Journal of Applied Phycology (2023) 35:1565–1574
https://doi.org/10.1007/s10811-023-03020-0

RESEARCH

Controlling the nitrogen environment for optimal *Rhodomonas salina* production

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Received: 21 March 2023 / Revised: 2 June 2023 / Accepted: 7 June 2023 / Published online: 29 June 2023

Abstract

The microalga *Rhodomonas salina* is a widely used species for rearing live feed organisms in the aquaculture feed market. A species-specific medium is an essential step towards enhancing productivity and decreasing production costs for microalgae cultivation. However, relevant aspects of medium composition such as nitrogen source and elemental ratio have not yet been characterized for this alga. This study aimed to optimize the following three aspects of culture media: 1) optimal ratio between nitrogen and phosphorus (N:P ratio); 2) preferred source of nitrogen; and 3) tolerance of *R. salina* towards free ammonia. To investigate this, we conducted a series of controlled laboratory experiments in shake flasks. Our experiments revealed a 45% increase in growth rate when an N:P ratio of 15:1 was used compared to the standard ratio of 25:1. Ammonium and nitrate were equally well accepted as a nitrogen source, however, a mix of ammonium and nitrate resulted in significant growth reduction. Free ammonia did not affect growth of the alga at the tested concentrations of up to 5 mg ammonia–nitrogen L^{-1}. We conclude that for optimal *R. salina* cultivation, an N:P ratio of 15:1 is strongly preferred, as it leads to a significant increase in growth rate. Further, media with a single source of nitrogen promote faster growth over media with mixed sources, and ammonium may safely be used as a nitrogen source, since *R. salina* tolerates certain levels of free ammonia. Overall, this work provides insights into the optimal cultivation conditions for *R. salina*, allowing for more efficient and reliable production of this relevant species.

Keywords Aquaculture · Cryptophyceae · Microalgae production · Medium optimization · Nutrient ratio · Nitrogen source · Ammonia toxicity

Introduction

Aquaculture has been the fastest growing food production sector of the last decade, recently surpassing wild fisheries in tonnage production of seafood (Ritchie 2019). The aquaculture sector is expected to play a key role in achieving the United Nations sustainable development goals No. 2: "End hunger", and No. 14: "Conserve marine life" (Ritchie and Roser 2021; EIT Food 2021). However, fed aquaculture today relies heavily on fish meal, which puts an additional burden on already depleted wild fish populations (Froehlich et al. 2018). Around 3 million tonnes of fish meal and 0.6 million tonnes of fish oil are consumed yearly by the aquaculture industry, which amounts to nearly 68% and 89% of all fish meal and fish oil produced worldwide (Tacon and Metian 2008; Jackson 2013).

Microalgae have been suggested as a promising alternative to conventional ingredients in aquaculture feed (Shah et al. 2017; Sarker et al. 2020a; Nagappan et al. 2021), with some species being known for their excellent digestibility and nutritional composition, exceeding even that of fish oil (Sarker et al. 2020b; Annamalai et al. 2021). Especially the high concentrations of polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) contribute to the high nutritional value of microalgae (Harwood 2019; Niccolai et al. 2019). A small range of algal species is produced commercially for the rearing of mollusks, crustaceans, and live feed organisms

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such as copepods, artemia, and rotifers (Brown & Blackburn 2013; de Moraes et al. 2022). For most fish produced in aquaculture, life feed organisms are essential because they are required in early larval development (Carter 2015). One of the most interesting species in this life feed production sector is *Rhodomonas salina* (Knuckey et al. 2005; Tremblay et al. 2007; Seixas et al. 2009).

Rhodomonas is a genus of fast-growing, mostly marine, cryptophyte microalga. The most apparent features of *Rhodomonas* are its red color (caused by the antenna pigment phycoerythrin 545), and the presence of a proteinaceous periplast in place of a cell wall (Kugrens et al. 1999). *Rhodomonas salina* is particularly popular in the aquaculture sector because it contains protein levels of up to 60%, is easily digestible, and contains both EPA and DHA in considerable quantities (Seixas et al. 2009; Thoisen et al. 2018). These qualities make it an ideal feed organism which is produced on the small scale in hatcheries for the rearing of rotifers, copepods, brine shrimp, and scallops (Knuckey et al. 2005; Seixas et al. 2009). Small scale localized production however is very cost-inefficient, with prices per kg being up to 10 times higher than for commercially produced microalgae (Oostlander et al. 2020a). Therefore the economic incentive for optimizing the cultivation of this alga and enabling its production on the large scale is large.

