RUC

Roskilde University

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

Thoisen, Christina Vinum; Thuy, Minh Vu Thi; Carron-Cabaret, Thibaut; Jepsen, Per Meyer; Nielsen, Søren Laurentius; Hansen, Benni Winding

1 **Small-scale experiments aimed at optimization of large-scale production of the microalga**

2 ***Rhodomonas salina***

3

4 Christina Thoisen[1*], Minh Thi Thuy Vu[1], Thibaut Carron-Cabaret[2], Per Meyer Jepsen[1], Søren

5 Laurentius Nielsen[1], Benni Winding Hansen[1]

6

7 [1]Roskilde University, Department of Science and Environment, Universitetsvej 1, Postbox 260,

8 Building 11.2, DK-4000 Roskilde, Denmark.

9 [2]Ecole Polytechnique Universitaire de Montpellier, Université Montpellier 2, Place Eugène Bataillon,

10 34095 Montpellier, France.

11

12 Contact: Christina Thoisen, thoisen@ruc.dk

13

14 Keywords: Seawater · fatty acids · growth medium modification · cell density · seawater treatment ·

15 sedimentation · initial density

16

17

Abstract

The cryptophyte *Rhodomonas* is an important feed item for live feed organisms in aquaculture and although large-scale cultivation 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 also 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 1.3 ± 0.7 d^{-1}. The treatment of 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) + autoclavation, 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 2,000 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 is solved and future large-scale production has become a great step closer.

1. Introduction

In marine aquaculture, microalgae are used as feed for larvae and benthic stages of filter feeders (Fernández-Reiriz et al. 2015; Tremblay et al. 2007) as well as for pelagic live feed organisms such as copepods, rotifers, and brine shrimp (McKinnon et al. 2003; Seixas et al. 2009; Srivastava et al. 2006). The microalgal cryptophyte *Rhodomonas* improves the survival, growth, lipid content, and reproduction of brine shrimp, copepods and scallop larvae (Arndt and Sommer 2014; Knuckey et al. 2005; McKinnon et al. 2003; Ohs et al. 2010; Seixas et al. 2009; Tremblay et al. 2007; Zhang et al. 2013), and contain the essential polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA, 20:5ω3), docosahexaenoic acid (DHA, 22:6ω3) and arachidonic acid (ARA, 20:4ω6) in ratios optimal for aquaculture organisms (Guevara et al. 2016; Jakobsen et al. 2018; Vu et al. 2016). 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., Arndt and Summer 2014; de Lima et al. 2013; Drillet et al. 2006; Jakobsen et al. 2018; Knuckey et al. 2005; McKinnon et al. 2003; Ohs et al. 2010; Seixas et al. 2009; Støttrup et al. 1999; Zhang et al. 2013). 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 (Huerlimann et al. 2010; Valenzuela-Espinoza et al. 2005). 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 (Chaloub et al. 2015; Eriksen et al.

61 1995; Vu et al. 2016). A recent small-scale study by Jepsen et al. 2018 evaluated the effect of salinity

62 and different commercial salts on *R. salina* and the copepod *Acartia tonsa* with positive outcomes for

63 large-scale cultivation located without access to seawater. Aside from Jepsen et al. 2018, studies

64 specifically regarding a meso- or large-scale production of *R. salina*, or optimization hereof, are not

65 found in the literature. The aim was therefore to study factors acting as barriers for large-scale

66 production of *Rhodomonas* as a microalgal diet for live feed organisms in aquaculture. This motivated

67 us to focus on: 1) the necessity of $CoCl_2$ (cobalt(II) chloride) in the f/2 growth medium, 2) the content

68 of PUFAs in five strains of *Rhodomonas* to identify the most suitable strain, 3) the temporal

69 sedimentation of the five strains of *Rhodomonas* to identify the one with the lowest sedimentation rate

70 which could potentially reduce biofouling of the PBR, 4) the effect on growth rate by adding nitrogen

71 as different sources to the growth medium, 5) the effect of different types of seawater treatment on the

72 growth rate, and finally 6) the growth rate of different initial inoculum densities..

73   Large-scale cultivation of microalgae in PBRs is extremely time and labor consuming to

74 conduct and therefore small-scale experiments were conducted to study the various factors and obtain

75 useful results within a short period. It can be problematic to transfer certain results from small- to large-

76 scale systems as there is a dimensional factor hindering an exact scale-up. Nonetheless, factors such as

77 nutrient requirement, commercial salts, and treatment of seawater is restricted to the organism and

78 results regarding these factors can therefore be transferred directly from small- to large-scale. Contrary,

79 the specific growth rate of initial cell densities will most likely be affected between scales but it still

80 provides a guidance to estimate the size and density of the inoculum for a desired production. In the

81 following sections each studied factor is introduced to clarify why these factors are important to study

82 when pursuing an ambition of optimizing the large-scale production of *Rhodomonas*.

83   Successful meso- and large-scale cultivation of *R. salina* in tubular and vertical PBRs has taken

84   place the last 4 years at Roskilde University and its project partners. One of the limiting factors for

85   cultivation is the need for cleaning of the PBR at a regular frequency due to biofouling. During

86   cleaning, the PBR is shut down which is an economic loss for the production. The period for

87   cultivation could potentially be prolonged by substituting with a *Rhodomonas* strain with a lower rate

88   of cell sedimentation (i.e., high motility) and thereby reduce the tendency of biofouling. Furthermore,

89   since closely related species and strains of a given microalga are known to have deviating biochemical

90   compositions it is important to compare the PUFA content between strains of *Rhodomonas* to identify

91   the most suitable strain as a microalgal diet for the aquaculture (Guevara et al. 2016; Lang et al. 2011).

