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The Effect of Nitrogen Starvation on Biomass Yield and Biochemical Constituents of *Rhodomonas* sp.

Christos Latsos[1], Jasper van Houcke[1] and Klaas R. Timmermans[1,2]*

[1] Research Group Aquaculture, HZ University of Applied Sciences, Vlissingen, Netherlands, [2] Department of Estuarine and Delta Systems, Royal Netherlands Institute for Sea Research (NIOZ), Utrecht University, Yerseke, Netherlands

The microalgae *Rhodomonas* sp. is known as an excellent feed source for live feed organisms such as copepods. The main benefits of feeding *Rhodomonas* to live feed animals are attributed to the relative high polyunsaturated fatty acid (PUFA) level, the combination of containing both docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), and the ratio between these fatty acids (FA). It has been shown that microalgae are able to accumulate valuable metabolites, such as lipids, under adverse conditions. The easiest and most inexpensive method to induce stress to microalgae is through nitrogen (N) starvation. In this study, the effect of N-starvation on biomass concentration, cell volume, and cellular composition, such as fatty acid concentration and composition, and phycoerythrin (PE) concentration of *Rhodomonas* sp. during a period of 8 days, was investigated. The research was divided into two stages. In the first (growth) stage, *Rhodomonas* sp. was cultivated in small 400 ml photobioreactors (Algaemist-S) under optimal conditions in turbidostat mode, which reached a biomass concentration of 1.5 gDW L^{-1} and dilution rate of 1.3 d^{-1}. Samples were taken every 24 h for cell density and volume and productivity measurements in order to ensure a healthy and stable culture. In the next stage (N-starvation), the biomass was washed and transferred in a reactor filled with N-depleted medium. During N-starvation, samples were taken for biomass concentration, cell volume, PE and FA composition. The results of this study demonstrate that the lipid content increased significantly from 9% (t = 0 h) to 30% (t = 120 h) of the dry weight. After 120 h of N-starvation, the total FA content of *Rhodomonas* sp. remained stable for the remainder of the experiment (next 72 h). The highest increase of the FA concentration was represented by C16:0, C:18:1, C18:2, and C18:3, with highest concentrations after 120 h of starvation. The maximum EPA and DHA concentrations were observed after 48 h of starvation, while the maximum DHA to EPA ratio was detected at the end of the starvation.

Keywords: ***Rhodomonas*** **sp., nitrogen starvation, cell volume, fatty acids, phycoerythrin, PUFA, DHA, EPA**

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****Correspondence:***
Christos Latsos
christos.latsos@hz.nl

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INTRODUCTION

In the aquaculture industry, microalgae are mainly used as a live feed. Microalgae are usually used in the cultivation of bivalves, crustaceans, marine fish and zooplankton. The main benefit attributed to using live microalgae is their high nutritional value (Borowitzka, 1997). The application for animal feed accounts for about 30% of the algal production in the world (Becker, 2007). The cost of feed

contributes significantly to the final cost price of aquaculture products. High priority is, therefore, given to improving feed efficiency in industrial systems (Patil et al., 2007). It is stated that algae could increase aquaculture production up to 4-fold and as a result decrease the fish production cost by €0.10 to €0.21 kg^{-1} (Brune, 2012).

Microalgae nutritional value as aquaculture feed needs to be high in order to guarantee the best production of cultured animals (Volkman et al., 1989). The nutritional quality is connected with cell size, digestibility, and biochemical composition of microalgae, especially the fatty acid concentration and composition and the relative proportions of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Chu and Dupuy, 1980; Watanabe et al., 1983; Brown et al., 1997; Muller-Feuga, 2000). Most aquatic animals are not able to synthesize polyunsaturated fatty acids (PUFAs), such as EPA and DHA (Kanazawa et al., 1979). PUFAs are crucial for growth and development in penaeid prawns and shrimps and oyster species (Langdon and Waldock, 1981; González-Araya et al., 2012). Even marine animals that do not require EPA or DHA have demonstrated higher growth rates and larval survival rates when these PUFAs were included in their diet (Brown and Blackburn, 2013). For instance, *C. gigas* larvae did not show any boosted growth when the feed was enriched with DHA more than 2% of total fatty acids (Thompson et al., 1993), while also clams do not require PUFAs in their diet (Helm and Bourne, 2004). Except for the quantity of DHA and EPA, the ratio of these fatty acids is nutritionally important in the larval performance of mussel and fish larvae (Rodríguez et al., 1998; Pettersen et al., 2010). Pettersen et al. (2010) study correlated positively the *Mytilus galloprovincialis* larvae settlement and metamorphosis with the EPA and DHA ratio of the microalgae diets. Rodríguez et al. (1998) reported that for fish larvae diet DHA/EPA ratio should be 1:1 to 2:1. Decreases of DHA/EPA ratios in rotifers feed resulted in lower larval growth performance, while the most deficient larval growth was obtained in rotifers fed with the lowest DHA/EPA ratio (Rodríguez et al., 1997).