Supplying the right amount of nutrients is a crucial factor for sustained and high productivity in microalgal cultivation (Spaargaren 1996; Markou et al. 2014). To optimize growth, the macronutrients nitrogen, phosphorus, and carbon need to be supplied in a suitable ratio and in a form that is readily available for algal uptake (Xin et al. 2010; An et al. 2020; Yaakob et al. 2021). If this is not the case, nutrient limitation quickly becomes an issue for growth and other organisms may outcompete the desired species (Wágner et al. 2021; Yaakob et al. 2021). For example, lake enrichment experiments have clearly demonstrated that the proportion of nitrogen to phosphorus can have a dramatic effect on the algal species composition and productivity (Stelzer and Lamberti 2001; Vrede et al. 2009). It follows that a nutrient medium tailored to the needs of an alga will selectively promote its growth rates.

Ample literature is available on the optimal conditions for *R. salina* with respect to light, temperature, salinity, and pH (Hammer et al. 2002; Chaloub et al. 2015; Vu et al. 2015; Guevara et al. 2016; Thoisen et al. 2018; Oostlander et al. 2020b; Latsos et al. 2021). However, little research exists on the optimal growth medium for this alga (Bi et al. 2012; Derbel et al. 2022). Instead, default growth solutions are used for its cultivation. These are for example the f/2 medium by Guillard, and the L1 medium, which is a variation of f/2 medium (Guillard 1975; NORCCA 2022; NCMA Bigelow 2021; CCAP 2020). The composition of an optimal nutrient medium should align with that of the biomass produced (Xin et al. 2010; An et al. 2020; Yaakob et al. 2021). All three nutrient media contain nitrogen (N) and phosphorus (P) at a molar ratio of 25:1, however, this ratio is not the same as the chemical composition of marine microalgae (Redfield 1934; King et al. 2015). King et al. (2015) investigated the composition of 7 marine microalgal species in seawater with f/2 medium. Only one species exhibited an N:P ratio of 25:1, the others had ratios closer to 10:1. Furthermore, the ratio most frequently found in marine life is around 16:1, known as the Redfield ratio (Redfield 1934). Since an unbalances stoichiometry of the available nutrients can inhibit algal growth,, it is important to investigate whether the given ratio of 25:1 is indeed suitable for sustaining *R. salina* growth.

Nitrate (NO_3^-) in the form of KNO_3 or $NaNO_3$ is the most common source of nitrogen used in nutrient media, while ammonium (NH_4^+) is less commonly used due to its potential toxicity under certain conditions (Lourenço et al. 2002; Collos and Harrison 2014). In other algae, ammonium has been shown to inhibit nitrate uptake by suppressing the activity of nitrate transport proteins (Florencio and Vega 1982; Fernández and Galván 2008). In a 1990 review, Dortch (1990) noted that while marine microalgae may prefer ammonium as a nitrogen source, growth rates may be higher in nitrate media.

Little literature is available with regards to the preferred nitrogen source of *R. salina*. Lourenço et al. (2002) suggested a low tolerance of cryptophyceae towards ammonium concentrations, leading to poor growth. By contrast, Thoisen et al. (2018) found in their experiment that ammonium promoted faster growth rates in *R. salina*. This apparent disparity may be explained by the fact that neither of the two studies provides information on the pH level in the experiments, despite the pH being especially relevant when working with ammonium. With increasing pH, ammonium is present in its de-ionized form free ammonia (NH_3) which is toxic to microalgae because it can diffuse across the cell membrane and disrupt cellular function, leading to metabolic stress and cell death (Rossi et al. 2020). Specifically, when algae and cyanobacteria are exposed to free ammonia, Photosystem II is being damaged, with the oxygen-evolving complex being affected most (Drath et al. 2008; Wang et al. 2019).

Exploring the biological limits of *R. salina* towards free ammonia is essential if ammonium is to be sincerely considered as an alternative source of nitrogen. Usually in commercial algal cultures the pH is maintained at the desired level of 7.5 at which concentrations of NH_3 are negligible. However, when CO_2 addition malfunctions, the pH can quickly rise to 8.5 or higher, leading to increased NH_3 concentrations. Treatment with free ammonia is further a common approach in algae culture to tackle protists contamination (Thomas et al. 2017) and its limitations must be clarified. Finally, ammonia tolerance becomes particularly

relevant when considering the usage of waste streams as a sustainable cultivation medium, which is becoming a more and more attractive option in the algae field (Salbitani and Carfagna 2021).

