampled from each treatment at the end of the population development experiment. Determinations of TAN levels were analyzed with a MERCK Spectroquant® (Darmstadt, Germany) test 1.14752, NO_3^- with test 1.14942, and NO_2^- with test 1.14776. Analyses were carried out using a MERCK Spectroquant NOVA 60 (Darmstadt, Germany).

Three commercial salts were analyzed, Red Sea Salt (RS), Red Sea – Coral Pro Salt (CP), and Blue Treasure Salt (BT), for their major cations and anions, nutrient compounds, and trace elements. Furthermore, the tap water, used to mix with the commercial salts, was also analyzed for major cations and anions, nutrient compounds, and trace elements, as well as inorganic carbon (Gran titration; Stumm and Morgan 1996). All commercial salts were mixed at 35 psu to be comparable with the SW. The cations and anions, nutrient compounds, and trace elements were analyzed by an ion chromatograph (Dionex ICS-1100, ThermoFisher Scientific, Roskilde, Denmark). The cations and anions were analyzed separately. The cations were analyzed using a DionexIonPacTMCS12A column, with eluent 20 mM H_2SO_4. The anions were analyzed using a DionexIonPacTMAS14A column, with eluent 8 mM Na_2CO_3/1 mM $NaHCO_3$.

Saltwater

The RS, CP, and BT used in experiments were all tested against a control of 0.2-μm filtrated SW that was usually used for successful production of both *R. salina* and *A. tonsa* at Roskilde University and DTU-Aqua (Støttrup et al. 1986; Drillet et al. 2006a; Jepsen et al. 2007). This SW originates from 30-m depth in the outer parts of the Kattegat (57°N, Denmark), with 35 psu. The salts were mixed with either tap water from Taastrup, Denmark (Table 1), or deionized (DI) water from Roskilde University, Denmark. Tap water in Denmark follows the legislation in BEK nr. 802 (Drikkevandsbekendtgørelsen 2016). Salinities were mixed to different psu by weighing salt on an AE163 Mettler scale (Glostrup, Denmark) and adding it to either tap or DI water to the psu given in the experimental setup. The mean salt strength was 30−35 psu for the SW used in the laboratory at Roskilde University. The salts and SW were left for at least 1 wk and mixed to ensure total dissolution of the commercial salt products. Salinity strengths were tested and adjusted prior to the experiments with a handheld refractometer with a resolution of 0.5 psu (ATAGO, Tokyo, Japan).

The salts RS and CP are both from the same producer, Red Sea (RedSea 2017). The salts are manufactured by natural evaporation of SW from the Red Sea, through three shallow ponds, increasing the salinity from 40 to 250 psu. During an evaporation process, sodium chloride crystals with ions of magnesium and potassium are formed. Thereafter, the salts are washed, dried, and finally RS and CP are elevated to different levels of calcium, magnesium, potassium, and other trace elements by the manufacturer (RedSea 2017).

The BT is a synthetic salt created from underground SW from the BoHai Bay in China. The manufacturing process is, according to the producer, first evaporation of the underground SW. Thereafter, the supernatant is removed and further evaporated in a pressure cooker. Then the refined sea salt is sieved and smashed before addition of trace elements and elevation of calcium, magnesium, and potassium by the manufacturer (Aquaseasalt 2017).

Cultivation of R. salina

For all experiments, *R. salina* strain K-1487, Scandinavian Culture Collection of Algae & Protozoa, was used. Before the experiments, *R. salina* was cultured at the exponential growth phase in 2-L round-bottom glass bottles, with 0.2-µm filtered SW fertilized with a modified f/2 media without cobalt (Guillard and Ryther 1962; Christina V. Thoisen, Roskilde University, Roskilde, unpublished data), at 17 ± 2 C and under constant light at $80 \, \mu mol/m^2/sec$ photosynthetically active radiation (PAR). The light intensity was measured with a Hansatech Instrument LTD Quantitherm light meter QRT1 (Norfolk, UK).

Optimal Salinity for Growth of R. salina

To investigate the optimal salinity for *R. salina* cultivation, an experimental setup was conducted in 0.5-L round-bottom glass bottles. The light intensity was between 100 and 115 $\mu mol/m^2/sec$ PAR and nutrients were added in saturation (modified f/2 media). The experiment was started with *R. salina* grown as described earlier and was inoculated into four replicate controls of 35 psu 0.2-µm filtrated SW and four replicate RS mixed with DI water to an ion strength of 35 psu. All replicates were initiated with a cell concentration of $50,000 \pm 1500$ cells/mL. All replicates were sampled and the cell concentration was measured using an electronic particle counter (Beckman Multisizer 4e Coulter Counter (Brea, CA) equipped with a 100-µm aperture tube). When the cell concentration reached 1 million cells/mL or more, the algae were diluted to 50,000 cells/mL in new salinity strengths of RSs by changing the salinity by ±5 psu. Hence, 35 psu was diluted into four new bottles with either 40 or 30 psu. This continued within the range from 35 to 0 and from 35 to 65 psu, respectively. For each of the salinities, growth rates were obtained and calculated by Equation (1).

$$\mu = \frac{\ln N_2 - \ln N_1}{t_2 - t_1} \qquad (1)$$

where μ is the growth rate (/d), N_1 is the number of cells at t_1, and N_2 is the number of cells at time t_2, respectively. To describe the relationship between salinity and growth rate in *R. salina*, we used a three-parameter Gaussian model (SigmaPlot version 10.0 build 10.0.0.54, Systat software, Chicago, IL).

$$f(x) = ae^{-0.05 \left(\frac{x - x_0}{b} \right)^2} \qquad (2)$$

where a is the height of the curves peak, x_0 is the center of the curves peak, and b is the SD of the mean.

Effect of Commercial Salts on R. salina *Growth*

Rhodomonas salina growth rates were evaluated in SW and in commercial salts at 20 and 30 psu. Each treatment was replicated three times in 1-L round-bottom glass bottles with aeration. The experiment was conducted with a light intensity of 80 $\mu mol/m^2/sec$ PAR and with nutrients in excess (modified f/2 media) (Vu et al. 2015). Growth rates were monitored each day, over 5 d, in all treatments, by daily measuring cell concentration using an electronic particle counter. The growth rates were calculated by Equation (1). A three-factor PERMANOVA was used to evaluate the effects of commercial salts (Factor A), tap or DI water (Factor B), and salinity (Factor C) upon the growth rate of *R. salina*. Euclidian distances were used for the resemblance matrix and the procedure "permdisp" was used to test if the assumptions for a PERMANOVA were fulfilled. The program Primer 6 with the add-on PERMANOVA was used (PRIMER-E Ltd., Plymouth, UK).

Cultivation of A. tonsa

The experiment was initiated with cold-stored *A. tonsa* eggs harvested from cultures kept at Roskilde University. The origin of the particular copepod culture is the DFH.AT1 culture isolated in Øresund in 1981 (Støttrup et al. 1986). The *A. tonsa* cultures were kept at 35 psu SW, no light, 17 ± 2 C, and fed a mono algal diet of *R. salina* (K-1487). The eggs were cold-stored by the method described by Drillet et al. (2006b), with the modification that the eggs were stripped of dissolved oxygen by bubbling with nitrogen gas, immediately after thoroughly cleaning

with SW in successive filters (200 and 65 μm). This treatment was applied to optimize storage conditions.

Optimal Salinity for A. tonsa *Egg Hatching Success*

Fresh eggs harvested from our culture within 4 h of spawning were used to initiate the optimal salinity and hatching experiment. In this experiment, SW was used and either concentrated by evaporation (>35 psu) or diluted with DI water (<35 psu). Salinities were 0, 5, 10, 15, 20, 25, 30, 35, 40, 50, 55, 60, 65, and 70 psu. Eggs were subsampled into 10×20-mL petri dishes for each treatment using a 10-mL kip automate (NS 29.2/32 Witeg, Wertheim, Germany). Each petri dish was stocked with a mean of 60 ± 3 eggs. Hatching successes were obtained after 48 h, by fixing nauplii and remaining eggs in 1% acid Lugol's solution and counted using a dissection microscope (SZ40 Olympus Optical GmBH, Germany at 20× magnification). The relationship between egg hatching success of *A. tonsa* eggs and salinity was determined by fitting the data to Equation (2).

Effect of Commercial Salts on A. tonsa *Egg Hatching Success*

Cold-stored eggs were used to evaluate the effects of tap, DI water, salinity, and commercial salts on 48 h hatching success. Three different salinities were used: 10, 20, and 30 psu, mixed with either tap or DI water. An aliquot of 10-mL water, adjusted to the relevant salinity, was subsampled into 10 replicate 20-mL petri dishes for each treatment with a 10-mL kip automate. Each Petri dish was stocked with a mean of 111 ± 11 eggs. Primer 6 was used to build a three-factor PERMANOVA design to statistically analyze the effects of commercial salts (Factor A), tap or DI water (Factor B), and salinity (Factor C) on the *A. tonsa* egg hatching success.

Effect of Commercial Salts on A. tonsa *Development*

Three commercial salts mixed with DI water were tested as growth media for *A. tonsa* and compared with 0.2-μm filtered SW, all at 30 psu. For each treatment, four replicate 600-mL bottles (Nalgene, ThermoFisher Scientific) were initiated with a total of $13,700 \pm 538$ cold-stored eggs/bottle. The eggs were subsampled with a 10-mL kip automate. For determination of initial hatching success, 10×20-mL petri dishes were subsampled for each of the eight treatments. The petri dishes were stocked with a mean of 217 ± 4 eggs/petri dish to determine initial hatching success. After 72 h, the hatched nauplii and remaining eggs were fixed in 1% acid Lugol's solution and counted using a dissection microscope. By multiplying the initial egg numbers (*d*0) with the hatching success, the actual numbers of starting individuals in each of the treatments are derived (*d*3). Each day, all bottles were fed in excess with *R. salina* (>950 μg C/L) (Kiørboe et al. 1985; Berggreen et al. 1988). At Days 5, 8, and 11, each of the four replicate bottles for all treatments were subsampled with a 10-mL kip automate. Animals from each subsample were fixated in 1% acid Lugol's solution and identified as either nauplii or copepodites/adults. At Day 14, the experiment was terminated and all animals were fixated, enumerated, and stage was determined. The enumerated animals from each treatment were used to estimate the instantaneous rates of mortality (Z/d) calculated using Equation (3) (Klein Breteler et al. 2004).

$$N_t = N_0 e^{-zt} \qquad (3)$$

where N_0 is the number of individuals at time 0 and N_t is the number of individuals at time *t*. N_t was corrected for sampling mortality using Equation (4) (Klein Breteler et al. 2004).

$$\frac{V n^{-1}}{(V - v_1)(V - v_2) \ldots (V - v(n^{-1}))} \qquad (4)$$

where *V* is the volume of the experimental bottle, *v* is the volume of the subsample, and *n* is the rank number of the subsample.

The instantaneous rates of mortality were calculated for each of the four replicate bottles within each of the treatments and presented as

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a mean mortality during the total experimental time for the total population.

To estimate the time duration (d) for change from nauplii to copepodites, data were divided into two fractions with nauplii as one fraction and copepodites as the other fraction. Plotting the developing fractions as a function of time showed where the majority of the population switched from nauplii to copepodites, because an intersection between the two lines happens. By assuming linearity between the lines in the intersection point, simple algebra can be used to solve the intersection point, and hence the time where the majority of the population was switching from nauplii to copepodites can be extracted.

Mortality, time for stage switch, and total population were each statistically tested for normal distribution and equal variances before further testing with a one-way ANOVA in SigmaPlot version 10.0 build 10.0.0.54 (Systat software).

Results

Water Quality

Mean dissolved oxygen was 5.65 ± 0.67 mg O_2/L, the mean pH was 8.12 ± 0.04, and the mean temperature was 15.7 ± 0.3 C during the experiment between all replicates. Mean concentrations of TAN were 0.06 ± 0.05 mg/L, mean NO_3^- at 0.42 ± 0.20 mg/L, and mean NO_2^- concentrations were all below a detection limit of 0.02 mg L^{-1} (Table 1).

The sum of major cations in RS resembled SW. Further high concentrations of Mg^{2+} was observed in RS. CP was the salt that was most similar to SW in terms of the composition of major cations. For major anions, all commercial salts (RS, CP, and BT) had twice as much SO_4^{2-} concentrations than SW. SW contained high amounts of nutrients (PO_4:P, NO_3:N, and NH_4:N) compared to the RS, CP, and BT. Trace elements were only detected in a small amount in SW and in RS but was below detection limit for both CP and BT.

Optimal Salinity for R. salina

The growth rate of *R. salina* was influenced by salinity, and the optimum growth rate was 1.13/d at 29.1 ± 2.3 psu. The model fits were:

peak $(a) = 1.13 \pm 0.07$, SD $(b) = 28.86 \pm 3.52$, and center of peak $(X_0) = 29.14 \pm 2.27$ ($n = 56$, $r^2 = 0.67$). The lowest growth rate was observed at each end of the salinity range at 0 and 55–65 psu. All salinities from 5 to 50 psu had a growth rate higher than 0.8/d (Fig. 1).

Effect of Salts on R. salina *Growth*

The effects of different salts showed that the *R. salina* growth rates in SW (from 1.11 to 1.21/d) were statistically significantly different and higher than the growth rates in both CP (from 0.73 to 1.04/d) and BT (from 0.83 to 1.03/d) ($P = 0.01$). When comparing salts prepared with tap water, *R. salina* grew better in SW (1.20/d at 20 psu and 1.12/d at 30 psu) than in RS (0.94/d at 20 psu and 0.93/d at 30 psu) ($P = 0.009$). Among the commercial salts, *R. salina* grew better in RS than in BT ($P = 0.01$). Comparing salts prepared with DI water revealed that *R. salina* grown in BT (1.02 and 1.03/d) had a significantly different lower growth rate than *R. salina* grown in both SW (1.21 and 1.11/d) and RS (1.22 and 1. 19/d) ($P = 0.01$). Furthermore, the results from the three-factor PERMANOVA showed statistically significant differences among the growth rates depending on the type of commercial salt used ($P = 0.0002$) and the type of water used ($P = 0.0008$). There was no statistical effect of different salinities at 20 and 30 psu ($P = 0.0579$) (Fig. 2 and Table 2).

Optimal Salinity for A. tonsa *Egg Hatching Success*

The salinity interval within which *A. tonsa* subitaneous eggs were capable of hatching ranged from 5 to 50 psu, with an optimum hatching success of 67.7% at 27.5 ± 0.5 psu. The model fits are: peak $(a) = 67.68 \pm 1.99$, SD $(b) = 14.28 \pm 0.54$, and center of peak $(X_0) = 27.51 \pm 0.54$ ($n = 170$, $r^2 = 0.81$). Within the range from 15 to 40 psu, more than 50% of the eggs hatched (Fig. 3).

Effects of Commercial Salts on A. tonsa *Egg Hatching Success*

Eggs exposed to RS had the highest hatching success (from 40.2 to 67.9%), closely followed
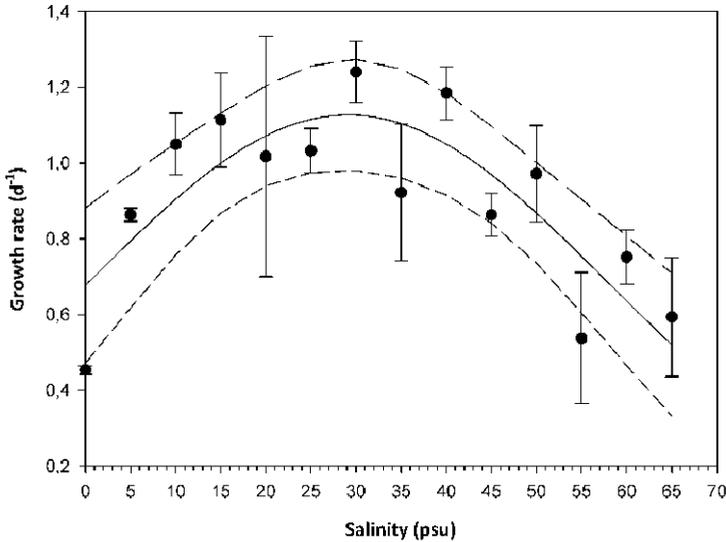
Figure 1. *The optimum salinity for growth of* R. salina *is 29.1 psu with a growth rate of 1.2/d. Symbols are mean± SE (n = 4). Solid line is a three-parameter Gaussian fit, and dashed lines are the 95% CL.*

by CP (38.5–60.9%) (Fig. 4A–C and Table 3). The overall hatching success was lowest in BT (25.3% in tap and 42.6% in DI water). The effects across all salinities confirmed this pattern where CP gave the highest hatching success regardless of whether tap (34.0%) or DI water (50.0%) was used for preparation and BT the lowest hatching success (Fig. 4D). In general, hatching success was higher in salt-water medium prepared with DI water than in tap water, except for at 30 psu (Fig. 4A–C, gray bars). The same pattern was observed across all salinities (Fig. 4D) and across all salts (Fig. 4E). In terms of salinity, the best hatching success was achieved at 30 psu (Fig. 4C, E, F). The type of water used to prepare the saltwater medium also had an effect at salinities from 20 psu and below, where the hatching success was lower when salts were mixed with tap water than with DI water (Fig. 4A–C, E, F).

Effects of Commercial Salts on A. tonsa Mortality, Development, and Culture Densities

The highest mean mortality was observed ($17.7 \pm 1.6\%$/d) in the population exposed to BT

and the lowest mean mortality was observed in SW ($14.2 \pm 0.4\%$/d) (Fig. 5).

The average shift in moulting time between the two major larval categories nauplii and cope-podites for copepods in SW was 7.02 ± 0.02 d, followed by CP (6.99 ± 0.04 d), RS (6.97 ± 0.06 d), and BT (6.91 ± 0.07 d) (Fig. 5).

The abundance showed the expected reverse pattern of mortality. The population with the highest survival rate was the one in SW with a mean of 1388 ± 68 individuals/L. The population with fewest animals at the end was the cope-pods exposed to BT, with a mean of 1167 ± 464 individuals/L (Fig. 5).

Discussion

Water Quality

We tested the water quality and the ions in different waters and salts to ensure the environ-mental comparability with our test organisms. Marcus et al. (2004) showed that dissolved oxygen concentrations of 1.5 mg/L did not affect the survival of *A. tonsa*; hence, we do not expect dissolved oxygen concentrations to

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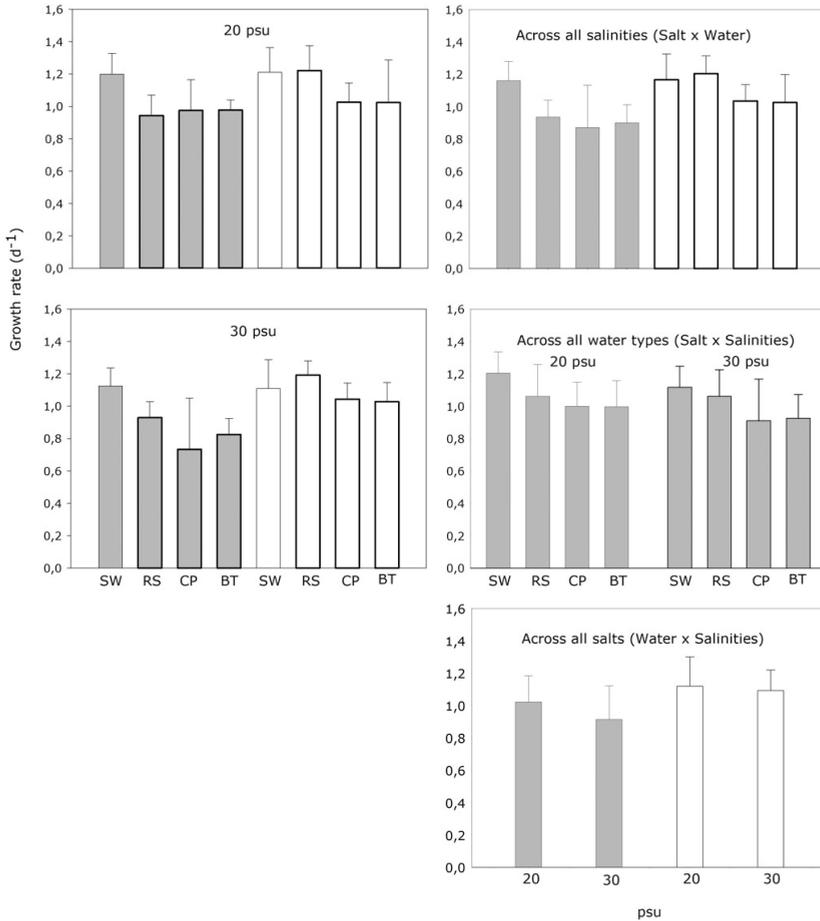
FIGURE 2. *Growth rate of* R. salina *as a function of commercial salt, water type, and salinity. Gray bars are salts mixed with ordinary tap water and white bars are salts diluted in deionized water. Bars are mean ± SD (*n *= 3 or 4). The two left graphs show single effects, whereas the three right graphs show the mixed effects.*

affect results of this study (ca. 5 mg/L). The pH level that copepods were exposed to in this study was 8.12, which in itself is not harmful and within the range of other experiments (Drillet et al. 2008; Jepsen et al. 2015; Hansen et al. 2017). TAN in the current experiment was 60 µg/L, whereas only 3–4% was NH_3 due to the NH_3/NH_4 pH-dependent equilibrium. Jepsen et al. (2015) has shown that this concentration of 2.4 µg NH_3/L has no effects on *A. tonsa* nauplii

or adults. NO_2^- was below the detection limit of 0.002 mg/L and is therefore not expected to have reached toxic levels for *A. tonsa*. NO_3^- was 0.42 mg/L in the current experiment (Table 1). The three salts, RS, CP, and BT, had two different origins, RS and CP were evaporated SW and BT was synthetically manufactured underground SW. Table 1 shows that RS has differences in major anion and cations with higher concentrations of Mg^{2+} and SO_4^{2-}, which was

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Tabl e 2.   *PERMANOVA results showing effects on algal growth rates.*[a]

Source	df	SS	MS	Pseudo-*F*	*P*(perm)	Unique perms
Salt	3	13.1740	4.3914	67.698	**0.0002**	9955
Water	1	8.9301	8.9301	13.767	**0.0008**	9813
Salinity	1	2.3784	2.3784	36.665	0.0579	9846
Salt × water	3	4.2008	1.4003	2.1586	0.1100	9953
Salt × salinity	3	0.4795	0.1599	0.2464	0.8604	9946
Water × salinity	1	0.9465	0.9465	1.4591	0.2318	9827
Salt × water × salinity	3	1.5656	0.5219	0.8045	0.4968	9958
Residual	44	28.5420	0.6487			
Total	59	59				

[a]Results from a three-way factual Permanova analyses. Effects of commercial salts and the water the salts are mixed and diluted in were observed. Statistical signif cances are marked in bold font (*P* < 0.05).
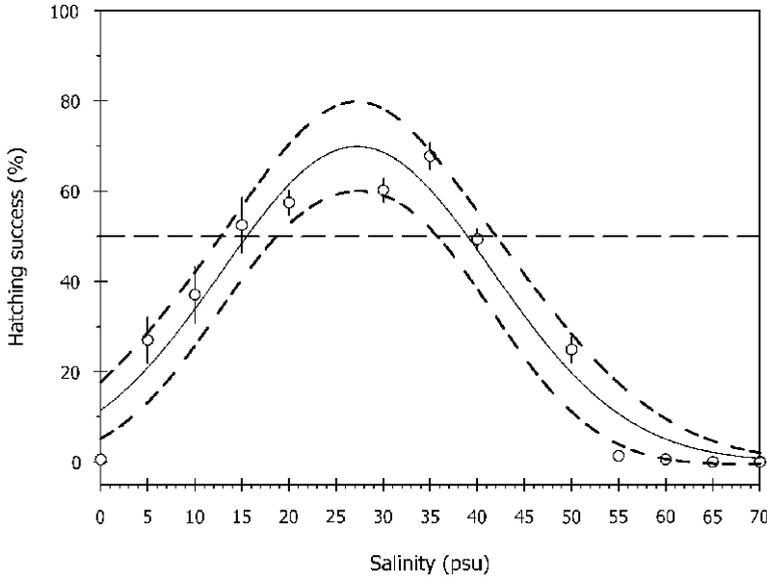
Figur e 3.   *The optimum salinity for hatching of A. tonsa eggs is 27.5 psu, with a 67.7% hatching success. Eggs from this A. tonsa strain are not capable of hatching at salinities above 55 psu. Symbols are mean values ± SE (n = 10 or 20). The horizontal dashed line shows a 50% cutoff for hatching success, we considered hatching of 50% and more as an acceptable hatching success in practice. The solid line is a three parameter Gaussian f t and dashed lines are 95% CL.*

probably related to that $MgSO_4$ was the source used as addition when formulating the salts by the manufacturer. Another observed difference was the Ca^{2+} content where especially RS has a low content which will potentially affect the buffering capacity and alkalinity of the saltwater when using this salt (Atkinson and Bingman 1997). Very few studies exist comparing dif-ferent commercial salts and no specif c study on microalgae and copepods has been found (Soundarapandian et al. 1994). However, dis-cussing rearing of microalgae and copepods with the scientif c community and aquarists all have a gut feeling that sea salts originating from evaporated SW have a better trace ion composition than rock salt and underground water extraction because ions are lost in the geological processes involved in the latter.