92   The cultivation of microalgae necessitates growth media, and numerous recipes are available

93   and generally target a broad range of species (Harrison et al. 1980; Keller et al. 1987). The growth

94   medium is therefore likely to contain unnecessary or excessive amounts of certain components for

95   cultivation of specific species. To our knowledge, there is no growth medium specifically defined

96   according to the nutrient requirements for *Rhodomonas*. The nitrogen source can be added as

97   ammonium (NH_4^+), nitrate (NO_3^-) or urea and the preferred source is species-specific (e.g., Giordano

98   1997; Lourenço et al. 2002). The increased growth rate of some microalgal species obtained when

99   cultivated on NH_4^+ (Giordano 1997) is assumed to be coupled to the lower demand of reductants for

100   assimilation (Dortch 1990). Growth media also contain different trace metals but the requirement of

101   various trace metals is species-specific and some microalgal species can substitute a given trace metal

102   with another (e.g., Timmermans et al. 2001; Xu et al. 2007). The compound $CoCl_2$ in growth media is

103   problematic for a large-scale production as it is widely recognized as a toxic substance. Exposure

104   limits, as well as limits for tolerated daily intake (TDI), have been established by both the European

105 Chemicals Agency (ECHA) in the European Union as well as by the National Institute of Occupational

106 Safety and Health (NIOSH) in the United States of America. In particular, the European Union

107 guidelines involve producing elaborated and detailed documentation for the use of $CoCl_2$. Producing

108 this necessary documentation is both manpower requiring and time consuming and since some

109 microalgal species are able to substitute cobalt (Co) with another trace metal, or simply does not

110 require Co, it is relevant to study if $CoCl_2$ can be excluded from the large-scale production of

111 *Rhodomonas* with no consequences for the yield.

112   During cultivation of microalgae it is essential that unwanted organisms are not introduced to

113 the culture. The treatments applied to eliminate unwanted organisms at small-scale (≤20L) are filtration

114 and autoclaving (e.g., Arndt and Sommer 2014; de Lima et al. 2013; Knuckey et al. 2005; Lourenço et

115 al. 2002; Vu et al. 2016) while larger volumes generally are treated by filtration and UV-radiation

116 (e.g., Bamba et al. 2014;Summerfelt 2003). Common for these types of treatments is no addition of

117 chemicals or production of toxic residues that may negatively affect the microalgae (Rhodes et al.

118 2008). However, filtration does not sterilize as small bacteria and certainly viruses can pass through

119 depending on the pore size of the filter material. Autoclaving is an effective sterilization method

120 although it can raise pH of seawater and cause precipitation of nutrients (Filip and Middlebrooks 1975;

121 Jones 1967). This, however, can easily be overcome by controlling pH during cultivation and adding

122 sterilized nutrients post autoclaving. Nevertheless, autoclavation is unrealistic in large-scale

123 productions and UV-radiation is widely used in, e.g., the aquaculture, where pre-filtration is crucial for

124 optimal effectiveness (Summerfelt 2003). The small-scale experiments in the present study use

125 autoclaved seawater to define the optimal cultivation conditions of *Rhodomonas* and a comparison of

126   the seawater treatments used at the different scales is therefore necessary for detecting possible effects

127   of a given treatment of the seawater used for cultivation of *Rhodomonas*.

128   The size and density of inocula used to initiate microalgae cultures are important, especially for

129   a large-scale production. A common rule of thumb is that the inoculum for a new culture should be

130   minimum 10% (v/v) of the original culture. However, there are to our knowledge no studies explaining

131   or confirming the validity of this rule, and it most likely depends on the species and the purpose of the

132   cultivation. Contrary, the cell density of a culture affects the growth rate as, e.g., self-shading may

133   reduce growth at higher densities. For a large-scale cultivation it is relevant to study the growth rate of

134   inocula at various initial cell densities to estimate when a given biomass for production is reached.

135   Furthermore, it is time consuming to maintain a large volume of inocula cultures for a large-scale

136   production and this can by reduced by merely maintaining the specific volume of inoculum necessary

137   for the production.

138

139   **2. Materials and methods**

140   **2.1 Algal strains and general culture conditions.** Five species / strains of *Rhodomonas* were obtained

141   from culture collections and are referred to their respective strain identity (Table 1). The strains were

142   cultivated in natural seawater (NSW with a salinity of 30-35 collected from > 30 m depth in the

143   Kattegat (DK) and filtered through a series of filters (terminal pore size of 0.2 μm). Equipment, NSW

144   and growth medium stock solutions were autoclaved (15 min at 125 °C) prior to use (CertoClav-Tisch-

145   Autoclav, Certoclav Sterilizer Gmbh). Irradiance was continuous (24:0 light:dark cycle) and measured

146   with a Hansatech Instruments LTD Quantitherm light meter QRT1 (see below for specific irradiance in

147   the separate experiments). The f/2 growth medium (without addition of silicate) was used for

148   cultivation (Guillard 1975; Guillard and Ryther 1962), except in experiment 5 'Seawater treatment'.

149   Cell concentration was enumerated on a Coulter Counter (Beckman) using the computer program

150   Multisizer 3, except experiment 4 'Nitrogen source' and experiment 6 ' Initial density' (see the

151   respective experimental sections below). Growth rates were calculated by fit of exponential growth

152   functions on either cell concentration or optical density (OD) over time.

153   **2.2 Experiment 1: CoCl₂.** Strain K-1487 was cultivated in two versions of f/2 growth medium; a

154   regular version and a version without addition of $CoCl_2$ (this strain is referred to as K-1487*).

155   Cultivation took place in a small-scale PBR (Multi-Cultivator MC1000, Photon System Instruments,

156   CZ) with 8 test tubes (each 85 mL) and aeration at 20°C and irradiance of 85 µmol m^{-2} s^{-1} (n = 4). The

157   experimental period was 5 days with a start concentration of 64,460 ±3,234 cells mL^{-1}. Samples for

158   fatty acids were taken on day 6 and analyzed as described in section 2.4. Nutrients were added daily.