The aim of our work was to develop a finely tuned nutrient medium, which specifically favors cultivation of *R.salina*. Understanding the effects of media composition on nitrogen uptake and assimilation in *R. salina* is crucial for the development of sustainable aquaculture and biotechnology applications, where the efficient use of nitrogen is essential for maximizing yields and minimizing environmental impacts. Therefore, we conducted a series of laboratory experiments. First, we determined the optimal N:P ratio. Second, we tested whether ammonium is preferred over nitrate as a nitrogen source at a stable pH of 7.5. Finally, we determined which concentration of free ammonia is toxic to *R. salina.* We hypothesized that (i) a lower nitrogen to phosphate ratio may be more suitable than the currently used N:P ratio of 25:1, (ii) that ammonium would be preferred over nitrate as a source of nitrogen, and (iii), that at a concentration of 9 mg L^{-1} of free ammonia in an ammonium based culture medium would become toxic and impede growth of *R. salina*.

Material and methods

Algal strain and media

The experiments were conducted at the facilities of AlgaSpring B.V. using the *Rhodomonas salina* strain "AS2003". Stock cultures of AS2003 were kept in 50-mL Erlenmeyer flasks in a laboratory shaker at 22 °C and at an irradiance of 80 µmol photons m^{-2} s^{-1} in f/2×2 medium. This is the f/2 medium with twice the concentration of all nutrients (see On-line Table A1).

Nutrient experiments

We experimentally investigated variations of the f/2×2 medium. Specifically we determined the optimal N:P ratio, preferred nitrogen source, and ammonia toxicity for the microalga *R. salina*. In all experiments and all groups, three biological replicates were used. To ensure equal starting conditions for all cultures, the initial optical density (OD) was measured at 750 nm (Griffiths et al. 2011). The media were prepared in tap water with 20 g L^{-1} marine salt (Reef crystal, Aquarium systems, France) for a salinity of 20 ppt (see On-line Table A2 for elemental composition).

Experiment 1: Optimal N:P ratio

To study the optimal N:P ratio for *R. salina* growth media, algal growth rates were determined experimentally at different ratios. Four treatments were tested: 5:1, 10:1, 15:1 and 25:1, the latter serving as the control group, since this corresponds to the default ratio found in f/2 medium (Guillard 1975). The varying N:P ratios were achieved by increasing the amount of phosphate added. Each treatment group and the control were prepared in three biological replicates in Erlenmeyer flasks of 100 mL. The media were prepared first and then inoculated from the same *R. salina* culture. The OD_{750} on day 1 was 0.1 in all groups. The 12 resulting flasks were then placed in a closed laboratory shaker at 100 rpm and 22 °C with 24 h light and an intensity of 80 µmol photons m^{-2} s^{-1}. The pH was recorded on the first and last day of the experiment, the nutrient media were refreshed every 7 days by adding 2 mL of concentrated nutrient stock solution. Cell counts were performed every 3–4 days. The total duration of the experiment was 25 days.

Experiment 2: Preferred nitrogen source

In the second experiment, the preferred source of nitrogen was investigated using a set up of three treatment groups: 1) 100% nitrate, 2) 50% nitrate and 50% ammonium, and 3) 100% ammonium. Sodium nitrate or alternatively ammonium sulfate were used to create the media. The total concentration of nitrogen in all groups was kept at 24.64 mg L^{-1}, equal to the one found in f/2×2 medium.

The experiment was conducted in triplicates in 500-mL Erlenmeyer flasks with an air infusion stone to ensure proper mixing and gas exchange. The cultures were sparged with air at atmospheric CO_2 concentration. The temperature was kept at a constant 21 °C and continuous light was provided by LED lamps. Over the course of the cultivation period, the flasks were moved closer to the light source to counteract self-shading through increasing cell densities. The incoming light increased over time from 100 to 180 µmol photons m^{-2} s^{-1}, but the outgoing light was kept stable at 80 ± 10 µmol photons m^{-2} s^{-1}. To maintain the pH at 7.5, HEPES (N-2-hydroxyethylpiperazine-N-2′-ethanesulfonic acid) was added to the culture media at a concentration of 0.7 g L^{-1}. The N:P ratio in the media was changed to 15:1 as this was determined optimal in the first part of the study. All media were first prepared and then inoculated from the same starting material at an initial OD_{750} of 0.2. Cell counts and concentrations of nitrate and ammonium were measured on days 0, 5, 6, 7, 11, and 14 of the experiment, to allow monitoring of the nutrient concentration and preferred source. By day five, the culture had depleted all nutrients and had to be refreshed with 2 mL of a concentrated stock solution to continue the experiment. After 14 days, the experiment ended.