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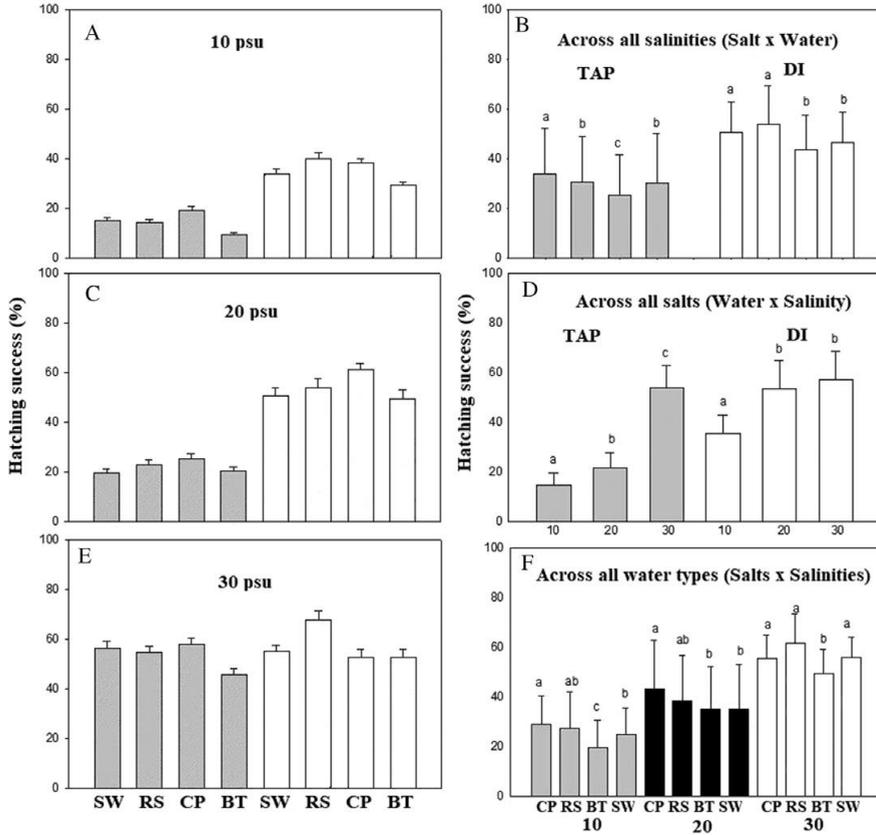
FIGURE 4. *Hatching success by* A. tonsa *eggs as a function of salt, water, and salinity. (A–E) Gray bars are salts mixed with ordinary tap water and white bars are salts diluted in deionized water. (F) Gray bars are 10 psu, black bars are 20 psu, and white bars are 30 psu. Bars are mean ± SD (*n = 4*). Panels (A–C) show single effects, whereas (D–F) show mixed effects.*

Microalgae

Rhodomonas salina can grow in all the tested salt products dissolved in both water types. Tap water varies in potential contaminants, alkalinity, pesticides, and volatile organic compounds depending on the source of the water (DeSimone 2009). Therefore, producers of commercial salts recommend that they are mixed with reverse osmosis water or DI water. In our experiment, we found that growth of *R. salina* was lower in tap water than in DI water and we speculate that this is an effect of various unknown impurities in the local tap water used, although no differences were observed in the ion composition analyses of the tap water (Table 1).

The *R. salina* growth rates (0.8–1.2/d) were similar or slightly higher than those reported in the literature (Lewitus and Caron 1990; Bartual et al. 2002; Hammer et al. 2002; Lafarga-De la Cruz et al. 2006; Vu et al. 2015). The important ions in relation to growth are the excess of NH_4 in the SW, CP, and BT as NH_4 is a state of nitrogen that is easy for the algae to take up and assimilate (Rückert and Giani 2004; Table 1).

TABLE 3. *Results from a three-way factual PERMANOVA analyzes, showing effects on copepod eggs hatching success.*[a]

Source	df	SS	MS	Pseudo-*F*	*P*(perm)	Unique perms
Salt	3	23439	0.7813	18349	**<0.0001**	9947
Water	1	22207	22207	521.51	**<0.0001**	9823
Salinity	2	31091	15545	365.08	**<0.0001**	9944
Salt × water	3	0.4023	0.13411	31496	**<0.0249**	9950
Salt × salinity	6	10071	0.16785	39418	**<0.0016**	9929
Water × salinity	2	91963	45981	107.99	**<0.0001**	9953
Salt × water × salinity	6	0.3799	633,13	14869	0.1827	9962
Residual	216	91975	425,81			
Total	239	75824				

[a]Results from a three-factual Permanova analyses effects on hatching success of commercial salts, which water the salts, were diluted in and salinity. Statistical significances are marked in bold font ($P < 0.05$).

Therefore, this extra nitrogen source could have an additive effect on the f/2 media (Guillard and Ryther 1962). Thoisen et al. (2018) showed that statistically significant higher growth rates of *R. salina* (K-1487) were obtained when cultivated with f/2 growth medium with ammonium compared to nitrate as the prime N-source. However, this does not explain the algae growth rates in water mixed with RS as only trace amounts of NH_4 were detected in this salt (Table 1). The primary environmental variables that control microalgae growth are irradiance levels and nutrient levels (Lafarga-De la Cruz et al. 2006). In the current experiment, both light and nutrients were supplied in excess, which will promote optimal growth rates. However, we are aware that it is difficult to compare growth rates between experiments as even small variations in growth conditions, algal strain selection, and abiotic conditions will render a different result.

Marine microalgae have different protection mechanisms to cope with changes in salinity of the surrounding environment (Gebser 2015). Adaptations to varying salinities therefore often depend on the ecological habitat the microalgae inhabit in nature. *R. salina* is ecologically a brackish water species; so a widespread tolerance to different salinities is to be expected, as shown by Cañavate and Lubian (1995), where the authors reported that *Rhodomonas baltica* was able to sustain rapid changes (24 h) from 36 to 20 psu. In short, the *R. salina* strain tested in this experiment had an optimal salinity of 29.1 psu but can be adapted to grow well in a very broad range of 5−50 psu.

Copepods

Optimal egg hatching success of 67.7% occurred at a salinity of 27 psu. The hatching success corresponded to values reported in the literature (Drillet et al. 2006b; Peck et al. 2015). Peck and Holste (2006) showed that besides salinity, the length of the period of cold storage of eggs had a negative effect on their hatching success. The optimal hatching in the present study corresponds to the response of eggs stored for between 100 and 150 days in the Peck and Holste (2006) study, although the eggs in the present study were less than 4 h old. However, we have often observed variation in egg hatching success among batches (Højgaard et al. 2008). Eggs exposed to a salinity below 15 psu still hatch and even eggs exposed to a salinity of 0 psu have earlier been shown to hatch when transferred to higher salinities (Højgaard et al. 2008). Another study showed that eggs were shock-treated with a high salinity (20 min of exposure) could sustain salinities up to 100 psu without a subsequent decrease in hatching success (Ohs et al. 2009). The capability of the eggs to survive in extreme salinities can be explained by the protecting function of the embryonic membrane and by the capability of the eggs to enter into a quiescent stage (Højgaard et al. 2008; Hansen et al. 2012). Within a salinity range from 15 to 40 psu, the hatching rate of the eggs was 50% or higher. Hatching rates above 50% are considered feasible in aquaculture and we recommend to culture *A. tonsa* within this salinity range. This salinity range was also considered where the egg hatching exhibited a large
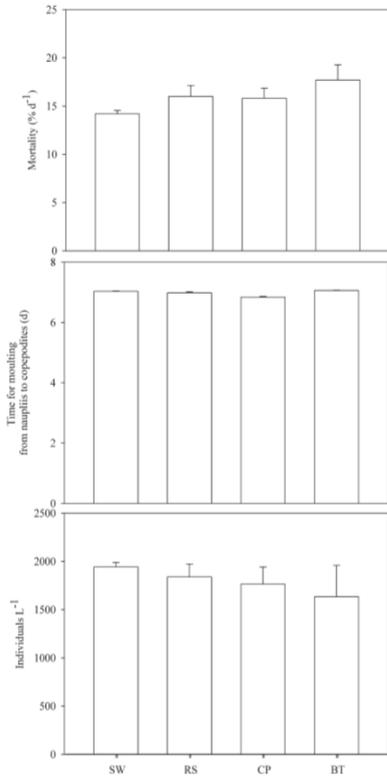
FIGURE 5. *Daily mortality by* A. tonsa *in different treatments expressed in %/d (mean ± SE, n = 4, top panel). Development time for switch from nauplii stages to copepodite stages, expressed in d (mean ± SE, n = 4, middle panel). Individuals/L at the end of the experiment (average ± SE, n = 4, bottom panel).*

plasticity, which did not cause any significant loss in viability of the eggs. Physiologically, it is reasonable to assume that the immobile egg stage is potentially more plastic than the mobile nauplii and copepodites stages to changes in salinity.

The results showed a decreased hatching effect of lower salinity regardless of salt type. The decreased hatching success was not observed at 30 psu with salts diluted with tap water. It is well known that high levels of ions present in SW act as a protecting factor against metal toxicity because of metal complexation with anions, especially chloride, and we speculate that this is the same type of effect as observed in the present study (Monteiro et al. 2013). So, the potential negative effects from tap water are diluted by increased salinity, hence adding more ions to the water. Investigating the effects of different salts on hatching success, BT was the salt that in most cases resulted in low hatching. We, therefore, recommend not using BT and for full precautionary effects no other mined and synthetic salts for hatching of *A. tonsa* eggs for aquaculture. BT was the salt with the lowest ion content and we therefore speculate that this slightly reduced amount of ions caused the observed effects (Monteiro et al. 2013). As we found such a profound effect of tap water on the hatching success, we excluded tap water for the further study on survival and development of nauplii and copepodites.

Table 1 showed that RS was the most unbalanced salt when compared to SW and the other two salts. The hardness of water is normally defined by the ion strength of Ca^{2+} and Mg^{2+}. RS had the lowest observed content of Ca^{2+} (2.9 mmol/kg), whereas three times higher content of Mg^{2+} was observed. Combined higher concentrations of these ions are known to lower metal toxicity and we therefore speculate if the more successful rearing of *A. tonsa* in RS than in the other salts is a result of reduced metal toxicity (Erickson et al. 1996). We are not aware of any study showing the single effect of Ca^{2+} on copepods. In *Daphnia*, low amounts of calcium have shown an effect on calcification and the same could be expected for freshwater copepods, but we do not expect this to be a problem for marine copepods as the general calcium pool there is significantly higher when compared to freshwater (Alstad et al. 1999).

The copepod mortality in the present study was approximately 15%/d and was not significantly different among treatments. Other studies have shown mortalities around 4–5%/d for this strain of *A. tonsa* in culture (Berggreen et al. 1988; Drillet et al. 2008, 2015). Although the mortality was relatively high in the present study, this has been reported before by our research team even with mortalities up to 17%/d (Jepsen et al. 2007). It has been speculated that elevated

mortality is a stress response from experimental handling (Jepsen et al. 2007). This hypothesis could be tested in future studies as recent scientific efforts have discovered stress-related proteins in *A. tonsa*, which is a relevant tool for embryonic and free-living *Acartia* stress monitoring (Tartarotti and Torres 2009; Nilsson et al. 2014). The copepod development rates were not different among the four treatments at approximately 7 d between moulting from nauplii to copepodite stage I, which is in agreement with previous studies that have reported time between moults of the same strain of *A. tonsa* in the range of 6.25−8 d with the same temperatures as in the present study (Berggreen et al. 1988; Drillet et al. 2008). No study has been identified that reports time for moult or stage development as a function of salinity. Instead, Peck and Holste (2006) reported egg production as a function of salinity to be optimal at 15 psu. As specific egg production is equal to specific somatic growth and thereby development (according to Berggreen et al. 1988), we anticipate that *A. tonsa* in the present experiment would have developed faster in lower salinity (15−20 psu). It can be hypothesized that salinities in this range are probably close to the osmolality of adult *A. tonsa* and they thereby save energy on hyporegulation and instead allocate it to somatic growth/egg production (Farmer 1980; Svetlichny and Hubareva 2014).

Conclusions

In conclusion, we found that water type, salts, and salinity had effects on the growth of both *R. salina* and *A. tonsa*.

Overall, it is indeed possible to produce both *R. salina* and *A. tonsa* with commercial salts, different water types, and various salinities. To ensure optimal outcome, we had best success using DI water mixed with RS and at a salinity of 28 psu as a compromise between the growth optimums of *R. salina* and *A. tonsa*.

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Paper 2

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Paper 3

<u>Title</u>

The effect of cell density on yield of biomass and fatty acids during meso-scale cultivation of the cryptophyte *Rhodomonas salina*.

<u>Status</u>

In prep.

The effect of cell density on yield of biomass and fatty acids during meso-scale cultivation of the cryptophyte *Rhodomonas salina*

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Abstract

The microalgae *Rhodomonas salina* is used in aquaculture as diet for the live feed calanoid copepod *Acartia tonsa*. The biomass of the microalgal diet needed to support sufficient food for a meso-scale production of *A. tonsa* necessitates a meso-scale production of *R. salina.* To achieve this, certain

production parameters need to be optimized for maximized yield. This study evaluates the effect of density (5×10^5, 1×10^6, 1.5×10^6 and 2×10^6 cells ml^{-1}) on the dilution rate (hence the growth rate), production yield and cell content of fatty acids for *R. salina* cultivated semi-continuously in a 250 L tubular photobioreactor. Densities of 1×10^6, 1.5×10^6 and 2×10^6 cells ml^{-1} are suitable for obtaining a durable yield while keeping the cell content of fatty acids at a promising level for meso-scale production under the conditions applied in this study. However, we recommend a cell concentration around 1×10^6 cells ml^{-1} to minimize biofouling of the photobioreactor.

1. Introduction

In the marine fish larviculture, rotifers and the brine shrimp *Artemia* are used as live feed despite their inadequacies as feed for many species of fish larvae due to a large size and deficiency in polyunsaturated fatty acids (PUFAs) (Evjemo et al. 1997, Drillet et al. 2006b). Inadequate live feed may negatively affect the survival rate, pigmentation and development of fish, however; this can be counteracted by supplementing with copepods as live feed for the first-feeding stages of the fish larvae (Bell et al. 2003, Schipp 2006). Copepods are the natural prey of many fish larvae species and the nauplii hatching from the copepod eggs have an adequate small consumable size. Despite this, the use

of copepods as live feed is virtually absent in aquaculture as cost efficient high density cultivation is challenging compared to rotifers and *Artemia*.

In attempting to implement the use of copepods in aquaculture, the meso-scale production of an adequate microalgal diet is essential. The cryptophyte *Rhodomonas* is an excellent microalgal diet for copepods due to the increase in copepod survival, growth and egg production as compared to when fed other microalga species (McKinnon et al. 2003, Knuckey et al. 2005, Zhang et al. 2013, Arndt et al. 2014). Furthermore, *Rhodomonas* contain the polyunsaturated fatty acids docosahexaenoic acids (DHA; C22:6 n-3), eicosapentaenoic acid (EPA; C20:5 n-3) and arachidonic acid (ARA; C20:4 n-6) in adequate amounts which are essential for finfish larviculture. These PUFAs are essential for the survival, development, growth and pigmentation of fish larvae and the content of fatty acids in then copepods is affected by the content of fatty acids in the microalgal diet (Dalsgaard et al. 2003, Caramujo et al. 2008, Lund et al. 2008, de Lima et al. 2013, Arndt et al. 2014).

Although *Rhodomonas* is known to be an ideal microalgal diet for copepods, it is not well studied how the density of a meso-scale culture of *Rhodomonas* affects growth rate, yield and cell content of DHA, EPA and ARA of the algae. The biochemical profile of microalgae can be manipulated

by altering the cultivation parameters. Generally, stressful conditions such as nutrient limitation result in an increase of lipids but with a decreased fraction of PUFAs (Qiao et al. 2016, Vu et al. 2016). High light intensities can result in a higher amount of total fatty acids (TFA) and PUFAs, and notably the production of EPA has been reported to increase (Lu et al. 2001, Vu et al. 2016). The effect of density on production yield and biochemical composition of microalgae is less studied, and results on *Rhodomonas* from meso- and large-scale experiments are lacking. Generally, growth rates are higher at lower densities due to limited self-shading, except in the case of light inhibition (Chrismadha et al. 1994, Courtois de Viçose et al. 2012). Assuming that the growth rate is higher at low densities, and high enough to compensate for the lower stating point, it could be expected that the production yield (in terms of biomass) is highest at low densities. However, given the low density of the culture, a trade-off could clearly be a production yield with an undesired large water volume of the microalgae culture creating higher costs to wasted inorganic nutrients and elevated maintenance of copepod cultures.

The purpose of this study is to evaluate the effect of density ($5x10^5$, $1x10^6$, $1.5x10^6$ and $2x10^6$ cells ml^{-1}) on the yield of biomass and fatty acids of a meso-scale semi-continuous cultivation of *R. salina* during a two-year period.

2. Materials and methods

2.1 Photobioreactor.

The cultivation system consisted of a horizontal tubular PBR (closed system) with a total volume of 250 L constructed from 2x12 transparent tubes (ø: 60 mm inner/64 mm outer, length 2000 mm) placed at a horizontal angle of 2,08 degrees. The tubes were acrylic glass (polymethyl methacrylate, PMMA) and connected by opaque bendings of polyvinyl chloride (PVC-U). Filtered air (0.3 μm polypropylene DOE filter; van Borselen B.V.) with CO_2 addition was supplied into the lowermost tube by an air pump (ALITA, model AL-60SB) providing an upwards water flow through the connected tubes (partially air-filled) into a 77 L dark collection tank (included in the total volume of 250 L), wherefrom the culture entered the lowermost tube again. The duration for obtaining total mixing in the PBR after addition of a substrate was 2 h and 13 min. The PBR was located in a controlled climate room and sensors were placed directly into the culture in the lowermost tube for measurements of pH, temperature and O_2 saturation. The pH and O_2 sensors (Mettler Toledo; Easy Sense pH 32 sensor and Easy Sense O_2 21 sensor) were connected to a transmitter (Mettler Toledo M 200Easy) connected to a data logger (CR1000, Campbell Scientific). Data from the data logger (CR1000, Campbell Scientific) provided measurements for each minute and was used to calculate

the average pH, temperature, dilution rate and light intensity. The light sources (6x SENMATIC, FL300 grow white LED Fixture) were placed 1 meter in front of the PBR and the intensity was adjusted via the Fionia Lighting Interface Software. A light measuring device (Li-Cor LI-193 Spherical Quantum Sensor) measuring the photosynthetic photon flux fluence rate was positioned centrally on the 6th lowest tube. Nutrients (f/2 growth medium) and harvested culture were kept in two separate 250 L tanks. Inflow of nutrients to the PBR and outflow of culture from the PBR was controlled by two separate dosing pumps (Grundfos ALLDOS, DDC). Nutrients and artificial seawater (ASW) were filtered (1 µm followed by 0.3 µm, Borospun filters) prior to entering the PBR tank.

2.2 Artificial seawater.

The commercial salt Red Sea Salt (Red Salt) 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).

2.3 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 a triple concentration of f/2 growth medium (Guillard et al. 1962, Guillard 1975) without cobalt as described in Thoisen et al. (*In review*). Inoculum cultures for the PBR were cultivated in five or seven 5 L glass flasks with aeration (0.2 µm filtered) at a light intensity of 100 mol m^{-2} s^{-1} and a temperature of 19 ˚C in the same climate room as the PBR.