159   **2.3 Experiment 2: Fatty acids.** Samples for comparing the fatty acid composition between the

160   *Rhodomonas* strains were taken from exponentially growing semi-batch cultures in 1 L round-bottom

161   flasks with aeration at 17˚C and irradiance of approximately 13 µmol m^{-2} s^{-1} (n = 3). The low irradiance

162   was chosen to maintain reduced, but still exponential, growth rates in these cultures. The cells were

163   filtered onto 0.2 µm glass microfiber filters (Whatman™ GF/C™) and stored at -80 ˚C until analyzed

164   according to Drillet et al. (2006) with minor adjustments: addition of 20 µL internal standard (C23-

165   methylester, 1000 µg mL^{-1}) and no sonication.

166   **2.4Experiment 3: Temporal sedimentation.** Each *Rhodomonas* strain was transferred to individual

167   250 mL beakers (n = 5) at a cell concentration of 418,423 ± 28,400 cells mL^{-1}, except CCAP 995/5 at

168 99,500 ± 3,536 cells mL^{-1}. Replicates were left undisturbed at room temperature in the stagnant water

169 and samples for cell enumeration were withdrawn 1 cm below the water surface after intervals of 1 and

170 6 hours.

171 **2.5 Experiment 4: Nitrogen source.** The nitrogen source in the f/2 growth medium was changed from

172 NO$_3^-$ (nitrate) to NH$_4^+$ (ammonium) and CO(NH$_2$)$_2$ (urea), and combinations of these with an equimolar

173 amount of N in all treatments (Table 2). Cultivation of strain K-1487 took place in the small-scale PBR

174 (described in section 2.2) at 20°C and irradiance of 100 µmol m^{-2} s^{-1} (n = 4). Start concentration was

175 141,500 ± 18,742 cells mL^{-1} and determined by a build-in OD measuring device measuring

176 automatically every 30 min for 30 hours. An equation between cell concentration (Coulter Counter -

177 Beckman) and absorbance (Spectrophotometer - Genesys 6, Thermo Scientific) was obtained by linear

178 regression to estimate the cell concentration by OD:

179
$$Cell\ concentration\ (mL^{-1}) = \left(\frac{abs_{550\,nm} - 0.0026}{0.0002}\right) * 100 \quad (1)$$

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

181 **2.6 Experiment 5: Seawater treatment.** Strain K-1487 was cultivated in filtered (0.2 µm) NSW

182 exposed to autoclavation or UV-radiation. For comparison, the growth rate of K-1487 in filtered NSW

183 without further treatment was included. Cultivation took place in aerated 2 L round-bottom flasks with

184 B1 growth medium added at experimental start and irradiance of 67 ± 7 µmol m^{-2} s^{-1} at 20°C (n = 4).

185 The experimental period was 4 days with a start concentration of 169,500 ± 2,500 cells mL^{-1}. Cell

186 concentrations were determined by OD as described in section 2.5.

187 **2.7 Experiment 6: Initial density.** Strain K-1487 was inoculated at increasing initial densities from

188 2,000 to 200,000 cells mL^{-1} in aerated 1 L round-bottom flasks at $18.5 \pm 0.7°C$ and irradiance of 103

189 µmol m^{-2} s^{-1} (n = 3). The specific initial densities were $2,000 \pm 0$; $7,000 \pm 0$; $9,500 \pm 4,183$; $43,667 \pm$

190 $2,582$; $104,500 \pm 7,583$ and $196,167 \pm 4,916$ cells mL^{-1}. Nutrients were added at experimental start.

191 Cell concentration during the exponential growth phase was determined daily by OD as described in

192 section 2.6. The cell concentration (N) over time was plotted as $\ln(N/N_0)$ where N_0 is the cell

193 concentration at the experimental start. Data was fitted to the modified Gompertz equation described in

194 Zwietering et al. (1990):

195
$$y = A \, exp\left(- \, exp\left(\frac{\mu_m e}{A}(\lambda - t) + 1\right)\right) \qquad (2)$$

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

197 obtained from the fit:

198
$$\mu_m = \frac{a*c}{e} \qquad (3)$$

199
$$\lambda = \frac{b-1}{c} \qquad (4)$$

200 **3. Statistical analysis.** Data on the cell content of fatty acids, growth rate on nitrogen sources, different

201 seawater treatments, and of different initial cell densities were subjected to one-way ANOVAs.

202 Significant results were followed by a Holm-Sidak post-hoc test to compare individual means across

203 significantly different levels.

204   Data on temporal sedimentation was recorded as percentage of cells remaining in the upper 1

205 cm of the water column after 1 and 6 hours and logit-transformed (Sokal and Rohlf 1995) prior to

206   analysis with one-way ANOVA followed by a Holm-Sidak post-hoc test to compare individual means

207   across significantly different levels.

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

209   tailed t-test.

210   Prior to ANOVAs and t-tests, data were tested for constant variance (Spearman's rank correlation) and

211   normality (Shapiro-Wilk test). All tests were carried out using SigmaPlot 12.0 (Systat Software) with α

212   = 0.05.

213

214   **4. Results**

215   **4.1 $CoCl_2$.** The growth rate of strain K-1487 in the two treatments (with and without $CoCl_2$ added to

216   the f/2 growth medium) was not statistically significant at an average of 0.69 ± 0.04 d^{-1} (p = 0.765)

217   (Figure 1). Likewise, the cell content of the PUFAs DHA, EPA and ARA were not statistically

218   significantly different between the two treatments with averages of 2.6 ± 0.1, 3.9 ± 0.4 and 0.2 ± 0.1 pg

219   cell^{-1}, respectively (One-Way ANOVA, p $\leq$ 0.453).

