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

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Abstract

The cryptophyte *Rhodomonas* is an important feed item for live feed organisms in aquaculture and although large-scale 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.

1995; Vu et al. 2016). A recent small-scale study by Jepsen et al. 2018 evaluated the effect of salinity and different commercial salts on *R. salina* and the copepod *Acartia tonsa* with positive outcomes for large-scale cultivation located without access to seawater. Aside from Jepsen et al. 2018, studies specifically regarding a meso- or large-scale production of *R. salina*, or optimization hereof, are not found in the literature. The aim was therefore to study factors acting as barriers for large-scale production of *Rhodomonas* as a microalgal diet for live feed organisms in aquaculture. This motivated us to focus on: 1) the necessity of $CoCl_2$ (cobalt(II) chloride) in the f/2 growth medium, 2) the content of PUFAs in five strains of *Rhodomonas* to identify the most suitable strain, 3) the temporal sedimentation of the five strains of *Rhodomonas* to identify the one with the lowest sedimentation rate which could potentially reduce biofouling of the PBR, 4) the effect on growth rate by adding nitrogen as different sources to the growth medium, 5) the effect of different types of seawater treatment on the growth rate, and finally 6) the growth rate of different initial inoculum densities..

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

Successful meso- and large-scale cultivation of *R. salina* in tubular and vertical PBRs has taken place the last 4 years at Roskilde University and its project partners. One of the limiting factors for cultivation is the need for cleaning of the PBR at a regular frequency due to biofouling. During cleaning, the PBR is shut down which is an economic loss for the production. The period for cultivation could potentially be prolonged by substituting with a *Rhodomonas* strain with a lower rate of cell sedimentation (i.e., high motility) and thereby reduce the tendency of biofouling. Furthermore, since closely related species and strains of a given microalga are known to have deviating biochemical compositions it is important to compare the PUFA content between strains of *Rhodomonas* to identify the most suitable strain as a microalgal diet for the aquaculture (Guevara et al. 2016; Lang et al. 2011).

The cultivation of microalgae necessitates growth media, and numerous recipes are available and generally target a broad range of species (Harrison et al. 1980; Keller et al. 1987). The growth medium is therefore likely to contain unnecessary or excessive amounts of certain components for cultivation of specific species. To our knowledge, there is no growth medium specifically defined according to the nutrient requirements for *Rhodomonas*. The nitrogen source can be added as ammonium (NH_4^+), nitrate (NO_3^-) or urea and the preferred source is species-specific (e.g., Giordano 1997; Lourenço et al. 2002). The increased growth rate of some microalgal species obtained when cultivated on NH_4^+ (Giordano 1997) is assumed to be coupled to the lower demand of reductants for assimilation (Dortch 1990). Growth media also contain different trace metals but the requirement of various trace metals is species-specific and some microalgal species can substitute a given trace metal with another (e.g., Timmermans et al. 2001; Xu et al. 2007). The compound $CoCl_2$ in growth media is problematic for a large-scale production as it is widely recognized as a toxic substance. Exposure limits, as well as limits for tolerated daily intake (TDI), have been established by both the European

Chemicals Agency (ECHA) in the European Union as well as by the National Institute of Occupational Safety and Health (NIOSH) in the United States of America. In particular, the European Union guidelines involve producing elaborated and detailed documentation for the use of $CoCl_2$. Producing this necessary documentation is both manpower requiring and time consuming and since some microalgal species are able to substitute cobalt (Co) with another trace metal, or simply does not require Co, it is relevant to study if $CoCl_2$ can be excluded from the large-scale production of *Rhodomonas* with no consequences for the yield.

During cultivation of microalgae it is essential that unwanted organisms are not introduced to the culture. The treatments applied to eliminate unwanted organisms at small-scale (≤20L) are filtration and autoclaving (e.g., Arndt and Sommer 2014; de Lima et al. 2013; Knuckey et al. 2005; Lourenço et al. 2002; Vu et al. 2016) while larger volumes generally are treated by filtration and UV-radiation (e.g., Bamba et al. 2014;Summerfelt 2003). Common for these types of treatments is no addition of chemicals or production of toxic residues that may negatively affect the microalgae (Rhodes et al. 2008). However, filtration does not sterilize as small bacteria and certainly viruses can pass through depending on the pore size of the filter material. Autoclaving is an effective sterilization method although it can raise pH of seawater and cause precipitation of nutrients (Filip and Middlebrooks 1975; Jones 1967). This, however, can easily be overcome by controlling pH during cultivation and adding sterilized nutrients post autoclaving. Nevertheless, autoclavation is unrealistic in large-scale productions and UV-radiation is widely used in, e.g., the aquaculture, where pre-filtration is crucial for optimal effectiveness (Summerfelt 2003). The small-scale experiments in the present study use autoclaved seawater to define the optimal cultivation conditions of *Rhodomonas* and a comparison of the seawater treatments used at the different scales is therefore necessary for detecting possible effects of a given treatment of the seawater used for cultivation of *Rhodomonas*.

