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Sustainable Innovation of Microbiome Applications in the Food System

Deliverable 3.2.

Description of growth characteristics of selected macro- and microalgae

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Summary

Micro- and macroalgal food-based products are experiencing a large increase in demand and are foreseen to be key sources of proteins and other beneficial compounds in the future. The algae can provide a nutritious, reliable, and sustainable food source that can be available in large amount for both, human consumption and feed stock for cattle and other aquaculture applications. Here we report on the growth characteristics of a selection of microalgal species including Cyanobacteria and of the macroalga *Ulva ohnoi* to be potentially used in the development of nutritious food. The authors, from the Norwegian institute for water research (NIVA) and the Centro Tecnológico de Acuicultura (CTAQUA), have tracked the growth and present the results obtained in this report.

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1. Objective

The objective of this deliverable is to document the growth characteristics of macro-, microalgae and cyanobacteria that can be promising candidates to produce high-quality food and feed ingredients. The microalgae were selected from the Nordic Culture Collection of Algae that NIVA hosts. With respect to the macroalgae, species of the genus *Ulva* have been preselected in the project's Description of Action as a model system. Specifically, the species selected is *Ulva ohnoi* M. Hiraoka & S. Shimada 2004, which has been isolated by CTAQUA in 2017 and has been under cultivation in its facilities since then. This species has been selected for its versatility, multitude of (potential) applications (food, feed, valuable extracts), ease of cultivation and high potential for upscaling of the production.

2. Background

Algal biomass as "food of the future" is still not fully exploited. Micro- and microalgae provide a large variety of products suitable for human and animal consumption (including the aquaculture sector) and are well-known sources of proteins and valuable components such as pigments, polyunsaturated fatty acids and triglycerides. This biomass offers therefore a large and almost untapped potential to produce food, feed and a whole set of nutraceutical components.

For seaweeds, this has been recently recognised by various public and private research institutions and companies and a call for more integrated research and policy has been launched in a Seaweed Manifesto, adopted by the UN Global Compact, Action Platform for Sustainable Ocean Business (Doumeizel & Aass, 2020). Despite faster growth rates than any land plant, their contribution to food production is still limited, partly due to production costs and stability of the biomass quality.

One of the unexplored features is the mostly bacterial microbiome associated with the micro and macroalgal cultures or on the surfaces of seaweeds. The SIMBA project will identify the associated microbiome and its dynamics under different cultivation conditions. Finally, the aim is to apply microbiomes in facilitating cultivation of micro-and macro-algae as direct or indirect food source.

The macroalga *Ulva ohnoi* is under culture at CTAQUA since 2017, when initial cultivation was performed in the framework of other projects, in particular, BIOSEA (project funded under the Bio Based Industries Joint Undertaking, Horizon 2020 Research and Innovation Programme under grant agreement No 745622). In the framework of SIMBA, studies have been started on the optimization of the cultivation and the testing of different systems in which growth of the alga has been evaluated. These systems form the basis for subsequent sampling and analysis of their microbiome. For the selection of the microalgae, NIVA has employed the NORCCA (Norwegian Culture Collection of Algae), that consist of more than 1700 strains.

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This collection, together with others from the Nordic Countries, are being explored under the frame of the Nordic Center of Excellence on Bioeconomy "NordAqua".

The SIMBA partners NIVA and CTAQUA will carry out cultivation of micro- and macroalgae respectively. Partners NIOZ (NWO-I) and MATIS will carry out analysis of the associated microbiome.

3. Methodology

3.1 Selection and growth of microalgae

A bibliographic screening was previously performed to identify the most promising strains with relevance to the scope and targets of the SIMBA project (sustainable high-quality food production). As biological source for the strains, the Nordic Culture Collection of Algae (NORCCA) was employed and the shortlisted microorganisms are presented in **Table 1**. The growth of microalgae was tracked on daily basis making use of a Coulter cell counter and the growth rate was calculated. 20 mL of the strains were freshly purchased from the Norwegian Culture Collection of Algae (NORCCA) and pre-incubated for 1 week prior to inoculation of the flasks where the growth was monitored. 30 mL of the acclimated strains where transferred to 40 mL transparent glass vials, having previously diluted in the appropriate culture medium to set an initial concentration about 100.000 cells/mL for all of them. The suspensions of the microalgae with the fresh culture media were rapidly shaken to ensure and immediate dispersion in the media and right after, they were placed in an incubator at 20 ºC under continuous illumination from day light-type fluorescent tubes (61 μmol.s^{-1}.m^{-2}). The strains were kept under orbital shaking (100 rpm) and their cell concentration was measured on a daily basis. The experiments were performed in triplicates.

All the microorganisms included in this selection were unicellular with the exception of *Desmodesmus subspicatus* and *Tetradesmus obliquus* that included multicellular structures.

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Table 1. Information on the different strains tested and used media.

Figure	Strain	Name	Culture medium	Environment	Type	Class
2	NIVA-CHL 163	*Dunaliella salina*	20% Z8 SW + Vit	Marine	Green algae	Chlorophyceae
3	K-0591	*Dunaliella tertiolecta*	20% Z8 SW + Vit	Marine	Green algae	Chlorophyceae
4	K-0297	*Tetraselmis suecica*	20% Z8 SW + Vit	Marine	Green algae	Chlorodendrophyceae
5	NIVA-3/10	*Tetraselmis suecica*	20% Z8 SW + Vit	Marine	Green algae	Chlorodendrophyceae
6	K-1281	*Nannochloropsis occulata*	20% Z8 + SW	Marine	Green algae	Eustigmatophyceae
7	NIVA-2/03	*Nannochloropsis oceanica*	20% Z8 + SW	Marine	Green algae	Eustigmatophyceae
8	NIVA-BAC 2	*Phaeodactylum tricornutum*	20% Z8 + SW	Marine	Diatom	Bacillariophyceae
9	NIVA-4/91	*Isochrysis galbana*	ES	Marine	Yellow-green	Coccolithophyceae/ Prymnesiophyceae
10	K-0633	*Isochrysis sp.*	ES	Marine	Yellow-green	Coccolithophyceae/ Prymnesiophyceae
11	NIVA-4/92	*Pavlova sp.*	ES	Marine	Green algae	Pavlovophyceae
--	K-1252	*Odontella aurita*	L1, 30 PSU	Marine	Diatom	Mediophyceae
12	K-1021	*Porphyridium aerugineum*	MV10, 10 PSU	Freshwater	Red algae - Rhodophytes	Porphyridiophyceae
13	NIVA-CYA 375	*Cyanobium sp*	Z8	Freshwater	Cyanobacteria (contains phycroerythrin)	Cyanobacteriaa
14	NIVA-CHL 70	*Chlorella sp*	Z8	Freshwater	Green algae	Trebouxiophyceae
15	NIVA-CHL 55	*Desmodesmus subspicatus*	Z8	Freshwater	Green algae	Chlorophyceae

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	Strain	**Name**	**Culture medium**	**Environment**	**Type**	**Class**
--	K-0084	*Haematococcus lacustris*	Z8	Freshwater	Green algae	Chlorophyceae
16	NIVA-CHL 90	*Monoraphidium sp*	Z8	Freshwater	Green algae	Chlorophyceae
17	NIVA-15/12	*Rhodomonas salina*	20% Z8 SW + Vit	Marine	Red algae (contains phycroerythrin)	Cryptophyceae
18	NIVA-5/91	*Rhodomonas baltica*	20% Z8 SW + Vit	Marine	Red algae (contains phycroerythrin)	Cryptophyceae
19	NIVA-8/92	*Cryptomonas sp*	ES	Marine	Blue-green algae	Cryptophyceae
20	NIVA-CHL 105	*Tetradesmus obliquus*	Z8	Freshwater	Green algae	Chlorophyceae
21	NIVA-CHL 19	*Chlorella vulgaris*	Z8	Freshwater	Green algae	Trebouxiophyceae
22	NIVA-CHL 168	*Chlamydomonas noctigama*	Z8 + Vit	Freshwater	Green algae	Chlorophyceae
23	NIVA-CHL 153	*Chlamydomonas reinhardtii*	Z8 + Vit	Freshwater	Green algae	Chlorophyceae
24	K-0493	*Chlamydomonas sp.*	Z8 + Vit	Freshwater	Green algae	Chlorophyceae
25	NIVA-CHL 131	*Chlorococcum*	Z8 + vitamin	Freshwater	Green algae	Chlorophyceae
26	NIVA-1/92	*Porphyridium purpureum*	Z8: SW - 1:1	Marine	Red algae – Rhodophytes (contains phycoerythrin)	Porphyridiophyceae
27	A1	*Synechocystis sp*	Z8	Freshwater	Cyanobacteria	Cyanophyceae
28	A2	*Acutodesmus obliquus*	Z8	Freshwater	Green algae	Chlorophyceae

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3.2 Selection and growth of macroalgae

U. ohnoi was isolated from earthen ponds in August 2017 and further grown in the laboratory. Initial cultivation works (previous to the SIMBA project) included the identification of the species by genetic analyses, consisting of sequencing of regions of the ITS1, the rbcL gene encoding a subunit of the chloroplast and the tufA gene encoding an elongation factor and comparison with existing sequences in Genbank / BLAST analysis following (Hayden & Waaland 2002, Saunders & Kucera 2010, Melton et al. 2016). In addition, base laboratory cultures were established in petri dishes, up-scaled first to 0.5 L and subsequently to 2 L flasks and maintained in a climate chamber under constant conditions (20 °C temperature, 14:10 L:D cycle at ± 100 µmol photons m^{-2} s^{-1} from white LED tubes). Experiments were carried out with treated (sand and mechanical filtration followed by 1 µm filtration through cartridge filter) natural seawater, standard f/2 was used as growth medium (Guillard & Ryther 1962, see annex). For optimization of cultivation and experimentation in the framework of the SIMBA project, two growth systems were used: indoor photobioreactors (PBRs) and outdoor tanks (**Figure 1).**

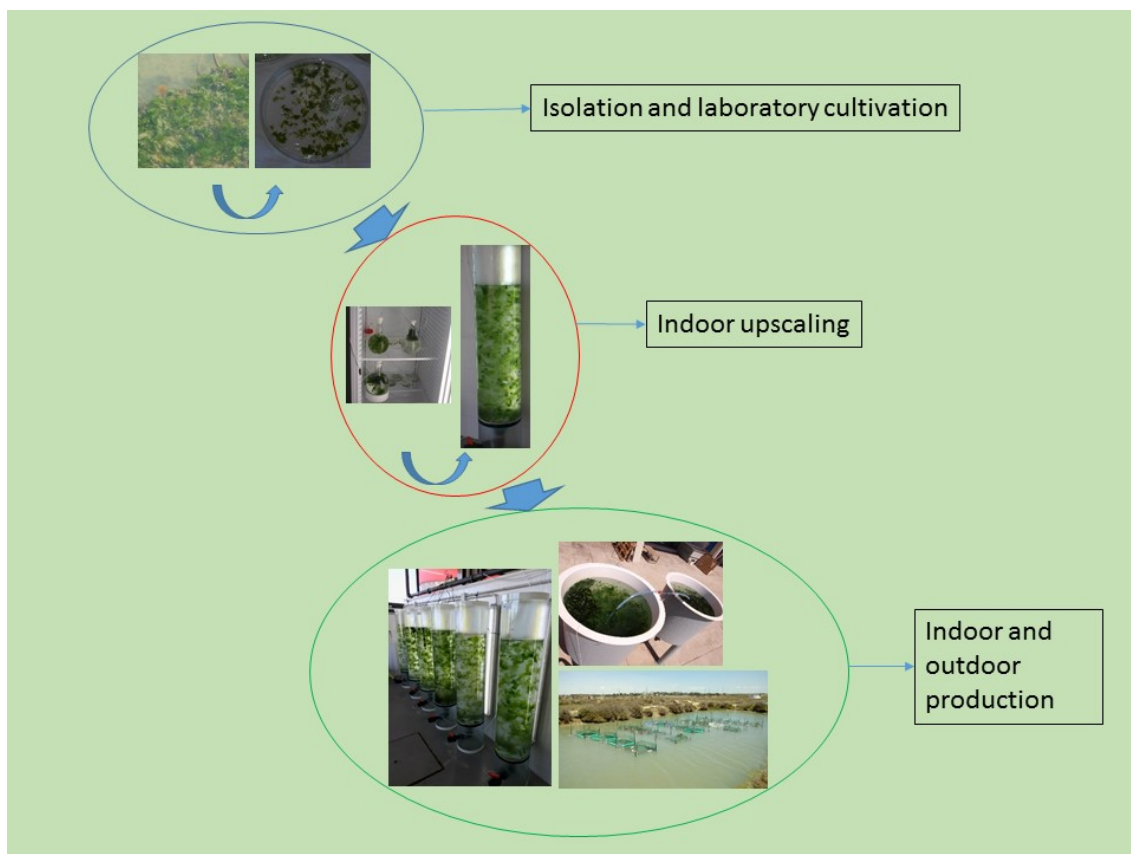

Figure 1. Phases in upscaling of cultivation of the macroalga *Ulva ohnoi*.