220   *Insert figure 1.*

221   **4.2 Fatty acids.** The cell content of the PUFAs DHA, EPA and ARA was compared in the five strains

222   of *Rhodomonas* (Figure 2). The strains' content of EPA and ARA was not statistically different ranging

223   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

224   ANOVA, EPA; p = 0.267 ARA; p = 0.156). However, ARA was either not present or below detection

225   limit in CCAP 995/5. The content of DHA was statistically significantly higher in CCAP 995/5 with

226   4.0 ± 0.1 pg cell^{-1} (One-Way ANOVA, p≤0.018). The cell content of EPA was higher than DHA for all

227   strains, except CCAP 995/5 where the opposite was observed.

228   The highest ratios of DHA/EPA were 1.3 and 1.0 for CCAP 995/5 and K-1487*, respectively

229   (Table 3). The remaining strains had similar DHA/EPA ratios of 0.7 and 0.8. For EPA/ARA, the

230   highest ratios were 5.1 and 4.4 for K-1487 and K-0435, respectively.

231   *Insert figure 2 and table 3.*

232   **4.3 Temporal sedimentation.** The cell sedimentation of the *Rhodomonas* strains was measured after 1

233   and 6 hours to identify the strain with the lowest sedimentation. The cell density (%) in the upper 1 cm

234   water column of undisturbed seawater was significantly different between the *Rhodomonas* strains at

235   the given time intervals (Figure 3). After 1 hour, the cell density of K-1487, K-1487* and K-0435 was

236   statistically highest with $\geq$ 80 % of the cells remaining in the water column. After 6 hours, the cell

237   density was still highest for K-1487 and K-1487* with 54±2 % and 63±3 %, respectively (One-Way

238   ANOVA, 1 hr; $p < 0.001$, 6 hr; $p < 0.001$). Merely the two significantly highest groups (A, a and B, b)

239   at each given time interval are considered here.

240   *Insert figure 3*

241   **4.4 Nitrogen source.** The effect of the nitrogen source on the growth rate of strain K-1487 was studied

242   by adding NO_3^-, NH_4^+, urea or combinations of these (Figure 4). Cultivation with NH_4^+ as the nitrogen

243   source yielded a significantly higher growth rate of 1.3 ± 0.07 d^{-1} compared to the growth rate for NO_3^-

244   of 1.0 ± 0.08 d^{-1} (One-Way ANOVA, $p = 0.046$). Contrary, urea and combinations of the nitrogen

245   sources gave no statistically significant difference in growth rate compared to both NO_3^- and NH_4^+.

246   *Insert figure 4*

247   **4.5 Seawater treatment.** The growth rate of K-1487 cultivated in NSW treated with 1) filtration (0.2

248   µm) + autoclavation, 2) filtration (0.2 µm) + UV-radiation, and 3) filtration (0.2 µm) was compared.

249   The results show that there were no significant differences on growth rates in the treatments with an

250   average of 0.7 ± 0.1 d^{-1} (One-Way ANOVA, p = 0.833). However, cultivation in simply filtered NSW

251   became contaminated after ~1 week of cultivation with an unidentified nanoflagellate (personal

252   observations) limiting the period of cultivation.

253   **4.6 Initial density.** The temporal cell concentration of initial densities of K-1487 in the range of 2,000

254   to 200,000 cells mL^{-1} was fitted to the modified Gompertz equation (Zwietering et al. 1990) to

255   calculate the exponential growth rate (Figure 5 and 6). The initial density of 2,000 and 7,000 cells mL^{-1}

256   obtained the highest growth rates at 1.4 d^{-1}. A trend was observed with the growth rate gradually

257   decreasing with increasing initial density to 0.7 d^{-1} at 200,000 cells mL^{-1}. The lag time was calculated

258   to be shorter than the sampling interval for cell enumeration and was therefore not considered further.

259

260   The time required for the initial densities to reach a biomass of 10^6 cells mL^{-1} successively

261   decreased with increasing initial density; 6.8 days for 2,000 cells mL^{-1} and 2.8 days for 200,000 cells

262   mL^{-1} (Table 4). However, initial densities of 40,000 and 100,000 cells mL^{-1} both reached 10^6 cells mL^{-1}

263   after just 3.8 days.

264   *Insert figure 5 and 6*

265

266   **<u>5. Discussion</u>**

267   The findings in this study represent a step towards a broader implementation of *Rhodomonas* as a

268   microalgal diet for live fed organisms in aquaculture as relevant practicalities for large-scale production

269   are sought clarified.

270   Various growth media recipes are available but, e.g., the the specific trace metal requirement of

271   phytoplankton varies and it depends on the species if a specific trace metal can be substituted with

272   another trace metal. Examples on Co from the literature showing this species-specificity are: the

273   coccolithophore *Emiliania huxleyi* substitute Co and Zn (zinc) with each other (Xu et al. 2007), the

274   diatoms *Thalassiosira pseudonana* and *T. ocenica* largely substitute Zn with Co (Sunda and Huntsman

275   1995; Yee and Morel 1996), the prymnesiophyte *Phaeocystis antarctica* substitute Zn with Co although

276   Zn is preferred (Saito and Goepfert 2008), the diatom *Chaetoceros calcitrans* lack a substitution of Zn

277   with Co (Timmermans et al. 2001), and the cyanobacteria *Synechococcus bacillaris* and

278   *Prochlorococcus* require Co for growth (Saito et al. 2002; Sunda and Huntsman 1995). Studies on the

279   trace metal requirements of *Rhodomonas* are lacking and our study did not seek to clarify the

280   requirement of all the trace metals in the f/2 growth medium . Nevertheless, it is a key finding for a

281   large-scale production of *Rhodomonas* that exclusion of Co from the growth medium does not affect

282   neither the growth rate nor the cell content of DHA, EPA and ARA as these parameters are essential

283   for aquaculture. Large quantities of growth medium are prepared during a large-scale production of

284   microalgae and exclusion of Co will ease production by bypassing the required elaborated and detailed

285   documentation required by, e.g., ECHA and NIOSH. However, it must be highlighted that NSW

286   contain small amounts of Co(0.00005 μm kg^{-1} according to Atkinson and Bingman 1997). Thus, either

287   strain K-1487* can substitute Co with another trace metal, or there is an adequate amount of Co present

288   in NSW.