The main benefits of feeding *Rhodomonas* sp. to live feed animals are attributed to the relative high PUFAs level, the combination of containing both DHA and EPA, and the ratio between these fatty acids. *Rhodomonas* sp. has a high content of essential fatty acids (PUFAs) and nutrients, lipid accounts for about 10–30%, and protein takes about 30–60% of the dry weight (Renaud et al., 1999; Seixas et al., 2009; Costard et al., 2012; Coutinho et al., 2020). The variability of lipid and protein concentration depends on cultivation conditions. The high protein concentration is attributed to by another characteristic of *Rhodomonas* sp., the high phycoerythrin (PE) content. PE is a water soluble, colored and fluorescent phycobiliprotein that harvests light in the green wavelength (λ_{max} = 545 nm) and provides energy to Photosystem II (PSII) (Doust et al., 2006). So, live *Rhodomonas* sp. can be an excellent feed for aquaculture. It has been previously proved to constitute a high-quality diet to rear calanoid copepods *Acartia sinjiensis* and *Artemia* sp. (Knuckey et al., 2005; Seixas et al., 2009; Coutinho et al., 2020).

It has been shown that microalgae are able to accumulate lipids under adverse conditions. The easiest and most inexpensive method to induce stress to microalgae is through N-starvation. The availability of nitrogen can influence the lipid quantity and quality of many microalgae (Richardson et al., 1969; Breuer et al., 2012; Simionato et al., 2013). A reduction of photosynthetic performance is also observed under N-depleted growth medium, because microalgae decrease their light harvesting efficiency and energy transduction (Berges et al., 1996; Young and Beardall, 2003). Especially cryptophytes under N-starvation lose their PE antenna entirely, while the chlorophyll a/c and PS II are affected as well (Rhiel et al., 1986; Bartual et al., 2002). *Rhodomonas* cells in N-starvation conditions decrease their PE concentration and their fluorescence capacity up to 90% (da Silva et al., 2009). As a result, PE acts as an internal N reserve for cells in order to ensure their continuation of growth.

The aim of our study is to investigate the effect of N-starvation on biomass yield cell volume, lipid concentration and composition, and PE concentration (as a proxy for protein concentration) of *Rhodomonas* sp. The effect of an N stress time period of 8 days on the total fatty acids (TFA) and PUFAs content of *Rhodomonas* sp. is explored. The change of the absolute and also the relative DHA and EPA levels during the N-starvation period is determined.

MATERIALS AND METHODS

Strain, Growth Medium, and Pre-cultivation

Rhodomonas sp. was supplied by the Dutch aquaculture industry, as a strain used in commercial application. The strain was characterized by 18S sequencing and confirmed as *Rhodomonas* sp. (data not shown). *Rhodomonas* sp. inoculum for the experiments was pre-cultured in 300 ml pre-sterilized (20 min at 120°C) Erlenmeyer flasks in a conditioned shaker incubator. Cool white TL fluorescent tubes continuously provided a photon flux intensity of 100 μmol photons m^{-2} s^{-1} and the temperature was kept stable at 20 ± 1°C. Algae were maintained in 20 times concentrated nutrients of filtrated (0.2 μm pore size) f/2 medium, to maintain a nutrient-rich condition. The final concentration of $NaNO_3$ and $NaH_2PO_42H_2O$ was 1.5 g L^{-1} and 113 mg L^{-1}, respectively, and a salinity of 30 g L^{-1}. Air enriched with 5% CO_2 V/V_{air} was supplied in the headspace of the Erlenmeyer flasks. The growth of the cultures was monitored by measuring the cell abundance with a Coulter counter (Beckman coulter Z1) in order to ensure that the inoculum was in the exponential phase before it was used in the experiments.