PBRs had a cultivation volume of 75 L and were initially placed in the aquaculture facilities of CTAQUA under semi-controlled conditions; i.e. receiving artificial LED light (130 – 150 µmol photons m^{-2} s^{-1}), biomass circulation using the airlift principle and addition of growth medium (f/2). Besides the light applied, PBRs are exposed to natural light with a maximum of 30 µmol

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photons m^{-2} s^{-1} around 11:00 a.m. in summer and very little (< 5 µmol photons m^{-2} s^{-1}) additional light in winter. The Algae in the PBRs were grown under ambient room temperature; during various episodes, water temperature was monitored using ibutton sensors (Maxim, USA). Since June 2020, PBRs have been relocated to a new building unit, allowing greater control of light (no outside light) and temperature conditions (air conditioning allowing for a temperature window of 20 - 27 °C). As limited growth data are available for the new situation, data from both locations will be presented. Trials are still ongoing with different biomass densities to determine the density that gives the optimum yield. In addition, experiments have been carried out with different salinities, nitrogen sources and phosphorous concentrations in the medium. For the salinity test, algae were grown at low (20 psu, natural seawater plus tap water), natural (37 psu) and high (50 psu, natural seawater plus NaCl added) salinity, a range that can be encountered in the natural environment of the algae. For the nutrient experiments, a first experiment was carried out using either nitrate or ammonium as nitrogen source in the f/2 medium and a second experiment experimenting with three different N:P ratios in the medium: low P (N:P molar ratio = 48:1, approximately twice the Redfield ratio), control (N:P = 24:1, standard ratio in f/2 and similar to Redfield ratio) and high P (N:P = 12:1, approximately half of Redfield ratio).

For controlled outdoor cultivation, a system was set-up in August 2019 consisting of two 430 L conical cultivation tanks. Tanks were filled with filtered natural seawater, seaweed circulation in the tanks was maintained using strong aeration and nutrients were added as f/2 in powdered form (Cell-Hi F2P, Varicon aqua, UK, 43 g per tank per week to final f/2 concentration). Nitrate concentration was measured weekly and used as a proxy for nutrient use; nitrate uptake was never more than 75% of the amount added, thus it was concluded that algae were never nutrient limited. Algae were harvested on a weekly basis. Cultures are being monitored continuously since September 2019, experimenting with different initial densities and constant monitoring of the water temperature and daily irradiance.

4. Results & Discussion

4.1. Tracking the growth of microalgae

The growth profiles were followed from the day of inoculation to day 10 and are presented in the **Figures 2** to **28**. For two of the pre-selected species, *Odontella aurita* and *Haematococcus lacustris* it was not possible to obtain useful data on cell number since the debris present in the sample provided a high interference signal in the Coulter cell counter. A flow cytometer was employed instead but again the characteristic signal of the cells was masked by the large inference of the matrix. This debris could have been originated by an excessive number of dead cells that have released their intracellular content to the media. In addition to the characteristic growth curves, the growth rate observed for each day has been calculated according to the equation below and plotted for each one of the strains. In this equation, N represents the cell concentration (cells/mL) and t, the time. The sub-index 0 and n represent initial and time n respectively.

$$\mu_{n-0} = \frac{\ln(N_n) - \ln(N_0)}{t_n - t_0} \times 24\ (\text{day}^{-1})$$

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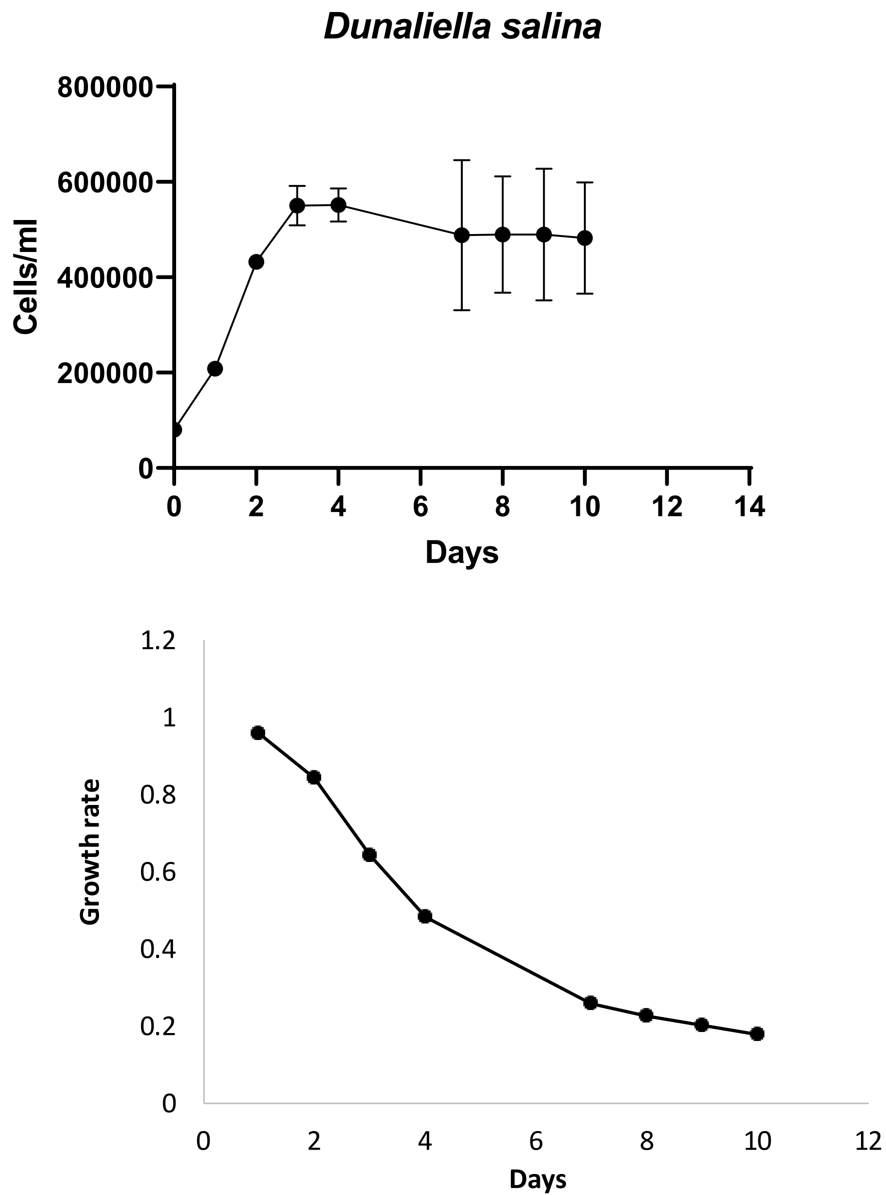

Figure 2. Characteristic growth and time-course profile of growth rate for *Dunaliella salina* (strain NIVA-CHL 163).

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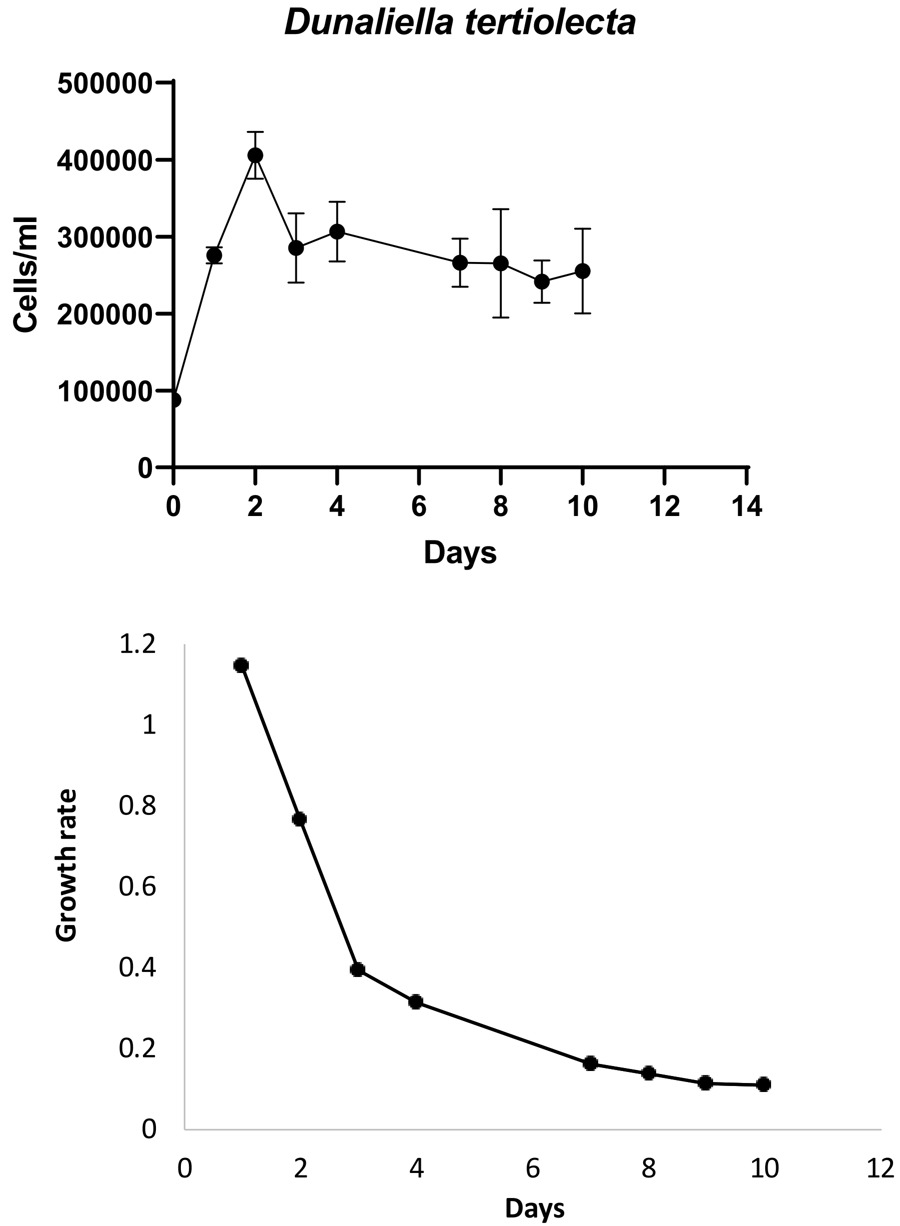

Figure 3. Characteristic growth and time-course profile of growth rate for *Dunaliella tertiolecta* (strain K-0591).

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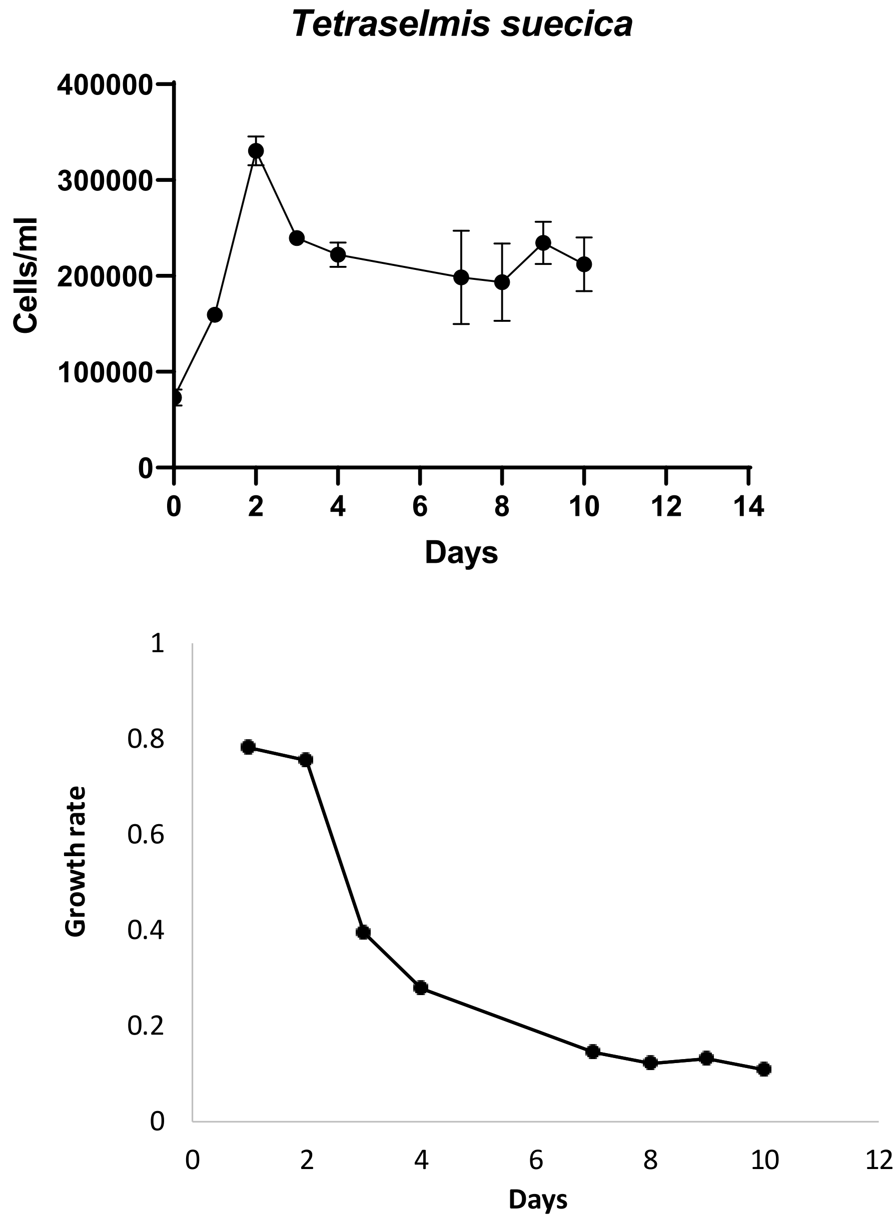

Figure 4. Characteristic growth and time-course profile of growth rate for *Tetraselmis suecica* (strain K-0297).