289

290   A further modification of the f/2 growth medium was the addition of various nitrogen sources.

291   The highest growth rate of strain K-1487 was obtained with NH_4^+ as the nitrogen source and similar to

292   the results reported by Lewitus and Caron (1990) for *Pyrenomonas* (now *Rhodomonas*) *salina* with a

293   growth rate at 1.2 d^{-1} (135 µmol, 21˚C). However, Lourenço et al. (1997) reported that the cryptophyte

294   *Hillea* sp. could not grow on NH_4^+ unless reduced to a concentration equal to half of that used in the

295   present study. The lower demand of reductants for assimilation of NH_4^+ is a plausible explanation for

296   the increase in growth rate observed in the present experiment (Dortch 1990). However, studies have

297   shown that the biochemical content of microalgae may be altered when supplied with different nitrogen

298   sources and future studies must clarify if NH_4^+ alters the biochemical profile (in particular the PUFAs)

299   of *Rhodomonas* (Fidalgo et al. 1998; Lourenço et al. 2002). Providing the nitrogen source in the form

300   of NH_4^+ may cause an acidification of the culture as NH_4^+ is taken up by the microalgae in the form of

301   NH_3, leaving a proton in the medium. However, when the cell concentration of microalgae increases

302   during cultivation the photosynthetic activity raises pH. In this experiment, pH was not controlled or

303   adjusted but the effect of acidification is assumed to be minor, as seawater is generally well buffered

304   due to its high content of carbonates (Goldman et al. 1982), and the cells in our experiment grew

305   exponentially during the experimental period indicating no negative effect of pH. It must be stressed

306   that the positive effect on growth rate of providing NH_4^+ as the nitrogen source obviously is larger than

307   any negative effects of growth medium acidification on growth rate. In many plant and algal growth

308   media (but not in the f/2 growth medium), the nitrogen source is provided as both NH_4^+ and NO_3^-

309   because the acidifying effect of NH_4^+ uptake counters the alkalizing effect of NO_3^- uptake (Asher and

310   Edwards 1983). In the present study, however, providing NH_4^+ as the only nitrogen source evidently

311 gave the highest growth rate despite any effects of NH_4^+-uptake on pH or of pH on the NH_3/NH_4^+-

312 equilibrium. When producing microalgae at large-scale, pH is usually controlled in a feedback system

313 by CO_2-addition as pH in the photobioreactor will increase during microalgal growth due to the

314 photosynthetic uptake of CO_2 and HCO_3^-.

315   The microalgal diet has been shown to affect the composition of fatty acids in copepods

316 (Caramujo et al. 2008; de Lima et al. 2013; Støttrup et al. 1999) and particularly *Rhodomonas* is

317 praised as an excellent diet for the copepod *Acartia* by improving the nauplii survival, development

318 rate and reproduction (Arndt and Sommer 2014; Knuckey et al. 2005; Zhang et al. 2013). While a

319 DHA/EPA/ARA ratio of 10 : 5 : 1 is considered optimal for some marine fish larvae (Sargent et al.

320 1999), studies on the specific nutritional requirement of PUFAs in copepods is limited (see references

321 in Camus and Zeng 2010), and some species, e.g., *Pseudodiaptomus annandalei*, *Tisbe furcate* and

322 *Nitokra lacustris*, may *de novo* synthesize certain fatty acids (Parrish et al. 2012; Raynar et al. 2015). A

323 short term study (96 hr) by Jakobsen et al. (2018) indicates that ARA is less important for *Acartia*

324 *tonsa* (Dana) as similar reproductive rates were obtained on a diet of *R. salina* (K-1487) compared to a

325 diet of the heterotrophic dinoflagellate *Crypthecodinium cohnii* with cell contents of ARA at 0.19 and

326 0.01 % TFA, respectively. However, it is most likely not the case in long term growth studies with the

327 copepod. All of our studied *Rhodomonas* strains, except CCAP 995/5, are suitable as a microalgal diet

328 for *A. tonsa* but to supply fish larvae with the essential ARA through the live feed (i.e. *A. tonsa*) a

329 *Rhodomonas* strain with high ARA content must be offered as the microalgal diet. This excludes strain

330 CCAP 995/5 unless ARA is supplied from another source. However, this would include another factor

331 in the production line which is undesirable. The cell content of PUFAs in the strains in the present

332 study can likely be increased and result in an improved nutritional value of *Rhodomonas* as a

333   microalgal diet for live feed organisms as studies have reported an effect on the content of PUFAs in

334   *Rhodomonas* when changing the temperature, light intensity and nutrient level (Guevara et al. 2016;

335   Renaud et al. 2002; Vu et al. 2016).

336   The strains exhibited different temporal sedimentation and strains K-1487 and K-1487* were

337   identified as most suitable for cultivation in PBRs due to a low sedimentation. However, this study did

338   not find an explanation for the low sedimentation in these two strains compared to the other strains.

339   Data analysis on cell length (data not presented), total fatty acid content (data not presented) and bio

340   volume (data not presented) showed no correlation with temporal sedimentation. The cultivation of a

341   strain with a low sedimentation is expected to reduce the inevitable biofouling and necessary cleaning

342   frequency of large-scale PBRs resulting in an increase of production.