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

2. Materials and methods

2.1 Algal strains and general culture conditions. Five species / strains of *Rhodomonas* were obtained from culture collections and are referred to their respective strain identity (Table 1). The strains were cultivated in natural seawater (NSW with a salinity of 30-35 collected from > 30 m depth in the Kattegat (DK) and filtered through a series of filters (terminal pore size of 0.2 µm). Equipment, NSW and growth medium stock solutions were autoclaved (15 min at 125 °C) prior to use (CertoClav-Tisch-Autoclav, Certoclav Sterilizer Gmbh). Irradiance was continuous (24:0 light:dark cycle) and measured with a Hansatech Instruments LTD Quantitherm light meter QRT1 (see below for specific irradiance in the separate experiments). The f/2 growth medium (without addition of silicate) was used for cultivation (Guillard 1975; Guillard and Ryther 1962), except in experiment 5 ‘Seawater treatment’. Cell concentration was enumerated on a Coulter Counter (Beckman) using the computer program Multisizer 3, except experiment 4 ‘Nitrogen source’ and experiment 6 ‘ Initial density’ (see the respective experimental sections below). Growth rates were calculated by fit of exponential growth functions on either cell concentration or optical density (OD) over time.

2.2 Experiment 1: $CoCl_2$. Strain K-1487 was cultivated in two versions of f/2 growth medium; a regular version and a version without addition of $CoCl_2$ (this strain is referred to as K-1487*). Cultivation took place in a small-scale PBR (Multi-Cultivator MC1000, Photon System Instruments, 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 experimental period was 5 days with a start concentration of 64,460 ±3,234 cells mL^{-1}. Samples for fatty acids were taken on day 6 and analyzed as described in section 2.4. Nutrients were added daily.

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

2.4Experiment 3: Temporal sedimentation. Each *Rhodomonas* strain was transferred to individual 250 mL beakers (n = 5) at a cell concentration of 418,423 ± 28,400 cells mL^{-1}, except CCAP 995/5 at

99,500 ± 3,536 cells mL^{-1}. Replicates were left undisturbed at room temperature in the stagnant water and samples for cell enumeration were withdrawn 1 cm below the water surface after intervals of 1 and 6 hours.

2.5 Experiment 4: Nitrogen source. The nitrogen source in the f/2 growth medium was changed from NO_3^- (nitrate) to NH_4^+ (ammonium) and $CO(NH_2)_2$ (urea), and combinations of these with an equimolar amount of N in all treatments (Table 2). Cultivation of strain K-1487 took place in the small-scale PBR (described in section 2.2) at 20°C and irradiance of 100 µmol m^{-2} s^{-1} (n = 4). Start concentration was 141,500 ± 18,742 cells mL^{-1} and determined by a build-in OD measuring device measuring automatically every 30 min for 30 hours. An equation between cell concentration (Coulter Counter - Beckman) and absorbance (Spectrophotometer - Genesys 6, Thermo Scientific) was obtained by linear regression to estimate the cell concentration by OD:

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

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

2.6 Experiment 5: Seawater treatment. Strain K-1487 was cultivated in filtered (0.2 µm) NSW exposed to autoclavation or UV-radiation. For comparison, the growth rate of K-1487 in filtered NSW without further treatment was included. Cultivation took place in aerated 2 L round-bottom flasks with B1 growth medium added at experimental start and irradiance of 67 ± 7 µmol m^{-2} s^{-1} at 20˚C (n = 4). The experimental period was 4 days with a start concentration of 169,500 ± 2,500 cells mL^{-1}. Cell concentrations were determined by OD as described in section 2.5.

2.7 Experiment 6: Initial density. Strain K-1487 was inoculated at increasing initial densities from 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 µmol m^{-2} s^{-1} (n = 3). The specific initial densities were 2,000 ± 0; 7,000 ± 0; 9,500 ± 4,183; 43,667 ± 2,582; 104,500 ± 7,583 and 196,167 ± 4,916 cells mL^{-1}. Nutrients were added at experimental start. Cell concentration during the exponential growth phase was determined daily by OD as described in section 2.6. The cell concentration (N) over time was plotted as ln(N/N_0) where N_0 is the cell concentration at the experimental start. Data was fitted to the modified Gompertz equation described in Zwietering et al. (1990):

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

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

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

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

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

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

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

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

4. Results

4.1 $CoCl_2$. The growth rate of strain K-1487 in the two treatments (with and without $CoCl_2$ added to the f/2 growth medium) was not statistically significant at an average of 0.69 ± 0.04 d^{-1} ($p = 0.765$) (Figure 1). Likewise, the cell content of the PUFAs DHA, EPA and ARA were not statistically significantly different between the two treatments with averages of 2.6 ± 0.1, 3.9 ± 0.4 and 0.2 ± 0.1 pg cell^{-1}, respectively (One-Way ANOVA, $p \leq 0.453$).

Insert figure 1.

4.2 Fatty acids. The cell content of the PUFAs DHA, EPA and ARA was compared in the five strains of *Rhodomonas* (Figure 2). The strains' content of EPA and ARA was not statistically different ranging from 1.9 ± 0.3 to 3.1 ± 0.1 pg EPA cell^{-1} and 0.07 ± 0.05 to 0.22 ± 0.05 pg ARA cell^{-1} (One-Way ANOVA, EPA; $p = 0.267$ ARA; $p = 0.156$). However, ARA was either not present or below detection limit in CCAP 995/5. The content of DHA was statistically significantly higher in CCAP 995/5 with

4.0 ± 0.1 pg cell^{-1} (One-Way ANOVA, $p \leq 0.018$). The cell content of EPA was higher than DHA for all strains, except CCAP 995/5 where the opposite was observed.