Equipment and 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 nutrients were added directly into the PBR tank corresponding to the total volume of ASW. Then, 25 to 35 L of dense inoculum culture was poured into the PBR tank. After inoculation the light intensity was set to 100 µmol m^{-2} s^{-1} to avoid light inhibition of the microalgal cells. Few days later, the light intensity was set to 200 µmol m^{-2} s^{-1} when the culture in the PBR reached a density of approximately 5×10^5 cells ml^{-1}. Dilution of the PBR (nutrient input and harvest output) was activated a few days after inoculation to avoid nutrient limitation, and was increased as needed to maintain the culture at a steady density.

2.4 Experimental conditions and sampling.

Densities of 5×10^5, 1×10^6, 1.5×10^6 and 2×10^6 cells ml^{-1} (n = 2, except

1.5×10^6 where n = 3) were cultivated in the PBR at a temperature of $19.0 \pm$ 0.1 ˚C, and pH 8.2 ± 0.3, except for 1.5×10^6 cells ml^{-1} at pH 9.0 ± 0.1. Light intensities were set to 100, 200 and 175 µmol m^{-2} s^{-1} for densities of 5×10^5, 1×10^6 and 1.5×10^6 cells ml^{-1}, respectively. For 2×10^6 cells ml^{-1} the light intensity for the first and second run was 200 and 250 µmol m^{-2} s^{-1}, respectively. The experimental duration of steady state at densities of 5×10^5, 1×10^6, and 2×10^6 cells ml^{-1} was roughly 4 to 5 days, and between 6 to 17 days at 1.5×10^6 cells ml^{-1}. The data presented is a compilation of experiments conducted in the described PBR from 2015 to 2017. Each run generally had duration of three weeks from inoculation to the end of a run and was adjusted to one or two densities throughout the run. Due to the wide temporal span of the data collected for this paper not all experimental parameters are exactly the same. Four runs in the PBR are illustrated on Fig. 1.
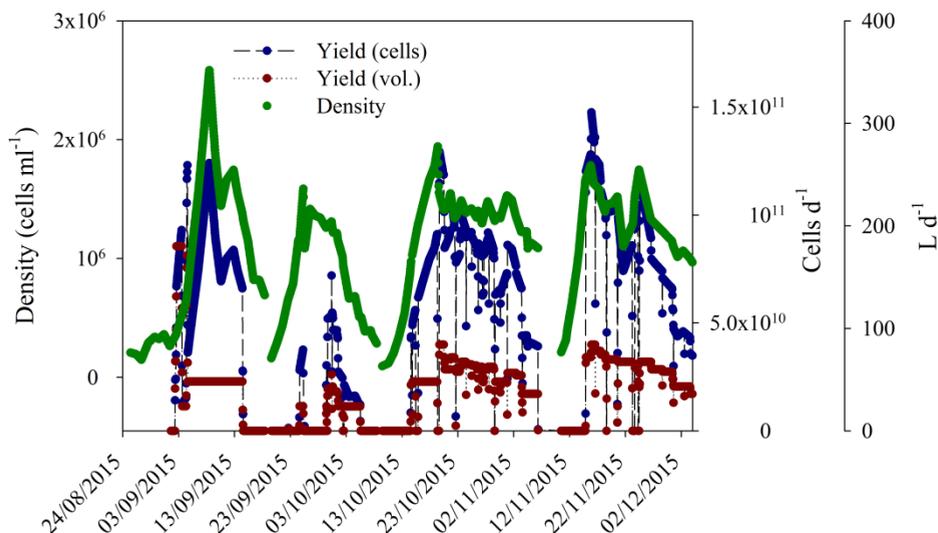
Fig. 1 Cultivation of *Rhodomonas salina* in a meso-scale PBR (250 L). Density (green, cells ml^{-1}) and production yield (blue, cells d^{-1}; red, L d^{-1}) is illustrated for four runs.

The density was determined from replicate samples (n = 3) tapped 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 sedimentation of cells. The optical density (OD) was measured and the density calculated using a constant obtained from a linear regression between OD$_{750\,nm}$ (Eppendorf BioSpectrometer) and cell enumeration on a Coulter Counter model 4E (Beckman):

$$Cell\ concentration\ (ml^{-1}) = 2969052.75 * OD_{750\,nm}$$

Samples for fatty acids were sampled from the same tap. 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, $L\ h^{-1}$):

$$Dilution\ rate\ (d^{-1}) = \frac{exchange\ rate\ (L\ h^{-1}) * 24\ h}{PBR\ vol.(L)}$$

2. 5 Fatty acids

Samples for comparing fatty acid composition between experimental conditions were filtered through 0.2 µm filters (Whatman[TM] 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. (2006a) 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) $NaHCO_3$ which 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 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.).

2.6 Light model

In order to be able to estimate the light conditions inside the PBR during

production a simple light model was established. With the empty PBR a series

of light measurements were made at corresponding locations outside as well

as inside the tubes. The light source was the actual LED lights used for

illuminating the PBR, and the light sensor was a LiCor 4π-sensor (LiCor, 192

Nebraska, USA). The attenuation coefficient (K_d) at various algal cell

densities was determined from a total of seven suspensions of *R. salina*

ranging from 5×10^5 to 9×10^6 cells ml^{-1} obtained by centrifugation. The light

attenuation across the full PAR spectrum was measured on a

spectrophotometer (Genesys 6, ThermoSpectronic) and used for calculation of

K_d. The relationship between K_d and density was determined by least-squares

linear regression (Sokal et al. 1995). The irradiance inside the PBR was

calculated as a function of algal cell density in sections of the PBR tube from

front to back based on values of LED light emission, light absorption in the

acrylic glass of the cylinder and K_d values. The amount of light received by

the cells in the culture in the PBR was calculated using a simple exponential

equation for light intensity at a given depth: $I_z = I_0 * e^{-K_d z}$ where I_z is the

light intensity at a depth of z (m) and I_0 is the light intensity just below the

surface. It must be emphasized that the light intensity inside the PBR

calculated this way is a minimum estimate, as the calculations are based on

the tubes being completely filled with algal suspension. This is not the case as

the design is a partially air-filled wavy tubular PBR but due to the chaotic

nature of the movement of the air and liquid phases it was not possible to take this into consideration. Furthermore, the following assumptions were made for this simple light model: 1) cells were homogenously distributed in the tube, and 2) light attenuation was exponential through the tube (Kirk 1994).

3. Results

The desired density in the experimental runs were 5×10^5, 1×10^6, 1.5×10^6 and 2×10^6 cells ml^{-1} with actual densities of $4.8 \times 10^5 \pm 2.3 \times 10^4$; $1.2 \times 10^6 \pm 3.8 \times 10^4$; $1.4 \times 10^6 \pm 1.0 \times 10^5$ and $2.1 \times 10^6 \pm 3.8 \times 10^5$, respectively (Fig. 2). The highest dilution rate was 0.52 ± 0.00 d^{-1} and obtained at 1×10^6 cells ml^{-1}. Lower but similar dilution rates between 0.28 and 0.36 were obtained at the other densities. pH was kept stabile close to pH 8, except at 1.5×10^6 where pH was 9.
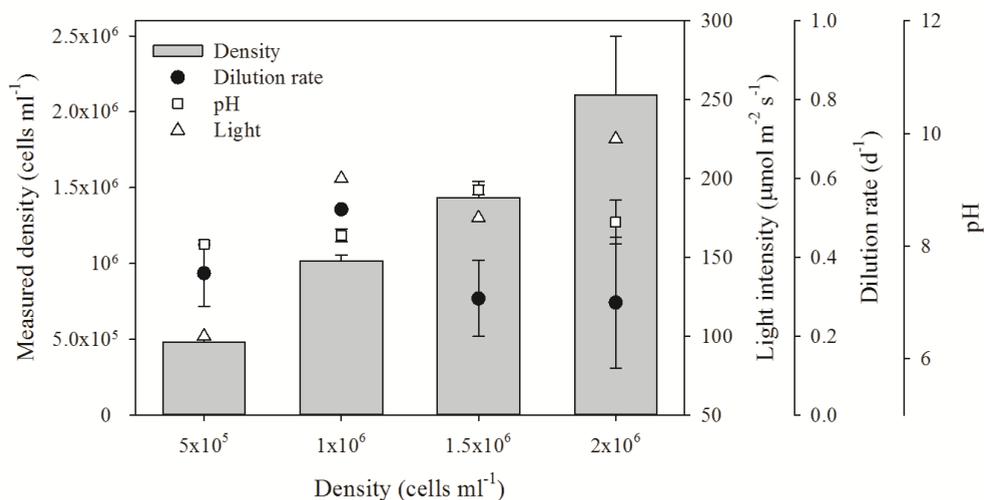
Fig. 2 Experimental parameters for meso-scale experiments with *Rhodomonas salina*; density

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(grey bars, cells ml^{-1}), light intensity ($\triangle$, µmol m^{-2} s^{-1}), dilution rate ($\bullet$, d^{-1}) and pH ($\square$). Mean values $\pm$ S.D. (n = 2, except 1.5x10^6; n = 3)

The amount of light received by the cells in the PBR is as much dependent on the density as the light intensity (Fig. 3). The light intensity at 1x10^6 cells ml^{-1} was set to 200 µmol m^{-2} s^{-1} but the calculated light received by the cells was 44 $\pm$ 2 µmol m^{-2} s^{-1} (78 % reduction). A similar light intensity (225 $\pm$ 35 µmol m^{-2} s^{-1}) at the highest density of 2x10^6 cells ml^{-1} resulted in a light received by the cells of 18 $\pm$ 4 µmol m^{-2} s^{-1} (92 % reduction). Even at the low density of 5x10^5 cells ml^{-1} the light was reduced by 63 % from 100 to 37 $\pm$ 1 µmol m^{-2} s^{-1}. The density receiving the highest amount of light in the culture (1x10^6) also had the highest dilution rate. There was a consistent pattern with decreasing light received by the cells in the culture resulting in a decreased dilution rate.
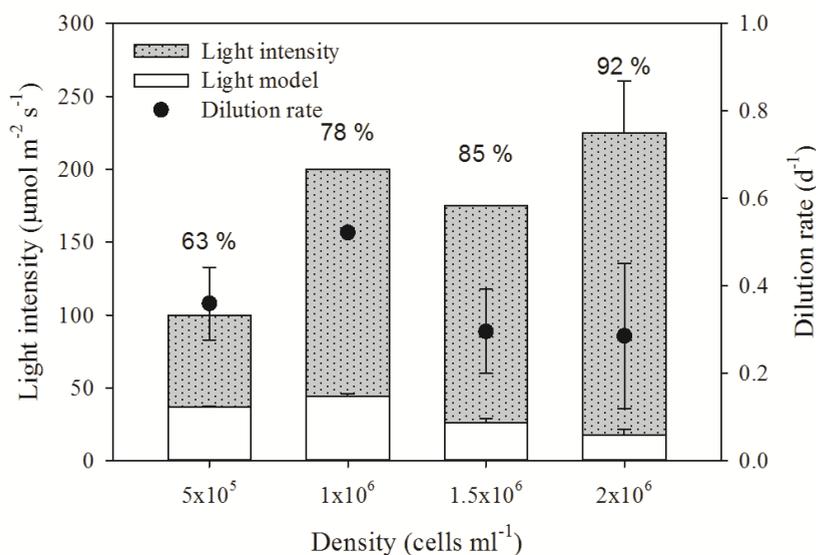
Fig. 3 The light received by *Rhodomonas salina* cells in the culture calculated with a simple model (white bars) compared to the light intensity (grey bars). Values above bars indicate the reduction (%) of light. Dilution rates ($\bullet$, d^{-1}) from figure 1 are shown for comparison. Mean value $\pm$ S.D. (n = 2, except 1.5×10^5; n = 3).

The yield in terms of number of cells and volume of culture was more or less within the same range for densities at 1×10^6, 1.5×10^6 and 2×10^6 cells ml^{-1} (Fig. 4). The highest yield of cells was $8.5 \times 10^{10} \pm 3.3 \times 10^9$ and $8.5 \times 10^{10} \pm 3.5 \times 10^{10}$ cells d^{-1} at densities 1×10^6 and 2×10^6 cells ml^{-1}. At 5×10^5 cells ml^{-1} the yield of cells was evidently lower ($2.6 \times 10^9 \pm 8.3 \times 10^9$). The yield of culture volume was lowest and highest at densities 1×10^6 and 2×10^6 cells ml^{-1} with 83.1 ± 0.1 and 42.4 ± 24.4 L d^{-1}, respectively. The yield in volume at

$5x10^5$ cells ml^{-1} was high (53.5 ± 14.6 L h^{-1}) considering the low yield of cells at this density.
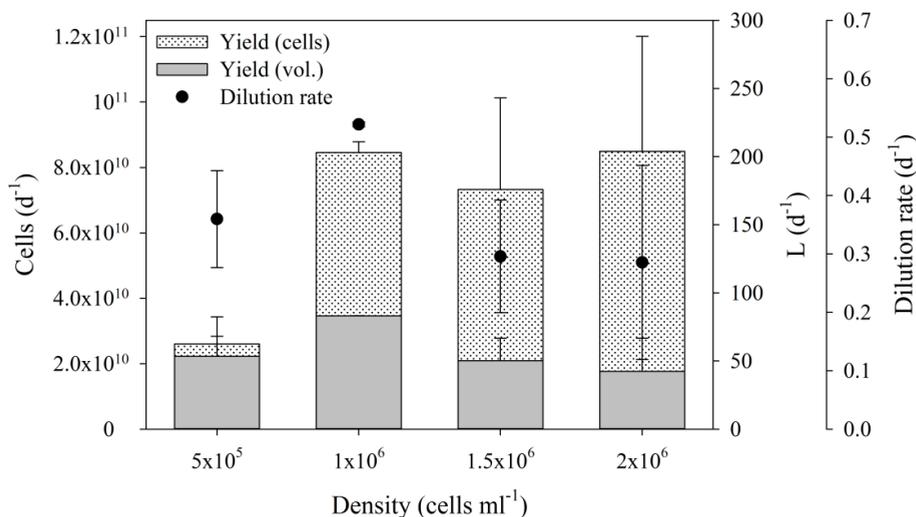
Fig. 4 Production yields from the different densities of *Rhodomonas salina* shown as cells (dotted bars, d^{-1}) and volume of culture (grey bars, L d^{-1}). Dilution rates ($\bullet$, d^{-1}) from figure 1 are shown for comparison. Mean values $\pm$ S.D. (n = 2, except $1.5x10^6$; n = 3). S.D. for volume at $1x10^6$ cells ml^{-1} is $\pm$ 0.13.

The cell content of TFA in *R. salina* showed a slight decrease from 13.1 ± 0.3 to 10.7 ± 0.4 pg cell^{-1} at $5x10^5$ and $2x10^6$ cells ml^{-1} (Table 1). This was reflected in the cell content of EPA and DHA with slightly higher values found at a density of $2x10^6$ cells ml^{-1} (12.9 ± 0.3 % EPA and 9.1 ± 0.3 % DHA). The ratio of DHA/EPA was similar for all densities at 0.7 to 0.8, while the ratio of EPA/ARA ranged from 14.1 at $5x10^5$ cells ml^{-1} to 21.5 at $2x10^6$ cells ml^{-1}.

Table 1 The cell content of EPA, DHA and ARA in *Rhodomonas salina* and the ratio of DHA/EPA and EPA/ARA. Unit of TFA is pg cell^{-1}. EPA, DHA and ARA are % of TFA. Mean values ± S.D. (n = 2, except 2x10^6; n = 1).

Treatment	TFA	EPA	DHA	ARA	DHA/EPA	EPA/ARA
5*10^5	13.1 ± 0.3	9.9 ± 0.3	8.1 ± 0.0	0.7 ± 0.1	0.8	14.1
1*10^6	11.4 ± 0.1	11.6 ± 1.0	8.6 ± 0.9	0.6 ± 0.0	0.7	19.3
2*10^6	10.7 ± 0.4	12.9 ± 0.3	9.1 ± 0.3	0.6 ± 0.0	0.7	21.5

4. Discussion

Production of microalgal biomass requires a thorough understanding of how various parameters affect a given microalgal species. The vast majority of studies report the light intensity in front of the cultivation vessel or the set value of the light intensity. However, this is highly insufficient as shown in the present study with the simple light model. The light received by cells is drastically reduced by the density. Although the light model is simple and somewhat underestimates the light received by the cells, there is an obvious coherency between the obtained dilution rates and the light received by the cells. A recent study by Vu et al. (*In preparation*) with the same strain of *R. salina* (K-1487) cultivated in a medium-scale (2x 47 L) bubble-column PBR (ø = 20cm) illuminated from both sides obtained a dilution rate of 0.42 d^{-1} at 2.2x10^6 cells ml^{-1} and 0.46 d^{-1} at 1.8x10^6 cells ml^{-1}. According to (Vu et al.

2016), the light compensation point for *R. salina* is approximately 12 μmol m^{-2} s^{-1} which is close to the light received by the cells in the culture at a density of 2x10^6 in the present experiment. Growth rates (equivalent to dilution rates) from small-scale experiments with *R. salina* are within a range of 0.6 to 1.4 d^{-1} (Bartual et al. 2002, Guevara et al. 2016, Vu et al. 2016, Thoisen et al. *In review*). This is higher compared to meso-scale experiments due to smaller cultivation vessels and no dark fraction of the system (i.e., bendings and PBR tank) allowing more light to enter the culture. Nonetheless, it is expected that the dilution rate of *R. salina* in the studied PBR can be increased by increasing the light received by the cells.

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) demonstrated that *R. salina* (K-1487) has a better biochemical profile for aquaculture when cultivated in excess of nutrients. The ratio of DHA/EPA in *R. salina* in the present study of 0.6 to 0.7 is in the low range of what has previously been reported for *R. salina* with ratios from 0.5 to 1.5 (Dunstan et al. 2005, Guevara et al. 2016, Vu et al. 2016). Contrary, the ratio of EPA/ARA is high compared to other studies (4 to 13.2 versus 14.1 to 21.5) (Dunstan et al. 2005, Guevara et al. 2016). It could be suggested that the density has a negative effect on TFA as a slight decrease was observed which

interestingly did not follow the pattern of light received by the cells calculated from the light model. Contrary, the cell content of EPA, DHA and ARA in pg cell^{-1} (data not shown) was similar between densities. Literature on the effect of density on fatty acids is limited and ambiguous. A study by Lu et al. (2001) reported an effect of density on TFA at 88 µmol m^{-2} s^{-1} but not at lower light (44 µmol m^{-2} s^{-1}) for the eustigmatophyte *Monodus subterraneus*, while Chrismadha et al. (1994) reported little but no clear effect for the diatom *Phaeodactylum tricornutum*.

A desirable outcome for a meso-scale production of microalgae as feed for copepods is a high number of cells in a low volume of culture to reduce the increase in volume of the copepod cultures. Thus, based on results within the experimental range in the present study, the optimal density of a meso-scale production of *R. salina* as a microalgal diet is 1.0×10^6 to 2×10^6 cells ml^{-1}. However, densities of 1×10^6 and 1.5×10^6 are more suitable due to a high increase of biofouling observed at 2×10^6 cells ml^{-1}. Biofouling of PBRs is a common problem as cleaning procedures are often tedious and may shut down production while in progress. Compared to many other microalgae species, *R. salina* is quite sensitive to stressful conditions which was also noted by Arndt et al. (2014). Thus, in the present study cultivation of *R. salina* was possible for no more than 3 weeks where after the PBR had to be emptied

and cleaned. When cultivation was prolonged >3 weeks the culture crashed despite sufficient nutrients. The sensitivity of *Rhodomonas* is a probably one of the main causes for its absence in the aquaculture as certain expertise is required to successfully cultivate this species. However, this study clearly shows that a semi-continuous production of *R. salina* is indeed achievable. By expanding the cultivation of *R. salina* in two or more PBRs the microalgal production can be optimized by sequentially cleaning one PBR while the other PBRs are running.

5. Conclusion

The successful semi-continuous meso-scale production of *R. salina* demonstrated here has great potential for aquaculture as it will allow the production of an ideal microalgal diet for copepods used as live feed for fish larvae. The cell content of fatty acids was more or less the same for the studied cell densities of *R. salina* but it is recommended to run the meso-scale production at a density of $1x10^6$ or $1.5x10^6$ to reduce negative effects of biofouling of the PBR. When cultivating microalgae the light received by the cells should be contemplated as an increase in external light intensity with increasing density does not necessarily result in increased dilution rate due to a high degree of shelf-shading of the cells.

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Paper 4

A comparison of five strains of the cryptophyte *Rhodomonas*: Temperature effects on growth rate and biochemical profile.

In prep.

A comparison of five strains of the cryptophyte *Rhodomonas*:
Temperature effects on growth rate and biochemical profile

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Abstract

Five strains of *Rhodomonas* were studied in a common garden setup to compare the effects of temperature on growth rate and biochemical composition among these closely related species. The strains did not differ in their content of carbon and nitrogen, and only strain CCAP 995/5 had a significantly longer cell length. However, the strains were different in terms of their content of fatty acids although the effect of temperature on polyunsaturated fatty acids (PUFAs) did not show a clear pattern other than an increase in ARA at 25 ˚C for all strains, except strain K-0435. Likewise, there was no clear pattern for their free amino acids (FAAs) composition and content at the experimental temperatures. We suggest that not only the growth rate but also the content of PUFAs and FAAs depends on the species and strain and most likely are genetically determined rather than controlled by the growth temperature.

Introduction

The growth rate and biochemical composition of microalgae is affected by, e.g., light intensity, temperature, salinity and nutrient availability (Bartual et al. 2002, Boussiba and Vonshak 1991, Durmaz et al. 2008, Durmaz et al. 2009, Flynn et al. 1992, Renaud et al. 1991, Renaud et al. 1995, Renaud et al. 2002, Thompson 2005, Vu et al. 2016). Microalgae can therefore, to a certain degree, be manipulated to increase their content of a desired biochemical compound by adjusting the parameters during cultivation. A well-known example is the pigment astaxanthin which is accumulated in high quantities during environmental and nutrient stress in the chlorophyte *Haematococcus pluvialis* (Boussiba and Vonshak 1991).