343   The seawater for large-scale cultivation of microalgae used as a diet for live feed organisms in

344   aquaculture requires a treatment without addition of chemicals and antibiotics as some organisms may

345   otherwise be negatively affected (Rhodes et al. 2008). Large volumes of water in PBRs should be

346   provided easy, cheap and effective to meet all practical requirements. Furthermore, the end-product of

347   the food chain, the fish, is intended for human consumption and must live up to high production

348   standards. Studies comparing the growth rate of microalgae in seawater treated by autoclaving,

349   filtration and UV-radiation are few. In the present study, the growth rate of *Rhodomonas* was not

350   affected by any of these treatments. This indicates that our results obtained from small-scale

351   experiments with *Rhodomonas* (using autoclaved seawater) can be directly implemented to a large-

352   scale production (typically using UV-radiated seawater). Contrary to our results, Jorquera et al. (2002)

353   obtained a lower growth rate of the prymnesiophyte *Isochrysis galbana* in UV-radiated seawater

354   compared to autoclaved seawater which may be due to differences in the sensitivity of microalgal

355 species to the toxic residues that can be produced during UV-radiation. It is therefore optimal to

356 combine filtration with UV-radiation as filtration improves the efficiency of UV by removing particles

357 shading the radiation (Liltved and Cripps 1999). Other examples from the literature on treatments used

358 in aquaculture include, e.g., electrolytic treatment (Jorquera et al. 2002) and ozone (Summerfelt 2003).

359   To start the cultivation of microalgae in a PBR, an inoculum of a given size and density is

360 required to obtain the desired production within a given time frame. The initial density was shown to

361 negatively affect the growth rate of strain K-1487with increasing density. The growth rate was

362 measured during the exponential phase, thus, limitation of nutrients is unlikely the cause for the

363 observed decreased growth rate with increasing initial density. Also, a density of 200,000 cells mL^{-1} is

364 by far a dense *Rhodomonas* culture and improbable to cause significant self-shading.

365   Generally, it is required to reach a desired cell density as fast as possible to produce sufficient

366 microalgal feed for the live feed organisms. An example based on the present findings, a cell density of

367 10^6 cells mL^{-1} is desired after approximately 3 days in a 500 L PBR. The PBR must then be inoculated

368 with an inoculum of 200 L with a density of 500,000 cells mL^{-1} which will result in an initial density of

369 200,000 cell mL^{-1} in the PBR. Contrary, if the PBR is inoculated with 10 L of the same inoculum as

370 above the initial density in PBR is 10,000 cells mL^{-1} and the desired cell density is not reached until

371 approximately 5 days after inoculation. Thus, the production efficiency must be adjusted depending on

372 the facility's capacity for maintaining inoculum cultures of a given volume and the time allowed before

373 cultivation at a desired cell density is reached. Knowledge on these parameters is valuable tools when

374 planning a large-scale production of any given microalgae.

6. 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 Co 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 is solved and future large-scale

production has become a great step closer.

398

399   **References**

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

403   Asher CJ, Edwards DG (1983) Modern solution culture techniques. In: Läuchli A, Bieleski RL (eds) Inorganic Plant
404   Nutrition. Encyclopedia of Plant Physiology (New Series), vol 15. Springer, Berlin, Heidelberg, pp 94-119

405   Atkinson MJ, Bingman C (1997) Elemental composition of commercial seasalts. J Aquaricult Aquat Sci 8(2):39-43

406   Bamba B, Yu X, Lozano P, Ouattara A, Vian MA, Lozano Y (2014) Photobioreactor-based procedures for reproducible
407   small-scale production of microalgal biomasses. Journal of Algal Biomass Utilization 5(1):1-14

408   Bartual A, Lubián LM, Gálvez JA, Niell F X (2002) Effect of irradiance on growth, photosyn thesis, pigment content and
409   nutrient consumption in dense cultures of *Rhodomonas salina* (Wislouch) (Cryptophyceae). Ciencias Marinas 28(4):381-
410   392

411   Bell JG, Sargent JR (2003) Arachidonic acid in aquaculture feeds: current status and future opportunities. Aquaculture
412   218:491-499

413   Boelen P, van Mastrigt A, van de Bovenkamp HH, Heeres HJ, Buma AGJ (2017) Growth phase significantly decreases the
414   DHA-to-EPA ratio in marine microalgae. Aquac Int 25:577-587

415   Camus T, Zeng C (2010) Roles of microalgae on total egg production over female lifespan and egg incubation time,
416   naupliar and copepodite survival, sex ratio and female life expectancy of the copepod *Bestiolina similis*. Aquacult Res
417   41:1717-1726

418   Caramujo MJ, Boschker HTS, Admiraal W (2008) Fatty acid profiles af algae mark the development and composition of
419   harpaticoid copepods. Freshwat Biol 53:77-90

420   Chaloub RM, Motta NMS, de Araujo SP, Aguiar PF, de Silva AF (2015) Combined effects of irradiance, temperature and
421   nitrate concentration on phycoerythrin content in the microalga *Rhodomonas* sp. (Cryptophyceae). Algal Res 8:89-94

422   de Lima LCM, Navarro DMAF, Souza-Santos LP (2013) Effect of diet on the fatty acid composition of the copepod *Tisbe
423   biminiensis*. J Crust Biol 33(3):372-381

424   Dortch Q (1990) The interaction between ammonium and nitrate uptake in phytoplankton. Mar Ecol-Prog Ser 61:183-201

425   Drillet G, Jørgensen NOG, Sørensen TF, Ramløv H, Hansen BW (2006) Biochemical and technical observations supporting
426   the use of copepods as live feed organisms in marine larviculture. Aquacult Res 37(8):756-772

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

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

431 Fidalgo JP, Cid A, Torres E, Sukenik A, Herrero C (1998) Effects of nitrogen source and growth phase on proximate
432 biochemical composition, lipid classes and fatty acid profile of the marine microalga *Isochrysis galbana*. Aquaculture
433 166:105-116

434 Filip DS, Middlebrooks EJ (1975) Evaluation of sample preparation techniques for algal bioassays. Water Res 9(5-6):581-
435 585