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

Insert figure 2 and table 3.

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

Insert figure 3

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

Insert figure 4

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

4.6 Initial density. The temporal cell concentration of initial densities of K-1487 in the range of 2,000 to 200,000 cells mL^{-1} was fitted to the modified Gompertz equation (Zwietering et al. 1990) to calculate the exponential growth rate (Figure 5 and 6). The initial density of 2,000 and 7,000 cells mL^{-1} obtained the highest growth rates at 1.4 d^{-1}. A trend was observed with the growth rate gradually decreasing with increasing initial density to 0.7 d^{-1} at 200,000 cells mL^{-1}. The lag time was calculated to be shorter than the sampling interval for cell enumeration and was therefore not considered further.

The time required for the initial densities to reach a biomass of 10^6 cells mL^{-1} successively decreased with increasing initial density; 6.8 days for 2,000 cells mL^{-1} and 2.8 days for 200,000 cells mL^{-1} (Table 4). However, initial densities of 40,000 and 100,000 cells mL^{-1} both reached 10^6 cells mL^{-1} after just 3.8 days.

Insert figure 5 and 6

5. Discussion

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

Various growth media recipes are available but, e.g., the the specific trace metal requirement of phytoplankton varies and it depends on the species if a specific trace metal can be substituted with another trace metal. Examples on Co from the literature showing this species-specificity are: the coccolithophore *Emiliania huxleyi* substitute Co and Zn (zinc) with each other (Xu et al. 2007), the diatoms *Thalassiosira pseudonana* and *T. ocenica* largely substitute Zn with Co (Sunda and Huntsman 1995; Yee and Morel 1996), the prymnesiophyte *Phaeocystis antarctica* substitute Zn with Co although Zn is preferred (Saito and Goepfert 2008), the diatom *Chaetoceros calcitrans* lack a substitution of Zn with Co (Timmermans et al. 2001), and the cyanobacteria *Synechococcus bacillaris* and *Prochlorococcus* require Co for growth (Saito et al. 2002; Sunda and Huntsman 1995). Studies on the trace metal requirements of *Rhodomonas* are lacking and our study did not seek to clarify the requirement of all the trace metals in the f/2 growth medium . Nevertheless, it is a key finding for a large-scale production of *Rhodomonas* that exclusion of Co from the growth medium does not affect neither the growth rate nor the cell content of DHA, EPA and ARA as these parameters are essential for aquaculture. Large quantities of growth medium are prepared during a large-scale production of microalgae and exclusion of Co will ease production by bypassing the required elaborated and detailed documentation required by, e.g., ECHA and NIOSH. However, it must be highlighted that NSW contain small amounts of Co(0.00005 µm kg^{-1} according to Atkinson and Bingman 1997). Thus, either strain K-1487* can substitute Co with another trace metal, or there is an adequate amount of Co present in NSW.

A further modification of the f/2 growth medium was the addition of various nitrogen sources. The highest growth rate of strain K-1487 was obtained with NH_4^+ as the nitrogen source and similar to the results reported by Lewitus and Caron (1990) for *Pyrenomonas* (now *Rhodomonas*) *salina* with a growth rate at 1.2 d^{-1} (135 µmol, 21˚C). However, Lourenço et al. (1997) reported that the cryptophyte *Hillea* sp. could not grow on NH_4^+ unless reduced to a concentration equal to half of that used in the present study. The lower demand of reductants for assimilation of NH_4^+ is a plausible explanation for the increase in growth rate observed in the present experiment (Dortch 1990). However, studies have shown that the biochemical content of microalgae may be altered when supplied with different nitrogen sources and future studies must clarify if NH_4^+ alters the biochemical profile (in particular the PUFAs) of *Rhodomonas* (Fidalgo et al. 1998; Lourenço et al. 2002). Providing the nitrogen source in the form of NH_4^+ may cause an acidification of the culture as NH_4^+ is taken up by the microalgae in the form of NH_3, leaving a proton in the medium. However, when the cell concentration of microalgae increases during cultivation the photosynthetic activity raises pH. In this experiment, pH was not controlled or adjusted but the effect of acidification is assumed to be minor, as seawater is generally well buffered due to its high content of carbonates (Goldman et al. 1982), and the cells in our experiment grew exponentially during the experimental period indicating no negative effect of pH. It must be stressed that the positive effect on growth rate of providing NH_4^+ as the nitrogen source obviously is larger than any negative effects of growth medium acidification on growth rate. In many plant and algal growth media (but not in the f/2 growth medium), the nitrogen source is provided as both NH_4^+ and NO_3^- because the acidifying effect of NH_4^+ uptake counters the alkalizing effect of NO_3^- uptake (Asher and Edwards 1983). In the present study, however, providing NH_4^+ as the only nitrogen source evidently gave the highest growth rate despite any effects of NH_4^+-uptake on pH or of pH on the NH_3/NH_4^+-equilibrium. When producing microalgae at large-scale, pH is usually controlled in a feedback system by CO_2-addition as pH in the photobioreactor will increase during microalgal growth due to the photosynthetic uptake of CO_2 and HCO_3^-.