Experimental Setup

In the experiments, *Rhodomonas* sp. was continuously cultivated in flat panel airlift-loop photobioreactor (PBR) (Algaemist-S, Technical Development Studio, Wageningen University, Netherlands) with 0.4 L working volume, 14 mm light path and 0.028 m^2 total illuminated area (Breuer et al., 2013). Warm light was continuously provided by Bridgelux LED lamps (BXRAW1200, Bridgelux, United States) from one side of the Algaemist-S system. Unintentional exposure to other light sources was prevented by a black cover on the other side of

the reactor. The primary light intensity was firstly set at around 100 μmol photons m^{-2} s^{-1} after the algae were inoculated to resemble the environment of the orbital incubator where the algae grew at the pre-culture phase. Then the light intensity was raised gradually until reaching 300 μmol photons m^{-2} s^{-1}, which is reported as non-limiting for *Rhodomonas* sp. (Vu et al., 2016). The temperature was maintained at 22°C using a water jacket, attached to the culture compartment of the photobioreactor. The pH was set at 7.5 ± 0.1 and maintained constant by mixing CO_2 with the airflow on demand. For the experiment, a two-phase N starvation strategy (growth and N-starvation, respectively) was applied. The experimental setup was performed in duplicate.

Growth Phase

The PBR was filled with 20 times concentrated nutrients of filtrated (0.2 μm pore size) f/2 medium, to ensure nitrogen sufficient conditions. After inoculation, the reactor was operated in batch mode until the outgoing light intensity equaled 15 μmol photons m^{-2} s^{-1} and the biomass concentration reached 1.4 g L^{-1}. Then, the turbidostat mode was applied and the secondary light PAR sensor of the systems ensured stable outgoing light of 15 μmol photons m^{-2} s^{-1} and continuous operation. The growth phase was continued for 5 days with a stable dilution rate. Samples were taken every 24 h in order to ensure a healthy and stable culture.

N-Starvation Phase

After the growth phase, the biomass of the reactor was harvested and centrifuged in 2000 rpm for 15 min. The supernatant was discarded and the cells were washed with N-depleted medium in order to remove nitrogen residuals. The procedure was repeated. After washing, the biomass was transferred in a PBR filled with N-depleted medium. Excepting the nitrogen concentration of the medium, the growth conditions were the same as in the growth phase. During N-starvation, samples were taken at 0, 8, 16, 24, 48, 72, 96, 120, 144, 168, and 192 h after changing to the N-depleted medium. Samples were taken for biomass concentration, cell density and volume, PE and FA composition.

Culture Analysis

Growth Phase

Optical density was measured at 750 nm ($OD_{750\ nm}$) in a spectrophotometer (DR 5000, HACH, United States), from which biomass concentration (C_x) was calculated according to Oostlander et al. (2020). Cell volume was measured in Coulter Counter (Multisizer 3, Beckman Coulter, United States) in the size range 7–14 μm. The growth rate (μ) was calculated when the PBR was running in turbidostat mode as the dilution rate (D) according to equation 1, where V_H is the harvested volume in a Δt period of time and V_R the reactor volume. The biomass production rate (r_x) for the turbidostat mode was calculated from the growth rate and the biomass concentration (C_x, eq. 2).

$$\mu = D = \frac{\frac{V_H}{\Delta t}}{V_R} \quad (1)$$

$$r_x = \mu \times C_x \quad (2)$$

N-Starvation Phase

In the N starvation phase, the cell density and volume and the biomass concentration were measured in triplicates as according to the method described in the section "Growth Phase." Additionally, the daily cell death rate (k_d) was calculated according to eq. 3.

$$k_d = -\frac{ln\frac{Cx_{(t+\Delta t)}}{Cx_t}}{\Delta t} \quad (3)$$

The biomass of each sample was centrifuged at 2500 rpm for 15 min, washed twice with 0.5 M ammonium formate, stored at −80°C and freeze-dried. The lyophilized biomass was used in PE and FA analysis.