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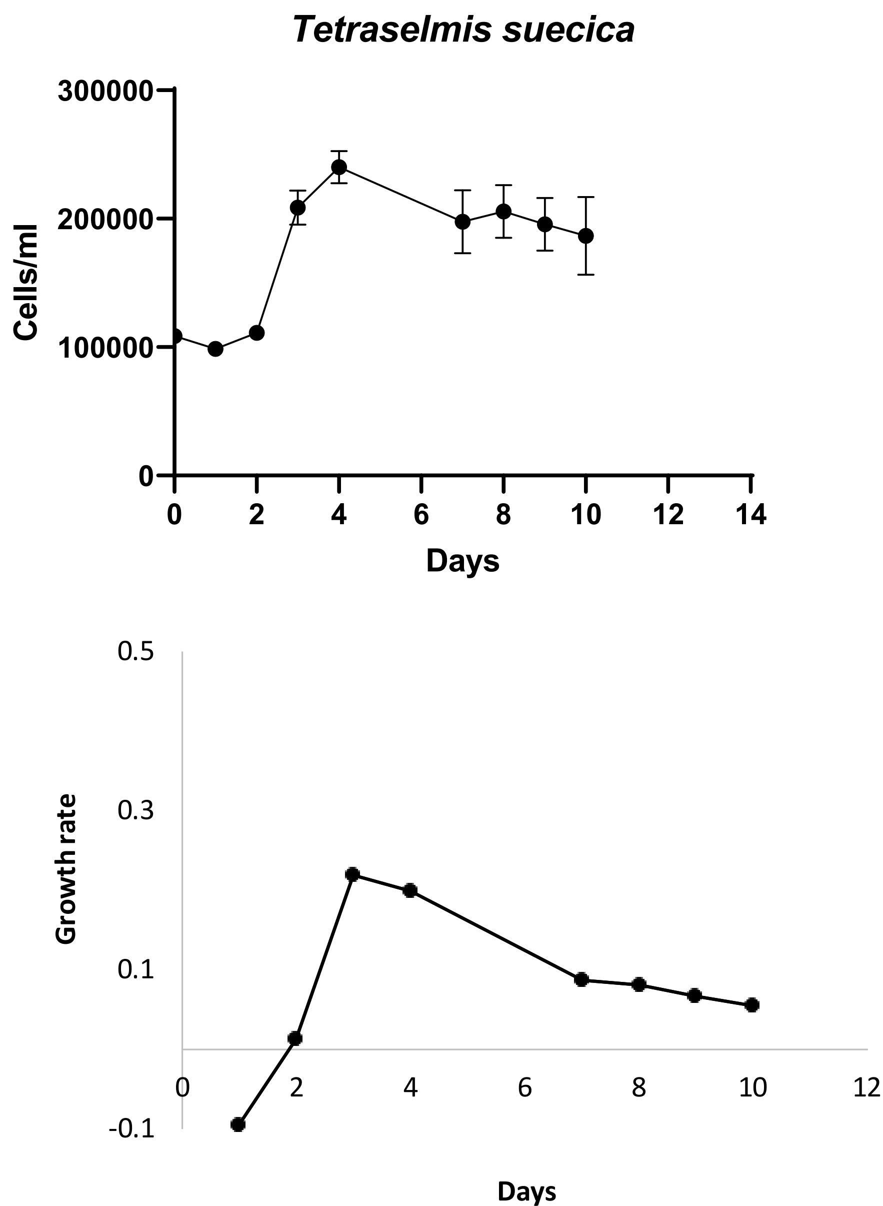

Figure 5. Characteristic growth and time-course profile of growth rate for *Tetraselmis suecica* (strain NIVA-3/10).

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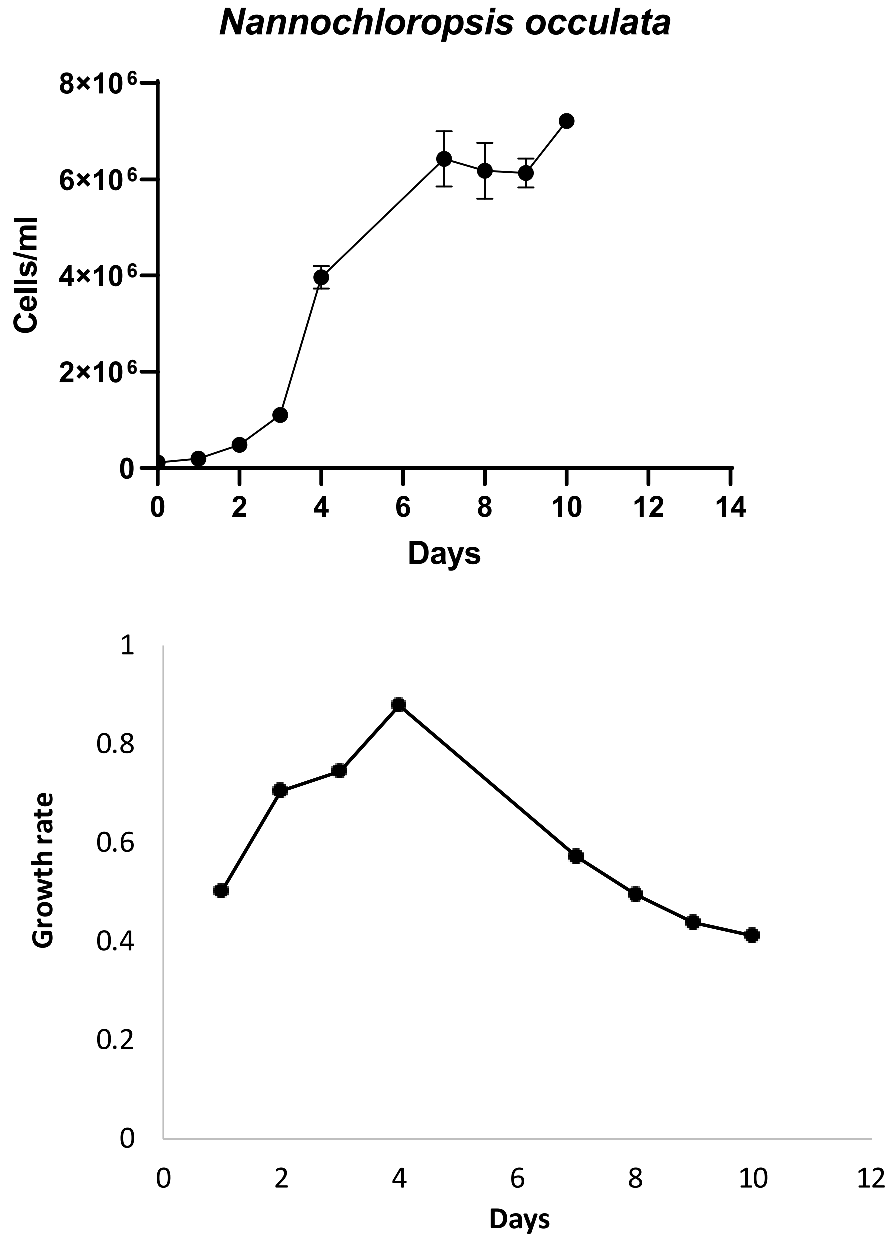

Figure 6. Characteristic growth and time-course profile of growth rate for *Nannochloropsis occulata* (strain K-1281).

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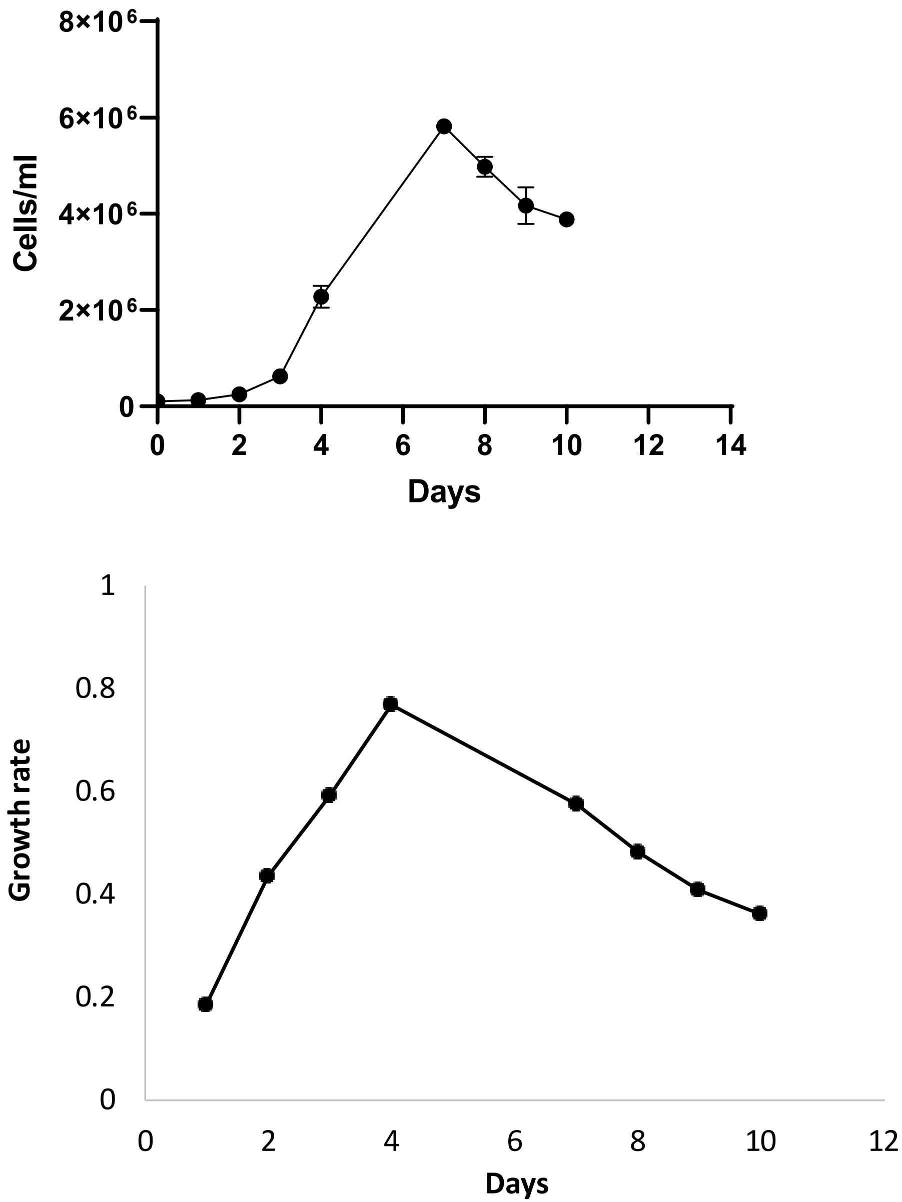

Figure 7. Characteristic growth and time-course profile of growth rate for *Nannochloropsis oceanica* (strain NIVA-2/03).