436 Giordano M (1997) Adaptation of *Dunaliella salina* (Volvacales, Chlorophyceae) to growth on NH_4^+ as the sole nitrogen
437 source. Phycologia 36(5):345-350

438 Goldman JC, Dennett MR, Riley CB (1982) Effect of nitrogen-mediated changes in alkalinity on pH control and CO_2
439 supply in intensive microalgal cultures. Biotechnol Bioeng 24:619-631

440 Guevara M, Arredondo-Vega BO, Palacios Y, Saéz K, Gómez PI (2016) Comparison of growth and biochemical parameters
441 of two strains of *Rhodomonas salina* (Crypthophyceae) cultivated under different combinations of irradiance, temperature,
442 and nutrients. J Appl Phycol 28(5):2651-2660

443 Guillard RRL (1975) Culture of phytoplankton for feeding marine invertebrates. In: Smith W and Chanley M (ed) Culture
444 of marine invertebrates animals, Plenum Press, New York, pp 26-60.

445 Guillard RRL, Ryther JH (1962) Studies of marine planktonic diatoms. I. *Cyclotella nana* Husted and *Detonula confervacea*
446 Cleve. Can J Microbiol 8:229-239

447 Harrison PJ, Rosemary EW, Taylor FJR (1980) A broad spectrum artificial seawater medium for coastal and open ocean
448 phytoplankton. J Phycol 16:28-35

449 Huerlimann R, de Nys R, Heimann K (2010) Growth, lipid content, productivity, and fatty acid composition of tropical
450 microalgae for scale-up production. Biotechnol Bioeng 107(2):245-257

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

453 Jepsen PM, Thoisen C, Carron-Cabaret T, Pinyol-Gallemí A, Nielsen SL, Hansen BW (2018). Effects of salinity,
454 commercial salts and water types on the cryptophyte *Rhodomonas salina* and the calanoid copepod *Acartia tonsa*. J World
455 Aquc Soc. https://doi.org/10.1111/jwas.12508

456 Jones GE (1967) Precipitates from autoclaved seawater. Limnol Oceanogr 12(1):165-167

457 Jorquera MA, Valencia G, Eguchi M, Katayose M, Riquelme C (2002) Disinfection of seawater for hatchery aquaculture
458 systems using electrolytic water treatment. Aquaculture 207:213-224

459 Keller MD, Selvin RC, Claus W, Guillard RRL (1987) Media for the culture of oceanic ultraphytoplankton. J Phycol
460 23:633-638

461 Knuckey RM, Semmens GL, Mayer RJ, Rimmer MA (2005) Development of an optimal microalgal diet for the culture of
462 the calanoid copepod *Acartia sinjiensis*: effect of algal species and feed concentration on copepod development.
463 Aquaculture 249:339-351

464 Lang I, Hodac L, Friedl T, Feussner I (2011) Fatty acid profiles and their distribution patterns in microalgae: a
465 comprehensive analysis of more than 200 strains from the SAG culture collection. BMC Plant Biol 11(124)

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

468 Liltved H, Cripps SJ (1999) Removal of particle-associated bacteria by prefiltration and ultraviolet irradiation. Aquacult Res
469 30:445-450

470 Lourenço SO, Barbarino E, Mancini-Filho J, Schinke KP, Aidar E (2002) Effects of different nitrogen sources on the
471 growth and biochemical profile of 10 marine microalgae in batch culture: an evaluation for aquaculture. Phycologia
472 41(2):158-168

473 Lourenço SO, Marquez UML, Mancini-Filho J, Barbarino E, Aidar E (1997) Changes in biochemical profile of *Tetraselmis*
474 *gracilis* I. Comparison of two culture media. Aquaculture 148:153-168

475 McKinnon AD, Duggan S, Nichols PD, Rimmer MA, Semmens G, Robino B (2003) The potential of tropical paracalanid
476 copepods as live feeds in aquaculture. Aquaculture 223:89-106

477 Ohs CL, Chang KL, Grabe SW, DiMaggio MA, Stenn E (2010) Evaluation of dietary microalgae for culture of the calanoid
478 copepod *Pseudodiaptomus pelagicus*. Aquaculture 307(3-4):225-232

479 Parrish CC, French VM, Whiticar MJ (2012) Lipid class and fatty acid composition of copepods (*Calanus finmarchicus*, *C.*
480 *glacialis*, *Pseudocalanus* sp., *Tisbe furcata and Nitokra lacustris*) fed various combinations of autotrophic and heterotrophic
481 protists. J Plankton Res 34(5):356-375

482 Raynar TA, Jørgensen NOG, Blanda E, Wu C-H, Huang C-C, Mortensen J, Hwang J-S, Hansen BW (2015) Biochemical
483 composition of the promising live feed tropical calanoid copepod *Pseudodiaptomus annandalei* (Sewell 1919) cultured in
484 Taiwanese outdoor aquaculture ponds. Aquaculture 441:25-34

485 Renaud SM, Thinh L-V, Lambrinidis G, Parry DL (2002) Effect of temperature on growth, chemical composition and fatty
486 acid composition of tropical Australian microalgae grown in batch cultures. Aquaculture 211:195-214

487 Rhodes MA, Sedlacek C, Marcus NH (2008) Large-scale batch algae cultivation: comparison of two seawater sterilization
488 techniques. Aquacult Res 39(10):1128-1130

489 Saito MA, Goepfert TJ (2008) Zinc-cobalt colimitation of *Phaeocystis antarctica*. Limnol Oceanogr 53(1):266-275

490 Saito MA, Moffett JW, Chisholm SW, Waterbury JB (2002) Cobalt limitation and uptake in *Prochlorococcus*. Limnol
491 Oceanogr 47(6):1629-1636

492 Sargent J, Bell G, McEvoy LA, Tocher DR, Estevez A (1999) Recent developments in the essential fatty acid nutrition of
493 fish. Aquaculture 177:191-199