The effect of various parameters on growth rate and biochemical composition is species-specific and even strain-specific (Brown 1991, Guevara et al. 2016, Renaud et al. 2002, Thompson 2005). In a study by Thompson et al. (1992), the growth rate of eight species of microalgae generally increased with temperature (10˚ C to 25 ˚C) but the content of PUFAs showed no clear relationship with temperature. However, when data for all eight species was pooled there was a weak trend for increased PUFA concentration at low temperatures. Contrary, Guevara et al. (2016) found similar growth rates of two strains of *Rhodomonas salina* at 19 and 29 ˚C and a strong influence of temperature on the content of PUFAs with a higher content also at the low temperature.

An extensive study by Lang et al. (2011) on > 2000 strains of microalgae showed that the fatty acid (FA) profiles of microalgae can be used as a chemotaxic marker as the FA distribution reflect the phylogenetic relationships at levels of both phylum and class. At lower taxonomic levels,

such as closely related species and strains, the FA content may be more variable and this is particularly interesting as, e.g., certain strains could potentially contain a more desirable biochemical composition than others. This was demonstrated for the two strains of *R. salina* in the study by Guevara et al. (2016) where one of the strains was found to be more suitable for use for aquaculture purposes due to higher growth rate and content of PUFAs.

Various marine microalgal species have been found to have similar amino acids profiles rich in essential amino acids (EAA). Generally high amounts of glutamic acid and aspartate are found, and low amounts of cysteine, methionine and histidine (Brown 1991, Brown et al. 1997, Fabregas and Herrero 1985, Kent et al. 2015). However, the effect of temperature on the content of amino acids is not well studied. A varying amino acid content affected by temperature (15 to 35 ˚C) was indicated for *Chlorella* and *Nannochloropsis* where the EAA content generally increased with temperature (James et al. 1989).

Several studies have shown that *Rhodomonas* is an excellent microalgal diet for copepods which are used as live feed in aquaculture (Arndt and Sommer 2014, Knuckey et al. 2005, Ohs et al. 2010, Støttrup and Jensen 1990, Zhang et al. 2013). This microalga has a good nutritional composition, particularly PUFAs, which allows a mono-algal diet for the copepods contrary to supplying with a mixed algal diet or even oil emulsion to obtain a sufficient nutritional value. The growth rate of *Rhodomonas* and content of fatty acids at different temperatures has previously been studied (Chaloub et al. 2015, Drillet et al. 2006, Guevara et al. 2016, Renaud et al. 2002, Vu et al. 2016) but aside from the study by Guevara et al. (2016) studies comparing several species and strains of the genus *Rhodomonas* are lacking.

This aim of this study was to investigate the biochemical profile and growth capacity of closely related species and strains of *Rhodomonas*, as well as the effects of varying temperature on these parameters. This was done with the underlying purpose to evaluate if some of the strains are more suitable for use in aquaculture than others.

Materials and methods

Rhodomonas **strains, cell lengths and CN elemental analysis.** The species and strains of the cryptophyte *Rhodomonas* were obtained from the culture collections listed in table 1 and referred to by their respective strain number. The strains were cultivated in filtered seawater (0.2 µm and salinity 32) from the Kattegat (DK) and a modified version of f/2 growth medium without cobalt(II)chloride ($CoCl_2$) and silicate (Thoisen et al. *Accepted*). The cell lengths of the strains were measured using an inverted microscope (Nikon Diaphot 300) connected to the program Nikon Software NIS-Elements (n = 40). The CN elemental analysis was carried out on a CE Instruments EA1110 CHNS (Wigan, UK) as described in Jakobsen et al. (2018) with strains cultivated at 20 ˚C at a light intensity of 85 µmol m^{-2} s^{-1}.

Table 1. The studied strains of *Rhodomonas* obtained from various culture collections.

Species	Strain no.	Culture collection
R. salina	K-1487	Scandinavian Culture Collection of Algae & Protozoa (SCCAP)
R. salina	K-0294	Scandinavian Culture Collection of Algae & Protozoa (SCCAP)
R. salina	LB 2763	The University of Texas at Austin (UTEX)
R. marina	K-0435	Scandinavian Culture Collection of Algae & Protozoa (SCCAP)
R. sp.	CCAP 995/5	Culture Collection of Algae and Protozoa (CCAP)

Experimental conditions. For the experiment on the effect of temperature, the strains were cultivated during five days in the small-scale photobioreactor (PBR) 'Multi-Cultivator MC1000-OD' (Photon System Instruments, CZ) which has eight vertical aerated test tubes each with a maximum culture volume of 85 ml. The experimental temperatures were 15, 20 and 25 ˚C at a light intensity of 85 µmol m^{-2} s^{-1}. The cell concentration at the beginning of the experiment was approximately 100,000 cells ml^{-1} and was enumerated daily on a Coulter Counter (Beckman) using the computer program Multisizer 3. The growth rates (µ, d^{-1}) were determined by linear regressions of logarithmic cell concentrations over time. f/2 growth medium was added daily to the test tubes ensuring nutrients in excess. Materials, seawater, stock solutions and vitamins for f/2 growth medium were autoclaved prior to use.

Fatty acids analysis. Samples for FA analysis were taken at the end of the experiment during the exponential algal growth phase. A known volume of each strain was filtered onto a 0.2 µm glass microfiber filter (Whatman^{TM} GF/C^{TM}), transferred to muffled Pyrex glass vials and stored at -80 ˚C until analysis. Prior to analysis the samples lyophilized for 24 hours and analyzed as described in Drillet et al. (2006) with minor adjustments. Each Pyrex glass vial was added 2 ml chloroform and 1 ml methanol followed by 20 µL of an internal standard (C-23-methylester, 1000 µg ml^{-1}). The vials were capped and allowed to extract for 24 hr at -20 ˚C. The extract was then transferred to a 2 ml GC vial and dried out at 60 ˚C under a flow of nitrogen. A volume of 1 ml of a mixture of toluene-MeOH-AcOCl (66ml: 85 ml: 15 ml) was added to each dried out GC vial and the vials were capped and heated at 90 ˚C for 2 hr. Hereafter, 500 µL of 5% $NaHCO_3$ was added to each vial creating an upper and lower phase. The two phases were mixed thoroughly with a glass pipette and separated by gently bubbling into the lower phase. The upper phase was

transferred to a new GC vial and the 'old' GC vial was re-extracted twice with 500 µL heptane, mixed and separated. The GC vials containing the upper phases were dried out at 60 ˚C under a flow of nitrogen, added 0.5 ml chloroform and capped until analysis. The samples were analyzed on an Agilent (Santa Clara, CA, US) GC 6890N connected to an Agilent Mass Selective Detedctor (MS) 5975 equipped with a 60 m Agilent J&W DB 23 column (internal diameter: 0.25 mm, film thickness: 0.25 µm).

Free amino acids analysis. Samples for FAA analysis were taken at the end of the experiment during exponential algal growth. A known volume of each strain was filtered onto a 0.2 µm glass microfiber filter (Whatman[TM] GF/C[TM]), transferred to muffled Pyrex glass vials and stored at -80 ˚C until analysis. The method used was a modified version of the method described in Drillet et al. (2006). Prior to analysis, the filters with the samples were lyophilized for 24 hours. Each sample was added 1 ml 6 µM of the internal standard α-aminobutyric acid (AABA 99%, obtained from Sigma and diluted in Milli-Q dH$_2$O) and heated at 95 ˚C for 15 min. The samples were then vortexed for 30 s and filtered through 0.2 µm GHP polypropylene membrane filters (13 mm) into a 2 ml HPLC vial, and a subsample of 100 µL was transferred to a 0.2 ml insert in a new HPLC vial. The 100 µL sample was evaporated at 40 ˚C under a flow of nitrogen, and added 100 µL borate buffer (0.2 M, pH 8.8) and 20 µL 10 mM AQC derivatization reagent (Synchem). The samples were then placed in a heating cabinet at 55 ˚C for 20 min.

The samples were analyzed on HPLC with a Waters Alliance 2695 separation module and a 3.9 x 150 mm Nov-Pak[TM] Silika C-18 column at 39 ˚C (injection volume: 10 µL). The samples were led through a UV detector (254 nm) and a spectrofluorometer (excitation: 250 nm, emission: 395 nm). Solvents for separation were acetate phosphate (87.4 g sodium acetate in 100

ml Milli-Q H_2O and 9.72 ml triethyleneamine) at pH 5.7 and pH 6.8, acetonitrile and Milli-Q H_2O. A serial dilution (1.25 − 160 µM) of a standard with amino acids (Waters) was analyzed as a reference. Results were obtained by integrating the peaks from the HPLC with the software Chromeleon[TM] 7.

Statistical analysis. Data on growth rate, cell content of FAs and FAAs were subjected to one-way ANOVAs and significant results were followed by a Bonferroni post-hoc test to compare individual means across significantly different levels. These tests were carried out using SigmaPlot 12.0 (Systat Software) with $\alpha = 0.05$.

Data for the multidimensional scaling (MDS) plot on the content of FAs in the strains was subjected to analysis of similarities (ANOSIM) and similarity percentage (SIMPER, Euclidian distance) to test for and identify differences between the strains. These tests were carried out using Primer 6 version 6.1.13 & Permanova version 1.0.3 (Primer-E Ltd.)

Results

Cell lengths and CN content. The cell lengths of the strains were similar at about 11.5 µm, except CCAP 995/5 which had a significantly longer cell length of 14.4 ± 1.6 µm ($p < 0.001$) (table 2). There was no statistical significant difference in the cell content of C and N between the strains with carbon ranging from 56.6 to 77.9 pg cell[-1] and nitrogen ranging from 11.8 to 14.5 pg cell[-1].

Table 2. The cell length and content of carbon (C) and nitrogen (N) of the *Rhodomonas* strains. Mean values ± S.D. (Cell length: n = 40, CN analysis: n= 4 to 8)

Strain	Cell length (μm)	C (pg cell^{-1})	N (pg cell^{-1})
K-1487	11.9 ± 1.3	56.6 ± 1.4[a]	12.7 ± 0.4[a]
K-0294	11.5 ± 1.3	73.5 ± 10.2	13.8 ± 1.2
LB 2763	11.6 ± 1.5	77.9 ± 21.3	14.5 ± 2.5
K-0435	11.5 ± 1.7	59.8 ± 7.6	11.8 ± 1.3
CCAP 995/5	14.4 ± 1.6	64.4 ± 7.0	12.4 ± 0.7

[a]CN results for strain K-1487 obtained from Jakobsen et al. (2018)

FA profiles. The FA profiles were found to differ significantly among strains (ANOSIM, Global R: 0.722) (Figure 1). In particular, strain CCAP 995/5 was highly separated from the other strains (ANOSIM, R = 1) due to differences in the content of FAs such as C18:4, C22:6 n-3 (DHA) and C16:0. Also strain K-0294 was highly separated from K-0435 and K-1487 (ANOSIM, R = 1) and from LB 2763(ANOSIM, R = 0.778) due to differences in the content of FAs such as C22:1, C18:3 n- 3, C18:4 and C16:0. The remaining strains (K-0435, K-1487 and LB 2763) were less separated (ANOSIM, R ≤ 0.5) but primarily differed in their content of C22:1. A full list of the FAs contributing to the dissimilarities between the strains is listed in table 4 in the appendix.
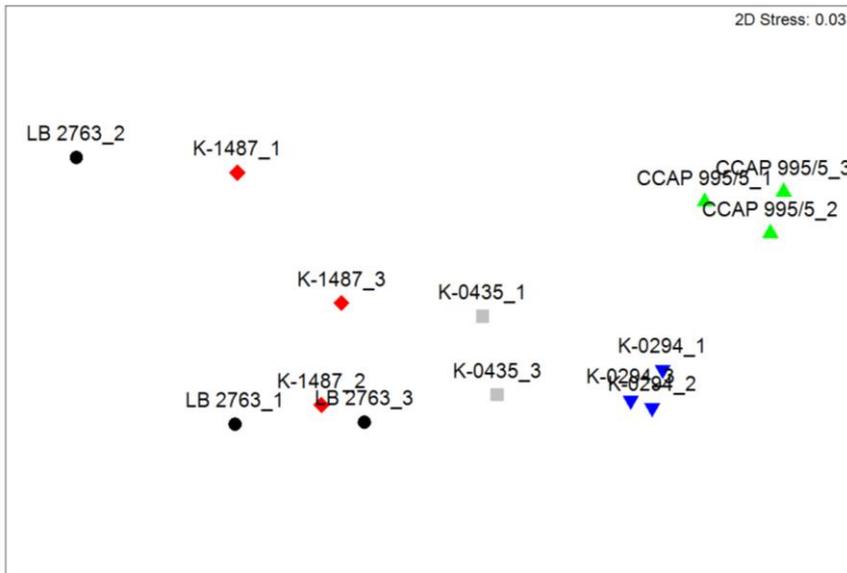
Figure 1. MDS plot of the FA profiles of the five strains of *Rhodomonas*.

Growth rates. The experimental temperatures had an effect on the growth rate of strains K-0435 and K-1487 (Fig. 2). The growth rate of K-0435 increased between 15 and 25 ˚C from 0.70 ± 0.02 d^{-1} to 0.82 ± 0.07 d^{-1} ($p <$ 0.001), while the growth rate of K-1487 was highest at 20 ˚C with 0.81 d$^{-1} \pm$ 0.02 d^{-1} ($p = 0.003$). None of the other strains were found to be affected by an increase in temperature within the studied 10°C interval.

There were no statistical significant differences in the growth rates between the strains at 20 ˚C with growth rates ranging from 0.75 ± 0.08 to 0.81 ± 0.02 d^{-1} or at 25 ˚C with growth rates ranging from 0.71 ± 0.12 to 0.87 ± 0.07 d^{-1}.Contrary, at 15 ˚C the growth rate of K-1487 was significantly lowest compared to the other strains ($p \leq 0.049$). The strains, except K-0294, were also
cultivated at 30 ˚C but evinced negative growth rates at this high temperature (data not shown).

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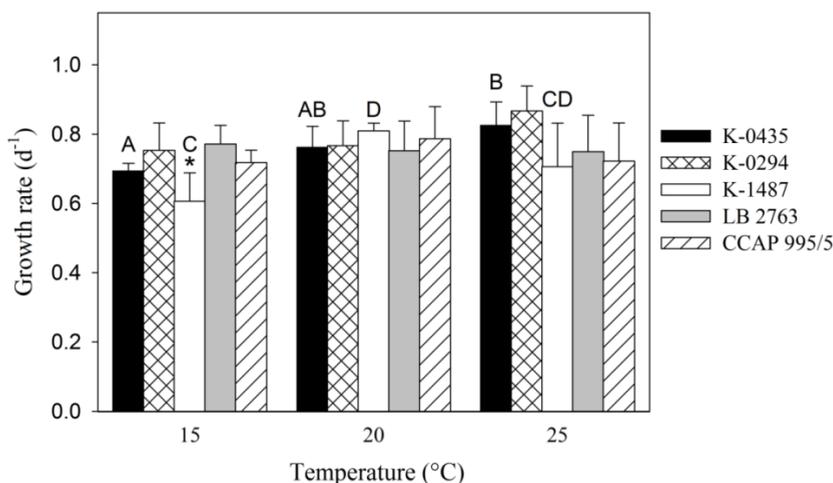
Figure 2. The growth rates of the five strains of *Rhodomonas* at temperatures 15, 20 and 25 ˚C. Symbol (*) indicates statistically significant different growth rates between strains at a given temperature. Letters (A, B, C, D) indicate statistically significant different growth rates of a given strain between temperatures. Mean values ± S.D. (n: see table 1 in the appendix).

Fatty acids. The amount of total fatty acids (TFA) did not vary statistically significant in any of the strains with increasing temperature or between the strains at a given temperature. The content of TFA ranged from 14.2 ± 1.6 to 26.4 ± 4.8 pg cell^{-1}(Figure 3).

There was a statistically significant lower cell content of EPA (% TFA) with increasing temperature for strains K-0294 ($p \leq 0.005$), LB 2763 ($p < 0.001$) and CCAP 995/5 ($p = 0.035$). At 25 ˚C the content of EPA in these strains had decreased to 10.02 ± 1.84 %, 10.49 ± 1.17 % and 11.33 ± 1.43 %, respectively. The content of EPA compared between the strains at a given temperature was not statistically significant at any of the experimental temperatures with values ranging from 10.02 to 14.11 % ($p \geq 0.065$) (figure 3).

An increase in temperature resulted in a statistically significant different content of DHA (% TFA) for strains K-0294 and K-0435. K-0294

had a lower content of 7.62 ± 0.44 % at 25 ˚C (p ≤ 0.025), and K-0435 had a lower content of 8.77 ± 1.03 % at 20 ˚C compared to 10.16 ± 0.37 at 15 ˚C (p = 0.014). The content of DHA was not statistically different between the strains at 20 ˚C with values ranging from 8.73 to 10.71 %. However, at 15 ˚C, there was a higher content of DHA in K-0435 (10.16 ± 0.37 %) compared to LB 2763 and K-0294 (p ≤0.010), and a lower content in LB 2763 (8.84 ± 0.44 %) compared to K-1487 and CCAP 995/5 (p ≤0.041). At 25 ˚C, the DHA content was higher in K-0435 (9.81 ± 0.23 %) compared to K-0294 and CCAP 995/5 (p ≤0.009). The overall tendency for the strains' content of ARA (% TFA) showed an increased content at 25 ˚C. Only strain K-0435 did not have a statistically significant higher content at this temperature. For K-0294, LB 2763 and CCAP 995/5 the content of ARA at 25 ˚C was higher compared to both 15 and 20 ˚C (p < 0.001), and for K-1487 there was a statistically different content between all the experimental temperatures (p ≤ 0.019). Also, the content of ARA between the strains at 25 ˚C was not statistically significant different but there was a difference between the strains at both 15 and 20 ˚C. K-0435 had the highest content of ARA at 15 ˚C (0.49 ± 0.23 %) compared to K-0294 and LB 2763 (p ≤ 0.023), and K-1487 had the lowest content (0.09 ± 0.18 %) at 20 ˚C compared to K-0435, K-0294 and CCAP 995/5. The statistical results are listed in table 3 in the appendix.

The statistical results for the content of EPA, DHA and ARA (% TFA) and the cell content of EPA, DHA and ARA expressed as pg cell[-1] are listed in table 2 and3 in the appendix.
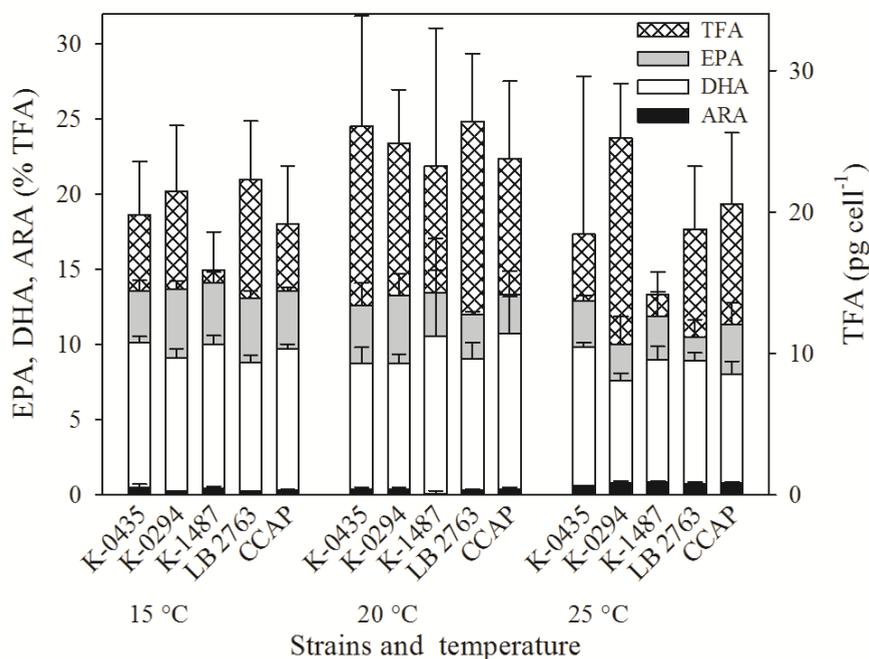
Figure 3. The content of EPA, DHA and ARA (% of TFA) and TFA (pg cell^{-1}) in the *Rhodomonas* strains. Mean values ± S.D. (n: see table 1 in the appendix).

The ratios of DHA / EPA and EPA / ARA in the strains are listed in table 2. The ratio of DHA / EPA was more or less similar at the experimental temperatures for K-0435 (0.69 to 0.75) and K-1487 (0.71 to 0.76). Contrary, a more pronounced increase in the ratio of DHA / EPA was found for LB 2763 and K-0294 at 25 ˚C (0.86 and 0.77, respectively). The highest ratio of DHA / EPA for CCAP 995/5 was found at 20 ˚C (0.85). The ratio was very similar among strains at 15 ˚C (0.67 to 0.74) but more diverse at 20 (0.65 to 0.85) and 25 ˚C (0.70 to 0.86).

The ratios of EPA / ARA ranged from 14 to 71 at the experimental temperatures. For K-0435 the ratio of EPA / ARA was quite similar at 15 and 20 ˚C (30 and 33) while the highest ratios were obtained at 15 ˚C for K-0294, LB 2763 and CCAP 995/5. Contrary, the highest ratio for K-1487 was

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obtained at 20 ˚C (71). Interestingly, all of the strains, except K-0294, obtained their lowest ratio of EPA / ARA at the highest temperature making 25 ˚C the temperature for the overall lowest ratio (14 to 21). At 15 and 20 ˚C the ratio of EPA / ARA was higher but somewhat similar (30 to 59 and 33 to 71, respectively).

Table 2. The ratios of DHA / EPA and EPA /ARA in the strains of *Rhodomonas* at 15, 20 and 25 ˚C. Values for the ratio of DHA / EPA are given with two decimals as these values were similar between strains and temperatures. The ratio of EPA / ARA is listed without decimals as these were more diverse. (n: see table 1 in the appendix).