Phycoerythrin analysis

The phycobilin pigments were extracted by a freeze-thawing process in 0.05 M phosphate buffer (containing equal volumes of 0.1M 0.1 M K_2HPO_4 and KH_2PO_4 and pH 6.7). The samples were kept in −80°C for 48 h and then for 24 h in 5°C for thawing. A centrifugation step at 4000 rpm for 10 min was followed to discard the biomass pellets from the tubes. The supernatant was analyzed using UV-VIS spectroscopy at 545 nm, according to Bennett and Bogobad (1973) and Lawrenz et al. (2011). PE was calculated in (g L^{-1}) according to eq. 4, where A is the absorbance at 545 nm, ε is the molar extinction coefficient (for PE: 2.41 × 10^6 $L{\cdot}mol^{-1}{\cdot}cm^{-1}$), *d* represents the path length of the cuvette and MW stands for the molecular weight of phycobilin (for PE: 240,000 g mol^{-1}).

$$\mathrm{PE} = \frac{A}{\varepsilon d}^{*} MW \quad (4)$$

Fatty acid analysis

Fatty acids were quantified in 10 mg of lyophilized biomass, according to Breuer et al. (2012). Cell disruption was performed in beat beater tubes ("Lysing matrix E," MP biomedicals, United States) using a Bead Beater (Precellys 24, Bertin Instruments, France) in the presence of glass beads (150–212 μm diameter). Three cycles of 60 s at 2500 rpm with 120 s interval were performed. The lipids were extracted using a chloroform:methanol (1:1.25 v/v) mixture and methylated in a MeOH solution containing 5% H_2SO4 for 3 h at 70°C in a block heater. The fatty acid methyl esters (FAME) quantification was performed in gas chromatography (7890, Agilent, United States) using a 30 m column (Supelco Nukol™) with Helium as carrier gas. FA/FAMEs were identified based on retention time data of known standards. Tripentadecanoin (C15:0 TAG) was used as internal standard for fatty acid quantification. TFA was calculated as the sum of all individual FA. FA were also processed as saturated fatty acids (SFA), monounsaturated fatty acids (MFA), PUFAs and DHA/EPA ratio.

Statistical Analysis

All data measurements are shown as average +/− standard deviation (±SD) of three independent replicates. Data were tested for normal distribution (Kolmogorov-Smirnov goodness of fit test) before being analyzed by one-way analyses of variance (ANOVA) with α = 0.05, using SPSS 15.0. Mann–Kendall test was

performed to determine whether the results have a monotonic trend over the period of N-starvation.

RESULTS

Growth Phase

During the growth phase, the PBR was in a steady state for 6 days (**Figure 1**). The volumetric productivity was stable, 1.5 ± 0.1 g L^{-1} d^{-1}, while the dilution rate was 1.3 ± 0.1 d^{-1}. The biomass concentrations in the PBR was 1.2 ± 0.1 g L^{-1}, which equals 9.2 ± 1.2 × 10^6 cells ml^{-1}. In the time of the growth phase, the cell volume was stable, 535 ± 18 μ m^3.

N-Starvation Phase

Biomass Yield: Cell Density and Cell Volume

The cell density and volume of *Rhodomonas* sp. changed when grown under N-starvation. The cell density reduced significantly ($P < 0.01$) from 6.3 × 10^6 cells ml^{-1} at 0 h until 32 h of N-starvation phase, with a death rate of 0.24 d^{-1}, to 4.6 ± 0.1 × 10^6 cells ml^{-1} (**Figure 2**). From 32 to 120 h of N-starvation, no significant difference ($P > 0.05$) was observed on the cell density. The highest death rate was observed at 144 h, 0.46 d^{-1}, where the cell density decreased to 3.1 × 10^6 cells ml^{-1}. From then on, the cell density continued declining significantly ($P < 0.01$) with a rate of 0.46 d^{-1}. When the experiment stopped, after 192 h in N-starvation phase, the cell density was 2.5 × 10^6 cells ml^{-1}. The cell volume increased significantly after N starvation from 556 ± 65 μm^3 at $t = 0$ h to 660 ± 67 μm at $t = 32$ h ($P < 0.01$). After 32 h the cell volume reduced gradually to 304 ± 36 μm at $t = 192$ h ($P < 0.01$).

Phycoerythrin

The PE concentration in *Rhodomonas* sp. when the N-starvation phase started was 19.1% of total DW and remained stable for the first 8 h (**Figure 3**). After 8 h, PE declined significantly ($P < 0.01$) until 32 h after N-starvation, when it was equal to 4.5% of total DW. From 32 until 74 h after N-starvation, there was no significant change in PE concentration ($P > 0.05$).