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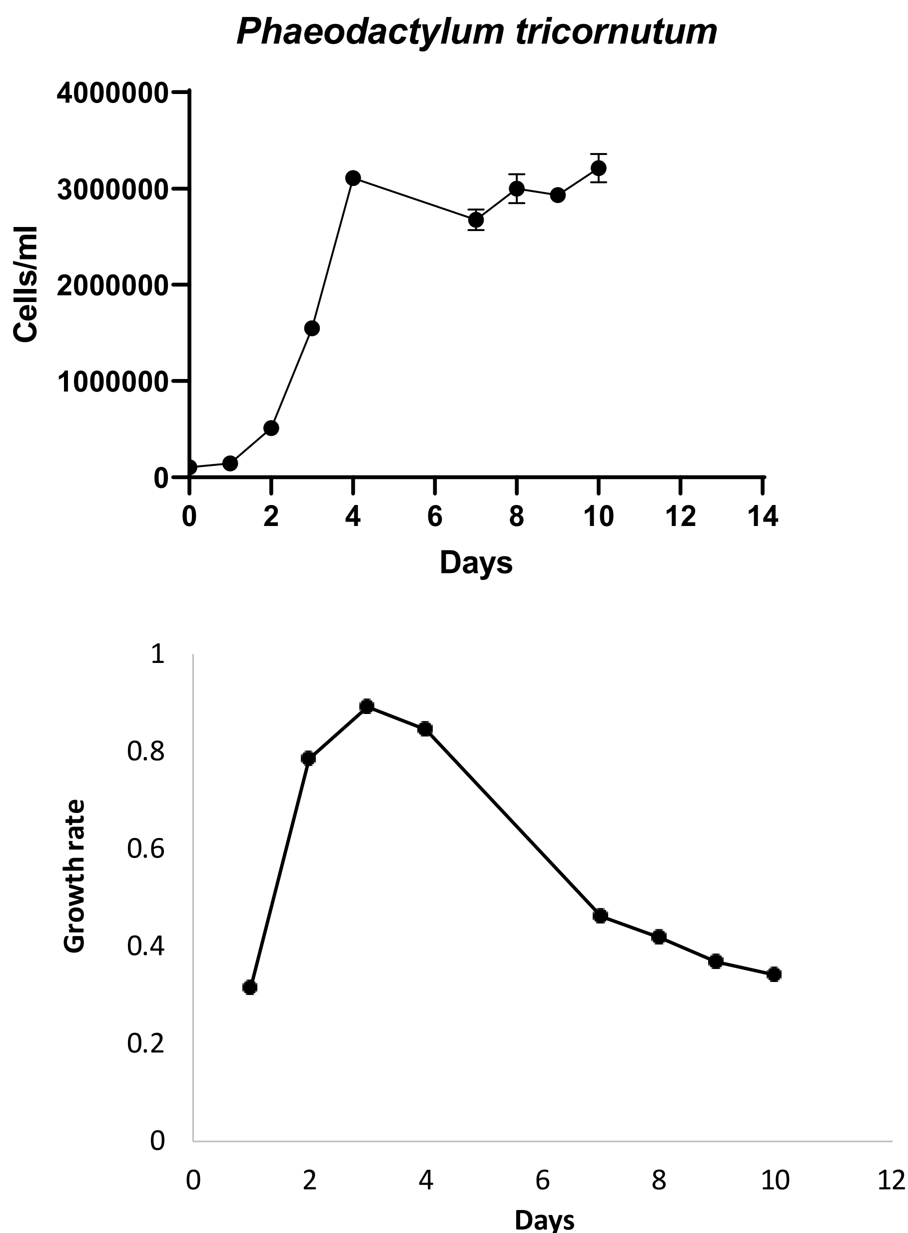

Figure 8. Characteristic growth and time-course profile of growth rate for *Phaeodactylum tricornutum* (strain NIVA-BAC 2).

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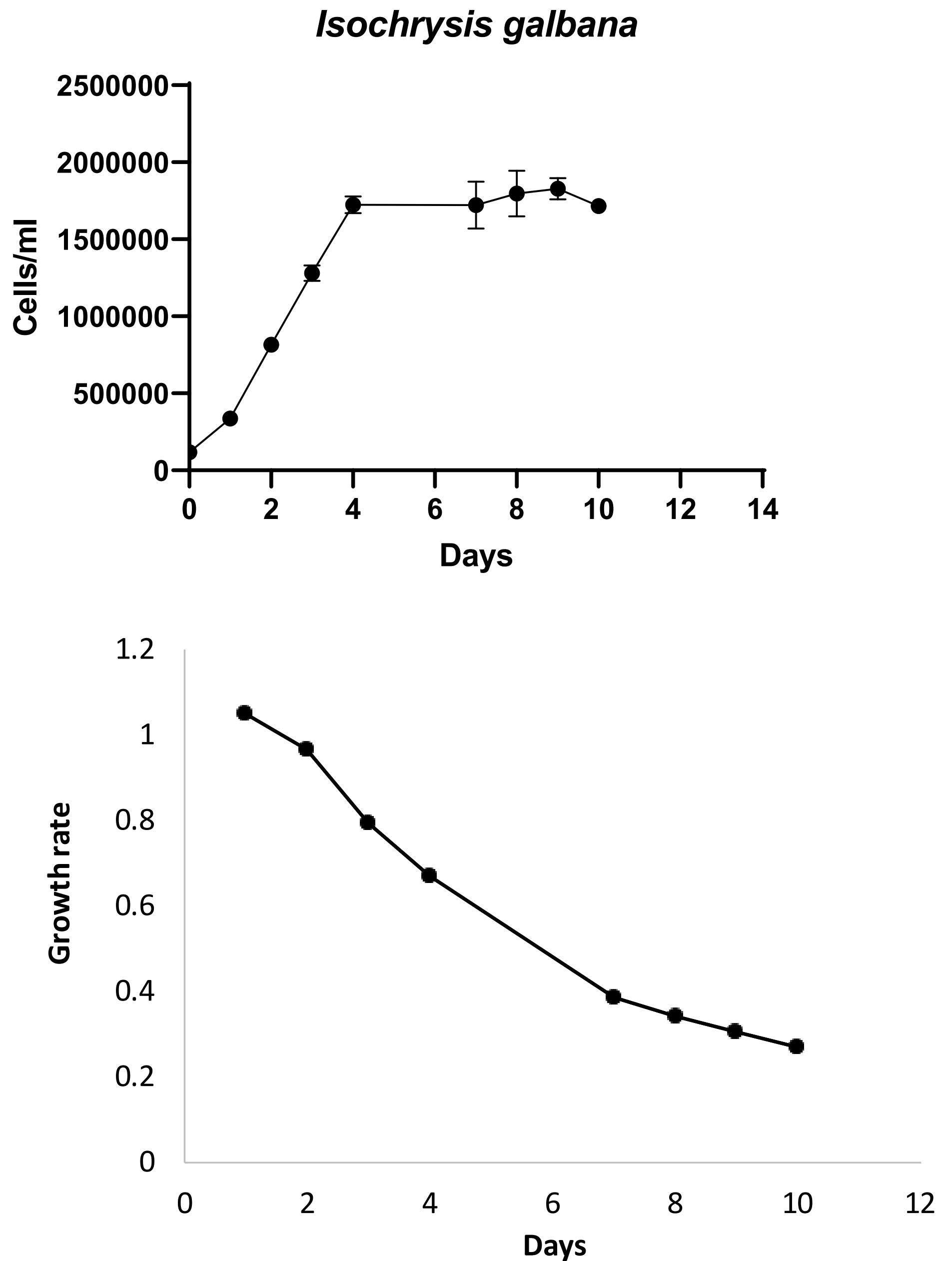

Figure 9. Characteristic growth and time-course profile of growth rate for *Isochrysis galbana* (strain NIVA-4/91).

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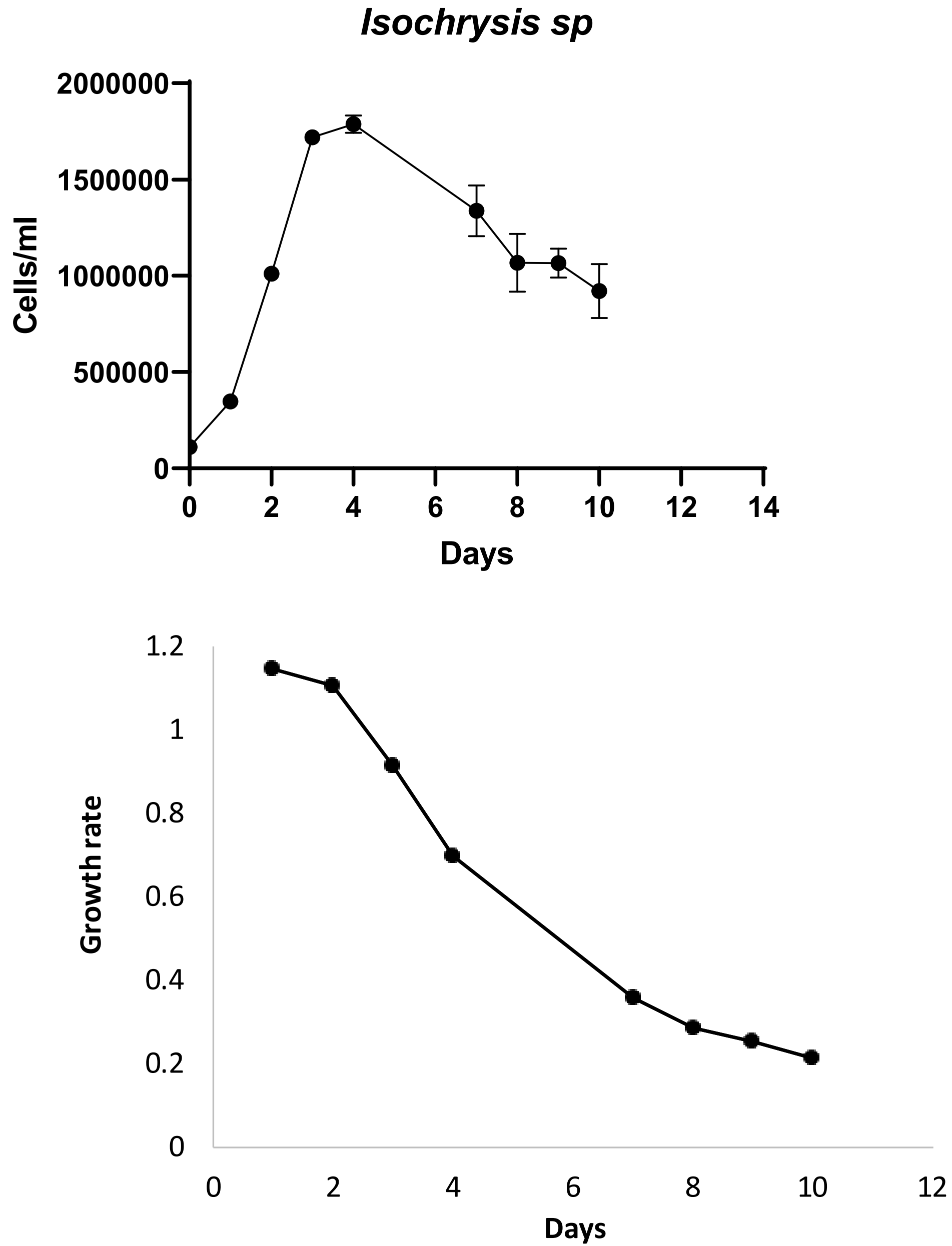

Figure 10. Characteristic growth and time-course profile of growth rate for *Isochrysis sp* (strain K-0633).

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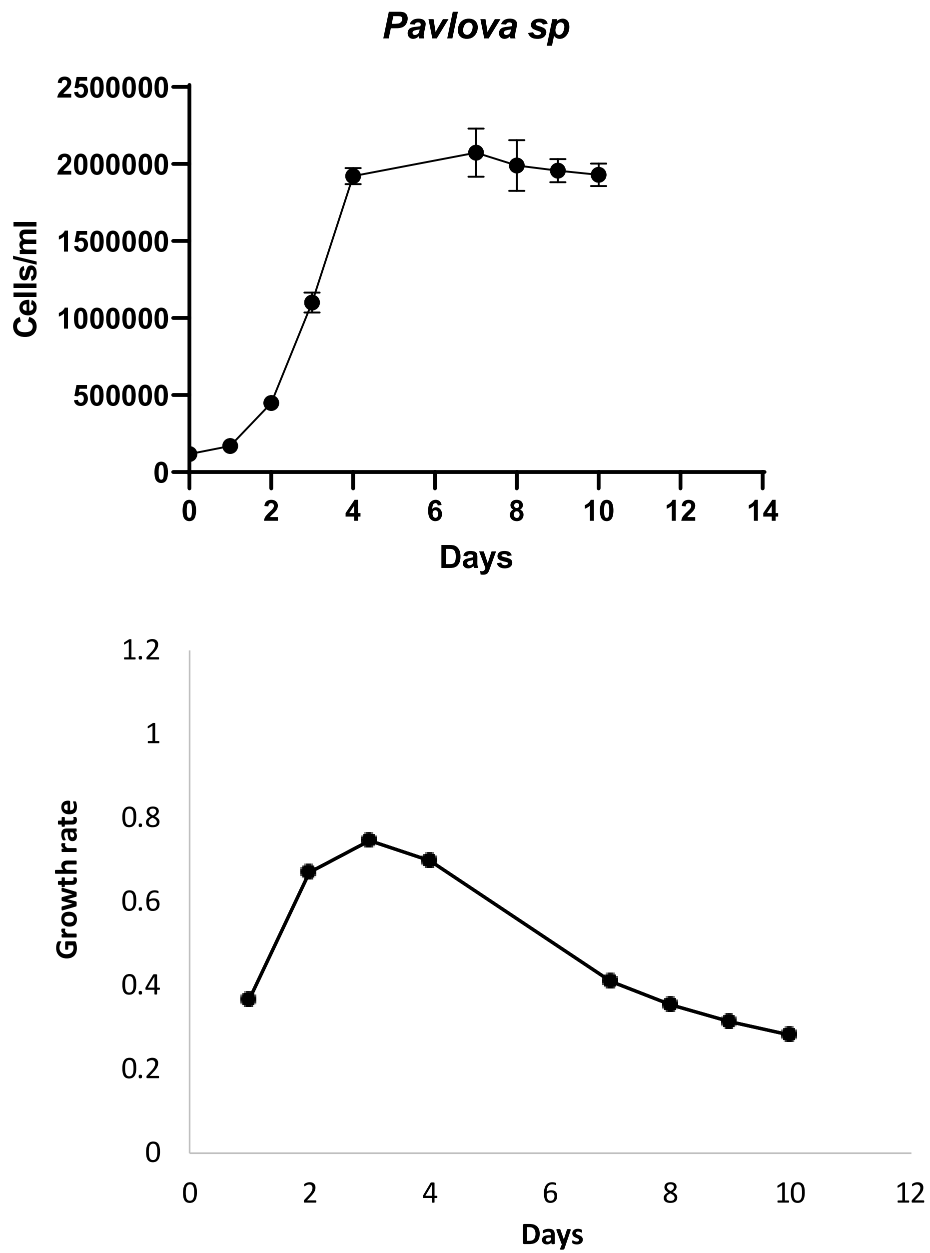

Figure 11. Characteristic growth and time-course profile of growth rate for *Pavlova sp* (strain NIVA-4/92).