Experiment 3: Ammonia toxicity

To test toxicity of ammonia on *R. salina*, an experiment with different concentrations of free ammonia was conducted. Three treatments with 0, 1, and 10 mg L^{-1} of NH_3 were set up in triplicates. These concentrations were chosen because they correspond to values frequently encountered during large scale cultivation. To reach these concentrations, ammonium was added as a nitrogen source instead of nitrate with a final concentration of 24.64 mg L^{-1}, then the pH was adjusted to values 7, 8, and 9, leading to the dissociation of ammonium into free ammonia and a proton. The pH values were buffered using 0.7 g L^{-1} of HEPES in groups 7 and 8, but not in group 9 as this pH exceeds the buffer capacity of HEPES. Instead, pH values were measured every 2–3 days and adjusted back to the desired value where needed by adding dropwise NaOH until the desired pH was reached. This experiment was also conducted in 500-mL Erlenmeyer flasks in modified f/2 × 2 medium with N:P ratio of 15:1. For details on setup and light see experiment 2. The total duration of the experiment was 20 days, the cell counts were performed every 3–5 days.

Sampling and data collection

The OD of each sample was measured at 750 nm. Since algae are photosynthetically active only below a threshold of 750 nm, at this value only the presence of cells is measured without the influence of algal pigments absorbing part of the light (Griffiths et al. 2011). Because *R. salina* cells sink to the bottom of the cuvette in a matter of minutes, the sample was pipetted up and down carefully with a 1 mL pipette tip immediately before taking the measurement.

The number of *R. salina* cells was counted under the microscope at 10 × magnification using a Neubauer counting chamber. In each of the two chambers, algal cells were counted in the top left and bottom right 0.5 mm^2 boxes, added together, and multiplied by 40,000 to attain the concentration per mL.

Colorimetric assays ammonium test kit "931,010" and nitrate test kit "931,041" by Visocolor® ECO were used to determine nutrient concentrations in the water. Tests for ammonium and nitrate concentrations were performed on the supernatant of culture samples after centrifugation at 3500 rpm for 10 min.

Statistical analysis

The effect of different media formulations on growth rates of *R. salina* was analyzed using linear regression models and analysis of variance (ANOVA). The average growth was determined by fitting linear regression models with the treatments as a grouping factor, cell count as the dependent, and time as the independent variable. Normal-QQ plots were used to ensure that the normality assumption for the regression models was met. To test for significant differences, the models were then compared using an ANOVA on an α level of 0.05. On the results of the ANOVA, a post-hoc correction for multiple comparisons was performed. Since the number of samples was small, it was important to choose the appropriate post-hoc test for each experiment to maintain statistical power: 1) For the experiment on N:P ratio, Dunnett's correction was used for comparing the treatments with the control group. 2) To compare the different N-source treatments, Tukey's HSD test was used because all differences between groups were of interest. Tukey's HSD test uses the assumption of equal variances, thus, Levene's test on equal variances was performed first to test the assumption. 3) To assess the ammonia toxicity experiment, again Dunnett's correction was used since treatments were compared with the control. Statistics were calculated using RStudio with the installed packages ggplot2, dplyr, and tidyverse (Wickham 2016, 2022a, b).

Results

Experiment 1: N:P ratio

Average growth rate was affected by treatments with different nitrate to phosphate ratios (F-value = 441.97, $p < 0.05$, Fig. 1). The control with the unchanged 25:1 ratio of the f/2 medium achieved the lowest growth rate with $0.23 \pm 0.05 \times 10^6$ cells mL^{-1} day^{-1}, while the highest was observed in the treatment with ratio 15:1 with $0.33 \pm 0.08 \times 10^6$ cells mL^{-1} day^{-1}. Groups with N:P ratios of 5:1 and 10:1 achieved growth rates of $0.24 \pm 0.095 \times 10^6$ cells mL^{-1} day^{-1}, and $0.31 \pm 0.03 \times 10^6$ cells mL^{-1} day^{-1}, respectively. The average growth rate of the treatment 15:1 was thus 45% higher than that of the control. For the 10:1 and the 5:1 treatment, the growth rates were 34% and 4% higher than the control respectively. On the last day, the cultures reached cell counts of 5.9 ± 1.95, 8.2 ± 0.67, 8.3 ± 1.69, and 5.4 ± 0.96 million cells mL^{-1} in rising order from 5:1 till 25:1. A one-way ANOVA with Dunnett's post-hoc test revealed significant differences between the control group (25:1) and groups 15:1 ($p = 0.027$) and 10:1 ($p = 0.046$) (see Appendix Table A3).