	15 ˚C		20 ˚C		25 ˚C	
	DHA / EPA	EPA / ARA	DHA / EPA	EPA / ARA	DHA / EPA	EPA / ARA
K-0435	0.74	30	0.69	33	0.75	21
K-0294	0.67	59	0.65	37	0.77	41
K-1487	0.71	32	0.76	71	0.76	14
LB 2763	0.68	58	0.74	40	0.86	14
CCAP 995/5	0.71	52	0.85	35	0.70	14

Free amino acids. The content of total FAA in strains LB 2763 and CCAP 995/5 was significantly affected at all of the experimental temperatures but the effect was not similar (Figure 4). Strain LB 2763 obtained its highest content at 25 ˚C (8.88 ± 0.47 pg cell^{-1}, $p < 0.001$) while CCAP 995/5 obtained its highest content at 15 ˚C (7.49 ± 0.58 pg cell^{-1}, $p \leq 0.002$). The content of total FAA in the remaining strains was not statistically affected by temperature. The content of total FAA between the strains at 20 ˚C did not differ with an average amount of about 5 pg cell^{-1}. However, there was a difference at 15 and 25 ˚C. At 15 ˚C, strain CCAP 995/5 had the highest content (7.5 ± 0.6 pg cell^{-1}) compared to the others strains ($p \leq 0.035$), except LB 2763. At 25 ˚C the highest content (8.8 ± 0.5 pg cell^{-1}) was found for LB 2763 ($p < 0.001$).
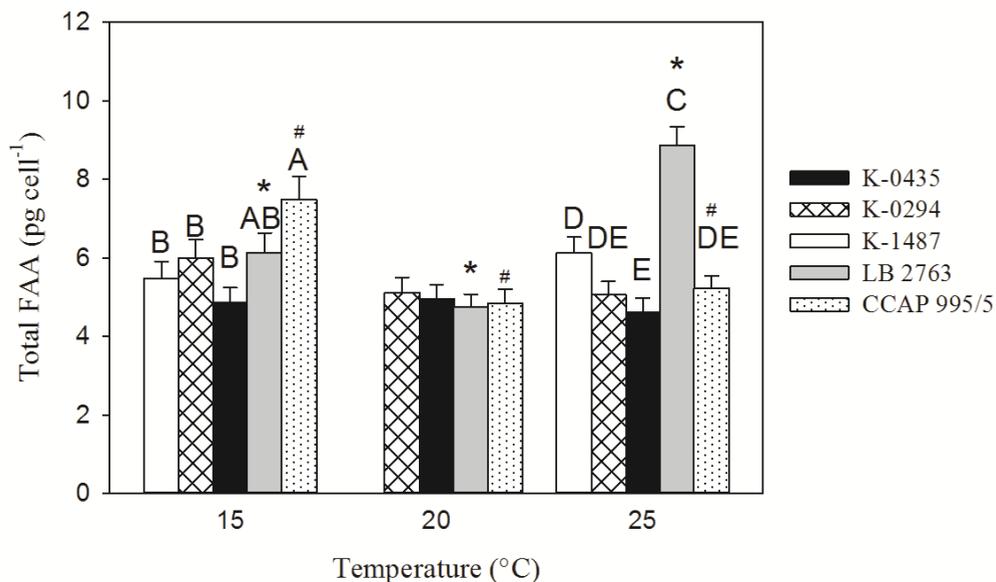
Figure 4. The cell content of total FAA in *Rhodomonas* strains at 15, 20 and 25 ˚C. Letters (A, B, C, D, E) indicate statistically significant differences between strains at a given temperature. Symbols (*, #) indicate statistically significant differences for a given strain between temperatures. K-1487: N/A at 20 ˚C. Mean values ± S.D. (n: 3).

Essential amino acids. The content of the EAA was not affected by temperature in strains K-1487 and K-0294. Contrary, strains K-0435, LB 2763 and CCAP 995/5 were affected significantly by temperature but obtained their highest content of EAA at various temperatures ($p \leq 0.014$) (Fig. 5 and table 3). There was no statistically significant difference in the content of free essential amino acids (% of total FAA) between the strains at 20 or 25 ˚C with the content ranging from 19.6 ± 1.9 to 25.7 ± 3.7 % at 20 ˚C ($p = 0.052$) and 12.3 ± 0.9 to 34.5 ± 6.5 % at 25 ˚C ($p = 0.080$). The highest content of EAA at 15 ˚C was found in CCAP 995/5 with 37.5 ± 2.9 % ($p \leq 0.022$).

The most general statistical significant patterns observed for the effect of temperature on the content of individual EAAs was a decrease in arginine (ARG) with an increase in temperature for all strains, except K-0435. An increase with temperature was found for tyrosine (TYR) (strains K-1487, K-

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0294 and CCAP 995/5), lysine (LYS) (K-0294, K-0435, LB 2763 and CCAP 995/5) and phenylalanine (PHE) (strains K-0294, K-0435 and CCAP 995/5).

The relative abundance (% total FAA) of FAA in the strains was dominated by glutamic acid (GLU), proline (PRO) and arginine. For the non-essential FAA, glutamic acid was highest at all of the experimental temperatures (ranging from 19.3 ± 1.5 % to 31.8 ± 5.8 %), whereas proline dominated at 15 and 20 ˚C (19.3 ± 2.3% to 13.0 ± 3.6 %) but decreased at 25 ˚C (< 8 %). For the essential FAA, arginine dominated at 15 ˚C (18.6 ± 7.0 to 25.6 ± 2.0 %) but decreased at 20 and 25 ˚C (≤ 11.0). Histidine (HIS) was low, absent or below detection limit for all strains, except CCAP 995/5, at 15 ˚C ($\leq 3.8 \pm 4.4$ %). A table listing all the FAAs found in the strains can be found table 5 in the appendix.
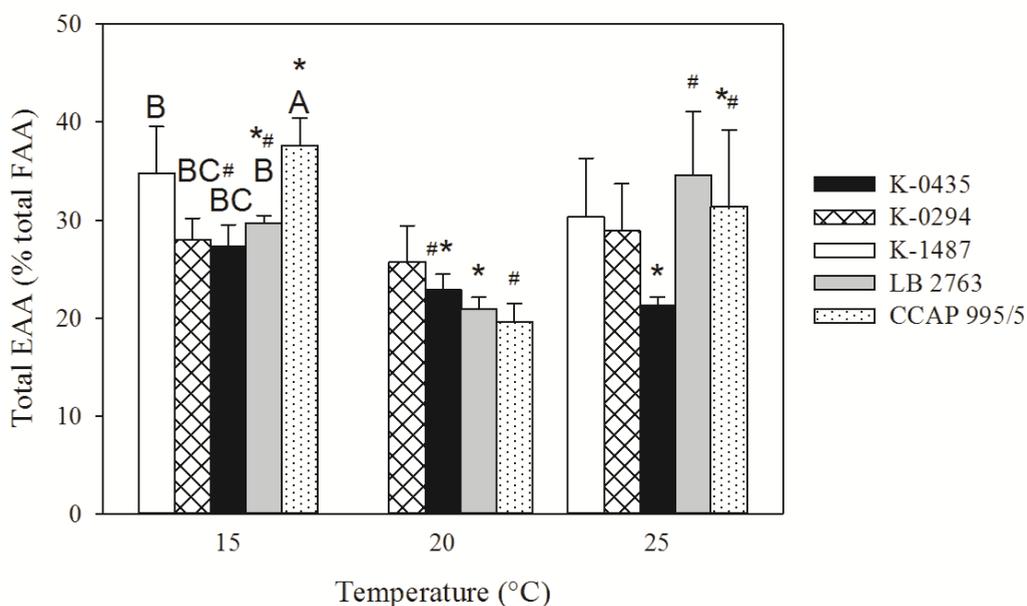
Figure 5. The content of essential amino acids (% of total FAA) in the *Rhodomonas* strains at 15, 20 and 25 ˚C. Letters (A, B, C) indicate ly significant differences between strains at a given temperature. Symbols (*, #) indicate statistically significant differences for a given strain between temperatures. K-1487: N/A at 20 ˚C. Mean values $\pm$ S.D. (n: 3).

Table 3. The content of individual EAAs, sub-total EAA and total FAA in the strains of *Rhodomonas* at 15, 20 and 25 ˚C. Units of EAA and sub-total EAA: % of total FAA. Units of total FAA: pg cell^{-1} Letters (A, B, C) indicate statistically significant differences for a given strain between temperatures. Numbers (1, 2, 3) indicate statistically significant differences between strains at a given temperature. K-1487: N/A at 20 ˚C. Mean values ± S.D. (n: 3).

eaa

Temp	Strain	HIS	THR	ARG	TYR	VAL	MET
15 ˚C	K-0435	0.00 ± 0.00	0.95 ± 0.06	18.10 ± 5.24	1.04 ± 0.13^{1}	2.42 ± 0.92	0.80 ± 0.05^{B,3}
	K-0294	0.02 ± 0.04	1.01 ± 0.27	21.69 ± 3.36^{A}	0.45 ± 0.03^{A,2}	1.02 ± 0.07^{A}	0.43 ± 0.18^{A,2,4}
	K-1487	0.00 ± 0.00	0.77 ± 0.05	23.74 ± 3.68^{A}	0.46 ± 0.08^{A,2}	1.81 ± 0.28	1.03 ± 0.14^{1,3}
	LB 2763	0.00 ± .00^{A}	0.89 ± 0.26	23.58 ± 1.19^{A}	0.42 ± 0.07^{2}	1.82 ± 0.50	0.41 ± 0.06^{2,4}
	CCAP 995/5	3.81 ± 4.43	1.25 ± 0.08^{A,B}	25.62 ± 1.97^{A}	0.54 ± 0.02^{B,2}	1.34 ± 0.18	0.75 ± 0.10
20 ˚C	K-0435	3.21 ± 1.36	1.34 ± 0.34	11.00 ± 1.85	0.96 ± 0.23	1.48 ± 0.44	0.90 ± 0.13^{B}
	K-0294	6.57 ± 3.88	1.44 ± 0.14	9.91 ± 2.71^{B}	1.12 ± 0.30^{B}	1.44 ± 0.23^{B}	1.14 ± 0.17^{B,1}
	K-1487	N/A	N/A	N/A	N/A	N/A	N/A
	LB 2763	2.89 ± 1.64^{A,B}	0.92 ± 0.13	10.94 ± 1.97^{B}	0.77 ± 0.22	1.02 ± 0.13	0.88 ± 0.04
	CCAP 995/5	3.15 ± 2.04	0.93 ± 0.02^{A}	9.66 ± 1.05^{B}	0.54 ± 0.11^{B}	1.58 ± 1.17	0.61 ± 0.19^{2}
25 ˚C	K-0435	1.06 ± 1.83	1.36 ± 0.21	10.08 ± 2.02	1.10 ± 0.24	1.57 ± 0.30	1.30 ± 0.15^{A}
	K-0294	3.37 ± 4.46	1.47 ± 0.33	12.82 ± 0.52^{B,1}	1.39 ± 0.28^{B}	1.85 ± 0.07^{B}	1.31 ± 0.18^{B}
	K-1487	3.61 ± 2.79	2.44 ± 1.40	8.91 ± 3.31^{B}	1.78 ± 0.69^{B}	2.33 ± 2.50	1.73 ± 0.78
	LB 2763	6.53 ± 1.99^{B}	2.70 ± 2.42	5.89 ± 0.91^{C,2}	3.45 ± 3.66	3.17 ± 3.52	2.48 ± 2.26
	CCAP 995/5	7.06 ± 3.54	2.42 ± 1.03^{B}	8.57 ± 2.25^{B}	1.44 ± 0.49^{A}	2.10 ± 1.07	1.49 ± 0.69

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Total FAA	Sub-total EAA	PHE	LYS	LEU	ILE
4.87 ± 0.37[2]	27.27 ± 2.23[A,2,3]	0.25 ± 0.14[A,2,4]	1.22 ± 0.34[A]	1.26 ± 0.47	0.38 ± 0.08
6.00 ± 0.4[2]	28.00 ± 2.16[2,3]	0.27 ± 0.05[A,2,4]	1.09 ± 0.17[B]	1.22 ± 0.19[B]	0.21 ± 0.10[A]
5.47 ± 0.44[2]	34.75 ± 4.77[3]	0.81 ± 0.11[1]	1.38 ± 0.29	1.62 ± 0.61	0.46 ± 0.14
6.13 ± 0.50[A,1,2]	29.70 ± 0.76[A,B,3]	0.14 ± 0.06[2,4]	1.11 ± 0.10[B]	1.11 ± 0.12	0.20 ± 0.05
7.49 ± 0.58[1,A]	37.54 ± 2.86[A,1]	0.56 ± 0.03[B,3]	1.45 ± 0.04[B]	1.90 ± 0.27[B]	0.43 ± 0.06[C]
4.96 ± 0.36	22.86 ± 1.61[A,B]	0.78 ± 0.17[B]	1.53 ± 0.32[A,B]	1.33 ± 0.23	0.42 ± 0.09
5.11 ± 0.39	25.71 ± 3.72	0.91 ± 0.19[B,1]	1.45 ± 0.21[B]	1.42 ± 0.30[B]	0.54 ± 0.09[B,1]
N/A	N/A	N/A	N/A	N/A	N/A
4.75 ± 0.33[B]	20.95 ± 1.14[A]	0.60 ± 0.08	1.55 ± 0.13[B]	1.11 ± 0.23	0.29 ± 0.07[2]
4.83 ± 0.37[B]	19.59 ± 1.86[B]	0.51 ± 0.06[B,2]	1.22 ± 0.20[B]	1.10 ± 0.06[A]	0.32 ± 0.05[B,C]
4.62 ± 0.36[2,4]	21.26 ± 0.87	0.92 ± 0.12[B]	2.11 ± 0.18[B]	1.39 ± 0.14	0.55 ± 0.05
5.05 ± 0.34[2,3]	28.94 ± 4.75[B]	1.12 ± 0.21[B]	3.24 ± 0.41[A]	1.86 ± 0.23[B]	0.68 ± 0.15[B]
6.12 ± 0.40[2,3]	30.30 ± 6.01	2.01 ± 1.05	3.15 ± 1.09	2.78 ± 1.42	1.46 ± 1.08
8.88 ± 0.46[C,1]	34.55 ± 6.48[B]	2.75 ± 3.25	4.84 ± 1.83[A]	4.05 ± 4.67	1.99 ± 2.45
5.23 ± 0.31[C,2,3]	31.38 ± 7.80[A,B]	1.56 ± 0.62[A]	2.73 ± 0.57[A]	2.61 ± 0.37[B]	1.25 ± 0.59[A,C]

Discussion

The studied strains of *Rhodomonas* are closely related and no statistical difference in their content of C and N was found. The cell lengths was also very similar and only CCAP 995/5 had a significantly longer cell length.

However, an analysis on the FA profiles of the strains showed that they are diverse in their amount of various fatty acids and exhibit unique fingerprints each of them. Nonetheless, all the strains contain the PUFAs essential for aquaculture.

The growth rate of microalgae changes with temperature within the upper and lower threshold for a given species (Thompson 2005). However, different strains of a species may exhibit variations in their growth rates and the associated temperature thresholds (Renaud et al. 1995, Thompson 2005). The growth rates of the *Rhodomonas* strains obtained in this study are comparable to those previously reported for *Rhodomonas* from small-scale experiments ranging from 0.6 to 1.1 (Bartual et al. 2002, Chaloub et al. 2015, da Silva et al. 2009, Eriksen et al. 1998, Guevara et al. 2016, Lafarga-De la Cruz et al. 2006). In particular, the growth rate of K-1487 is similar to that obtained in Vu et al. (2016) using the same strain, and quite similar temperature and light intensity (20 ˚C,100 μmol m^{-2} s^{-1}). Interestingly, the growth rates did not differ for the majority of the studied strains within the experimental temperatures of 15, 20 and 25 ˚C while 30 ˚C evinced negative growth rates. A similar result was reported by Guevara et al. (2016) where two strains of *R. salina* had a growth rate of about 0.6 d^{-1} at both 10 and 29 ˚C. Contrary to our results and the results reported by Guevara et al. (2016), different growth rates for *R.* sp. were obtained with increasing temperature (20 to 32 ˚C and 25 to 33 ˚C, respectively) resulting in Q_{10} values of approximately 0.7 and 0.3 by Chaloub et al. (2015) and Renaud et al. (2002). Thus, the effect of temperature on growth rate and the temperature threshold is indeed species- and strain-specific, although operating within similar intervals.

An overview of relevant literature with various microalgae (including a heterotrophic dinoflagellate) show a general pattern with decreasing content of EPA and DHA with increasing temperature while ARA is increasing, and a lower content of PUFAs with increasing temperature (Durmaz et al. 2008, Durmaz et al. 2009, Guevara et al. 2016, Jiang and Chen 2000, Qiao et al. 2016, Renaud et al. 1995, Renaud et al. 2002, Thompson et al. 1992). Studies on the effect of temperature on the FA content of *Rhodomonas* are scarce. Renaud et al. (2002) studied the effect of temperature (25 to 33 ˚C) on *R.* sp. and found a decrease of EPA from 7.7 to 4.7 %, a decrease of DHA from 3.8 to 2.6 %, and an increase of ARA from 0.3 to 0.6 % in good agreement with the general pattern found in the literature. Noticeably, the content of EPA, DHA and ARA in our five *Rhodomonas* strains did not show a consistent pattern with increasing temperature. Contrastingly, K-0435 had a lower content of DHA at 20 ˚C compared to 15 ˚C, and K-0294 had a lower content of DHA at 25 ˚C. The most pronounced effect of temperature was that the highest content of ARA for the strains was found at 25 ˚C, except for K-0435. Similar results were obtained by Renaud et al. (1995) who also did not find a consistent pattern for the effect of temperature on the content of FAs in tropical microalgae in temperature intervals of 10 to 15 ˚C, and Thompson et al. (1992) who found no consistent pattern for FAs in a temperature interval of 15 ˚C for eight microalgal species. However, if the results for EPA, DHA and ARA for all the *Rhodomonas* strains in the present study are pooled at each temperature, EPA slightly decreases from 15 to 25 ˚C, DHA slightly decreases at 25 ˚C, and ARA is highest at 25 ˚C which corresponds to the general pattern in the literature. The TFA (pg cell^{-1}), EPA and DHA (% of TFA) found by Vu et al. (2016) for strain K-1487 at 20 ˚C are lower compared to those obtained in the current study. The content of EPA, DHA

and ARA (% of TFA) in the strains are very similar to those reported for *R. salina* in (Drillet et al. 2006).

The strains were cultivated under saturated light intensity according to Vu et al. (2016) as this parameter has been shown to have a significant effect on quantity and composition of free amino acids (FAAs) with the total FAA being highest at light limitation but the EAA highest at saturated light intensity. The content of total FAA in our strains at 20 ˚C are comparable to those reported by Vu et al. (2016) at the same temperature (4.75 ± 0.33 to 5.11 ± 0.39 pg cell^{-1} versus 4.38 ± 1.12 pg cell^{-1}, respectively) and to those reported by Drillet et al. (2006) at 17 ˚C. The total EAA in K-1487 was found to be 32 % by Vu et al. (2016) and Drillet et al. (2006) compared to 29 to 37 % at 15 ˚C and 19 to 26 % at 20 ˚C in the present study. The content of FAA in microalgae are of importance as they are crucial for fish larvae and may be transferred through the fish prey, i.e., copepods (Rayner et al. 2017, Rønnestad et al. 1999). Similar to our results, Rayner et al. (2017) also found that glutamic acid and proline were the dominating non-essential, and arginine the dominating essential FAAs in *R. salina* (K-1487). However, there was no clear pattern on the effect of temperature on the content of FAA in our strains. An overall increase of methionine with increasing temperature was observed for K-0435 and K-0294 and the same trend was found for *Chlorella* by (James et al. (1989)). It should be noted that there was no detectable amount of histidine present at 15 ˚C in the majority of our strains making this temperature unfavorable for cultivation of these strains if the microalga is to be used as a microalgal diet.. Studies on the effect of temperature on the content of FAA in microalgae are lacking but according to (Bermúdez et al. (2015)) the content of protein and EAA content may increase with temperature until a certain threshold.

Final remarks

The effect of temperature on the growth rate and biochemical composition of the five strains of *Rhodomonas* did not follow a clear pattern. The growth rate of some species was affected while others were not affected. However, all the strains were able to grow within the experimental temperature range with an adequate growth rate, which is promising for large-scale cultivation as this allows more flexibility in the temperature setting during cultivation. Overall, the PUFAs EPA, DHA and ARA at the experimental temperatures were that the content of EPA and DHA slightly decreased while ARA increased. However, on strain level the effect of temperature on PUFAs as well as EAA was highly diverse with no clear pattern. However, histidine was absent or below detection limit for some of the strains at 15 ˚C which could be problematic if these strains are to be used as a microalgal diet in aquaculture. Nonetheless, all strains have a good nutritional composition of PUFAs and free EAAs making them suitable as microalgal diet for copepods in the aquaculture. It must be emphasized that this study clearly shows that the effect of temperature on growth rate and biochemical composition is species- and strain-specific although the results are within the same range. This suggests that fundamental traits are genetically programmed why next step should be to link genetical profiling with other traits like growth and biochemical composition.

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Appendix

Table 1. The number of replicates of each strain analyzed in the study.

	Growth rates			Free amino acids			Fatty acids		
	15 ˚C	20 ˚C	25 ˚C	15 ˚C	20 ˚C	25 ˚C	15 ˚C	20 ˚C	25 ˚C
K-0435	8	6	6	3	3	3	6	5	4
K-0294	6	5	5	3	3	3	6	5	5
K-1487	10	4	4	3	N/A	3	5	12	3
LB 2763	6	5	5	3	3	3	6	3	4
CCAP 995/5	6	6	4	3	3	2	6	6	4

Table 2. The statistical results on the content of EPA, DHA and ARA in the *Rhodomonas* strains at 15, 20 and 25 ˚C. No statistically significant differences were found for the content of EPA between strains at any of the experimental temperatures and is therefore not included.

		EPA	DHA	ARA
Strain	**Temperature (˚C)**		**p**	
K-0435	15 vs. 20		0.014	
	20 vs. 25	0.005		
	25 vs. 15	0.001		
K-0294	15 vs. 20			
	20 vs. 25		0.025	< 0.001
	25 vs. 15		0.002	< 0.001
K-1487	15 vs. 20			0.002
	20 vs. 25			< 0.001
	25 vs. 15			0.019
LB 2763	15 vs. 20			
	20 vs. 25			< 0.001
	25 vs. 15	< 0.001		< 0.001
CCAP 995/5	15 vs. 20			
	20 vs. 25			< 0.001
	25 vs. 15	0.035		< 0.001

Temperature (˚C)	**Strain**	**DHA (p)**
15	K-0435 vs. LB 2763	< 0.001
	K-0435 vs. K-0294	0.010
	LB 2763 vs. K-1487	0.005
	LB 2763 vs. CCAP 995/5	0.041
20		
25	K-0435 vs. K-0294	< 0.001
	K-0435 vs. CCAP 995/5	0.009

Temperature (˚C)	**Strain**	**ARA (p)**
15	K-0435 vs. K-0294	0.021
	K-0435 vs. LB 2763	0.023
20	K-1487 vs. K-0435	0.005
	K-1487 vs. CCAP 995/5	0.003
	K-1487 vs. K-0294	0.009
25		

Table 3. The cell content of EPA, DHA and ARA in the *Rhodomonas strains*. Unit: pg cell^{-1} ± S.D. (n: see table 1 in the appendix).