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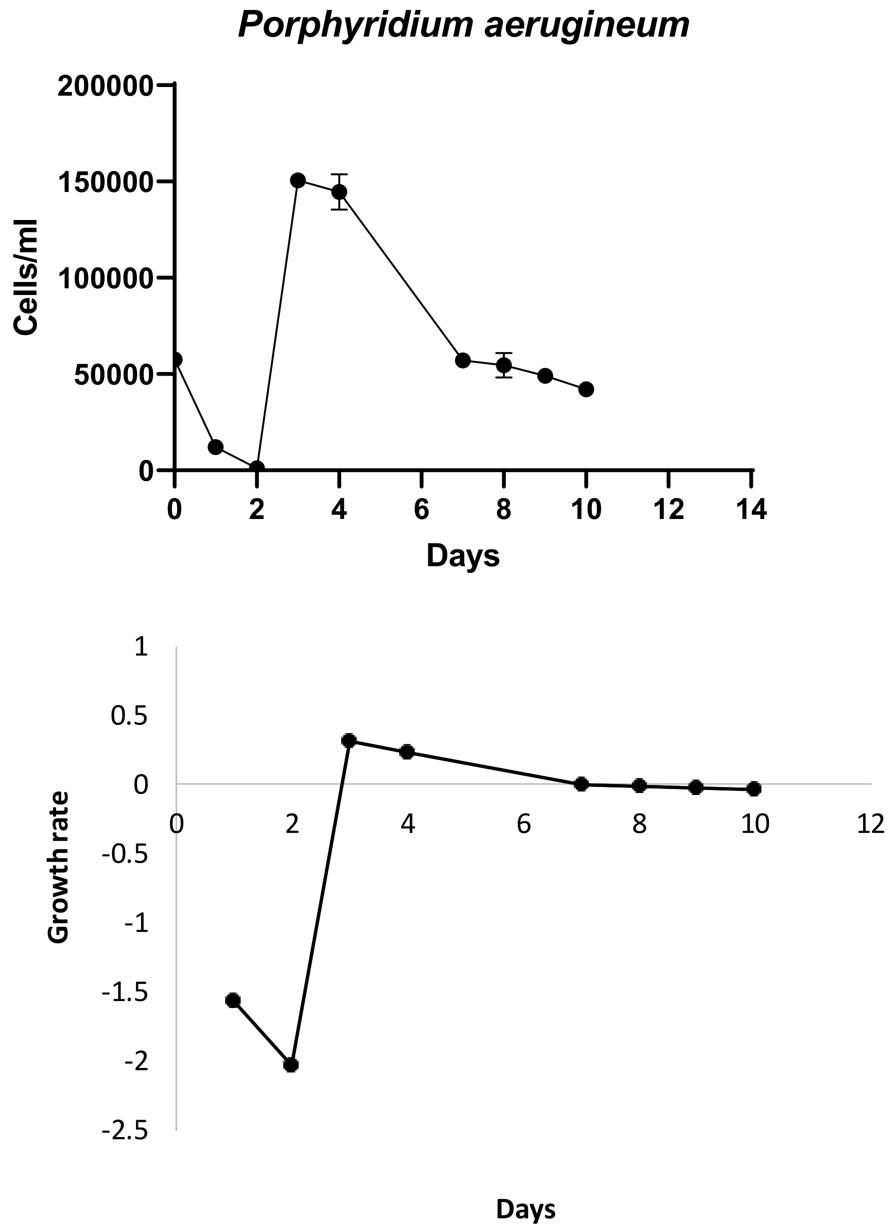

Figure 12. Characteristic growth and time-course profile of growth rate for *Porphyridium aerugineum* (strain K-1021).

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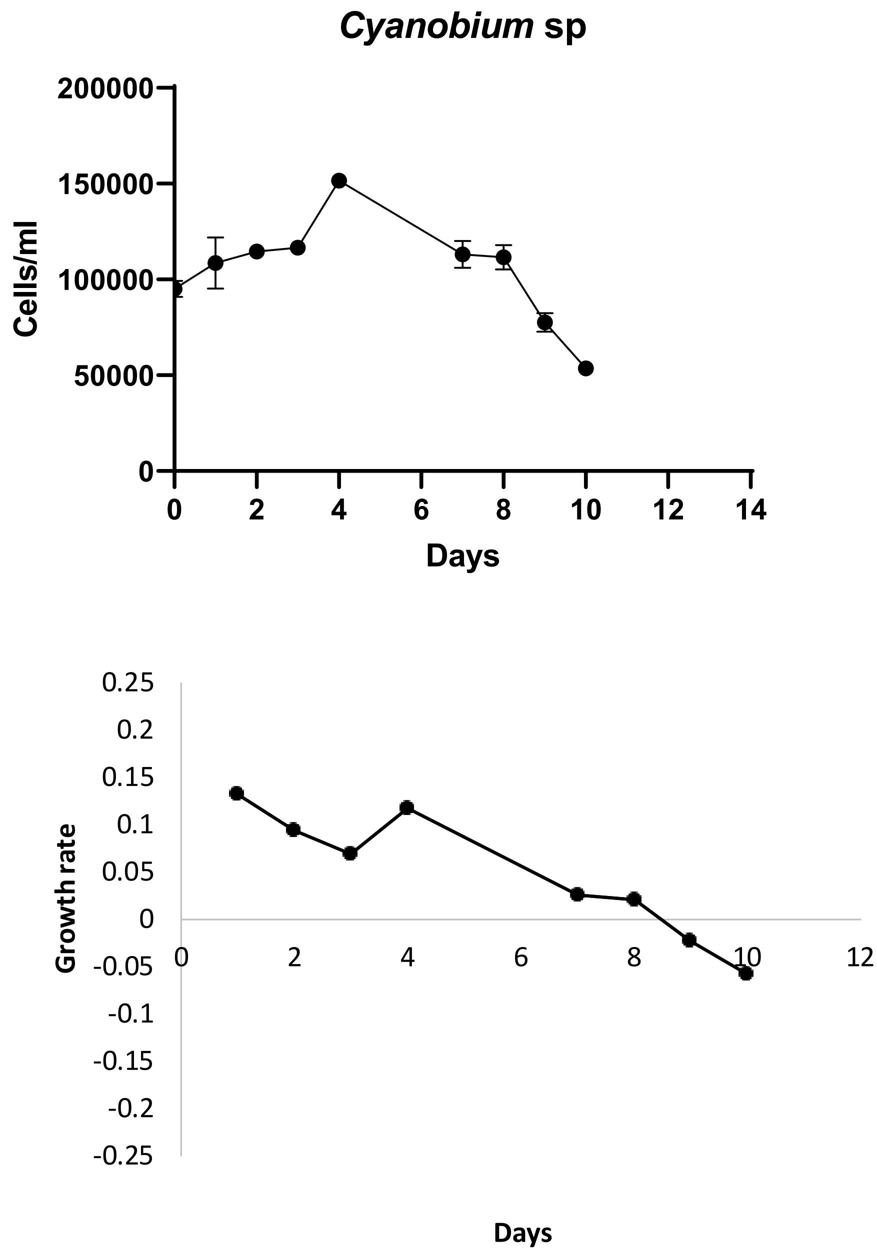

Figure 13. Characteristic growth and time-course profile of growth rate for *Cyanobium sp* (strain NIVA-CYA 375).

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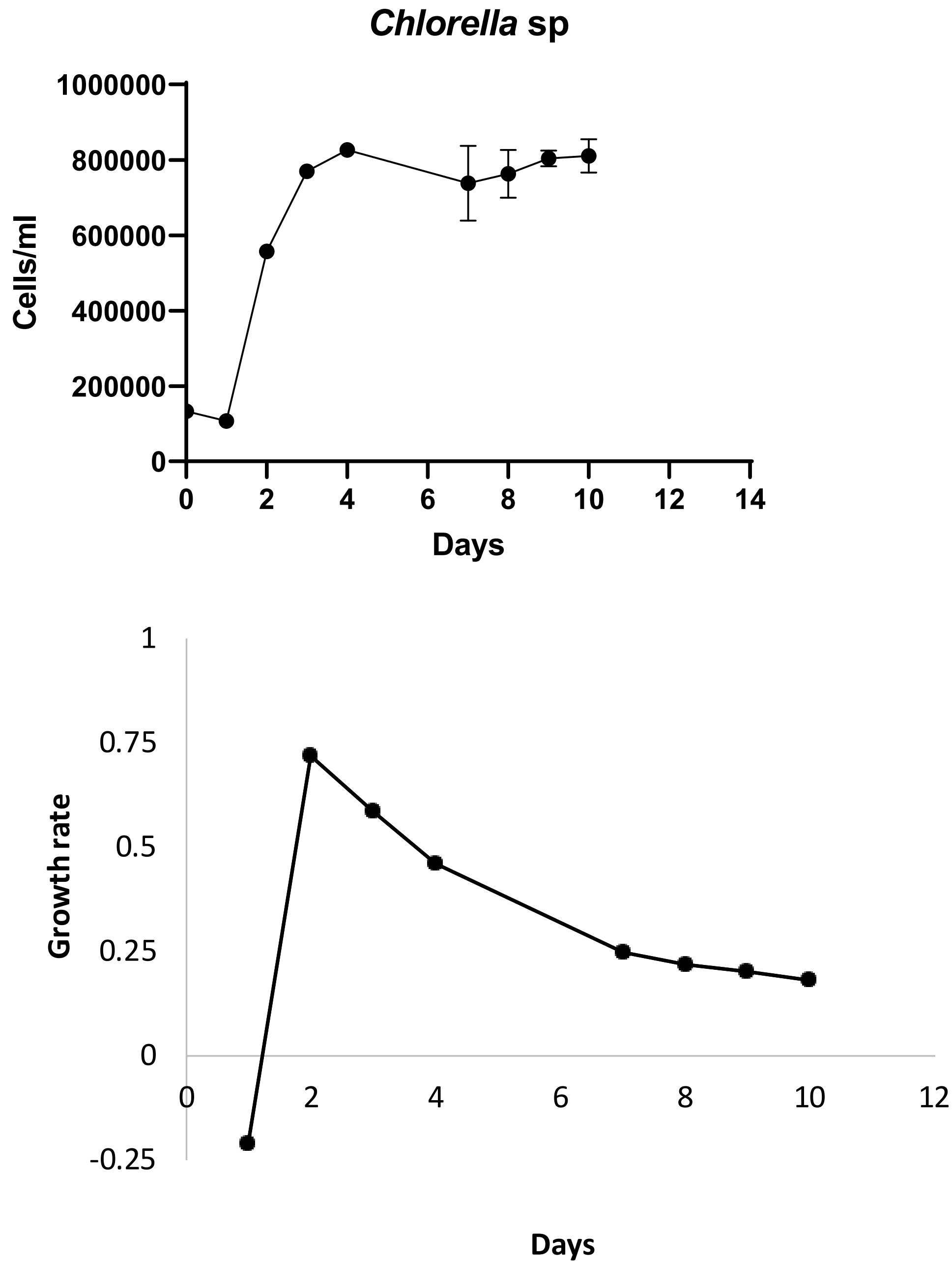

Figure 14. Characteristic growth and time-course profile of growth rate for *Chlorella sp* (strain NIVA-CHL 70).