Experiment 2: Optimal nitrogen source

Growth rates were affected by the nitrogen source used (F-value = 111.27, $p < 0.05$, Fig. 2). Specifically among the three tested substrates, regression analysis showed

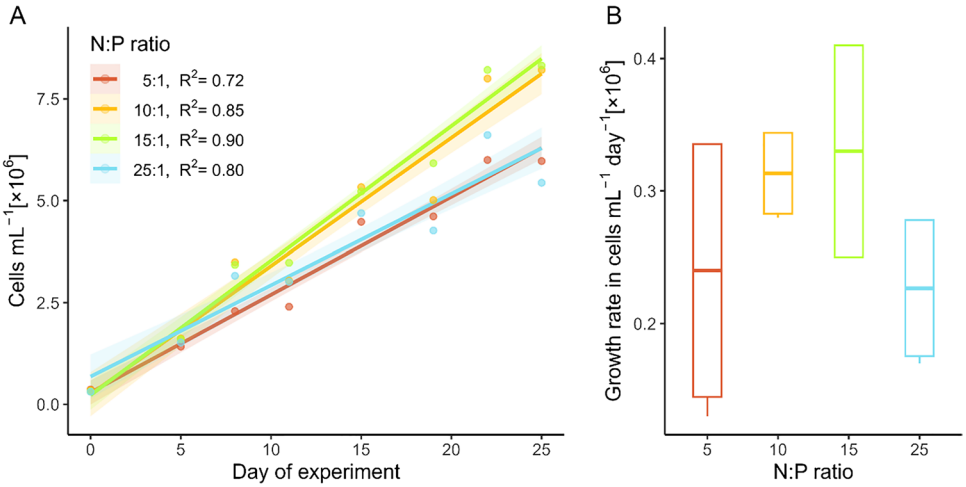

Fig. 1 **A** Linear models describing growth of *R. salina* in 10^6 cells mL^{-1} $day.^{-1}$ at different N:P ratios. Treatment and fit are shown in legend. **B** Boxplot shows median and SD of growth rate (the slopes found through linear regression) for different N:P treatments ($n=3$)

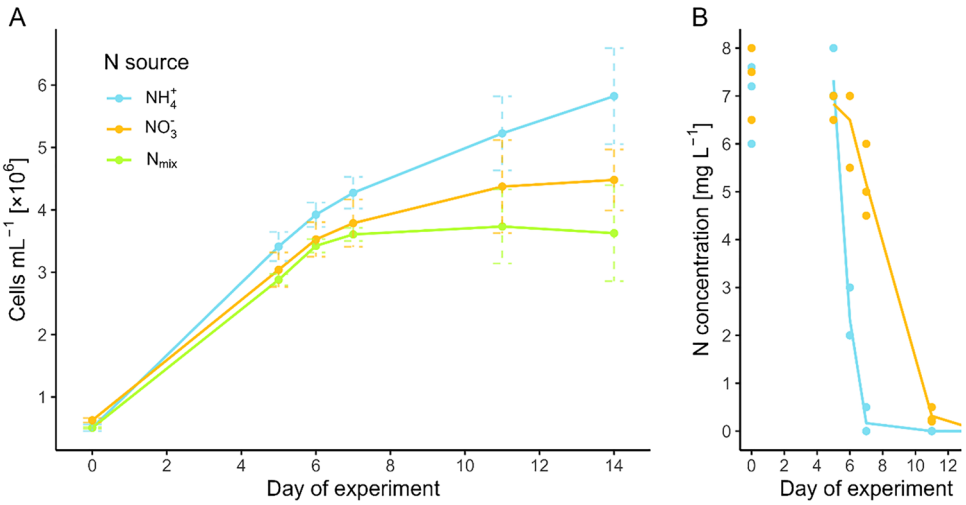

Fig. 2 **A** Growth of *R. salina* in growth media with different sources of nitrogen: orange = NO_3^-, blue = NH_4^+, and green = 50:50 NH_4^+ and NO_3^-. The error bars represent the standard error ($n=3$). **B** Consumption of NH_4^+ (blue line) and of $NO_3.^-$ (orange line) in the 3 replicates of the treatment group with mixed nitrogen sources over seven days. On day 5,all nutrients had been depleted and were restored by adding 2 mL of concentrated stock

the highest trend in growth rate in the ammonium media ($0.37 \pm 0.1 \times 10^6$ cells mL^{-1} day^{-1}), though this was found not to be statistically significant. In the nitrate media the growth rate was 37% lower ($0.27 \pm 0.01 \times 10^6$ cells mL^{-1} day^{-1}) and in cultures with a combination of ammonium and nitrate it was 76% lower ($0.21 \pm 0.1 \times 10^6$ cells mL^{-1} day^{-1}) compared with pure ammonium. Lastly, growth in the nitrate group was 29% higher than that in the mixed media group.