The microalgal diet has been shown to affect the composition of fatty acids in copepods (Caramujo et al. 2008; de Lima et al. 2013; Støttrup et al. 1999) and particularly *Rhodomonas* is praised as an excellent diet for the copepod *Acartia* by improving the nauplii survival, development rate and reproduction (Arndt and Sommer 2014; Knuckey et al. 2005; Zhang et al. 2013). While a DHA/EPA/ARA ratio of 10 : 5 : 1 is considered optimal for some marine fish larvae (Sargent et al. 1999), studies on the specific nutritional requirement of PUFAs in copepods is limited (see references in Camus and Zeng 2010), and some species, e.g., *Pseudodiaptomus annandalei*, *Tisbe furcate* and *Nitokra lacustris*, may *de novo* synthesize certain fatty acids (Parrish et al. 2012; Raynar et al. 2015). A short term study (96 hr) by Jakobsen et al. (2018) indicates that ARA is less important for *Acartia tonsa* (Dana) as similar reproductive rates were obtained on a diet of *R. salina* (K-1487) compared to a diet of the heterotrophic dinoflagellate *Crypthecodinium cohnii* with cell contents of ARA at 0.19 and 0.01 % TFA, respectively. However, it is most likely not the case in long term growth studies with the copepod. All of our studied *Rhodomonas* strains, except CCAP 995/5, are suitable as a microalgal diet for *A. tonsa* but to supply fish larvae with the essential ARA through the live feed (i.e. *A. tonsa*) a *Rhodomonas* strain with high ARA content must be offered as the microalgal diet. This excludes strain CCAP 995/5 unless ARA is supplied from another source. However, this would include another factor in the production line which is undesirable. The cell content of PUFAs in the strains in the present study can likely be increased and result in an improved nutritional value of *Rhodomonas* as a microalgal diet for live feed organisms as studies have reported an effect on the content of PUFAs in *Rhodomonas* when changing the temperature, light intensity and nutrient level (Guevara et al. 2016; Renaud et al. 2002; Vu et al. 2016).

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

The seawater for large-scale cultivation of microalgae used as a diet for live feed organisms in aquaculture requires a treatment without addition of chemicals and antibiotics as some organisms may otherwise be negatively affected (Rhodes et al. 2008). Large volumes of water in PBRs should be provided easy, cheap and effective to meet all practical requirements. Furthermore, the end-product of the food chain, the fish, is intended for human consumption and must live up to high production standards. Studies comparing the growth rate of microalgae in seawater treated by autoclaving, filtration and UV-radiation are few. In the present study, the growth rate of *Rhodomonas* was not affected by any of these treatments. This indicates that our results obtained from small-scale experiments with *Rhodomonas* (using autoclaved seawater) can be directly implemented to a large-scale production (typically using UV-radiated seawater). Contrary to our results, Jorquera et al. (2002) obtained a lower growth rate of the prymnesiophyte *Isochrysis galbana* in UV-radiated seawater compared to autoclaved seawater which may be due to differences in the sensitivity of microalgal species to the toxic residues that can be produced during UV-radiation. It is therefore optimal to combine filtration with UV-radiation as filtration improves the efficiency of UV by removing particles shading the radiation (Liltved and Cripps 1999). Other examples from the literature on treatments used in aquaculture include, e.g., electrolytic treatment (Jorquera et al. 2002) and ozone (Summerfelt 2003).

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

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

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.

Acknowledgements

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Table legends

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

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

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

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

Figure legends

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

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

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

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

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

Fig. 6 The growth rates (d^{-1}, ●) of different initial densities of K-1487 calculated with parameters from the fit to the 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_4Cl)	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