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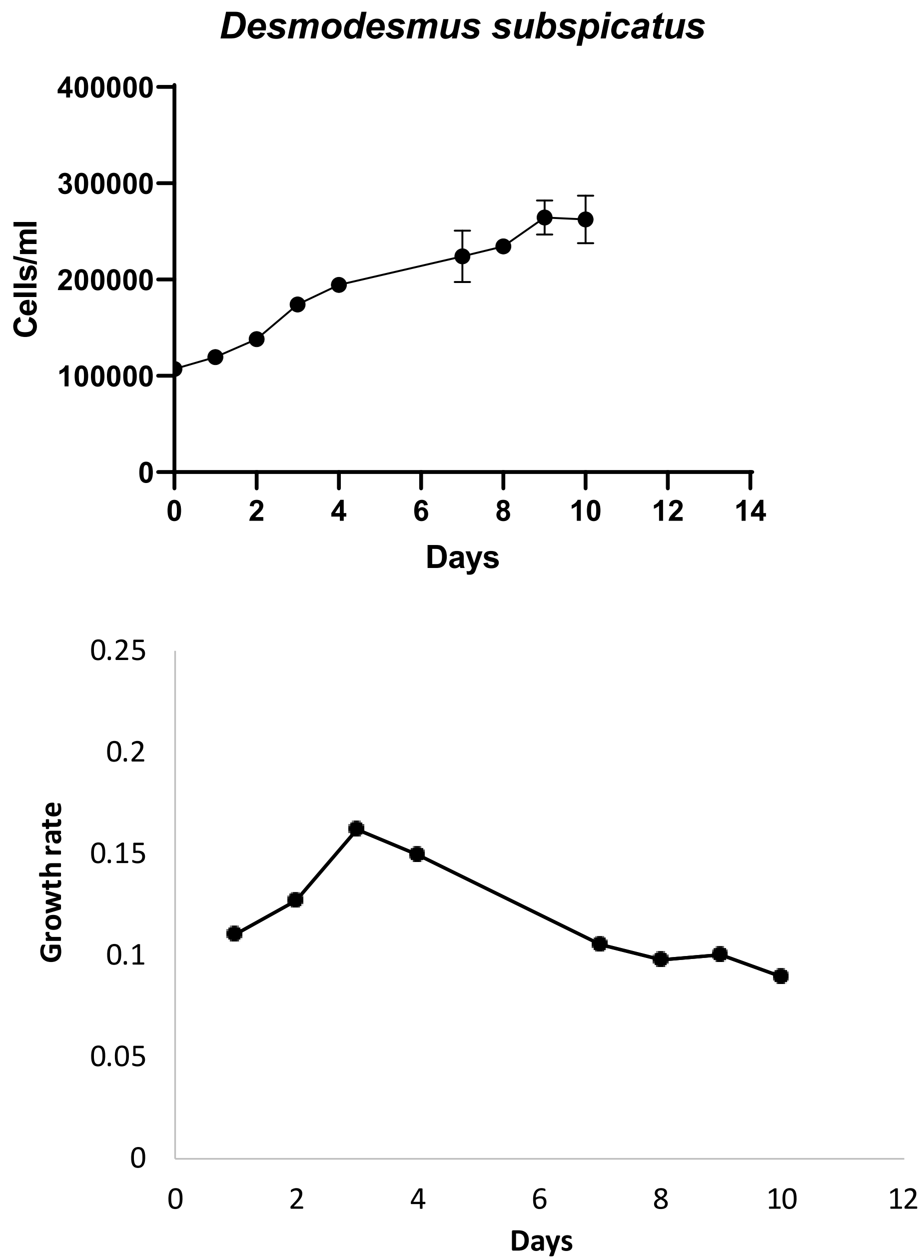

Figure 15. Characteristic growth and time-course profile of growth rate for *Desmodesmus subspicatus* (strain NIVA-CHL 55).

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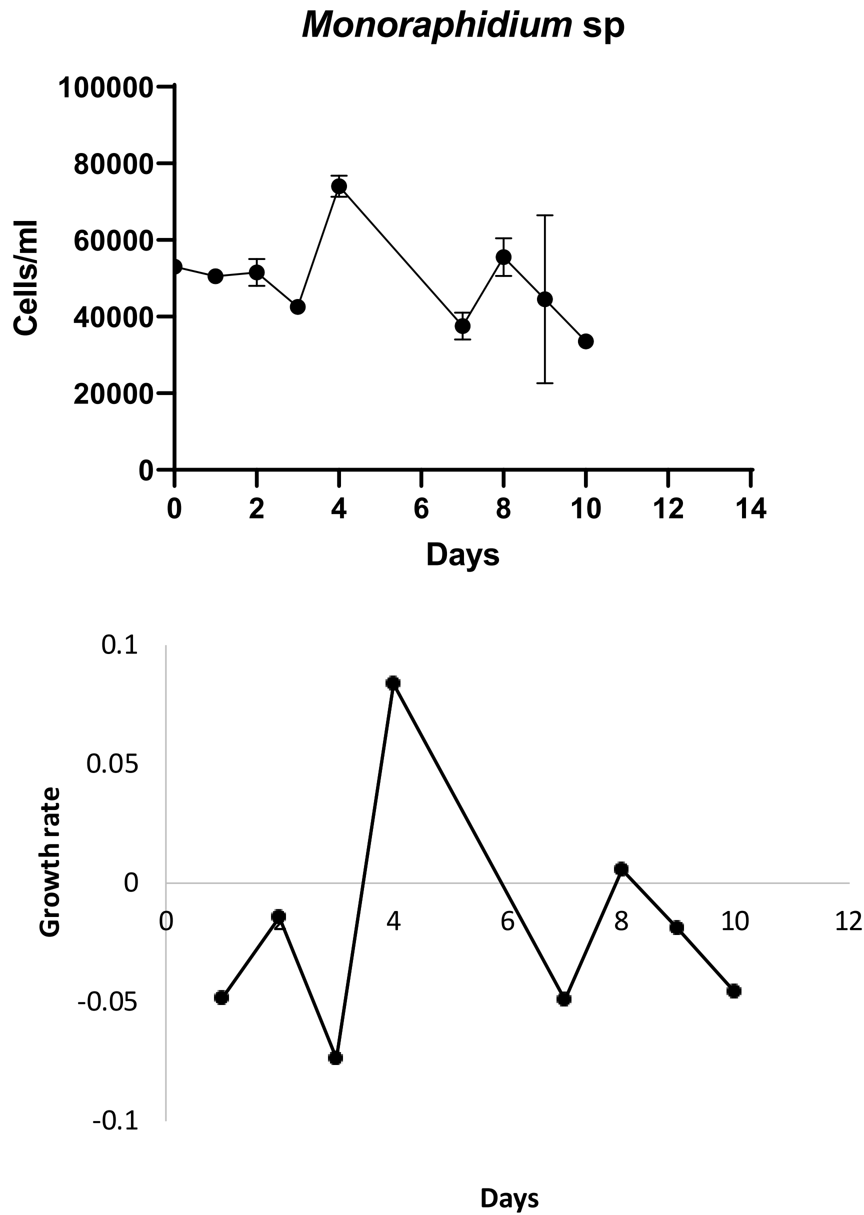

Figure 16. Characteristic growth and time-course profile of growth rate for *Monoraphidium sp* (strain NIVA-CHL 90).

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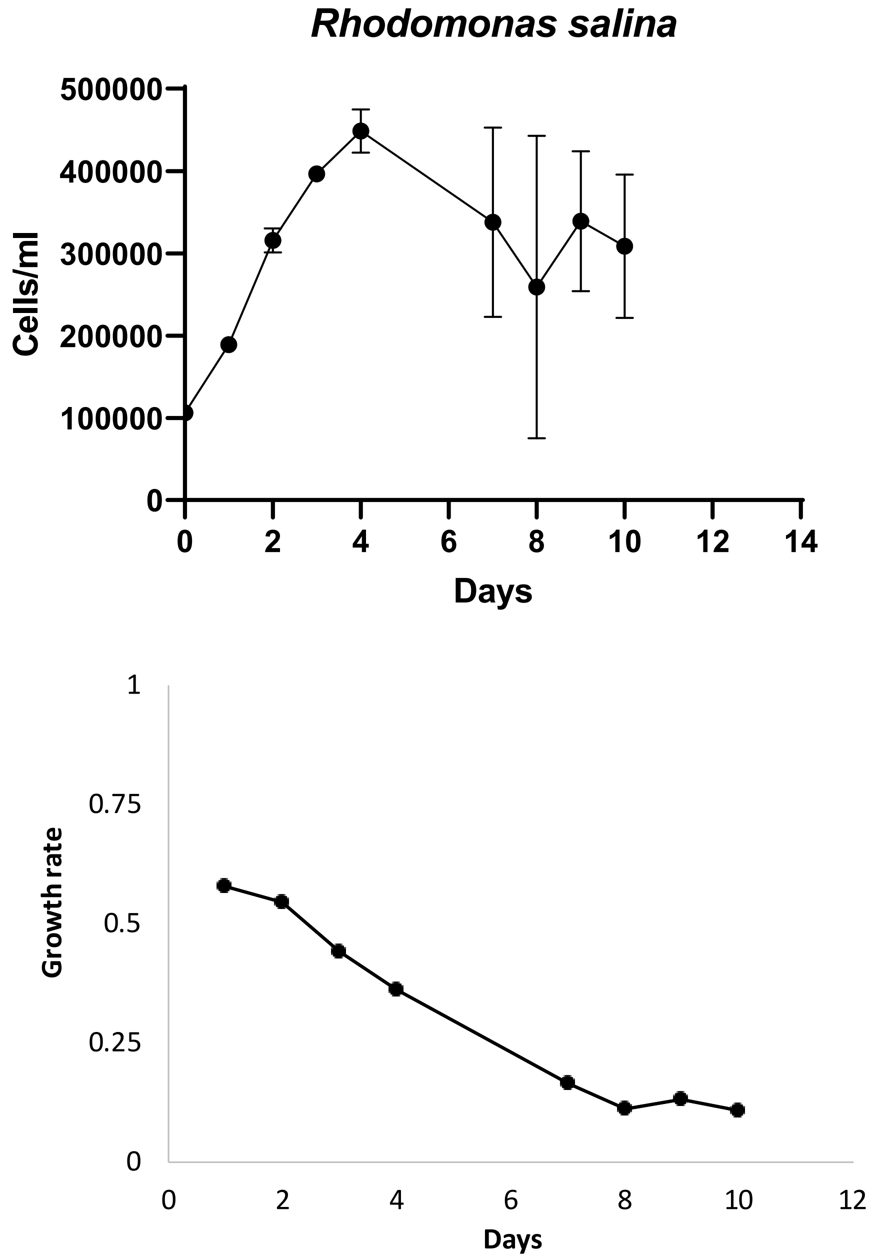

Figure 17. Characteristic growth and time-course profile of growth rate for *Rhodomonas salina* (strain NIVA-15/12).

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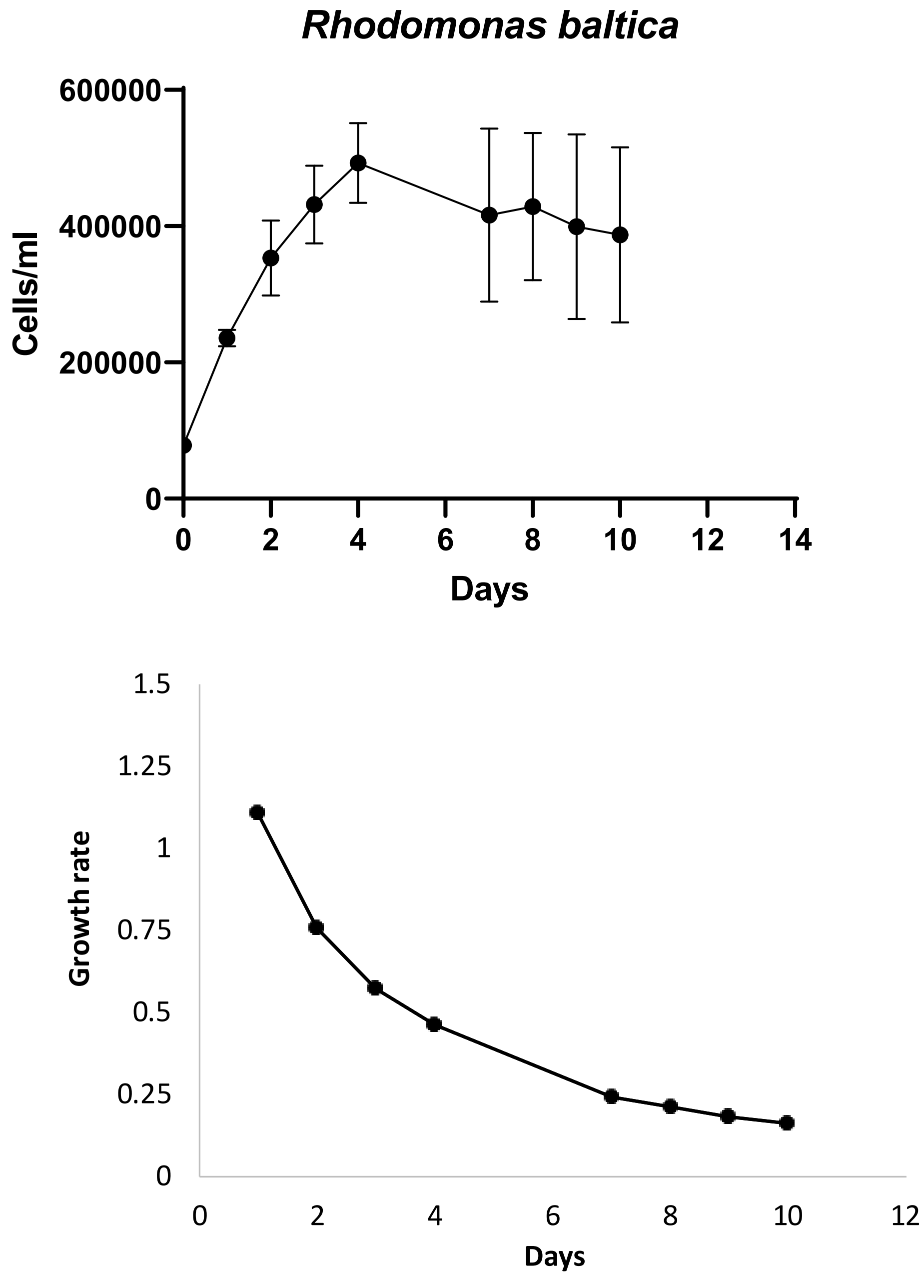

Figure 18. Characteristic growth and time-course profile of growth rate for *Rhodomonas baltica* (strain NIVA-5/91).