		K-0435	K-0294	K-1487	LB 2763	CCAP 995/5
15 °C	EPA	2.70 ± .63	2.96 ± .73	2.22 ± 0.28	2.92 ± 0.62	2.60 ± 0.56
	DHA	2.01 ± 0.36	1.98 ± 0.53	1.57 ±0.21	1.98 ± 0.47	1.86 ± 0.41
	ARA	0.09 ± 0.04	0.05 ± 0.01	0.07 ± 0.01	0.05 ± 0.01	0.05 ± 0.00
	TFA	19.8 ± 3.8	21.5 ± 4.6	15.9 ± 2.7	22.3 ± 4.2	19.1 ± 4.1
20 °C	EPA	3.27 ± 0.90	3.30 ± 0.54	2.85 ± 0.33	3.17 ± 0.58	3.12 ± 0.62
	DHA	2.25 ± 0.53	2.16 ± 0.27	2.16 ± 0.61	2.36 ± 0.23	2.66 ± 1.25
	ARA	0.10 ± 0.04	0.09 ± 0.03	0.04 ± 0.08	0.08 ± 0.04	0.09 ± 0.02
	TFA	26.10 ± 3.80	24.85 ± 3.82	23.26 ± 9.76	26.42 ± 4.77	23.77 ± 5.48
25 °C	EPA	2.36 ± 1.38	2.49 ± 0.41	1.67 ± 0.14	1.94 ± 0.36	2.32 ± 0.62
	DHA	1.78 ± 1.00	1.91 ± 0.22	1.27 ± 0.04	1.66 ± 0.30	1.63 ± 0.33
	ARA	0.11 ± 0.06	0.06 ± 0.05	0.12 ± 0.02	0.14 ± 0.04	0.16 ± 0.06
	TFA	18.43 ± 11.15	25.24 ± 3.83	14.16 ± 1.61	18.80 ± 4.43	20.58 ± 5.03

Table 4. The contribution of individual fatty acids (%) to the dissimilarities between the strains of *Rhodomonas* found by the SIMPER analysis. Cut off for low contributions: 90 %. (n = 3, except K-0435 n =2). A list with names of the FAs according to their numbers given in the table is listed at the end.

	CCAP 995/5	K-0294		
FA	Average value	Average value	Contribution (%)	Cumulative (%)
35	4.14	3.06	35.38	35.38
21	4.31	5.19	23.46	58.84
27	0	0.711	15.23	74.07
6	1.46	0.956	11.50	85.57
31	1.17	0.842	3.95	89.52
30	3.65	3.36	2.60	92.12
	CCAP 995/5	K-0435		
FA	Average value	Average value	Contribution (%)	Cumulative (%)
21	4.31	5.79	43.99	43.99
35	4.14	3.06	23.55	67.54
27	0	0.666	9.24	76.78
10	3	2.42	6.84	83.62
6	1.46	1.08	5.44	89.06
18	2.08	1.63	4.19	93.25
	CCAP 995/5	K-1487		
FA	Average value	Average value	Contribution (%)	Cumulative (%)
21	4.31	6.07	35.85	35.38
10	3	1.82	16.31	52.16
31	1.17	2	14.30	66.47
20	5.44	4.63	7.60	74.07
35	4.14	3.37	7.33	81.40
6	1.46	0.85	6.57	87.97
27	0	0.605	4.78	92.75
	CCAP 995/5	LB 2763		
FA	Average value	Average value	Contribution (%)	Cumulative (%)

21	4.31	6	26.26	26.26
10	3	1.7	17.48	43.74
31	1.17	2.03	14.54	58.28
6	1.46	0.388	11.74	70.02
20	5.44	4.36	10.88	80.90
27	0	0.939	8.42	89.32
35	4.14	3.57	3.26	92.57
	K-0294	**K-0435**		
FA	**Average value**	**Average value**	**Contribution (%)**	**Cumulative (%)**
18	2.34	1.63	26.54	26.54
21	5.19	5.79	18.76	45.30
16	1.8	1.21	18.13	63.43
31	0.842	1.14	7.80	71.23
10	2.78	2.42	6.92	78.15
30	3.36	3.71	6.51	84.66
20	5.6	5.28	5.36	90.02
	K-0294	**K-1487**		
FA	**Average value**	**Average value**	**Contribution (%)**	**Cumulative (%)**
31	0.842	2	29.75	29.75
20	5.6	4.63	14.93	44.68
10	2.78	1.82	14.88	59.56
21	5.19	6.07	12.31	71.87
16	1.8	0.95	11.60	83.47
30	3.36	3.89	4.79	88.26
18	2.34	1.84	4.20	92.46
	K-0294	**LB 2763**		
FA	**Average value**	**Average value**	**Contribution (%)**	**Cumulative (%)**
31	0.842	2.03	27.43	27.43
20	5.6	4.36	19.03	46.46
10	2.78	1.7	16.86	63.31
21	5.19	6	8.16	71.47
16	1.8	1.21	7.06	78.54

FA	Average value	Average value	Contribution (%)	Cumulative (%)
30	3.36	4.03	5.96	84.50
6	0.956	0.388	4.14	88.64
35	3.06	3.57	3.66	92.29
	K-0435	**K-1487**		
FA	**Average value**	**Average value**	**Contribution (%)**	**Cumulative (%)**
31	1.14	2	44.83	44.83
20	5.28	4.63	14.52	59.35
10	2.42	1.82	12.66	72.01
35	3.06	3.37	4.89	76.90
6	1.08	0.85	4.58	81.48
11	1.27	0.925	4.12	85.60
21	5.79	6.07	2.71	88.31
12	0.265	0	2.54	90.85
	K-0435	**LB 2763**		
FA	**Average value**	**Average value**	**Contribution (%)**	**Cumulative (%)**
31	1.14	2.03	33.80	33.80
20	5.28	4.63	17.60	51.39
10	2.42	1.7	14.69	66.08
6	1.08	0.388	9.89	75.98
35	3.06	3.57	6.04	82.01
16	1.21	1.21	4.50	86.52
11	1.27	0.962	3.52	90.04
	K-1487	**LB 2763**		
FA	**Average value**	**Average value**	**Contribution (%)**	**Cumulative (%)**
31	2	2.03	48.35	48.35
5	0.85	0.388	10.41	58.75
16	0.95	1.21	10.31	69.06
10	1.82	1.7	7.87	76.94
27	0.605	0.939	6.80	83.74
35	3.37	3.57	3.96	87.70
20	4.63	4.36	3.18	90.88

FA number	FA name
6	C14:0
10	C16:0
11	C16:1
16	C18:1 cis
18	C18:2 cis
20	C18:3 n-3
21	C18:4
27	C20:4 n-6 (ARA)
30	C20:5 n-3 (EPA)
31	C22:1
35	C22:6 n-3 (DHA)

Table 5. Total free amino acids (FAAs) and free essential amino acids (EAAs) in the strains of *Rhodomonas* cultivated at 15, 20 and 25 ˚C. Units of FAA and sub-total EAA: % of total FAA. Units of total FAA: pg cell^{-1}. (n: see table 1 in the appendix).

Temp	Strain	ASP	GLU	ASN	GLY
15 ˚C	K-0435	4.77 ± 1.76	25.39 ± 6.61	10.48 ± 5.40	8.85 ± 2.41
	K-0294	4.50 ± 0.53	24.80 ± 4.10	12.80 ± 0.11	8.36 ± 0.54
	K-1487	3.26 ± 0.93	22.46 ± 4.48	10.62 ± 3.09	7.80 ± 2.09
	LB 2763	4.44 ± 0.25	25.68 ± 1.98	11.17 ± 1.24	8.31 ± 0.51
	CCAP 995/5	3.65 ± 0.15	22.27 ± 0.23	9.24 ± 0.32	7.17 ± 0.01
20 ˚C	K-0435	4.69 ± 4.11	25.93 ± 4.27	14.28 ± 3.69	11.15 ± 1.76
	K-0294	6.46 ± 1.56	31.80 ± 5.82	8.47 ± 7.46	10.02 ± 1.32
	K-1487	N/A	N/A	N/A	N/A
	LB 2763	10.05 ± 0.93	22.76 ± 0.86	14.72 ± 2.63	11.74 ± 0.35
	CCAP 995/5	5.92 ± 0.59	27.58 ± 6.31	17.52 ± 3.03	10.52 ± 0.20
25 ˚C	K-0435	4.86 ± 1.07	24.75 ± 1.431	25.43 ± 3.72	10.79 ± 0.87
	K-0294	6.09 ± 0.57	25.23 ± 3.63	16.87 ± 7.27	10.36 ± 1.32
	K-1487	5.20 ± 0.88	24.13 ± 4.52	20.39 ± 9.78	9.90 ± 1.535
	LB 2763	4.90 ± 0.99	24.13 ± 6.80	8.80 ± 4.25	10.20 ± 4.03
	CCAP 995/5	6.83 ± 0.82	19.32 ± 1.53	20.68 ± 10.03	9.32 ± 0.72

TYR	PRO	ALA	ARG	THR	HIS	GLN
1.04 ± 0.13	18.10 ± 5.24	2.38 ± 0.31	18.10 ±	0.95 ± 0.06	0.00 ± 0.00	2.20 ± 0.83
0.45 ± 0.03	17.77 ± 2.12	1.82 ± 0.31	21.69 ± 3.36	1.01 ± 0.27	0.02 ± 0.04	2.12 ± 0.28
0.46 ± 0.08	19.33 ± 2.26	2.00 ± 0.34	23.74 ± 3.68	0.77 ± 0.05	0.00 ± 0.00	1.95 ± 0.73
0.42 ± 0.071	16.34 ± 1.26	1.78 ± 0.11	23.58 ± 1.19	0.89 ± 0.26	0.00± 0.00	2.14 ± 0.17
0.54 ± 0.02	16.09 ± 0.60	1.71 ± 0.11	25.62 ± 1.97	1.25 ± 0.08	3.81 ± 4.43	1.79 ± 0.25
0.96 ± 0.23	16.25 ± 3.45	2.41 ± 0.59	11.00 ± 1.85	1.34 ± 0.34	3.21 ± 1.36	1.51 ± 0.52
1.12 ± 0.30	12.96 ± 3.64	2.32 ± 0.50	9.91 ± 2.71	1.44 ± 0.14	6.57 ± 3.88	1.38 ± 0.34
N/A	N/A	N/A	N/A	N/A	N/A	N/A
0.77 ± 0.22	15.46 ± 0.46	1.99 ± 0.56	10.94 ± 1.97	0.92 ± 0.13	2.89 ± 1.64	1.70 ± 0.11
0.54 ± 0.11	14.55 ± 2.33	2.16 ± 0.85	9.66 ± 1.05	0.93 ± 0.02	3.15 ± 2.04	1.49 ± 0.02
1.10 ± 0.24	7.71 ± 1.75	2.85 ± 0.27	10.08 ± 2.02	1.36 ± 0.21	1.06 ± 1.83	1.62 ± 0.42
1.39 ± 0.28	7.86 ± 1.33	2.24 ± 0.14	12.82 ± 0.52	1.47 ± 0.33	3.37 ± 4.46	1.49 ± 0.16
1.78± 0.69	3.78± 3.28	3.85 ± 1.71	8.91 ± 3.31	2.44 ± 1.40	3.61 ± 2.79	1.27± 0.23
3.45 ± 3.66	3.85 ± 1.57	5.84 ± 5.98	5.89 ± 0.91	2.70 ± 2.42	6.53 ± 1.99	1.01 ± 0.39
1.44 ± 0.49	6.9 5 ±0.79	2.86 ± 0.13	8.57 ± 2.25	2.42 ± 1.03	7.06 ± 3.54	1.24 ± 0.46

LYS	LEU	ILE	MET	VAL	CYS
1.22 ± 0.34	1.26 ± 0.47	0.38 ± 0.08	0.80 ± 0.05	2.42 ± 0.92	0.87 ± 0.22
1.09 ± 0.17	1.22 ± 0.19	0.21 ± 0.10	0.43 ± 0.18	1.02 ± 0.07	0.41 ± 0.02
1.38 ± 0.29	1.62 ± 0.61	0.46 ± 0.14	1.03 ± 0.14	1.81 ± 0.28	0.48 ± 0.11
1.11 ± 0.10	1.11 ± 0.12	0.20± 0.05	0.41 ± 0.06	1.82 ± 0.50	0.47 ± 0.11
1.45 ± 0.04	1.90 ± 0.27	0.43 ± 0.06	0.75 ± 0.10	1.34 ± 0.18	0.41 ± 0.12
1.53 ± 0.32	1.33 ± 0.23	0.42 ± 0.09	0.90 ± 0.13	1.48 ± 0.44	0.82 ± 0.38
1.45 ± 0.21	1.42 ± 0.30	0.54 ± 0.09	1.14 ± 0.17	1.44 ± 0.23	0.64 ± 0.02
N/A	N/A	N/A	N/A	N/A	N/A
1.55 ± 0.13	1.11 ± 0.23	0.29 ± 0.07	0.88 ± 0.04	1.02 ± 0.13	0.60 ± 0.11
1.22 ± 0.20	1.10 ± 0.06	0.32 ± 0.05	0.61 ± 0.19	1.58 ± 1.17	0.62 ± 0.31
2.11 ± 0.18	1.39 ± 0.14	0.55 ± 0.05	1.30 ± 0.15	1.57 ± 0.30	0.56 ± 0.02
3.24 ± 0.41	1.86± 0.23	0.68 ± 0.15	1.31 ± 0.18	1.85 ± 0.074	0.76 ± 0.12
3.15 ± 1.09	2.78 ± 1.42	1.46± 1.08	1.73 ± 0.78	2.33 ± 2.50	1.26 ± 1.09
4.84 ± 1.83	4.05 ± 4.67	1.99 ± 2.45	2.48 ± 2.26	3.17 ± 3.52	3.41 ± 4.09
2.73 ± 0.57	2.61 ± 0.37	1.25 ± 0.59	1.49 ± 0.69	2.10± 1.07	1.54 ± 0.57

Total FAA	Sub-total EAA	PHE
4.87 ± 0.37	27.27 ± 2.23	0.25 ± 0.14
6.00 ± 0.48	28.00 ± 2.16	0.27 ± 0.05
5.47 ± 0.44	34.75 ± 4.77	0.81 ± 0.11
6.13 ± 0.50	29.70 ± 0.76	0.14 ± 0.06
7.49 ± 0.58	37.54 ± 2.86	0.56 ± 0.03
4.96 ± 0.36	22.86 ± 1.61	0.78 ± 0.17
5.11 ± 0.39	25.71 ± 3.72	0.91 ± 0.19
N/A	N/A	N/A
4.75 ± 0.33	20.95 ± 1.14	0.60 ± 0.08
4.83± 0.37	19.59 ± 1.86	0.51 ± 0.06
4.62 ± 0.36	21.26 ± 0.87	0.92 ± 0.12
5.05± 0.34	28.94 ± 4.75	1.12 ± 0.21
6.12± 0.40	30.30 ± 6.01	2.01± 1.05
8.88± 0.46	34.55 ± 6.48	2.75 ± 3.25
5.23± 0.31	31.38 ± 7.80	1.56 ± 0.62

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Paper 5

<u>Title</u>

Crypthecodinium cohnii: a promising prey toward large-scale intensive rearing of the live feed copepod *Acartia tonsa* (Dana).

<u>Status</u>

CrossMark

Crypthecodinium cohnii: a promising prey toward large-scale intensive rearing of the live feed copepod Acartia tonsa (Dana)

H. H. Jakobsen[1] & C. Thoisen[1,2] & B.W. Hansen[2]

Abstract Autotrophic microalgae are in general used as prey for copepods in laboratory experiments and in aquaculture mass culturing. We tested the suitability of using the osmotrophic thecate dinoflagellate Crypthecodinium cohnii as an alternative prey for the live prey organism for fish larvae, the planktonic calanoid copepod Acartia tonsa. We found that A. tonsa fed and transformed ingested C. cohnii into new production well, although the gross growth efficiency was somewhat lower (~22%) than those reported in the literature when fed the autotrophic microalgae Rhodomonas salina (> 36%). We also compared the egg hatching success of eggs produced by the copepod when fed C. cohnii and R. salina and found a slightly lower hatching in eggs produced based on C. cohnii (60%) than on R. salina (89%)-fed copepods. The dinoflagellate C. cohnii is reared in the dark in bioreactors where it can obtain by far higher cell concentrations and biomasses per unit time and volume than the autotrophic prey R. salina reared in photobioreactors. Biochemical composition among the two prey showed that the carbon and nitrogen content was not very different; however, their fatty acid content deviated. The total fatty acids were ~ 17% of the cell carbon in R. salina whereas ~ 9% of the total cell carbon in C. cohnii. Moreover, ~ 18% of the fatty acids were EPA in R. salina, whereas EPA was mostly absent in C. cohnii. In contrast, ~ 63% of the fatty acids were DHA in C. cohnii compared to 12% DHA in R. salina. The trade-off of switching to the heterotrophic dinoflagellate diet is that the copepod performance is about 40% lower. Still, we propose that eliminating light in the rearing of copepod feed makes C. cohnii an interesting alternative and an economical feasible feed worth pursuing in large-scale rearing of copepods.

Keywords Acartia tonsa · Copepod · Fatty acids · Live feed · PUFA · Rhodomonas

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Paper 5

Introduction

Intensive aquaculture of marine finfish is largely based on live prey in rearing of the first feeding fish larval stages. The industry has traditionally adopted rotifers and brine shrimps as the primary prey. Rotifers are in particular used, because of their fast growth and high reproductive rates, whereas the brine shrimp *Artemia* can be collected in nature and stored as cysts until needed. Unfortunately, rotifers and *Artemia* are not a part of the natural diet for most marine fish larvae and their nutritional values do not always meet the nutritional requirement needed for the fish larvae development as compared to copepods (e.g., Barroso et al. 2013; Shields et al. 1999). The high and fluctuating cost of *Artemia* needed for mass-producing marine fish has in addition made it attractive to search for alternative live prey such as copepods (Abate et al. 2015; Drillet et al. 2011).

Copepods are rich in polyunsaturated fatty acids (PUFAs) (Støttrup et al. 1999). Among the important PUFAs are the highly polyunsaturated fatty acids (HUFAs). The HUFAs include fatty acids such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and arachidonic acid (ARA). DHA and EPA are regarded essential for copepod reproduction and growth (Klein Breteler et al. 1999; Støttrup and Jensen 1990), whereas the role of ARA remains less clear. However, copepods reared on laboratory-grown algae seem to contain less HUFAs than copepods collected in the wild (Barroso et al. 2013). HUFAs are needed by copepods in their ontogenetic development (Tang et al. 2001) and positively influence egg production rates, and these constituents are therefore regarded key for fish larval performance (Kattner et al. 2007).

The fatty acid composition of the prey is reflected in the copepods, which in turn also reflects the growth and development of the fish larvae that prey upon the copepods (McKinnon et al. 2003). There is an intensive ongoing research focused on the optimal microalgae prey that support nauplii survival, growth, and later egg production and viability in domesticated copepods which at the same time must fulfill the dietary requirements in fish larvae. A short and incomplete list of studies on optimal microalgae prey for aquaculture-reared copepods published over the past decade include Arndt and Sommer (2014), Barroso et al. (2013), Camus and Zeng (2010), Camus et al. (2009), Gusmao and McKinnon (2009), Huang et al. (2011), Martinez-Cordova et al. (2012), Matias-Peralta et al. (2011), Ohs et al. (2010), Pan et al. (2014), Tzovenis et al. (2009), and Zhang et al. (2013). These studies focused at identifying optimal phytoplankton diets suitable for copepods used in the aquaculture industry. One of the main bottlenecks, however, is to provide the copepods with a mass-produced microalga of sufficient biochemical quality.

Large-scale productions of copepods demand a stable supply of a large amount of phytoplankton, which can be met by culturing the microalgae by various photobioreactor systems (PBRs). However, PBRs are challenged by several issues, which unless solved, may limit the biomass production. These challenges are potential light limitation, excess oxygen, and OH^- produced during photosynthesis by high-concentration cultures (Hansen 2002; Molina et al. 2001). This can be counteracted by adding CO_2 to the PBR (Brennan and Owende 2010) or less likely by increasing light (Vu et al. 2016), whereas removal of excess oxygen is more difficult.

Replacing autotrophic prey by heterotrophic prey may eliminate many of the issues associated with photosynthesis because no light is needed and, therefore, no oxygen and OH^- is produced. Whereas there are many thoroughly studied phytoplankton species to choose from, when designing the ideal diet for copepods, there are much fewer studies (if any) on heterotrophic species available.

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The nonphotosynthetic DHA-producing osmotrophic thecate dinoflagellate *Cryptecodinium cohnii* is here used as an alternative to phototrophic microalgae and contrasted with *Rhodomonas salina* as the prey organism. Heterotrophic organisms such as *C. cohnii* can reach final biomass densities exceeding those of phototrophic organisms as light is not a limiting factor during cultivation as is the case for phototrophic organisms such as *R. salina*.

The main organic constituents in *C. cohnii* are highly dependent on culture conditions such as substrate and pH (Pleissner and Eriksen 2012) but are in general terms made up by starch (ca. 50%), lipid (ca. 12–14%), and protein (7–15%). The PUFAs constituted almost solely of DHA that made 2–4% (ca. 20–40% of PUFAs) of the dry weight in *C. cohnii* (de Swaaf et al. 2003; Pleissner and Eriksen 2012). Vu et al. (2016) studied the nutritional quality of *R. salina* under varying resources and found similar PUFA content (ca. 32%) in cells grown under resource-replete conditions. *C. cohnii* has an ideal prey size for the late nauplii and copepodite stages of *Acartia tonsa* (Berggreen et al. 1988). Moreover, it is very motile (Fenchel 2001) in its vegetative life stage, thus making it conspicuous for rheotactic planktonic grazers such as calanoid copepods (Kiørboe et al. 1999).

The aim of the present study was to investigate whether *C. cohnii*, either as a single diet or as a supplement to *R. salina*, could improve or replace a single diet of *R. salina* for *A. tonsa* cultures. The chosen evaluation endpoints were copepod grazing rates, egg production, gross growth efficiency, and egg hatching success when offered *C. cohnii* in mixtures with *R. salina* or in single diet trials.