It was observed that R. salina first consumed ammonium and then nitrate in the nitrogen mix during seven days (Figs. 2B). By day five, the culture had depleted all nutrients and was resupplied with fresh stock solution. Six days after that, the nutrients were again fully depleted. The concentration of ammonium (light blue line) decreased rapidly from day five onwards, while the nitrate concentration remained more stable until day seven and only decreased after that (see Appendix Table A4). There was thus a lag in the consumption on nitrate when ammonium was present compared to the pure nitrate media (see also Appendix Fig. A5).

The difference in growth rates between the ammonium group and the nitrate group was not statistically significant ($p=0.10$). A significant difference was found between the ammonium group and the mixed media group ($p=0.028$).

Experiment 3: Ammonia toxicity

Average growth rates were not affected by addition of free ammonia. The regression models revealed growth rates of $0.21 \pm 0.02 \times 10^6$ cells mL^{-1} day^{-1} for the control, $0.15 \pm 0.01 \times 10^6$ cells mL^{-1} day^{-1} for a low ammonia treatment and $0.13 \pm 0.01 \times 10^6$ cells mL^{-1} day^{-1} for a high ammonia treatment (Fig. 3). The pH values in the treatment groups with low and high ammonia fluctuated widely between 7.2 and 8.1, and 7.8 and 9.5, respectively (see Appendix Table A6). This also led to a fluctuation of the actual concentration of free ammonia found in solution, as it is dependent on the pH. Average pH, the corresponding NH_4^+ concentrations and achieved growth rates are shown in Table 1.

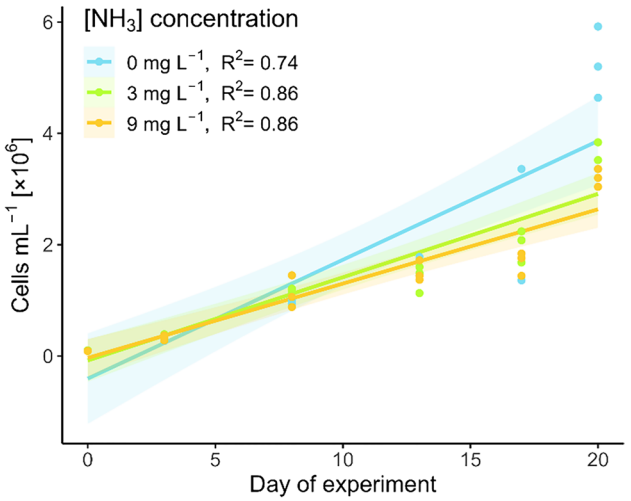

Fig. 3 Linear models showing growth of *R. salina* in 10^6 cells mL^{-1} day^{-1} at different concentrations of free ammonia. Colored bands show that standard deviations are relatively wide

The differences between treatment and control groups were small and standard deviations were large. In an ANOVA no significant differences were found between the groups ($F = 8.58$, $p = 0.07$).

Discussion

In this study an optimized nutrient medium was devised for faster growth rates of *R. salina*. Our data provide new insights into the requirements and optimal growth conditions of this species. These findings can aid microalgae producers in producing *R. salina* more reliably and efficiently, leveling the way towards a cost-effective production for the aquaculture feed market.

Optimal ratio of nitrogen to phosphorus

Our experiment revealed that an N:P ratio of 15:1 leads to a 45% increase in growth rates compared to the standard ratio of 25:1 and is thus to be preferred to increase production. Increasing the relative P content has led to higher growth rates, implying that at the standard ratio the algae were P limited.