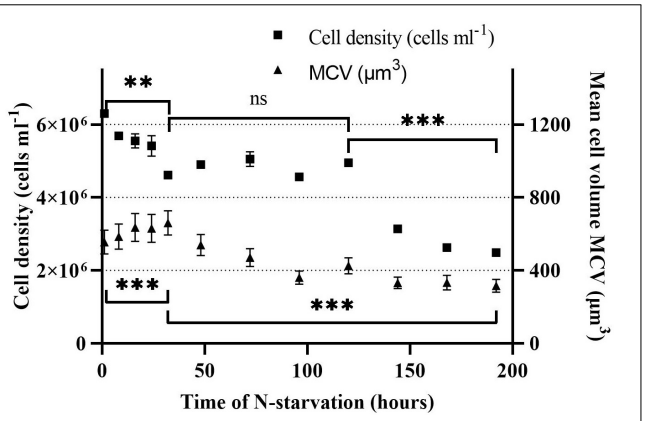

FIGURE 2 | Cell density and mean cell volume (MCV) of *Rhodomonas* sp. during a 192 h N-starvation period of time. Data are expressed as the average of three replicates ± SD. *Indicates the significant difference after ANOVA *post hoc* test. ns $P > 0.05$, **$P < 0.01$, ***$P < 0.001$.

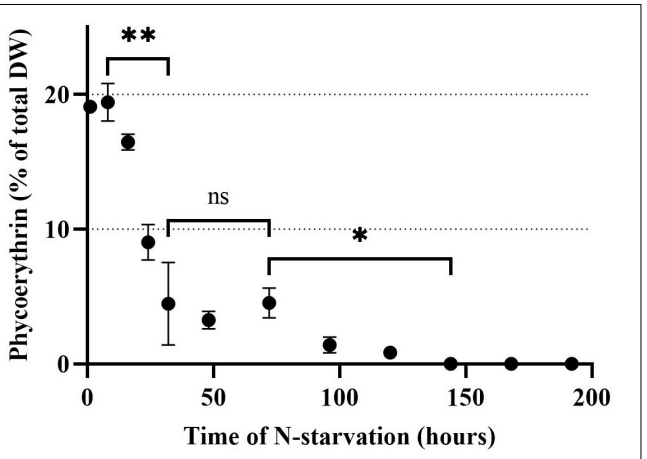

FIGURE 3 | Phycoerythrin concentration of *Rhodomonas* sp. during a 192 h N-starvation period of time. Data are expressed as the average of three replicates ± SD. *Indicates the significant difference after ANOVA *post hoc* test. ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$.

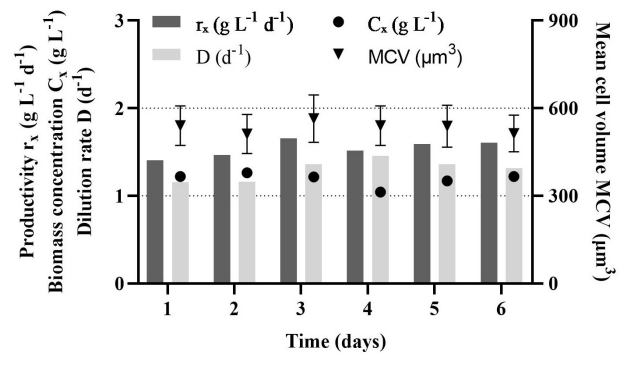

FIGURE 1 | Steady state volumetric productivity (rx), dilution rate (D), biomass concentration (Cx) and mean cell volume (MCV) of *Rhodomonas* sp. in a photobioreactor during the growth phase.

After 74 h of N-starvation, PE concentration declined further significantly ($P < 0.01$) until 144 h after N starvation, when no PE could be measured.

Fatty Acid Analysis

Rhodomonas sp. accumulated FA 32 h after the transfer into the N-depleted medium (**Figure 4**). There was no significant difference in TFA the first 24 h of N-starvation phase ($P > 0.05$). TFA increased significantly from 9.2% of total DW at 24 h to 30.3% of total DW at 120 h ($P < 0.01$). After 120 h no significant ($P > 0.05$) increase of TFA was measured up until 192 h of N- starvation. SFA did not change significantly in the first 24 h of N-starvation ($P > 0.05$). A significant increase ($P < 0.01$) of SFA concentration was observed after 24 h and continued until 120 h of N-starvation, from 2.3% to 12.4% of total DW, respectively. PUFA content followed the same pattern as SFA