Material and methods

Culturing The copepod *Acartia tonsa* used in the current experiment is a descendant from the original culture (DFH-ATI) used by Støttrup et al. (1986). This particular clone has been given the designation DIFRES by Drillet et al. (2008) and was cultured following the outline by Berggreen et al. (1988). An inoculum of ca. 10×10^4 to 20×10^4 cold-stored eggs (sensu Drillet et al. 2006) was added to 100-L tanks filled with 0.2 µm filtered seawater (FSW; salinity 28–32) and maintained at 15 °C. *Rhodomonas salina* as prey (2×10^4 to 5×10^4 cells mL^{-1}) was added at the same time as the eggs. The rearing tanks were gently aerated to maintain prey alga in suspension. The algae were frequently monitored and new prey was added whenever needed and, typically, when prey concentration fell below 1×10^4 mL^{-1}, thus supporting the copepods well fed until needed. The time from starting the culture until the adultness of the copepods was reached and they were ready for the experiments usually required ca. 2 to 3 weeks. No copepod cultures older than 3 weeks after maturity was reached were used.

The autotrophic prey *R. salina* (Scandinavian Culture Collection of Algae & Protozoa; https://SCCAP.dk/; strain no. K-1487) was maintained in L1 medium (Guillard and Hargraves 1993) in aerated 2-L clear glass bottles under a PAR irradiance of ca. 100 µmol photons m^{-2} s^{-1} provided by daylight fluorescent bulb in a day:night illumination cycle of 14:10. The *R. salina* cultures were frequently diluted maintaining algae fresh and always in exponential growth phase.

The heterotrophic thecate dinoflagellate *Cryptecodinium cohnii* (Seligo) Javornicky, 1962 (National Center for Marine Algae and Microbiota (NCMA) at Bigelow Laboratory; https://ncma.bigelow.org/; strain CCMP 316) was maintained axenic in 30 PSU sterile FSW-

added L1 media and 10% NPM growth media (Guillard 1960), in 50-mL blue cap bottles. Storage cultures were reinoculated every week to avoid the culture to enter lag phase and dormancy. The cultures were maintained on a shaker table at 20 rpm at 20 °C under dim light of ca. 10–20 μmol photons m^{-2} s^{-1} until needed for the experiments. Because there is a risk of carryover of excess dissolved NPM media to feed suspensions creating bacterial growth causing low oxygen concentration, we removed NPM media by centrifuging the cells at 800 g for 15 min and removed the supernatant when preparing food suspensions for the copepods. The prey *C. cohnii* were made ready for experiments by resuspending the pellet in 0.2 μm filtered seawater and counted. Microscopic examination revealed that cells were motionless right after centrifugation but full motility was regained within 12 h.

Counting Cells of *R. salina* or *C. cohnii* were harvested from exponentially growing cultures and fixed in acid Lugol's solution (final 2%). Then, a known concentration was added to 3-mL multidishes, filled with FSW, and sealed by a coverslip to eliminate optical distortion. The cells were left for the next day to sediment and then counted on an inverted Nikon microscope. The cells were counted using software (NIS ver. 4.2, Nikon microscopes) with an automated separation of gray scale difference between cells and background by field of view calibrated to ×10 or ×20 objectives. Cells for the elemental and fatty acid analysis were counted in a Beckman Coulter multisizer 4e (https://www.beckmancoulter.com).

Grazing rates were estimated over 21 h and obtained by measuring the change in prey concentration in replicate incubations ($n = 4$) relative to the control without prey (triplicate bottles). Female *A. tonsa* copepods were manually pipetted and used in the feeding trials with *C. cohnii* as single prey or in mixed diet experiments of *C. cohnii* and *R. salina*. Five females were added to 500-mL blue cap bottles in 0.2 μm FSW added to capacity and L1 media. Females were incubated in a range of prey concentrations on a slow rotation plankton wheel (1 rpm) at 15 °C in dim light.

The numbers of live females were recorded after each experiment and their numbers used in the calculation of clearance and ingestion. The eggs were counted and used in the calculation of egg production rates. Clearance and ingestion rates were calculated for each prey concentration according to Frost (1972) after verification that the terminal prey concentrations in grazing bottles always were lower than those in the controls. The reduction in prey concentration in experimental bottles was not allowed exceeding 40%. Grazing rates in mixed diet/prey experiment were calculated by counting each prey and estimating grazing separately on each prey.

In two mixed experiments was *R. salina*:*C. cohnii* offered in biomass ratios of 2.5 and 2.7 at total concentrations ~ 800–1100 μg C L^{-1} and incubated as above on a plankton wheel.

Carbon-specific rates Adult females of *A. tonsa* do not grow somatically. Instead, ingested food is sequestered into respiration, defecation, and egg production. Secondary production can therefore be estimated as egg biomass produced divided by the biomass of *A. tonsa*. Biomass of *A. tonsa* was estimated by determining the prosome length of 20 randomly collected copepods after 21 h grazing. The average prosome length of 20 females was used as input values (791 μm) in the length versus weight regression for *A. tonsa* (Berggreen et al. 1988), yielding a mean female biomass of 3.22 μg C female^{-1}. *A. tonsa* egg carbon biomass was obtained from Kiørboe et al. (1985) as 46 ng C egg^{-1}. Cellular carbon biomass of *R. salina* and *C. cohnii* was estimated experimentally as outlined below.

The functional response in invertebrates follows a sigmoid function Holling type III (Holling 1966; Real 1977). The sigmoid shape is a result of arrested feeding at low food

availability, i.e., "feeding threshold," where grazers arrest feeding activities in order to save resources rather than investing energy in searching for limited prey resources. We were unable to identify any low prey feeding threshold in our data and therefore used the simpler Holling type II (Eq. 1) to describe ingestion rate and egg production rate.

$$R = \frac{R_{\max} \cdot C}{K_m + C} \tag{1}$$

Where R is either ingestion or specific egg production at prey concentration (C); $R_{\max}$ is the maximal achievable R; and K_m is the prey concentration required to meet $R_{\max}/2$.

Egg hatching success was determined on a diet of *C. cohnii* and compared to that of *R. salina*. Adult female *A. tonsa* were pipetted into six 600-mL bottles (~15 adult females in each bottle), filled with 0.2 μm FSW. The bottles were divided into two treatments. One set of triplicates was added *C. cohnii* and one set of triplicates was added *R. salina*. Both prey types were initially ad libitum (>1000 μg C L^{-1}). The bottles were incubated on a plankton wheel (1 rpm) at 15 °C in dim light. The treatments were pretreated to the designated diet for 96 h to let the copepods adapt to the diet and achieve balanced growth. During the pretreatment, the diet was replaced every 24 h by gently screening the copepods by a 200-μm mesh to remove eggs and hatched nauplii. After 96 h pretreatment, the eggs were harvested, counted, and incubated for hatching during 72 h followed by fixation in 2% acid Lugol's solution. Nauplii and unhatched eggs were then counted and diet-specific hatching success was estimated.

CN elemental analysis A known amount of cells were collected on precombusted (450 °C) 10 mm GF/C filters corresponding to an estimated final cell biomass (Menden-Deuer and Lessard 2000) for analysis of ~50–150 μg C per filter. The filters were folded, placed in tin capsules, and dried at 105 °C for 24 h. Then, the samples were placed in the autosampler of a CE Instruments EA 1110 CHNS (Wigan, UK) elemental analyzer using a methionine standard curve to obtain concentrations of C and N.

Fatty acids A known amount of cells was collected on precombusted (450 °C) 10 mm GF/C filters corresponding to an estimated final cell biomass (Menden-Deuer and Lessard 2000) for analysis of ~500–1000 μg C per filter. Filters were freeze dried (lyophilization) and individually extracted for 24 h at −20 °C in 3 mL of a chloroform:methanol mixture (2:1 vol:vol) and added an internal standard of 20 μL C23-methylester (concentration of 1000 μg mL^{-1}). The extract from each sample was transferred to individual clean GC vials, and the chloroform:methanol was dried out by evaporation at 60 °C under a flow of nitrogen. A reagent solution (1 mL) composed of a mixture of toluene:methanol:acetyl chloride (66 mL:85 mL:15 mL) was added to the vials which were capped and then heated for 2 h at 90 °C. Aqueous NaHCO$_3$ (5% by weight, 500 μL) was added to the vials. After vigorous mixing, the upper fatty acid methyl ester solvent layer was transferred to a new GC vial. The original vial was extracted twice with heptane (2 × 500 μL), and the combined organic layers collected were evaporated under a gentle flow of nitrogen. Finally, the methyl esters were resuspended in chloroform and analyzed by GC-MS.

The GC-MS instrument was composed of an Agilent 6890 series gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a PTV inlet and an Agilent 5973 mass selective detector. The column was a 60m Agilent DB23 with an inner diameter

of 250 mm and a film thickness of 0.3 μm. The carrier gas was helium at a constant flow rate of 1 mL min^{-1}. The oven temperature program was initially at 60 °C with a temperature ramp of 24.6 °C min^{-1} until 200 °C, which was maintained for 10 min followed by a second temperature ramp of 5 °C min^{-1} until 250 °C which was maintained for 3 min. The mass spectrometer was run in selective ion monitoring mode (SIM) by the application of the masses m/z 55, 74, 79, and 81. The programmable temperature vaporization (PTV) inlet was operated in splitless mode and with the evaporation program increasing from 60 to 300 °C, with a temperature ramp of 720 °C min^{-1} and maintained for 2 min.

Statistical analysis Treatment means were compared using SigmaStat 12.5™ by t test when data were normally distributed, whereas a Mann-Whitney rank sum test was used when data failed the test for normality. We used a significance level of 0.05. Nonlinear as well as linear regressions were conducted by the statistical software package SAS 9.4 using the PROC model fitting library for the orthogonal linear regression PROC MODEL fitting library. Test parameters were tested against fixed values, e.g., whether intercept was different from zero by the likelihood test included in the PROC MODEL fitting library.

Results

Mixed experiment We found no statistically significant difference between clearances in the two experiments or between prey species (t test, $P > 0.1$) (Fig. 1a). In terms of carbon-specific ingestion, we found statistically significant difference between prey at *Rhodomonas salina*:*Crypthecodinium cohnii* ratio of 2.5 (Mann-Whitney rank sum test, $P = 0.029$), whereas no statistically significant difference in the ingestion of *R. salina* and *C. cohnii* was found at a *R. salina*:*C. cohnii* ratio of 2.7 (t test, $P = 0.115$) (Fig. 1b).

Feeding on *C. cohnii* Carbon-specific clearance rate peaked at ~ 16 mL μg female C^{-1} day^{-1} at a concentration of 40 μg *C. cohnii* C L^{-1} followed by an asymptotic decrease yielding 8 mL μg female C^{-1} day^{-1} at 100 μg *C. cohnii* C mL^{-1} followed by a further asymptotic decrease toward 2 mL μg female C^{-1} day^{-1}. In absolute terms, clearance rates ranged from 39 to 6 mL female day^{-1} (Fig. 2).

Ingestion rate increased asymptotically from ~ 0.45 to ~ 4.2 μg C μg C^{-1} day^{-1} reached at 1100 μg *C. cohnii* L^{-1} (Fig. 3). This range in ingestion is equivalent to 46–208% bodily carbon day^{-1} (data not shown).

Egg production in the two mixed diet experiments was not statistically significantly different (t test, $P > 0.3$), and we, therefore, pooled the egg production of the two ratio experiments into one set of data. Egg production (egg female^{-1} day^{-1}) was 20 ± 2.6 (± standard deviation). When egg production was converted to carbon-specific egg production, then the *Acartia tonsa* females were producing 25.2 ± 0.8% (± standard deviation) bodily carbon as eggs corresponding to 0.9 μg C female^{-1} day^{-1} (Table 1).

Egg production increased asymptotically from ca. 5 eggs female^{-1} day^{-1} at ~ 40 μg *C. cohnii* C L^{-1} to ~ 13 eggs female^{-1} day^{-1} at prey concentrations > 900 μg *C. cohnii* C L^{-1} (Fig. 4). In female carbon body terms, then daily carbon-specific egg production covered a range of 6 to 16% (data not shown).

We fitted ingestions and egg production rates to Eq. 1. The fit parameters are found in Table 2.
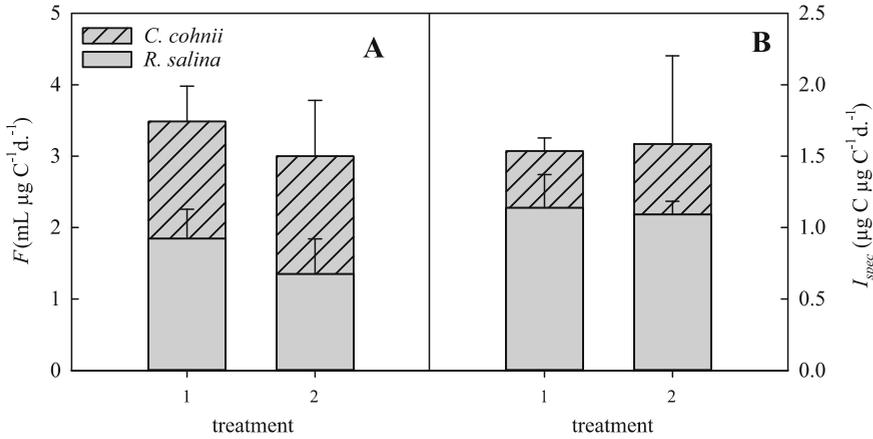
Fig. 1 Mixed diet grazing experiment. **a** Clearance rate specific to grazer biomass (F, mL µg C^{-1} day^{-1}). **b** Specific ingestion (µg C µg C^{-1} day^{-1}). *Gray bar* is *R. salina* and *hatched bar* is *C. cohnii*. Treatments 1 and 2 refer to the *R. salina*:*C. cohnii* biomass ratio of 2.5 and 2.7, respectively

Scaling of egg production in terms of carbon-specific egg production (CEP) and carbon-specific ingestion (CSI) increased linearly (Fig. 5). The linear regression of the line CEP = GGE + i × CSI, where the slope (GGE) is the gross growth efficiency and i is the intercept with the ordinate. The relationship between the two measured variables was fitted by orthogonal regression (type II regression sensu Sokal and Rohlf 1998). We found that CEP equalled 22 ± 3.8% (±1 standard error, $P < 0.001$), whereas c equalled 0.03 ± 0.07 (±1 standard error) and was significantly different from 0 (likelihood test; $P < 0.0001$). The
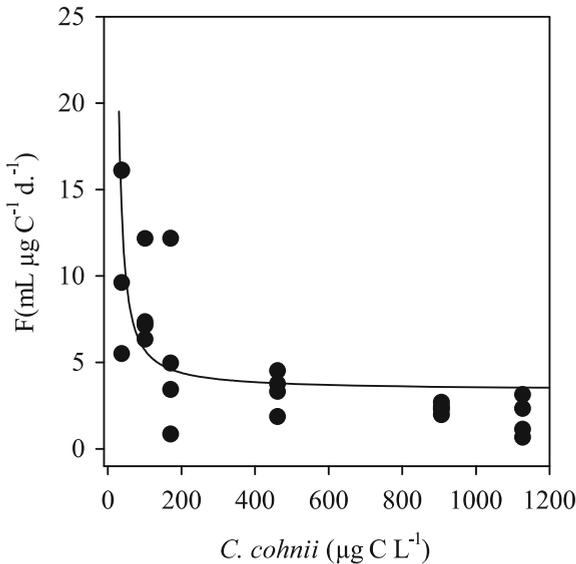
Fig. 2 Clearance rate specific to grazer biomass (F, mL µg C^{-1} day^{-1}) as a function of prey concentration (*C. cohnii*, µg C L^{-1}). The fitted curve follows the line $F = 3.39e^{(52.5/c)}$
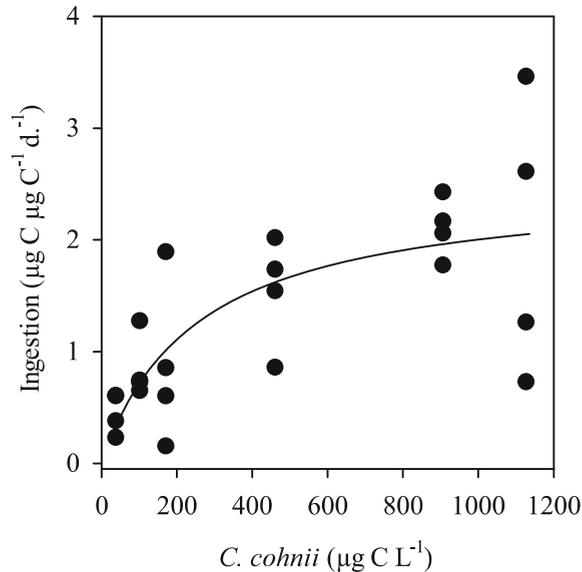
Fig. 3 Specific ingestion (I_{spec}, μg C μg C^{-1} day^{-1}) as a function of prey concentration (c, μg *C. cohnii* L^{-1}). The full line is the fit made by Eq. 1

orthogonal regression estimates the uncertainty of the linear regression corresponding to ± 0.95 μg C female^{-1} day^{-1} for the ingestion and ±0.22 μg C female^{-1} day^{-1} for the egg production.

Egg hatching success was 60% for the diet of *C. cohnii* and 89% for the *R. salina* diet (Table 3). The difference between the two treatments was statistically significantly different (*t* test, *P* = 0.025).

Cellular content The results from the CN elemental analysis and the main fatty acids are outlined in Table 4. The cell volume of *C. cohnii* was ~4.4 larger than that of *R. salina*. The volume-specific carbon content was 0.24 pg C μm^{-3} for *R. salina* and 0.23 pg C μm^{-3} for *C. cohnii*. The volume-specific N content was 0.054 and 0.048 pg N μm^{-3} for *R. salina* and *C. cohnii*, respectively. The total fatty acids were ~17% of the cell carbon in *R. salina* whereas ~9% of the total cell carbon in *C. cohnii*.

The most conspicuous difference between the two prey organisms was that ~18% of the fatty acids were EPA in *R. salina* whereas EPA was mostly absent in *C. cohnii* (Table 4). In contrast was ~63% of the fatty acid DHA in *C. cohnii*, whereas only 12% of the fatty acids were DHA in *R. salina*. When converted to cell-specific terms, this corresponds to 0.0046 and

Table 1 Secondary production in the mixed diet experiment. Egg production expressed in absolute, percentage of carbon per day and as carbon female per carbon per day. Numbers are given as means ± 1 standard deviation

Egg prod. (egg female^{-1} day^{-1})	20.0 ± 2.6
Egg prod. (% day^{-1})	25.2 ± 0.8
Egg prod. (C female C^{-1} day^{-1})	0.91 ± 0.1
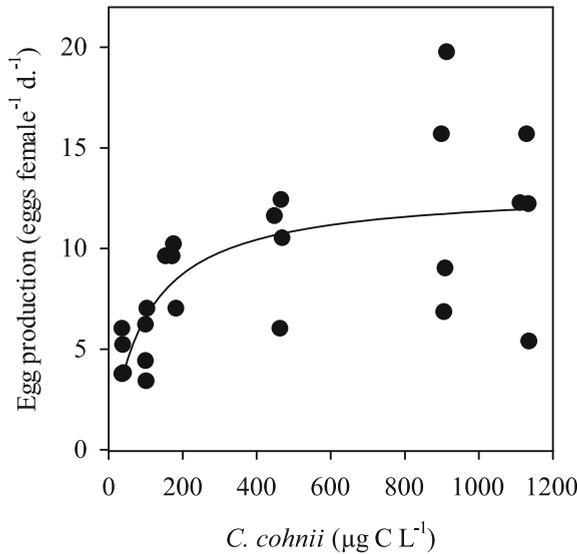
Fig. 4 Absolute egg production (egg female^{-1} day^{-1}) as a function of prey. The full line is the fit made by Eq. 1

0.0131 pg DHA μm^{-3} for *R. salina* and *C. cohnii*, respectively. The fatty acid ARA was not abundant in any significant amount in neither of the prey organisms.

Among the unsaturated fatty acids did *Rhodomonas salina* have 43% of its total fatty acids as C18:4, whereas C18:4 in *Crypthecodinium cohnii* was 0.13% of the total fatty acids (Table 4).

Discussion

In general terms, starch usually makes 50%, lipids ca. 12–14%, protein 7–15%, fatty acids ca. 10%, and DHA 2–4% of the dry weight in *Crypthecodinium cohnii* (Pleissner and Eriksen 2012). In this study, the cellular content of EPA, DHA, and ARA in *Rhodomonas salina* is very similar to that found in the same strain by Vu et al. (2016). That is, *R. salina* had an almost twice as high lipid content as in *C. cohnii* (17 versus 9%). However, this figure is somewhat deceiving because only 30% was HUFAs (EPA + DHA) in *R. salina*, whereas 64% was HUFA in *C. cohnii*. Because the absences of chloroplasts are one of the most conspicuous differences between the two preys, it is tempting to suggest that fatty acids ARA and C18:4 are directly linked to chloroplasts in the autotrophic *R. salina*. The required thresholds of HUFAs in the diet for optimum growth remain unknown for most copepod species including *Acartia tonsa*, and it is likely to differ between species and life stages (Ahlgren et al. 2009). Yet,

Table 2 Fit parameters ± 1 standard error of ingestion, egg production, and specific egg production versus *C. cohnii* concentrations obtained by Eq. 1

	Max. rate (B_{max})	$K/2$ (μg C L^{-1})	R^2	P
I (μg C μg C^{-1} day^{-1})	2.51 ± 0.56	251 ± 138	0.52	< 0.0001
Egg prod. (egg female day^{-1})	13.0 ± 1.46	97.1 ± 43.9	0.46	< 0.0001
Specific egg prod. (% day^{-1})	17.8 ± 2.00	97.1 ± 43.9	0.46	< 0.0001
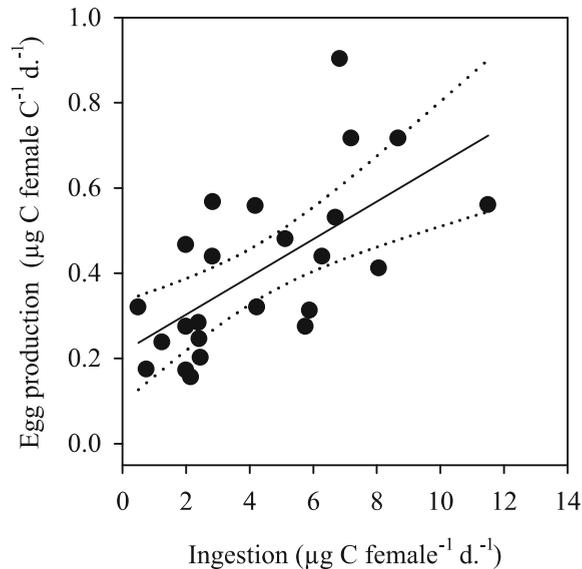
Fig. 5 Carbon-specific egg production (CEP) as a function of carbon-specific ingestion (CSI). Data are derived from Figs. 3 and 4. The full regression line follows the equation CEP = 0.04 + 0.22 × CSI. The dotted lines identify the 95% confidence limit of the linear estimate

secondary production seems negatively affected by small amounts of HUFAs available in the diet and several examples in the literature exist. As an example, decreased fish egg viability by 10% followed by a decrease in fish larval survival was caused by just a 0.6% decrease in the dietary HUFA content offered to the parental fish (Izquierdo et al. 2000). Similarly, small additions of dinoflagellates to a copepod diet consisting mainly of cyanobacteria increased the HUFAs in the diet from about 10–12 to 16–18%, causing significantly increased egg production in the copepods (Ianora et al. 2003).

