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

2 ***Rhodomonas salina***

3

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15 sedimentation · initial density

16

17

18 **Abstract**

19 The cryptophyte *Rhodomonas* is an important feed item for live feed organisms in aquaculture and
20 although large-scale cultivation of *Rhodomonas* in photobioreactors (PBRs) is feasible, the production
21 needs to be optimized through further studies of specific factors. Through small-scale experiments
22 several factors relevant for an on-going large-scale production of *Rhodomonas* were studied and the
23 results presented here provide a useful insight on factors that can help future large-scale production.

24 The content of polyunsaturated fatty acids (PUFAs) and the temporal sedimentation was
25 compared in five strains of *Rhodomonas*. Strain K-1487 (*R. salina*) was chosen as the most suitable for
26 cultivation in PBRs due to a good biochemical content of PUFAs and low cell sedimentation. The f/2
27 growth medium used for cultivation was modified by excluding CoCl_2 which did not affect either
28 growth rate or cell content of the PUFAs DHA, EPA and ARA. Furthermore, the growth medium was
29 also modified by adding the nitrogen source as ammonium (NH_4^+), nitrate (NO_3^-), urea or combinations
30 of these, with NH_4^+ yielding a significantly higher growth rate of $1.3 \pm 0.7 \text{ d}^{-1}$. The treatment of the
31 seawater used for cultivation was exposed to three types of treatments which gave no significant
32 difference in the growth rate; 1) filtration (0.2 μm) + autoclavation, 2) filtration (0.2 μm) + UV-
33 radiation, and 3) filtration (0.2 μm). Finally, the results for growth rates of inocula at initial densities
34 ranging from 2,000 to 200,000 cells mL^{-1} showed that growth rate decreased with increasing density
35 but a final density of 10^6 cells mL^{-1} was obtained fastest with the highest initial density. With the
36 present findings several barriers for effective cultivation is solved and future large-scale production has
37 become a great step closer.

38

39 **1. Introduction**

40 In marine aquaculture, microalgae are used as feed for larvae and benthic stages of filter feeders
41 (Fernández-Reiriz et al. 2015; Tremblay et al. 2007) as well as for pelagic live feed organisms such as
42 copepods, rotifers, and brine shrimp (McKinnon et al. 2003; Seixas et al. 2009; Srivastava et al. 2006).
43 The microalgal cryptophyte *Rhodomonas* improves the survival, growth, lipid content, and
44 reproduction of brine shrimp, copepods and scallop larvae (Arndt and Sommer 2014; Knuckey et al.
45 2005; McKinnon et al. 2003; Ohs et al. 2010; Seixas et al. 2009; Tremblay et al. 2007; Zhang et al.
46 2013), and contain the essential polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA,
47 20:5 ω 3), docosahexaenoic acid (DHA, 22:6 ω 3) and arachidonic acid (ARA, 20:4 ω 6) in ratios optimal
48 for aquaculture organisms (Guevara et al. 2016; Jakobsen et al. 2018; Vu et al. 2016). These PUFAs
49 are essential for the survival and development of fish larvae (Bell and Sargent 2003; Sargent et al.
50 1997; Sargent et al. 1999) and are transferred to the fish larvae through the live feed.

51 The existing literature on *Rhodomonas* primarily discuss the nutritional value of the microalga
52 as a diet for live feed organisms in aquaculture based on its biochemical composition with the majority
53 focusing on copepods (e.g., Arndt and Summer 2014; de Lima et al. 2013; Drillet et al. 2006; Jakobsen
54 et al. 2018; Knuckey et al. 2005; McKinnon et al. 2003; Ohs et al. 2010; Seixas et al. 2009; Støttrup et
55 al. 1999; Zhang et al. 2013). The biochemical composition of *Rhodomonas* has also been studied at
56 different temperatures (Renaud et al. 2002), irradiances and nutrient levels (Guevara et al. 2016; Vu et
57 al. 2016), at different growth phases (Boelen et al. 2017), and when cultivated in various growth media
58 (Huerlimann et al. 2010; Valenzuela-Espinoza et al. 2005). In addition, the content of the pigment
59 phycoerythrin has been studied at different temperatures (Chaloub et al. 2015), irradiances (Bartual et
60 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 ($\leq 20\text{L}$) 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, autoclaving 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 be 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 CoCl₂ (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 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (n = 4). The
157 experimental period was 5 days with a start concentration of 64,460 \pm 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 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (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 $\mu\text{g mL}^{-1}$) and no sonication.