The preferred ratio is markedly lower than what is used in the standard marine culture media (Guillard 1975). We believe this mismatch results from the fact that these media are used for a wide variety of microalgae and are thus not optimized for *R. salina*. The found ratio is instead much closer to the elemental composition of algae in natural ecosystems (Redfield 1934; King et al. 2015). The balancing of nutrients in cultivation media is important because in an optimal media, nutrients are taken up in synchronization and no surplus is left over. If however a stoichiometric imbalance exists, nutrients will remain in the media and will accumulate over time for other organisms to use (Yaakob et al. 2021). As such, a careful balance of nutrient composition may contribute to keeping contaminants under control and maintaining a clean culture. Different nutrient conditions can also have an impact on the composition of algal biomass (Latsos et al. 2020; Yamamoto et al. 2020). If the production goal is not maximum productivity, but instead the relative content of certain compounds, such as lipids, or PUFAs the ratio should be chosen carefully (Lari et al. 2016).

Growth rates observed in this experiment were lower than those of Vu et al. (2015). Possible reasons for this include differences in the strains used, the agitation method (in the current study no CO_2 enriched air was used), and the method of biomass determination.

Preferred nitrogen source

Our second experiment showed that the algal growth rate did not differ significantly between nitrate and ammonium sources, suggesting that both may be equally well used for cultivation of *R. salina*. Growth differences between treatment groups were slightly more pronounced during stationary phase compared to the exponential phase. This could reflect the increased energy requirements for nitrate assimilation which lead to lowered productivity and

Table 1 *R. salina cultures* were grown in media with 32.8 mg L^{-1} of ammonium (NH_4^+) at different pH values, causing different concentrations of free ammonia (NH_3). Intended pH and actual pH differed substantially as is shown in the table

Treatment	Intended pH	Actual pH	Intended NH_3 (mg L^{-1})	Actual NH_3 (mg L^{-1})	Final cell count	Growth rate in 10^6 cells mL^{-1} day^{-1}
Control	pH 7	7.10 ± 0.2	0	0.0 ± 0	5.25 * 10^6	0.21 ± 0.02 day^{-1}
Low NH_3	pH 8	7.62 ± 0.4	3	0.8 ± 0.6	3.57 * 10^6	0.15 ± 0.01 day^{-1}
High NH_3	pH 9	8.26 ± 0.7	9	5.0 ± 4.5	3.20 * 10^6	0.13 ± 0.01 day^{-1}

Culture conditions, growth rates and final cell count are shown as mean ± standard deviation of the three replicates

capacity for cell production in the long run (Huppe and Turpin 1994). However, with the exception of the mixed nitrogen treatment group, it should be noted that these differences were not significant. Using daily measurements of algal growth and nutrient uptake, future research could provide more detailed information on the temporal dynamics of these processes. It is further intriguing to note that the cultures in the mixed nitrogen group reached a plateau in cell density between day 7 and day 14, despite continuing to consume nitrate. Possible explanations for this could include maintenance losses equaling out growth rates, or changes in the composition of the microbial community.

In the mixed media treatment, growth was also significantly lower compared to both pure media groups (76% and 29% compared with ammonium and nitrate respectively). A possible explanation is that all ammonium in the media was consumed first, after which the algae had to readjust their metabolism in order to use nitrate (Pritchard et al. 2015). Then, when on day five the media was refreshed in order to continue monitoring the cultures behavior, the metabolic adjustment had to be reversed. This repeated change between nitrogen sources may create an artificial lag phase and slow down algal growth, as new proteins need to be synthesized. Such a continued lag phase could explain the poor performance of the cultures in mixed media. This may be a relevant insight in cases where the makeup water contains noteworthy amounts of ammonium. If this is the case, a nitrate-based media may be detrimental to the culture as the algae are forced to switch from one source to the other every time the culture is refreshed with new water after a harvest or to increase the volume.

After day five in the mixed media group we observed that ammonium was consumed first and nitrate was used only after its depletion. We conclude that ammonium is preferred by the algae for uptake of nitrogen, and that nitrate will only be used in the absence of ammonium. This is likely due to ammonium imposing a lower demand for reductants on the cells compared to nitrate (Huppe and Turpin 1994; Giordano 1997). In other algae it has been shown that ammonium can suppress nitrate uptake through direct inhibition of nitrate transport proteins (Florencio and Vega 1982; Fernández and Galván 2008). Our results suggest that this inhibitory effect of ammonium also exists in *R. salina*, explaining the successive uptake of the two nutrients.

Tolerable levels of free ammonia

It was expected that in the treatment with high ammonia, the algal growth would be impaired. We did not observe significant effects in growth rates at any of the free ammonia concentrations. We believe that this was not because of an unusually high resistance of *R. salina* to free ammonia, but rather due to the fluctuations in pH. Over the course of the experiment, major pH fluctuations occurred and the intended concentration of 9 mg L^{-1} for the high ammonia treatment could not be maintained. Another limitation was that adjusting the pH manually via addition of acid and base may have caused stress to the cells, thus using another form of pH control or buffer would have been preferred.