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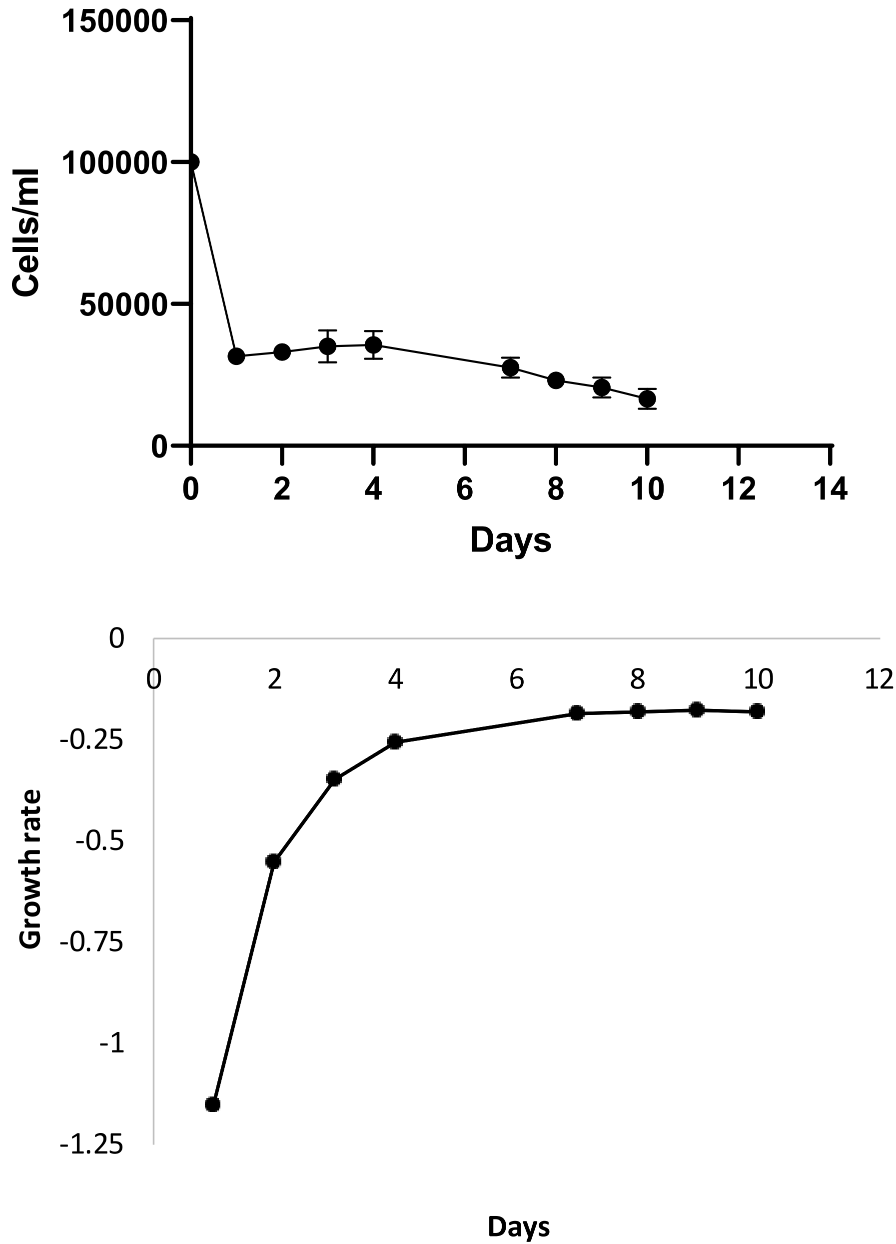

Figure 19. Characteristic growth and time-course profile of growth rate for *Cryptomonas sp* (strain NIVA-8/92).

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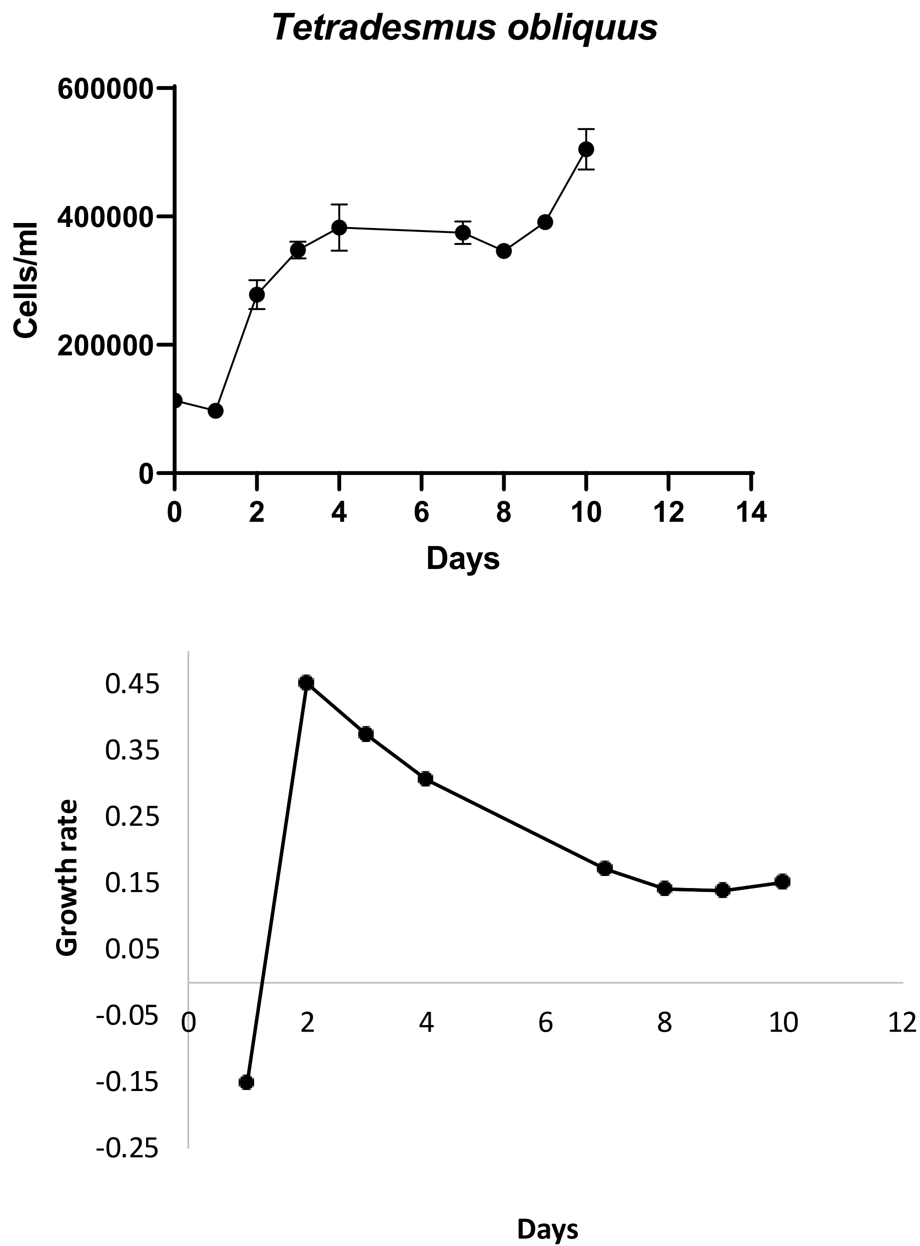

Figure 20. Characteristic growth and time-course profile of growth rate for *Tetradesmus obliquus* (strain NIVA-CHL 105).

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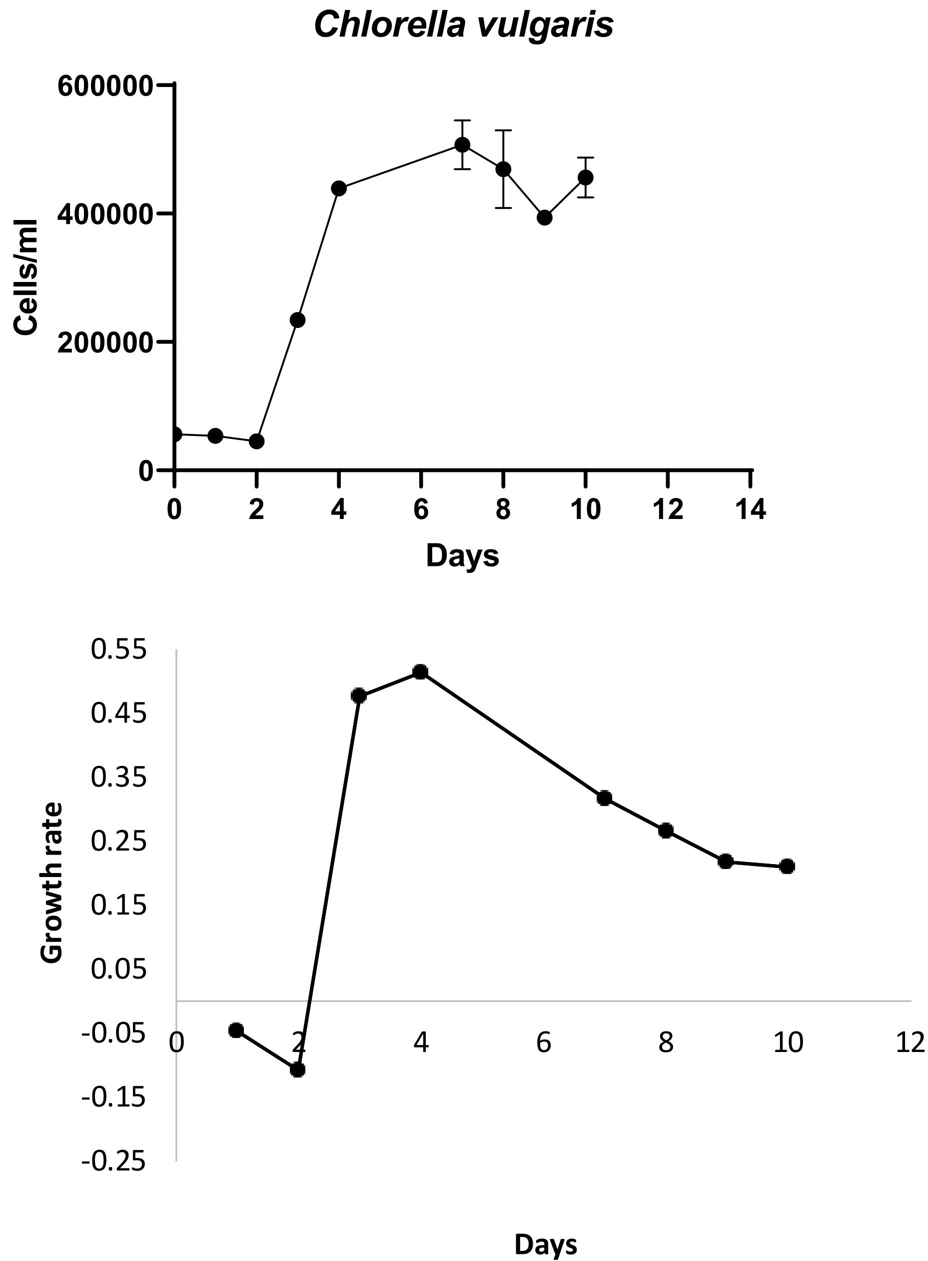

Figure 21. Characteristic growth and time-course profile of growth rate for *Chlorella vulgaris* (strain NIVA-CHL 19).