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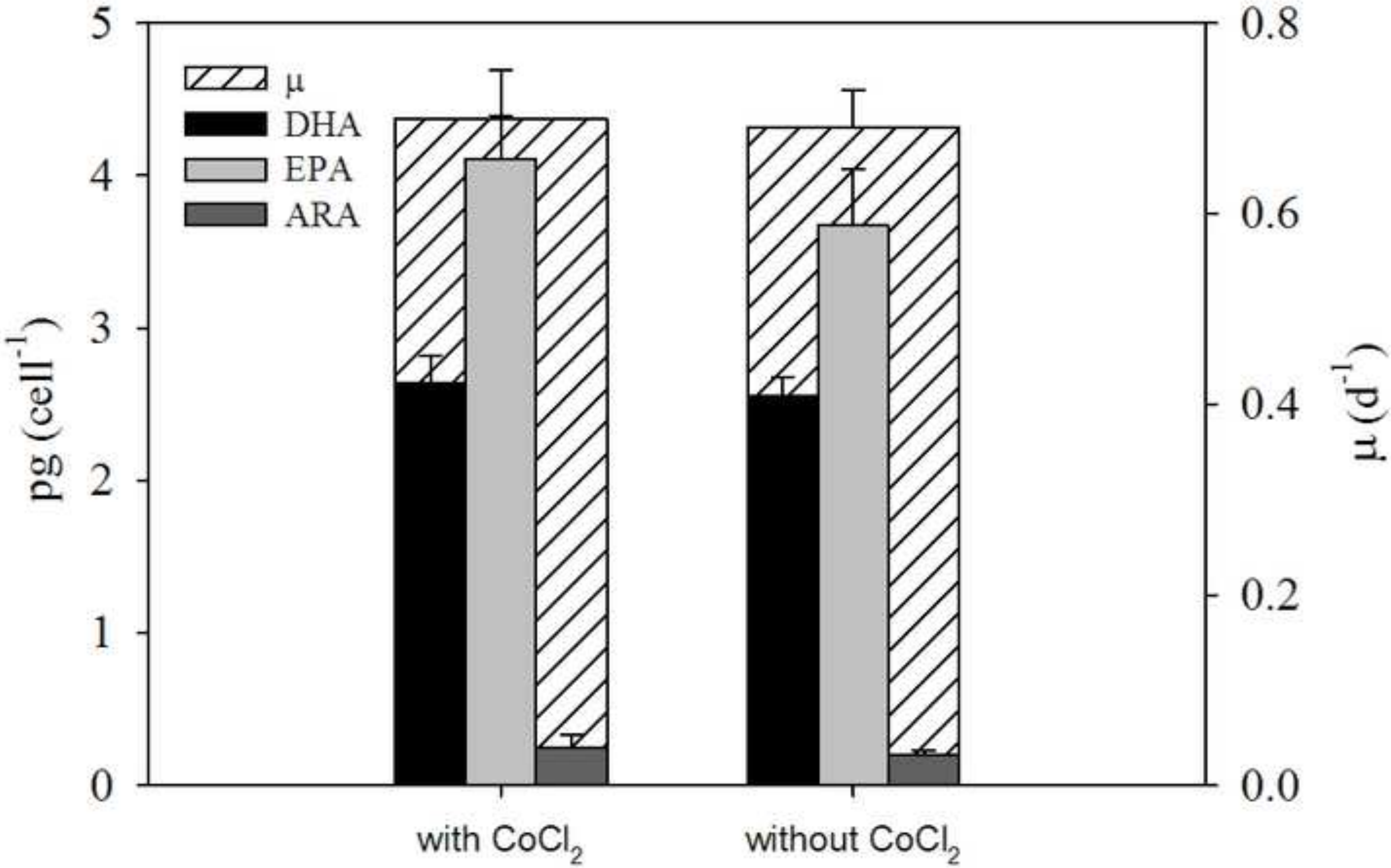

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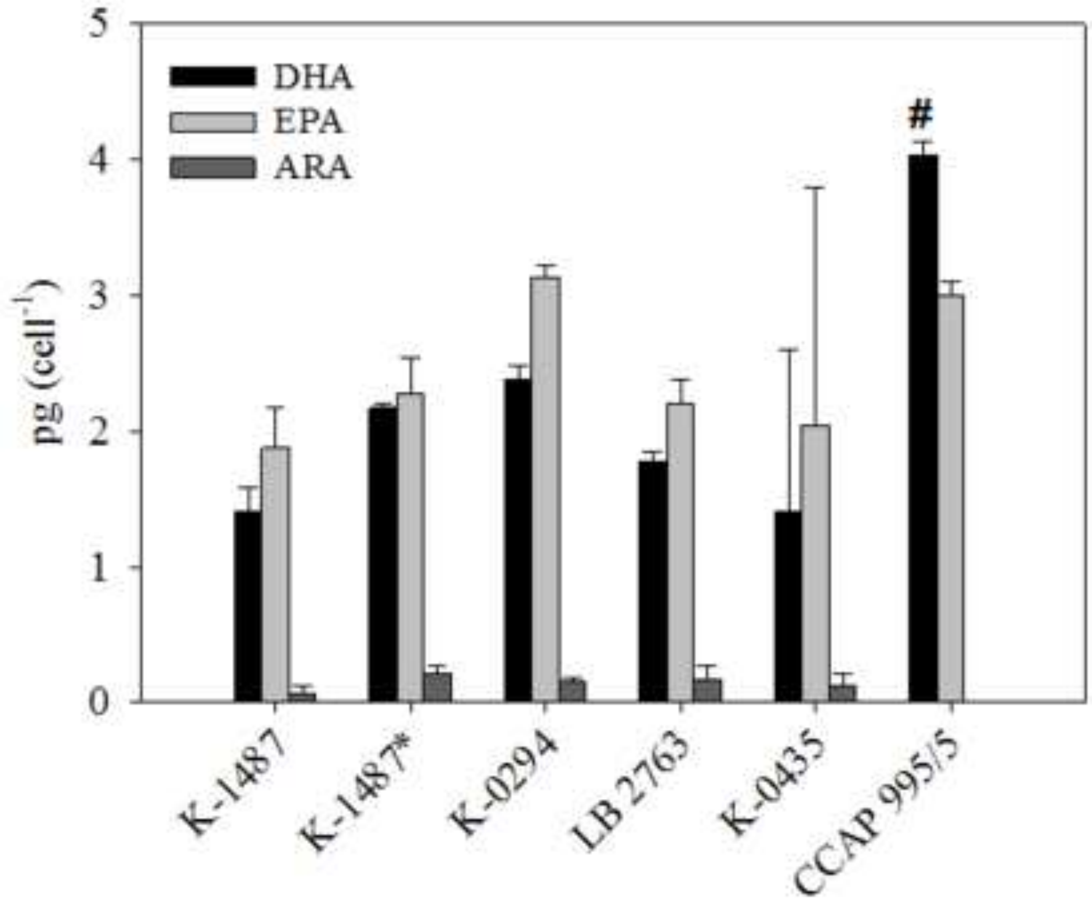