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

496 Seixas P, Coutinho P, Ferreira M, Otero A (2009) Nutritional value of the cryptophyte *Rhodomonas lens* for *Artemia* sp. J
497 Exp Mar Biol Ecol 381(1):1-9

498 Sokal RR, Rohlf FJ (1995) Biometry. W.H. Freeman, 3rd edition. New York, USA

499 Srivastava A, Hamre K, Stoss J, Chakrabarti R, Tonheim SK (2006) Protein content and amino acid composition of the live
500 feed rotifer (*Brachionus plicatilis*): with emphasis on the water soluble fraction. Aquaculture 254:534-543

501 Støttrup JG, Bell JG, Sargent RJ (1999) The fate of lipids during development and cold-storage of eggs in the laboratory-
502 reared calanoid copepod, *Acartia tonsa* Dana, and in response to different algal diets. Aquaculture 176:257-269

503 Summerfelt ST (2003) Ozonation and UV irradiation - an introduction and examples of current applictions. Aquacult Eng
504 28:21-36

505 Sunda WG, Huntsman SA (1995) Cobalt and zinc interreplacement in marine phytoplankton: biological and geochemical
506 implications. Limnol Oceanogr 40(8):1404-1417

507 Timmermans KR, Snoek J, Gerringa LJA, Zondervan I, de Baar HJW (2001) Not all eukaryotic algae can replace zinc with
508 cobalt: *Chaetoceros calcitrans* (Bacillariophyceae) versus *Emiliania huxleyi* (Prymnesiophyceae). Limnol Oceanogr
509 46(3):699-703

510 Tremblay R, Cartier S, Miner P, Pernet F, Quéré C, Moal J, Muzellec M-L, Mazuret M, Samain J-F (2007) Effect of
511 *Rhodomonas salina* addition to a standard hatchery diet during the early ontogeny of the scallop *Pecten maximus*.
512 Aquaculture 262(2-4):410-418

513 Valenzuela-Espinoza E, Lafarga-De la Cruz F, Millán-Núñez R, Núñez-Cebrero F (2005) Growth, nutrient uptake and
514 proximate composition of *Rhodomonas* sp. cultured using f/2 medium an agricultural fertilizers. Ciencias Marinas
515 31(1A):79-89

516 Vu MTT, Douëtte C, Raynar TA, Thoisen C, Nielsen SL, Hansen BW (2016) Optimization of photosynthesis, growth, and
517 biochemical composition of the microalga *Rhodomonas salina* - an established diet for live feed copepods in aquaculture. J
518 Appl Phycol 28:1485-1500

519 Xu Y, Tang D, Shaked Y, Morel FMM (2007) Zinc, cadmium, and cobalt interreplacement and relative use efficiencies in
520 the coccolithophore *Emiliania huxleyi*. Limnol Oceanogr 52(5):2294-2305

521 Yee D, Morel FMM (1996) In vivo substitution of zinc by cobalt in carbonic anhydrase of a marine diatom. Limnol
522 Oceanogr 41(3):573-577

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

526 Zwietering MH, Jongenburger I, Rombouts FM, van Riet K (1990) Modeling of the bacterial growth curve. Appl Environ
527 Microbiol 56(6):1875-1881

528   **Table legends**

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

530   **Table 2** The concentration (g L^{-1}) of the specific nitrogen sources in the modified f/2 stock solutions and the volume (mL L^{-}

531   ^{1}) of each stock solution added to 1 L seawater for cultivation of K-1487. All N-sources had an equimolar amount of N

532   **Table 3** The cell content of DHA, EPA and ARA in the *Rhodomonas* strains expressed as % of TFA, and the ratios of

533   DHA/EPA and EPA/ARA. ARA was not present or below detection limit in CCAP 995/5. Mean values ± S.D. (n = 3)

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

535 **Figure legends**

536 **Fig. 1** The growth rate (d^{-1}, striped bars) and cell content (pg) of the PUFAs DHA (black bars), EPA (light grey bars) and
537 ARA (dark grey bars) of K-1487 cultivated with and without $CoCl_2$ added to the f/2 growth medium. Mean values $\pm$ S.D. (n
538 = 4)

539 **Fig. 2** The cell content (pg) of DHA (black bars), EPA (light grey bars) and ARA (dark grey bars) in the *Rhodomonas*
540 strains. Symbol (#) indicates a statistical difference. ARA was not present or below detection limit in CCAP 995/5. Mean
541 values $\pm$ S.D. (n = 3)

542 **Fig. 3** The cell density (%) of the *Rhodomonas* strains in the upper 1 cm water column after 1 (black bars) and 6 hours
543 (white bars) in undisturbed water. Letters A and B indicate the two statistically significant groups at each given time
544 interval with the highest percentage of cells remaining (1 hr; uppercase, 6 hrs; lowercase). Statistically significant
545 differences at lower densities are not indicated. Mean values $\pm$ S.D. (n = 5)

546 **Fig. 4** The growth rate (d^{-1}) of K-1487 cultivated with the nitrogen sources NO^{3-}, NH^{4+}, urea, and combinations of these
547 (1:1). Letters A and B indicate statistically significant groups. Mean values $\pm$ S.D. (n = 4)

548 **Fig. 5** The temporal cell concentration ($\ln(N/N_0)$) of different initial densities (cells mL^{-1}) of K-1487* fitted to the modified
549 Gompertz equation. Mean values $\pm$ S.D. (n = 3)

550 **Fig. 6** The growth rates (d^{-1}, ●) of different initial densities of K-1487 calculated with parameters from the fit to the
551 modified Gompertz equation

Table 1

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)

Table 2

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

Table 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	-

Table 4

Initial density (cells mL^{-1})	2,000	7,000	10,000	40,000	100,000	200,000
Time (d)	6.8	6.0	5.3	3.8	3.8	2.8