166 **2.4 Experiment 3: Temporal sedimentation.** Each *Rhodomonas* strain was transferred to individual
167 250 mL beakers (n = 5) at a cell concentration of 418,423 \pm 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 NO₃⁻ (nitrate) to NH₄⁺ (ammonium) and CO(NH₂)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:

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

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 ± 0.7°C and irradiance of 103
 189 μmol m⁻² s⁻¹ (n = 3). The specific initial densities were 2,000 ± 0; 7,000 ± 0; 9,500 ± 4,183; 43,667 ±
 190 2,582; 104,500 ± 7,583 and 196,167 ± 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/N₀) where N₀ is the cell
 193 concentration at the experimental start. Data was fitted to the modified Gompertz equation described in
 194 Zwietering et al. (1990):

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

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:

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

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

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 CoCl₂ 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 CoCl₂.** The growth rate of strain K-1487 in the two treatments (with and without CoCl₂ added to
216 the f/2 growth medium) was not statistically significant at an average of $0.69 \pm 0.04 \text{ d}^{-1}$ ($p = 0.765$)
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 $0.2 \pm 0.1 \text{ pg}$
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 $3.1 \pm 0.1 \text{ pg EPA cell}^{-1}$ and 0.07 ± 0.05 to $0.22 \pm 0.05 \text{ pg ARA cell}^{-1}$ (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, $p \leq 0.018$). 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 ≥ 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 $0.7 \pm 0.1 \text{ d}^{-1}$ (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 **5. Discussion**

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 *Emiliana huxleyi* substitute Co and Zn (zinc) with each other (Xu et al. 2007), the
274 diatoms *Thalassiosira pseudonana* and *T. oceanica* 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($0.00005 \mu\text{m kg}^{-1}$ 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} ($135 \mu\text{mol}$, 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 $\text{NH}_3/\text{NH}_4^+$ -
 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 furcata* 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 *Cryptocodinium 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-1487 with 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
367 10⁶ 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.

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

390

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398

399 **References**

- 400 Arndt C, Sommer U (2014) Effect of algal species and concentration on development and fatty acid composition of two
 401 harpacticoid 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, Bielecki 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, photosynthesis, 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 of algae mark the development and composition of
 419 harpacticoid 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 JLL (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* (Volvocales, Chlorophyceae) to growth on NH₄⁺ 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 CO₂
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* (Cryptophyceae) 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) *Cryptocodinium 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 *Aquac 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, Whitticar 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 applications. *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 *Emiliana 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-Cabrero F (2005) Growth, nutrient uptake and
 514 proximate composition of *Rhodomonas* sp. cultured using f/2 medium and agricultural fertilizers. *Ciencias Marinas*
 515 31(1A):79-89
- 516 Vu MTT, Douët 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 *Emiliana 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^{-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 \pm 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
<i>R. salina</i>	K-1487	Scandinavian Culture Collection of Algae & Protozoa (SCCAP)
<i>R. salina</i>	K-0294	Scandinavian Culture Collection of Algae & Protozoa (SCCAP)
<i>R. salina</i>	LB 2763	The University of Texas at Austin (UTEX)
<i>R. marina</i>	K-0435	Scandinavian Culture Collection of Algae & Protozoa (SCCAP)
<i>R. sp.</i>	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 ₃)	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