Some insights are derived from our study in terms of the ratios between DHA, EPA, and ARA. That is, the fatty acid ARA appears to be less important in feeding and egg production in *A. tonsa* because the high reproductive rates are achieved on a diet of both *R. salina* and *C. cohnii* that contain < 0.2% of this fatty acid. In terms of fish growth, Barroso et al. (2013) found that wild populations of copepods with low ARA concentrations supported growth and development of fish larvae. In addition, the fish larvae in the experiment by Barroso et al. (2013) contained much higher ARA than the prey copepod, indicating that this fatty acid is either retained or de novo produced by the fish and may have a functional or structural role in fish that is different from that in copepods. In addition, ARA was also identified as a key structural component in the development and survival of captivity-kept cod (*Gadus morhua*) (Røjbek et al. 2014). This observation corroborates with a mesocosm study that suggested that

Table 3 Hatching success (%) ± 1 standard deviation

Diet	Hatching success (%) ± 1 standard deviation
C. cohnii	60 ± 18
R. salina	89 ± 2

Table 4 Main constituents of the prey items used. Fatty acids are given as % ± standard deviation of total fatty acids content. Fatty acids of <C18 make only a minor fraction and not shown

	R. salina	C. cohnii
Cell vol (μm^3)	240	1060
pg N cell^{-1}	12.70 ± 0.43	50.80 ± 2.43
pg C cell^{-1}	56.60 ± 1.43	246.00 ± 9.40
Total fatty acids (pg cell^{-1})	10.10 ± 2.35	22.10 ± 0.95
Total fatty acids (%)	17	9
C20:4 n-6 (ARA; %)	0.19 ± 0.04	0.01 ± 0.01
C20:5 n-3 (EPA; %)	18.40 ± 4.43	0.19 ± 0.02
C22:6 n-3 (DHA; %)	11.80 ± 2.93	63.60 ± 3.05
C24:1 (%)	0.03 ± 0.01	0.22 ± 0.28
C24:0 (%)	0.08 ± 0.01	0.11 ± 0.00
C22:2 (%)	0.01 ± 0.01	0.08 ± 0.05
C22:1 (%)	2.25 ± 0.15	2.42 ± 0.54
C22:0 (%)	0.15 ± 0.04	0.11 ± 0.03
C21:0 (%)	0.02 ± 0.00	0
C20:3 n-6 (%)	0.05 ± 0.01	0.01 ± 0.00
C20:3 n-3 (%)	0.03 ± 0.01	0.07 ± 0.07
C20:2 *trans* (%)	0.01 ± 0.01	0.01 ± 0.01
C20:1 (%)	0.14 ± 0.12	0.13 ± 0.03
C20:0 (%)	0.07 ± 0.01	0.09 ± 0.00
C18:4 (%)	42.90 ± 10.30	0.13 ± 0.03
C18:3 n-6 (%)	0.12 ± 0.03	0.01 ± 0.01
C18:2 *cis* (%)	0.89 ± 0.20	0.05 ± 0.01
C18:2 *trans* (%)	0.10 ± 0.02	0.05 ± 0.02
C18:1 *trans* (%)	0.19 ± 0.04	0

imbalance between HUFAs derived from different phytoplankton communities induced reproductive imbalance in Atlantic salmon (*Salmon salar*) (Ahlgren et al. 2005). Hence, there is evidence that ARA is a key biochemical component throughout the entire life cycle of fish whereas ARA seems less important in copepods.

In the mixed diet experiment at prey concentrations of 800–1100 μg C L^{-1}, the average copepod egg production was 20 eggs female^{-1} day^{-1}, corresponding to a GGE of 25% per day. Other studies on *A. tonsa* feeding *R. salina* as single prey have found higher GGE values of 36% (Kiørboe et al. 1985) and 33% (Berggreen et al. 1988).

This is further confirmed when *C. cohnii* was offered as a single prey where the highest GGE was 22%. It is also noteworthy that when even *R. salina* was available at biomass ratios exceeding that of *C. cohnii* by a factor of 2.5, the GGE remained lower than that reported in the literature; thus, their synergy, in terms of copepod egg production in adding *R. salina* as in the mixed feeding experiment, seems limited.

Acartia tonsa displayed a clearance on *C. cohnii* of ~ 50% (18 μm) of the maximal clearance rate reported on *R. salina* (12 μm). However, the clearance rate on *C. cohnii* was similar to that on the larger dinoflagellate *Akashiwo sanguinea* (45 μm) (Berggreen et al. 1988). Ingestion rates by *A. tonsa* on *C. cohnii* varied between 0.5 and 12 μg C female^{-1} day^{-1} and exceeded the highest values found on *R. salina*. However, since clearance rates are dependent on prey size, it supports prey size-specific clearance rates found in Berggreen et al. (1988) by *A. tonsa* on *Rhodomonas baltica* besides larger prey particles resembling the cell size of *C. cohnii*. Consumer food quality is not only restricted to essential fatty acids but also includes other quality properties such as amino acids, sterols, vitamins, elemental stoichiometry, and morphological characteristics (Sommer et al. 2012; Wacker and Martin-Creuzburg 2012). However, the similar amounts of nitrogen between the two preys suggest that also *C. cohnii* meets the required amino acid threshold for secondary production in *A. tonsa*.

Rhodomonas salina versus Cryptecodinium cohnii as prey in domesticated copepods

The growth rate and biochemical composition of the photosynthetic algae *Rhodomonas* spp. depend on factors such as temperature, light availability, pH, and cell density. For batch cultures of *Rhodomonas* spp., growth rates depend on initial cell concentration. As an example, an initial cell density of 2000 cells mL^{-1} resulted in a higher growth rate (1.4 day^{-1}) compared to 200,000 cells mL^{-1} (0.7 day^{-1}) (Thoisen pers. comm.). Published data on the growth rate of *Rhodomonas* in continuous PBR production is scarce, but similar growth rates of *Rhodomonas* ($\sim$ 0.9–0.95 day^{-1}) were achieved at 10^7 cells mL^{-1} (Eriksen et al. 1998) and at 2.2 × 10^6 cells mL^{-1} (Vu et al. 2016) in PBRs with a volume of 1.7 and 2 × 47 L, respectively.

Suitable diets of *C. cohnii* are various sources of organic dissolved carbon (DOC) (Tuttle and Loeblich 1975), and the content of DHA in *C. cohnii* can be altered depending on the carbon source and concentration, as well as the culture temperature and inorganic nutrients. For comparison, the biochemical composition of *C. cohnii* is likewise plastic and can be modified in various ways by the composition of the offered substrate (Pleissner and Eriksen 2012). As an example, de Swaaf et al. (2003) estimated that DHA exceeded 30% of the total fatty acid content, whereas other PUFAs are found in small quantities. This is in contrast to our study where 9% of the fatty acids were DHA (Table 4).

The optimal irradiance for growth in batch cultures of *Rhodomonas* has been found in the range of 60–150 μmol m^{-2} s^{-1}, an irradiance range also optimal for the cell content of essential fatty acids (Chaloub et al. 2015; Vu et al. 2016). As an example, the cellular content of PUFAs in *Rhodomonas* spp. is affected by temperature, and literature reports indicate that a temperature around 20 °C is preferable compared to higher temperatures. As examples, Guevara et al. (2016) found higher contents of DHA, EPA, and ARA in *Rhodomonas* grown at 19 °C compared to 29 °C; similarly, Renaud et al. (2002) observed decreased contents of DHA and EPA when temperatures were elevated from 25 to 35 °C in *Rhodomonas* sp., whereas ARA content increased.

The literature clearly shows that even when cultivated under optimal conditions, the content of DHA in *Rhodomonas* spp. exceeds that of *C. cohnii*. However, *C. cohnii* has several advantages as opposed to autotrophic phytoplankters. *C. cohnii* produces an exceptionally high concentration of DHA (Jiang et al. 1999), which otherwise is difficult to obtain in large-scale settings of autotrophic microalgae (Grima et al. 1993). Moreover, it divides several times per day as opposed to less than once a day, which is typical for phytoplankton such as *R. salina*. On the downside, *C. cohnii* requires sterile bioreactor culturing which requires initial investment and training of the personnel and access to an axenic nutritious DOM diet. In these terms, *R salina* is much less demanding.

The highest reported growth rate of *C. cohnii* in the literature is 2.2 day^{-1} from experiments in 250-mL flasks (Jiang and Chen 2000; Jiang et al. 1999). Cultivation is possible within a wide temperature range from 15 to 30 °C, and a wide pH range of 5.5 to 9 can be applied with optimal pH reported between 6.6 and 7.2 (Jiang and Chen 2000; Jiang et al. 1999; Tuttle and Loeblich 1975). Hence, *C. cohnii* is considered a very tolerant organism considering abiotic conditions.

Our study suggests that the fatty acid ARA is less important in the early life strategy in *A. tonsa* because ARA seems mostly absent in *C. cohnii* and in very low quantities in *R. salina*. That is, in a projected aquaculture setting with *A. tonsa*, feeding on *C. cohnii* needs to be tested whether the low ARA + EPA affects the fish species in question. Hence, if any negative effects are observed in the growth or survival of the fish larvae that feed on *C. cohnii*-reared copepods, then the diet of the copepods can be balanced by the addition of supplemental alternative prey such as *R. salina*. Prey

size preference in *A. tonsa* increase with size (Berggreen et al. 1988), and initial feeding newly hatched nauplii with *R. salina* instead of *C. cohnii* therefore also has the advantage of a better predator prey ratio. That is, alternating between prey of *R. salina* and *C. cohnii* either to optimize nutritional quality of nauplii or copepodites shortly before feeding them to the fish larvae, or to optimize growth of nauplii, may be a path to follow in large-scale rearing systems. In this fashion is the demand for energy requiring large-scale rearing of *R. salina* lowered.

Conclusion

Using *Crypthecodinium cohnii* as a diet for *Acartia tonsa* and potentially for other copepods presents a promising alternative strategy for mass cultivation of prey for copepods in culture for the benefit of edible and ornamental fish larvae hatcheries.

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Paper 6

<u>Title</u>

A simple and fast method for extraction and quantification of cryptophyte phycoerythrin.

<u>Status</u>

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MethodsX

Method Article

A simple and fast method for extraction and quantification of cryptophyte phycoerythrin

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GRAPHICAL ABSTRACT
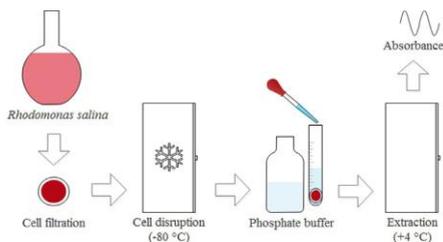
ABSTRACT

The microalgal pigment phycoerythrin (PE) is of commercial interest as natural colorant in food and cosmetics, as well as fluoroprobes for laboratory analysis. Several methods for extraction and quantification of PE are available but they comprise typically various extraction buffers, repetitive freeze-thaw cycles and liquid nitrogen, making extraction procedures more complicated. A simple method for extraction of PE from cryptophytes is described using standard laboratory materials and equipment. The cryptophyte cells on the filters were disrupted at $-80\,^{\circ}C$ and added phosphate buffer for extraction at $4\,^{\circ}C$ followed by absorbance measurement. The cryptophyte *Rhodomonas salina* was used as a model organism.
• Simple method for extraction and quantification of phycoerythrin from cryptophytes.
• Minimal usage of equipment and chemicals, and low labor costs.
• Applicable for industrial and biological purposes.

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Paper 6

Background

Phycoerythrin (PE) is a light harvesting pigment belonging to the phycobiliproteins, which also include phycocyanin, allophycocyanin and phycoerythrocyanin. Phycobiliproteins are found in red algae, cryptophytes and cyanobacteria [1], and are used as natural colorant in food and cosmetics. In particular, phycoerythrin is used as a fluoroprobe for clinical and biological analysis due to its high fluorescence [1].

The cell content of PE in microalgae depends on species and cultivation conditions. Generally, microalgae sustain cellular growth/metabolism during nitrogen limitation by degradation of phycobiliproteins [2,3] which are nitrogen-rich. As an example, a study by Eriksen and Iversen [4] showed that nitrogen-sufficient cells of the cryptophyte *Rhodomonas* sp. were red and contained PE, while nitrogen-limited cells were green and without detectable amounts of PE. Also, the cell content of PE in *R. salina* was lower during nutrient limited cultivation compared to nutrient excess in a study by Vu et al. [5]. According to Kathiresan et al. [6], the content of PE in the red microalgae *Porphyridium purpureum* depends not only on nitrogen but on various specific macro nutrients. Light intensity and temperature can also affect the cell content of PE as demonstrated by Chaloub et al. [7] where PE in *Rhodomonas* sp. increased at low light intensity ($15 \, \mu\text{mol m}^{-2}\text{s}^{-1}$, 12:12 light: dark cycle) combined with increased temperature ($26 \, ^\circ\text{C}$). Thus, quantification of the microalgal cell content of PE has relevant purposes such as being a proxy for the nutrient status during cultivation, and optimizing cultivation conditions to yield a higher cell content of PE.

The extraction efficiency of PE from microalgae depends on the rigidity of the cell wall, if present. The most suitable cell disruption method is therefore species dependent [8]. Cryptophytes do not possess a cell wall but a periplast of thin and fragile rectangular plates underneath the plasma membrane, which is very fragile (see references in Goldman and Dennett [9]) and easily disrupted. Numerous methods for extraction of PE are available but they are based on various species and comprise unnecessary chemicals, working steps and equipment for the extraction of PE from species without a cell wall [6,10,7]. The methods are often too comprehensive and time consuming for simple purposes such as comparing the cell content of PE whether it is between species/strains or between different cultivation conditions. For such comparisons it is beneficial with a simple and low labor cost method to obtain fast results.

This paper describes a simple and fast method for extraction and quantification of PE from the cryptophyte *R. salina* using only few materials and equipment easily available in standard laboratories at a low labor cost.

Method details

Materials

- Culture of *Rhodomonas salina*
- Whatman™ GF/C filter (0.2 μm)
- Pyrex glass vials
- Phosphate buffer (0.1 M, pH 6.7)
- Pasteur glass pipettes
- Syringe with 25 mm syringe filter (0.2 μm cellulose acetate membrane)
- Plastic cuvettes

Equipment

- Filtration apparatus
- Refrigerator (+4 °C)
- Freezer (−80 °C)
- Spectrophotometer

Pigment extraction and absorbance measurement

Filter the microalgae cells onto Whatman^{TM} GF/C filters (0.2 µm) under a pressure of approximately 34 kPa. Fold the filters midway with the cells inside and transfer each filter to a Pyrex glass vial. Add 3 ml of the extraction solvent 0.1 M phosphate buffer (pH 6.7, 0.05 M K_2HPO_4, 0.05 M KH_2PO_4) and freeze at −80 °C for 24 h to disrupt the cells. Hereafter place the Pyrex glass vials with the filters in a refrigerator (4 °C) and extract for 24 h. Then transfer the extraction solvent with a Pasteur glass pipette (150 mm) to a 5 ml syringe with a 25 mm filter (0.2 µm, cellulose acetate membrane) and filter into a disposable plastic cuvette (1 cm path length). Measure the absorbance at 455, 564, 592 and 750 nm on a spectrophotometer using phosphate buffer as a blank. Scatter-correct the absorbance values by subtracting the absorbance at 750 nm. Avoid excess light exposure of the samples during the entire process from filtration to absorbance measurement by wrapping in, e.g., tin foil.

Calculate the content of phycoerythrin (PE) according to Beer and Eshel [11]:

PE (mg/ml) = $[(A_{564} − A_{592}) − (A_{455} − A_{592})*0.2]*0.12$

Where A refers to absorption at the indicated wave lengths.

Additional information

To identify the easiest and fastest method for extraction and quantification of PE from *R. salina*, several treatments were compared (Table 1); freezing at −80 °C, lyophilization at −10 °C (Christ Alpha 1–2), and extraction in phosphate buffer each had a duration of 24 h. Sonication (Bransonic, Branson 1210, Struers KEBO Lab, model B1210E-MT) in an ice-bath with a frequency of 47 kHz ± 6% had a duration of 10 min. All samples were extracted for 24 h at 4 °C, and measured and calculated according to Beer and Eshel [11] as described in the previous section. All treatments used few materials and equipment available in standard laboratories. Replicates of all treatments were obtained at the same time from the same culture of *R. salina* cultivated at a low light intensity of 13 µmol m^{-2} s^{-1} and 17 °C.

There was a statistically significant difference in the yield of PE depending on the treatment (one-way ANOVA, $F_{7.16}$ = 36.6, $p < 0.001$) (Fig. 1). Treatment 5 yielded a statistically significant higher

Table 1
The treatments of filters with *R. salina* (1–8) and their processing time (days) used to identify the easiest and fastest method for extraction and quantification of PE.

Treatment	Day 0	Day 1	Day 2	Day 3
1	Extract	Measure absorbance		
2	Sonicate, extract	Measure absorbance		
3	Lyophilize	Extract	Measure absorbance	
4	Lyophilize	Sonicate, extract	Measure absorbance	
5	Freeze	Extract	Measure absorbance	
6	Freeze	Sonicate, extract	Measure absorbance	
7	Freeze	Lyophilize	Extract	Measure absorbance
8	Freeze	Lyophilize	Sonicate, extract	Measure absorbance
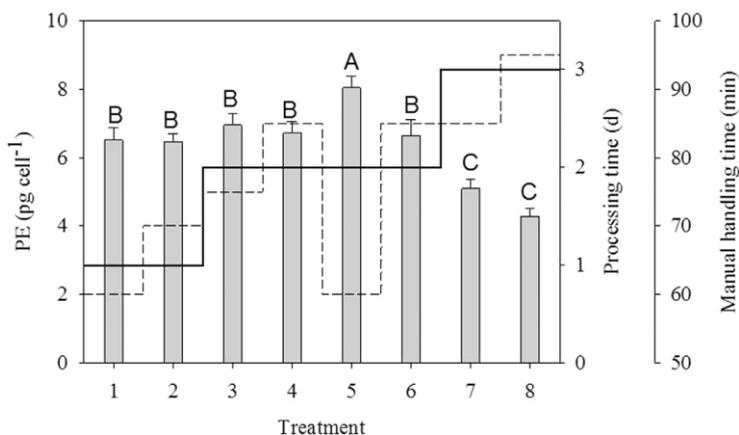
Fig. 1. The cell content of PE (bars) in *Rhodomonas salina* from the treatments in Table 1. Treatment 5 resulted in the highest amount of PE, while treatment 7 and 8 resulted in the lowest amounts of PE (see text). Different letters indicate statistically significant differences between treatments. Bars are mean values $\pm$ S.D. (n = 3). The total processing time (d) for replicates of each treatment is indicated by the solid line. The estimated manual handling time (min) for replicates of each treatment is indicated by the dashed line. Manual handling time includes working steps such as filtrating cells onto filters and transferring filters to Pyrex glass vials.

amount of PE (8.04 ± 0.34 pg cell^{-1}) compared to the other treatments (p $\leq$ 0.002, Holm-Sidak) with a processing time of 2 days. Therefore, treatment 5 is the recommended method described in the section above. However, the yield in treatment 5 was merely 19% higher compared to treatments 1 and 2 with a processing time of 1 day. Thus, methods with a processing time of 1 day are also applicable. A statistically significant lower cell content of PE (p $\leq$ 0.001, Holm-Sidak) was obtained with treatments 7 and 8.

Extraction of microalgal pigments often includes sonication and/or repetitive freeze-thaw cycles to disrupt the cell wall. Sonication, however, is unnecessary for cryptophytes since they do not have a cell wall. In fact, the presented results of PE for treatments exposed to sonication were lower compared to their counterpart without sonication (Treatment 1 versus 2, treatment 3 versus 4, etc.). Whether sonication has a direct negative effect on the yield of PE, or if this pattern is merely a coincidence, is unknown. Also, one freeze-thaw cycle using liquid nitrogen was sufficient for maximal PE extraction in *Pyrenomonas* (now *Rhodomonas*) *salina* [2], and a direct extraction of PE from *Rhodomonas* without any prior processing is possible as indicated by the results in Table 1 and Fig. 1.

Estimates of the manual handling time of triplicate samples of the treatments in Table 1 are shown in Fig. 1. This is defined as the time from which the cells are filtrated onto the filter to the PE results are obtained. The manual handling time includes working steps such as filtrating cells onto filters and transferring the filters to Pyrex glass vials, removing the lid from the Pyrex glass vials and adding phosphate buffer, etc. Based on the estimates, treatment 1 and 5 requires the lowest time of manual handling with 60 min. The total processing time of treatment 1 and 5 is 1 and 2 days, respectively (Fig. 1). In addition, the minimum extraction time (after one freeze-thaw cycle and 4 °C) of PE from *R. salina* was found to be 4 h by [12]. Thus, the processing times given above could likely be reduced by 20 h.

Based on the results for cell content of PE in the different treatments, the total processing time, and the manual handling time, we recommend the method for treatment 5. This method is a simple and fast method for obtaining results on the cell content of PE whether it is for comparing the content of PE in microalgal species, finding cultivation conditions resulting in a higher cell production of PE, or other comparative studies on the content of PE.

Acknowledgement

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