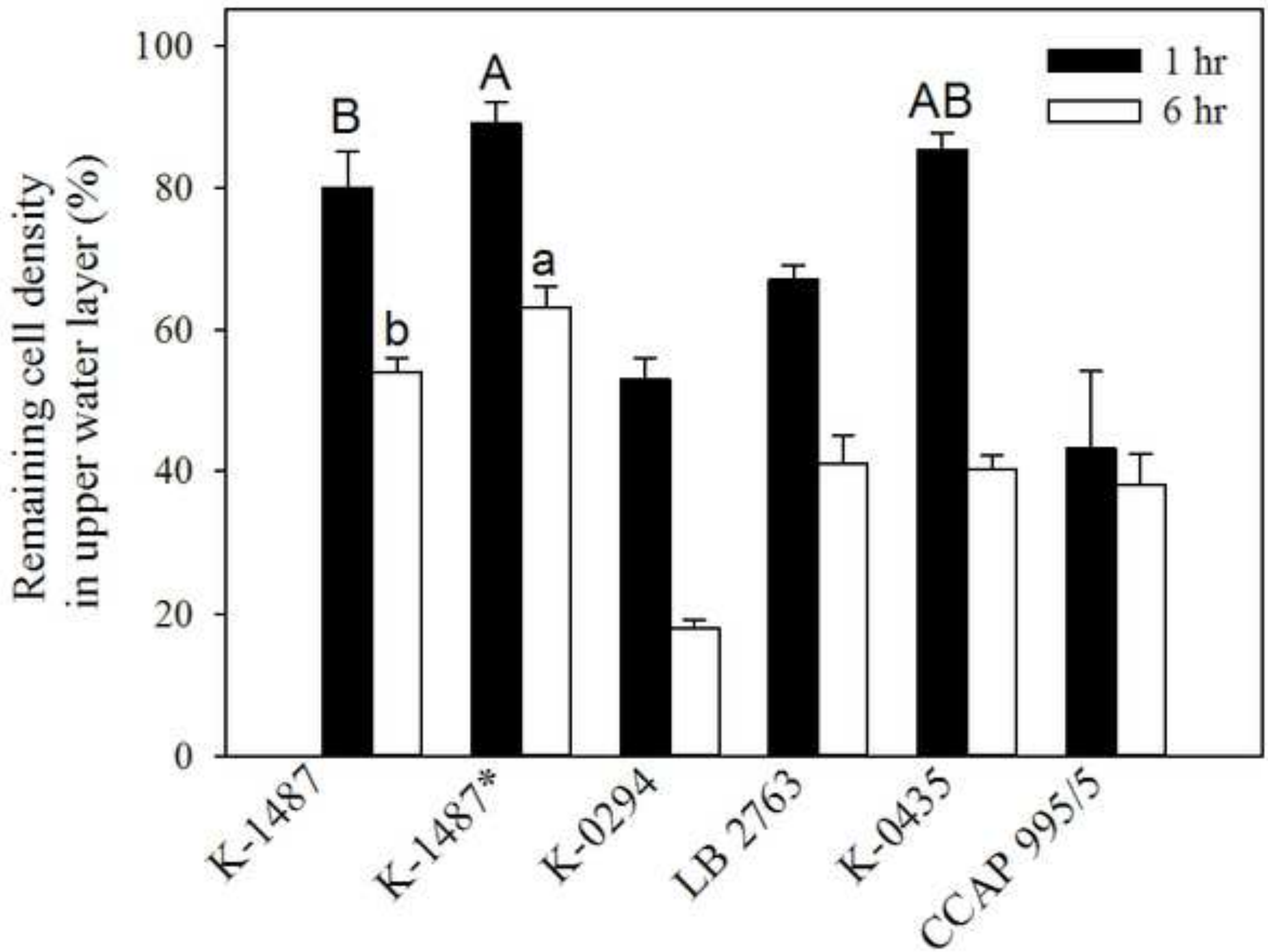
Remaining cell density in upper water layer (%)