At the given conditions, the concentrations of free ammonia on average reached 0.8 ± 0.6 mg L^{-1} in the "low ammonia" treatment and 5.0 ± 4.5 mg L^{-1} in the "high ammonia" treatment. At these concentrations, no impact on algal growth was observed. To put these numbers in context, Collos and Harrison (2014) suggest free ammonia levels may become toxic to some marine microalgae from 1.9 mg L^{-1} with highly resilient species tolerating up to 62 mg L^{-1}.

Next to the ammonium:ammonia balance, changes in pH also affect the way in which CO_2 is dissolved in water. At a low pH (< 4.2), inorganic carbon is present in dissolved CO_2 form, and at higher pH, this shifts to bicarbonate (HCO_3^-), and finally carbonate (CO_3^{2-}). While *Rhodomonas* is capable of direct HCO_3^- uptake (Camiro-Vargas et al. 2005), speed and efficiency are affected since CO_2 can freely diffuse over the cell membrane, while the transport of HCO_3^- is an enzymatic process that consumes energy (Moheimani and Borowitzka 2011). The equilibrium point between CO_2 and HCO_3^- is at a pH of 6.3, and for HCO_3^- and CO_3^{2-} at pH 10.3; this means that the form of inorganic carbon available to the cells changes with pH. In the control group at pH 7 the algae experienced the highest concentration of dissolved CO2 of around 20% of the total inorganic carbon, while in the treatment groups, inorganic carbon was predominantly present in bicarbonate form. In a recent study, Umetani et al. (2021) investigated the carbon uptake of a different alga and did not report differences in growth between the two sources CO_2 or HCO_3^-. Yet we cannot rule out the possibility that the altered ratio of carbon sources may have affected algal growth.

The highest, though not significant, growth rate of $0.21 \pm 0.02 \times 10^6$ cells mL^{-1} day^{-1} was reached in the control group without any free ammonia. As was discussed in great detail above, these elevated rates may result from the fact that the optimal growth condition for *R. salina*—also with respect to CO_2 availability—is found at pH 7.0 (Latsos et al. 2021). The differences in growth may thus be related purely to the pH preference. Further work is required to investigate the toxicity and tolerance of *R. salina* towards free ammonia, with a method for controlling the pH being a critical aspect for experimental success.

Conclusions and implications

In this study, we were able to develop a more efficient nutrient medium for cultivating *R. salina*. We found that a finely adjusted ratio of macronutrients (N:P ratio of 15:1) can promote growth rates up to 45% higher compared to the frequently used f/2 medium. Meeting *R. salina* with a precisely matched growth medium will significantly enhance its yields, possibly reducing issues caused by contaminants and grazers, and overall leading to a more economical and stable production. The optimization of macronutrients also promotes a more efficient use of resources, reducing surface water pollution and thus making cultivation more sustainable. With regard to the nitrogen source, the medium with ammonium promoted an advantage of 37% in growth speed over the nitrate medium (though not significant) and a 76% advantage over a medium with mixed sources. The effect of free ammonia toxicity was found to be negligible at concentrations up to 5 mg L^{-1}. Thus the alga can be safely cultivated when ammonium is used as the nitrogen source and when pH fluctuations lead to formation of free ammonia. This insight may also unlock other possible use cases for *R. salina*, such as nutrient reclamation from wastewater, which is often high in ammonium, or cultivation in a setting where the make-up water contains noteworthy levels of ammonium. The maximum tolerance of *R. salina* towards free ammonia was not found and should be addressed by future research.

Overall, we were able to show how specific aspects of medium optimization can enhance cultivation of *R. salina* and thus bring this relevant species one step closer to large-scale commercial production.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10811-023-03020-0.

Acknowledgements I would like to thank Ralph Temmink and Marco la Russa for their excellent supervision, as well as my colleagues at AlgaSpring for their support and input towards this study. Specifically Belén Toribio and Milou Schuurmans have been a vital help in the preparation and completion of the lab work and the interpretation of the results.

Authors contribution Antonia A. Fichtbauer: Methodology, Investigation, Formal analysis, Visualization, Writing original draft.

Ralph J.M. Temmink.: Methodology, Supervision, Reviewing and Editing of draft.

Marco la Russa: Conceptualization, Resources, Supervision, Reviewing and Editing of draft.

Data availability The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors declare no competing interests.

Conflicts of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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