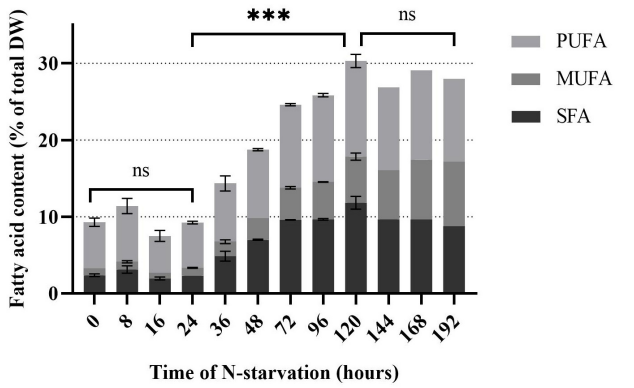

FIGURE 4 | Fatty acid concentration of *Rhodomonas* sp. during a 192 h N-starvation period. SFA: Saturated fatty acids, MUFA: Monounsaturated fatty acids and PUFA: Polyunsaturated fatty acids. Data are expressed as the average of three replicates ± SD. *Indicates the significant difference after ANOVA *post hoc* test. ns $P > 0.05$, ***$P < 0.001$.

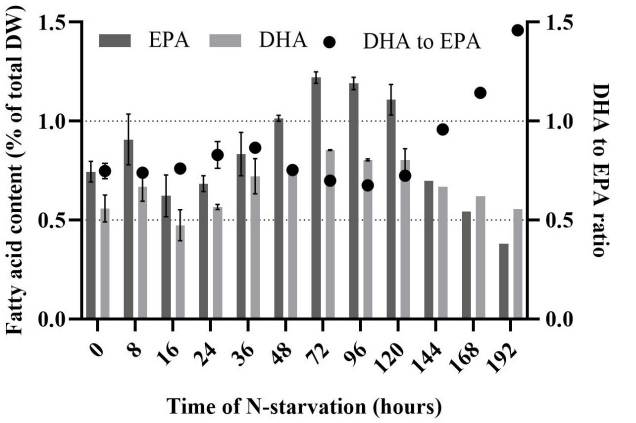

FIGURE 5 | Eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and DHA to EPA ratio of *Rhodomonas* sp. during a 192 h N-starvation period. Data are expressed as the average of three replicates ± SD.

(**Figure 4**). PUFAs content started at 6% of total DW before the N-starvation phase and raised to 12.5% of total DW 120 h after the N-starvation started. The same pattern was followed by the MUFA rising from 1% at 24 h to 6.4% of total DW at 120 h of N-starvation. After 120 h the SFA and PUFA showed a decrease, while the MUFA kept increasing until 192 h of N starvation period to 8.4% of total DW. EPA and DHA showed a peak after 72 h, 1.2 and 0.9% of total DW, respectively. The initial concentration was 0.7% and 0.6% of total DW for EPA and DHA (**Figure 5**). After 192 h, the EPA and DHA concentration dropped to 0.4% and 0.6% of total DW, respectively. The DHA to EPA ratio did not show a significant difference ($P > 0.05$) the first 120 h after N-starvation and remained around 0.72. At 144 h, the DHA to EPA ratio more than doubled ($P < 0.01$), to 1.46 at 192 h.

The FA with the highest concentration were linolenic (18:3, 1.8% of total DW), stearidonic (18:4, 1.5% of total DW), palmitic acid (16:0, 1.2% of total DW) and linoleic (18:2, 1.2% of total DW) acids before the starvation period. After 120 h of N-starvation the highest FA in *Rhodomonas* sp. were palmitic acid (6.1% of total DW), oleic acid (18:1, 5.5% of total DW) and linolenic acid (3.7% of total DW) (**Supplementary Table S1**).

DISCUSSION

The nutritional value of microalgae as aquaculture feed is an essential key factor for the aquaculture sector. This study established that *Rhodomonas* sp. quality can be maintained stable in a PBR, but it can also be manipulated. An N-starvation application can modify the quality of *Rhodomonas* sp. by inducing the FA accumulation and protein consumption (PE as proxy).