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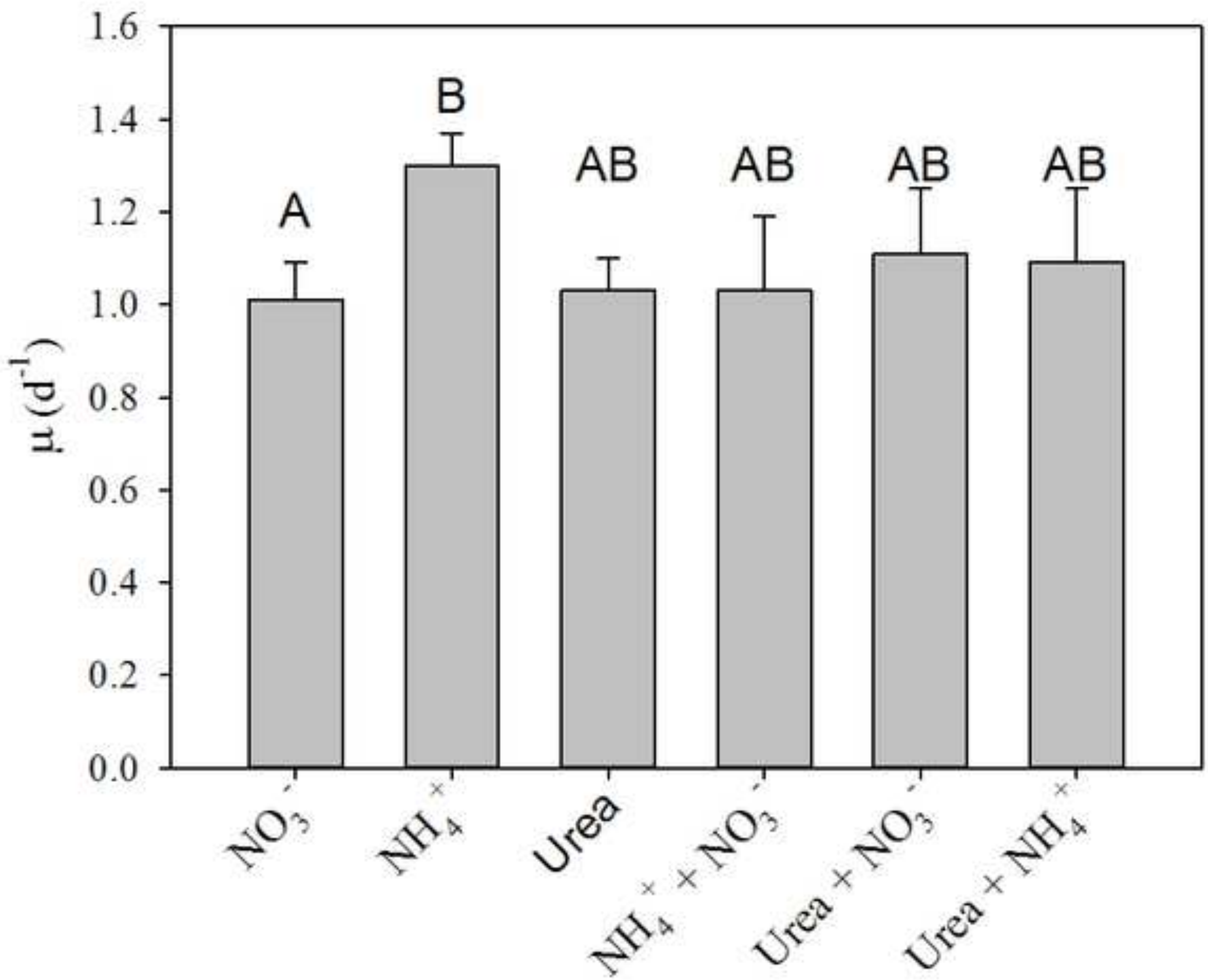