Growth Phase

During the growth phase *Rhodomonas* sp. was stable in the PBR in the sense of biomass productivity and cell volume. The growth rate that was observed during a period of 6 days, 1.3 ± 0.1 d^{-1}, was higher than most of the maximum growth rates that have been reported for *Rhodomonas* species in the literature, 0.8 to 1.0 d^{-1} (Bartual et al., 2002; Lafarga-De la Cruz et al., 2006; Vu et al., 2016). Only Fernandes et al. (2016) reported a higher growth rate of 1.6 d^{-1} for the strain of *Rhodomonas marina*. It has to be noted that the maximum growth rate data from these studies were obtained in batch cultures during exponential phase. In our study, we present the steady state growth rates during a turbidostat mode, not the maximum growth rate of the strain. Moreover, interspecific variation can explain the variation of the growth phase data of this study with the data given in other studies. For example, Guevara et al. (2016) studied two *Rhodomonas salina* species and obtained significant differences in growth and nutritional quality between the species (protein, total lipids, EPA, and DHA).

N-Starvation Phase

Cell Volume and Phycoerythrin

The cell volume of *Rhodomonas* sp. changed under N-starvation, with cells being bigger the first 32 h of N-starvation phase, thereafter observing a significant cell decrease. The volume increase at the beginning of the N-starvation phase could be explained by carbohydrate accumulation, which is reported to start earlier than lipid accumulation. Jia et al. (2015) cultivated *Nannochloropsis oceanica* in N-deplete medium for 14 days and observed a 2-fold increase of free glucose and mannitol the first and second day of starvation, respectively. Previous research on *Rhodomonas* sp. (da Silva et al., 2009) illustrated that N-starved cells increased their volume in the first 3 days of the N-starvation phase. The decrease of cell volume that followed after 48 h in N-free medium in our experiment is supported in many studies. Kilham et al. (1997) found that the volume per cell of *Ankistrodesmus falcatus* decreased as a function of N-limitation compare to non-limited cells. Lynn

et al. (2000) also observed smaller cell sizes for N-limited *Stephanodiscus minululus* cultures as compared to non-limited cultures. Rhee (1978) observed that *Scenedesmus* sp. cells size was smaller when growing in N-limited medium and explained it in terms of lower protein content. The decrease of protein content has been reported for many algae species grown under N-starvation conditions (Harrison et al., 1990; Lynn et al., 2000). Jia et al. (2015) reported a decrease in protein concentration of *Nannochloropsis oceanica*, which started 48 h after N-starvation phase.

The reduction of cell volume and the correlation with protein content can be explained in our study by the decrease of PE concentration (here used as a proxy for cellular protein concentration), which started 16 h after the N-starvation started. It has also been demonstrated in other studies that cells of *Rhodomonas* sp., as in some other cryptomonads, showed a drastic decrease of the protein and phycoerythrin content upon N-starvation (Sciandra et al., 2000; Bartual et al., 2002; Vu et al., 2016). Yamamoto et al. (2020) reported for *Rhodomonas* sp. a 75% reduction of PE concentration between the exponential and late stationary phase, where no N-source is available. In our research, the PE concentration was reduced by 75% 32 h after N-starvation, while after 4 days of starvation, the PE reduction was 93%. The different light intensity conditions that were used in Yamamoto et al. research can clarify the differences in the PE concentrations with our results. Consistent with Vu et al. (2016) study, N-starvation conditions caused a reduction in PE. Proteolysis of phycobiliproteins in cyanobacteria under N-starvation maintain the protein turnover (Grossman et al., 1994). These studies suggest that PE behaves as cell reserves in N-deprivation conditions providing amino acids for new protein synthesis.

FA Concentration

Many publications support that microalgae decrease their cell division and start to accumulate lipid under nutritional limitation (Shifrin and Chisholm, 1981; Piorreck et al., 1984) or starvation (Tornabene et al., 1983; Converti et al., 2009). Illman et al. (2000) showed the response of five *Chlorella* species to N-limitation. Lipid content doubled for all *Chlorella* species in low N medium. PUFA content increases relative to the increasing nutrient limitation (Janssen et al., 2019). In our study, the FA (SFA, MUFA, and PUFA) increase is in line with the literature for *Rhodomonas* sp. in N-starvation phase (Yamamoto et al., 2020). The highest increase during N-starvation was obtained for oleic (C18:1), palmitic (C16:0), and linoleic (C18:2) acid, 10-fold, 5-fold and 4-fold, respectively. PUFAs, which are the most important FA for aquaculture, indicated a 2-fold increase in the first 96 h of N-starvation. Similar increased PUFA content (65% of total FA) was reported by Coutinho et al. (2020) for *Rhodomonas lens* maintained under nitrate saturated conditions. DHA and EPA presented a peak in concentration 72 h after N-starvation. However, at the end of the experiment (120 h in N-starvation), DHA concentration returned to the initial value (t = 0 h), while EPA showed a 2-fold decrease compared to the initial concentration.

Fatty Acid Composition

The major FA's of *Rhodomonas* sp. in this study were palmitic (C16:0), linoleic (C18:2), linolenic (C18:3), and stearidonic (C18:4) acid. These FA's are in line with the literature results for *Rhodomonas* species (van Houcke et al., 2017; Vu et al., 2019; Coutinho et al., 2020). However, differ from lipid profiles of other microalgae that are used in aquaculture, for example, *Tisochrysis lutea*, *Chaetoceros neogracile*, *Skeletonema marinoi*, *Pavlova lutheri*, *Nitzschia* sp., *Thalassiosira* sp. *Tetraselmis* sp., *Dictyosphaerium pulchellum, Stichococcus* sp., *Chlorella* sp. and *Scenedesmus* sp. (Pratoomyot et al., 2005; González-Araya et al., 2012; González-Araya and Robert, 2018). The SFA composition or *Rhodomonas* sp. varies from 25 to 39% of TFA, which is similar to Yamamoto et al. (2020) research on *Rhodomonas* sp. The most significant difference is noticed in PUFA, which is 65% of TFA in *Rhodomonas* sp., while in other marine algae varies from 25 to 47% of TFA. This difference is defined mainly by the higher DHA content, 6% of TFA, while the EPA concentration is 8% of TFA, higher than *Tisochrysis lutea* and *Tetraselmis* sp., but lower than *Pavlova lutheri*, *Chaetoceros neogracile*, *Skeletonema marinoi*, *Thalassiosira* sp. and *Nitzschia* sp. This comparison confirms the interspecific nutritional value variability due to the differences in absolute amounts of TFA, EPA and DHA between algae species (Boelen et al., 2013).

DHA to EPA Ratio

Due to the changes in the absolute value of DHA and EPA, the DHA to EPA ratio is affected. In our study, the DHA to EPA ratio the first 120 h in N-starvation remained relatively stable, 0.75 $\pm$ 0.05. This ratio is similar to the ratio that has been reported before for *Rhodomonas* species (Dunstan et al., 2005; Drillet et al., 2006; Vu et al., 2016). Boelen et al. (2017) reported that DHA to EPA ratio of *Rhodomonas salina* decreased from exponential to stationary and late stationary phase, from 0.72 to 0.56 and 0.46, respectively. In contrast to Boelen et al. (2017) results, our research showed a twofold increase of DHA to EPA ratio of *Rhodomonas* sp. to 1.5 after 192 h in N-starvation. The effect of N-starvation on DHA and EPA can affect the nutritional quality of *Rhodomonas* sp., as it is reported that the nutritional value of microalgae in aquaculture depends not only on the quantity of DHA and EPA but also on the DHA to EPA ratio, which has proved essential for fish larvae development (Rodríguez et al., 1998).

CONCLUSION

This study has illustrated that *Rhodomonas* sp. adapts rapidly to changes in N availability by changes in cell density and volume and biochemical composition and as a result, N availability can strongly enhance the nutritional value of *Rhodomonas* sp. The high PUFA concentration of *Rhodomonas* sp. and the increase of PUFA under N-starvation makes it good nutritional feed for aquaculture animals. This research demonstrates that in the application of *Rhodomonas* sp. as aquaculture feed, the time of

harvest (and the starvation applied) is essential in order to obtain the desired quality as a feed.

In order to benefit from our results on a (semi-)commercial scale cultivation systems of management should be adapted. A cultivation system could be divided into two phases. The first phase enables a steady and high production rate of *Rhodomonas* sp. in optimal cultivation conditions and N-sufficient medium (20 × f/2 medium, 300 μmol photons m^{-2} s^{-1} and 22 °C). The second phase induces N-starvation, where *Rhodomonas* sp. accumulates lipids. In the second phase, there is a critical point for harvesting, and it depends on the purpose of the feed. PUFAs have the highest concentration 120 h after N-starvation, while EPA and DHA present maximum concentration 72 h after N-starvation and DHA to EPA ratio rises above 1 after 144 h in N-starvation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

CL contributed to the design and implementation of the research, analysis of the results, and writing of the original draft of the manuscript. JH and KT supervised the project and results, and contributed to the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2020.563333/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.