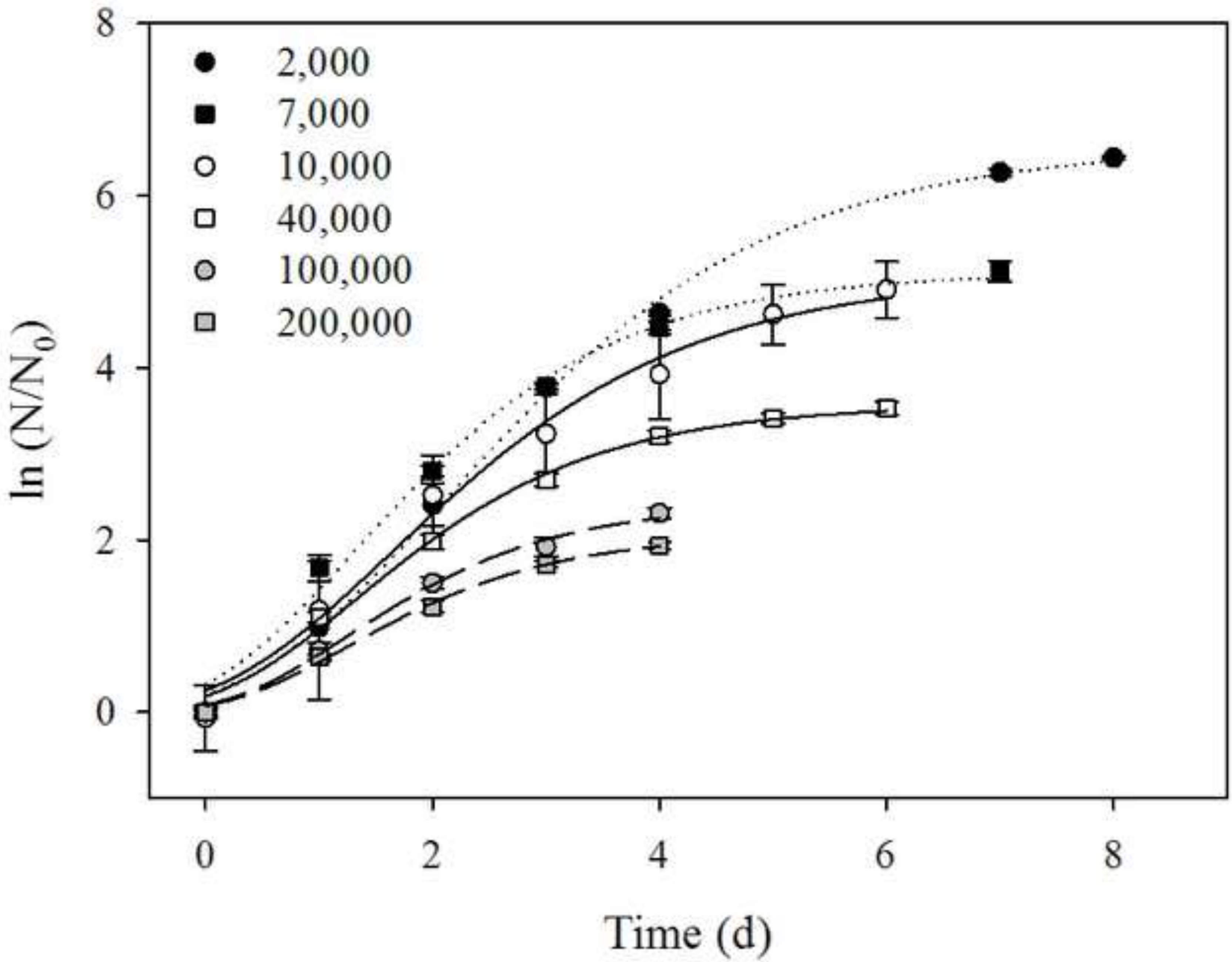
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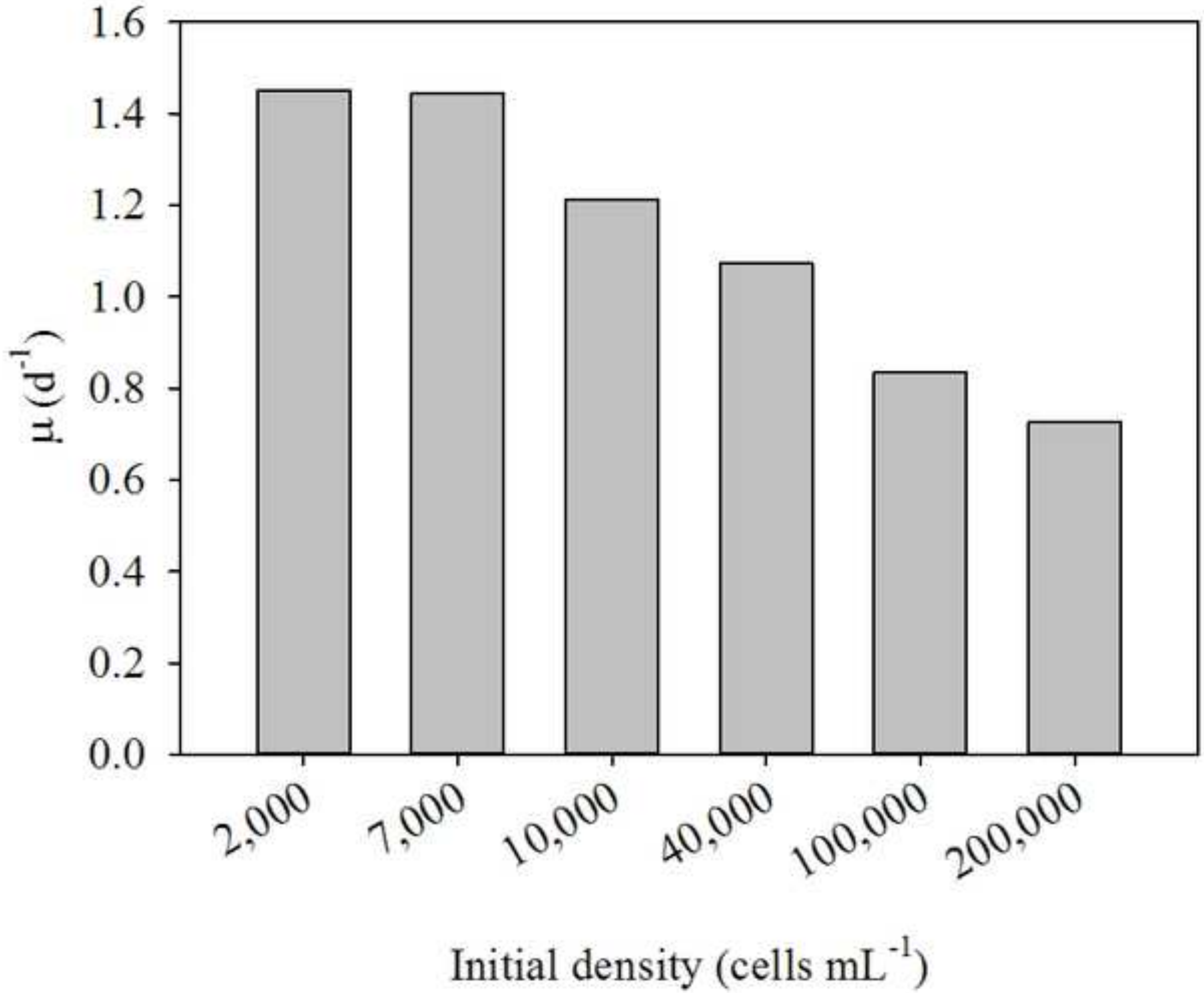
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Initial density (cells mL^-1)