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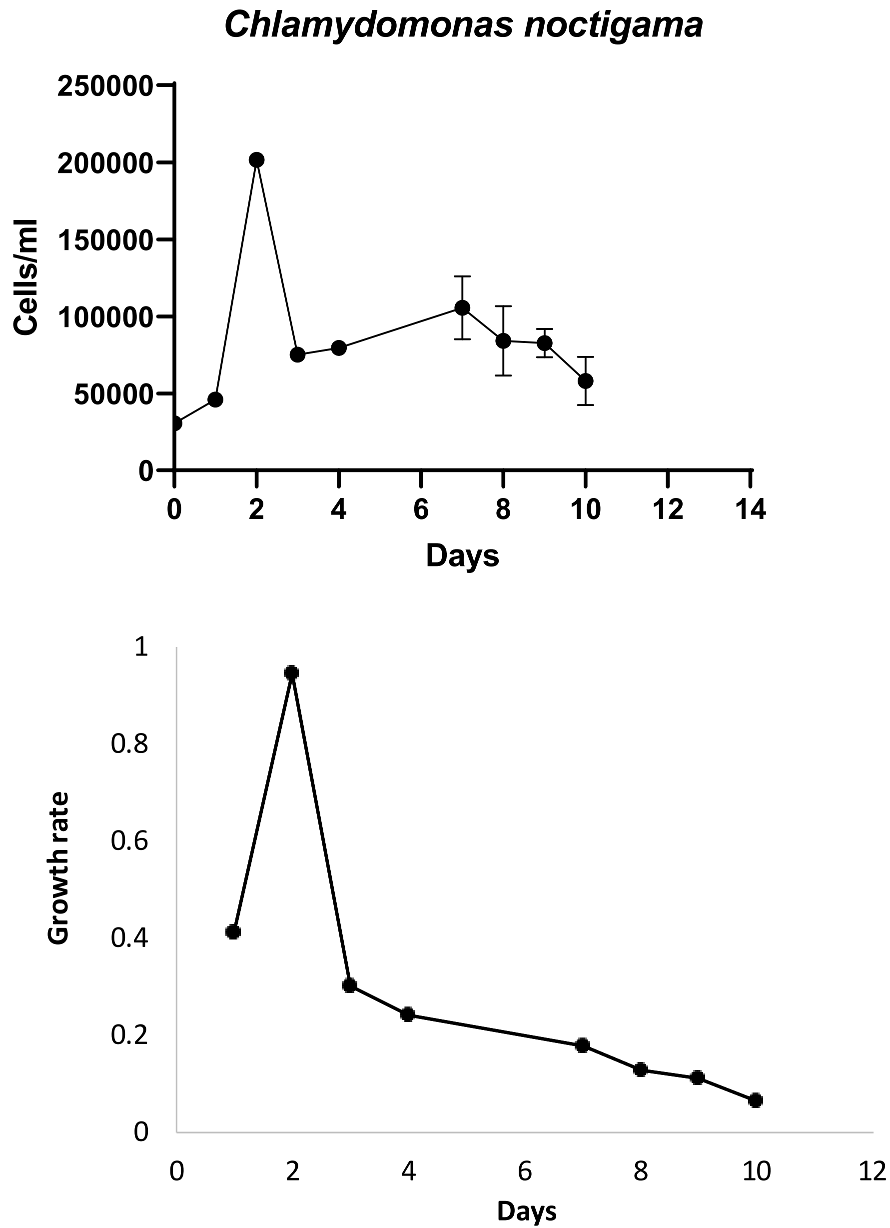

Figure 22. Characteristic growth and time-course profile of growth rate for *Chlamydomonas noctigama* (strain NIVA-CHL 168).

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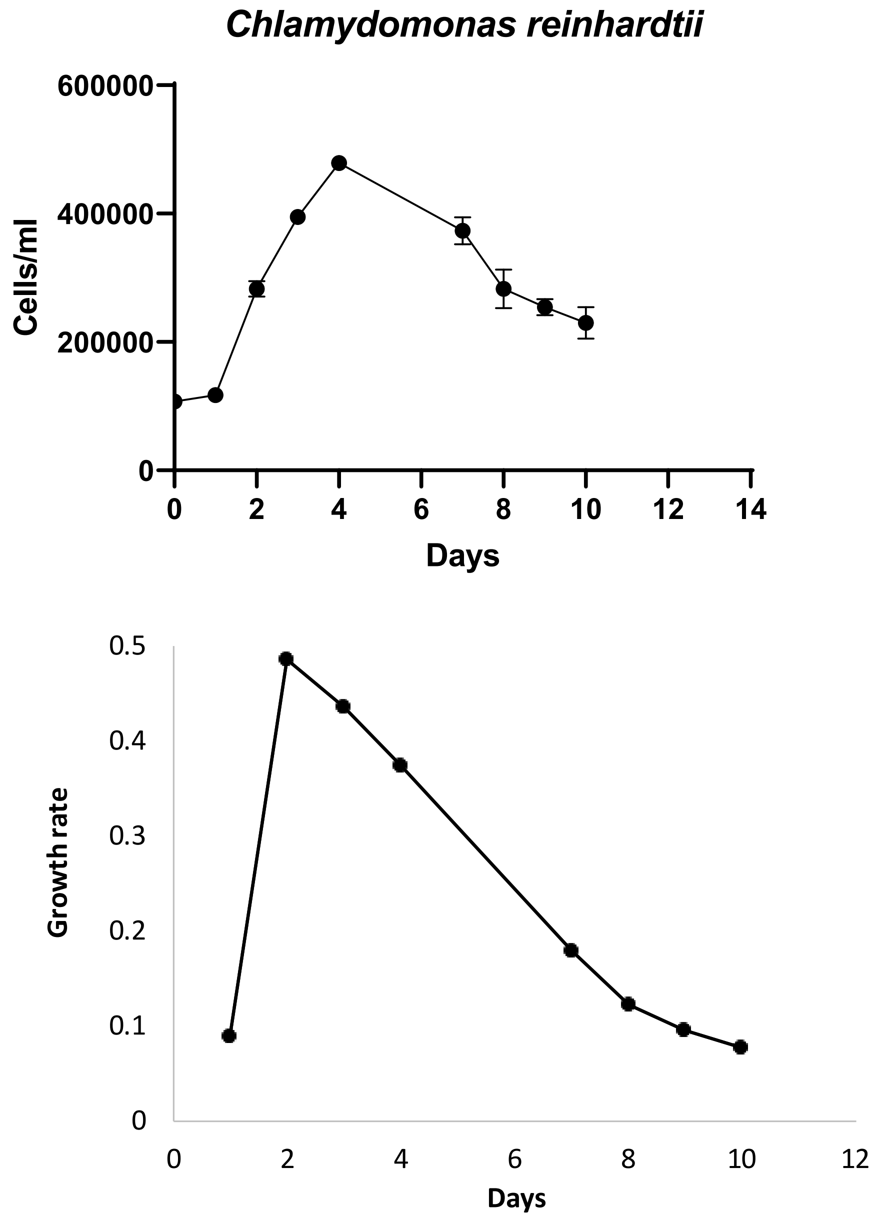

Figure 23. Characteristic growth and time-course profile of growth rate for *Chlamydomonas reinhardtii* (strain NIVA-CHL 153).

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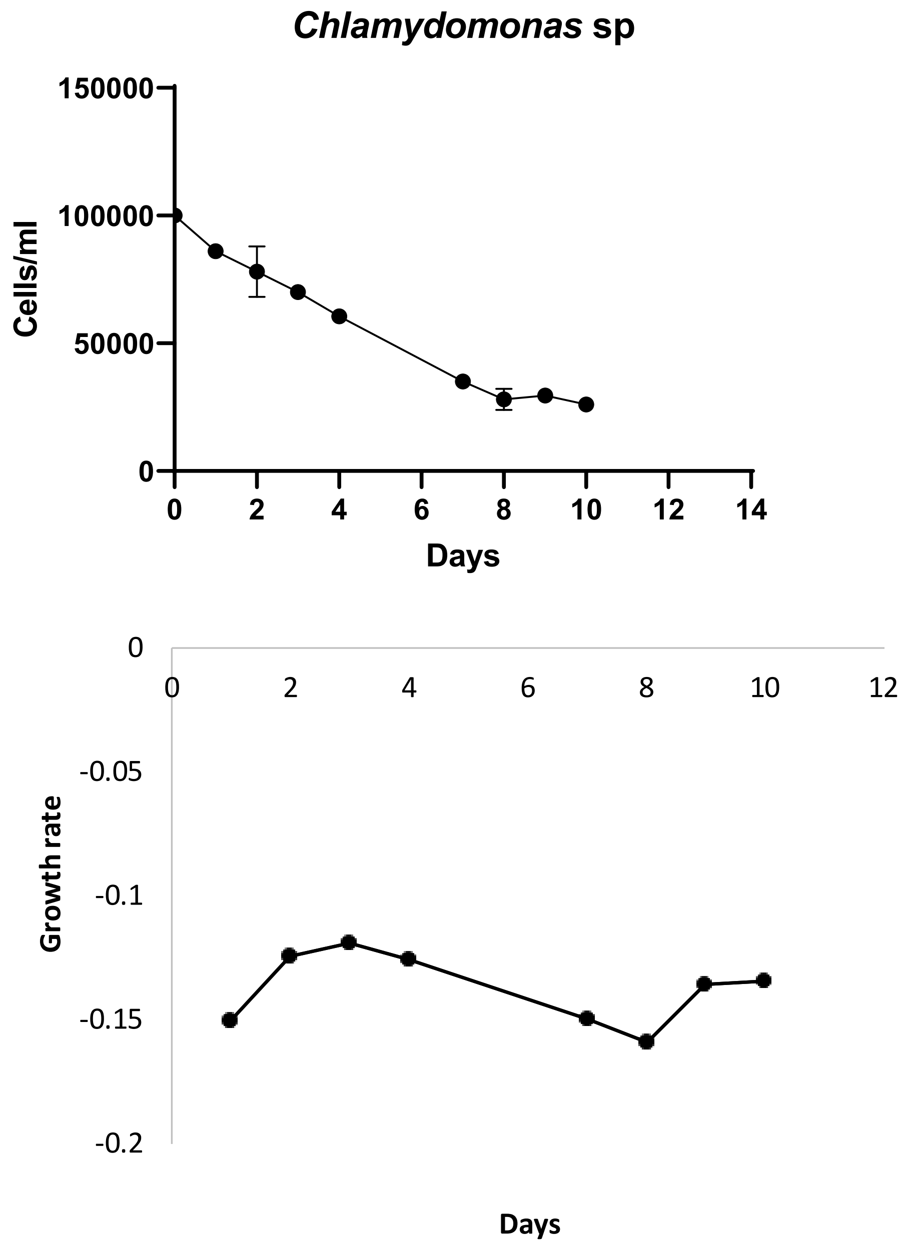

Figure 24. Characteristic growth and time-course profile of growth rate for *Chlamydomonas sp* (strain K-0493).

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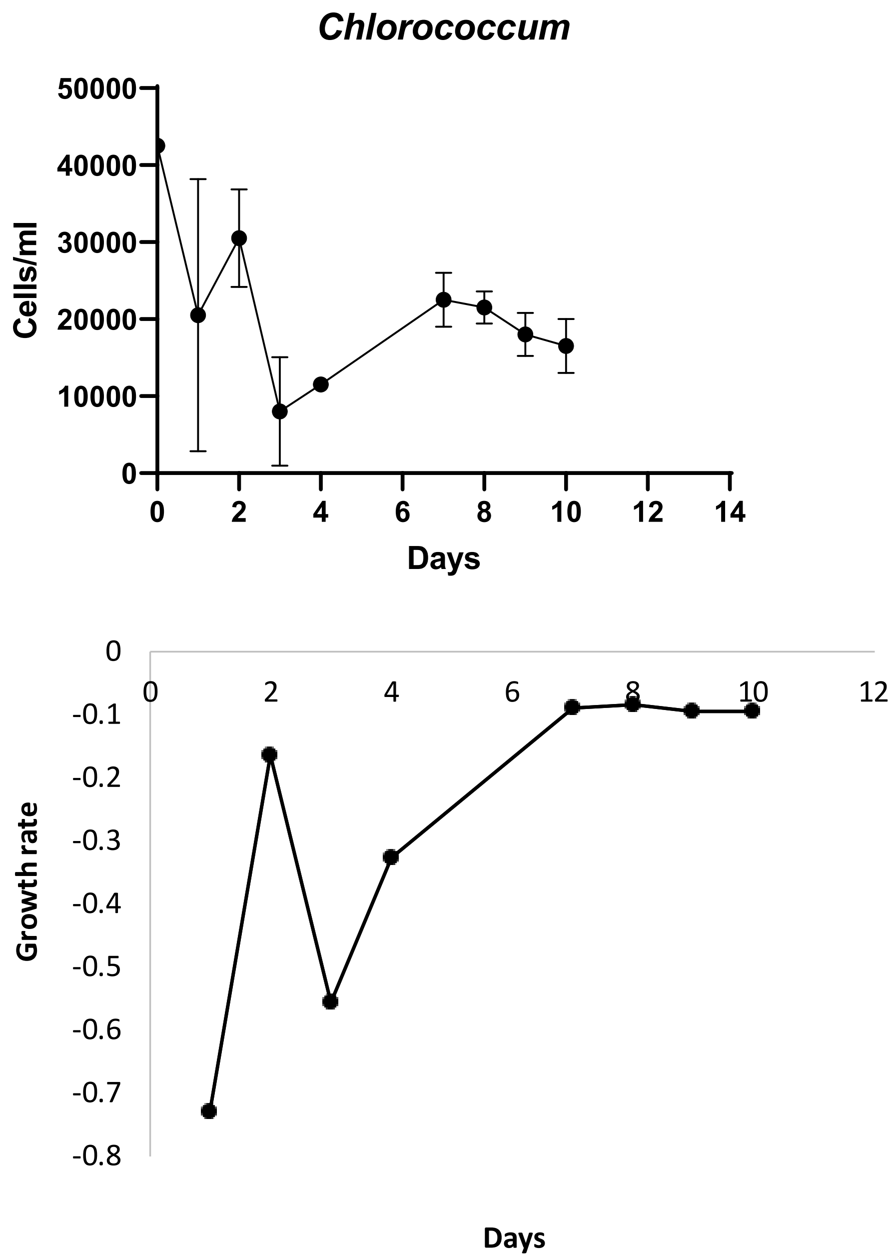

Figure 25. Characteristic growth and time-course profile of growth rate for *Chlorococcum* (strain NIVA-CHL 131).

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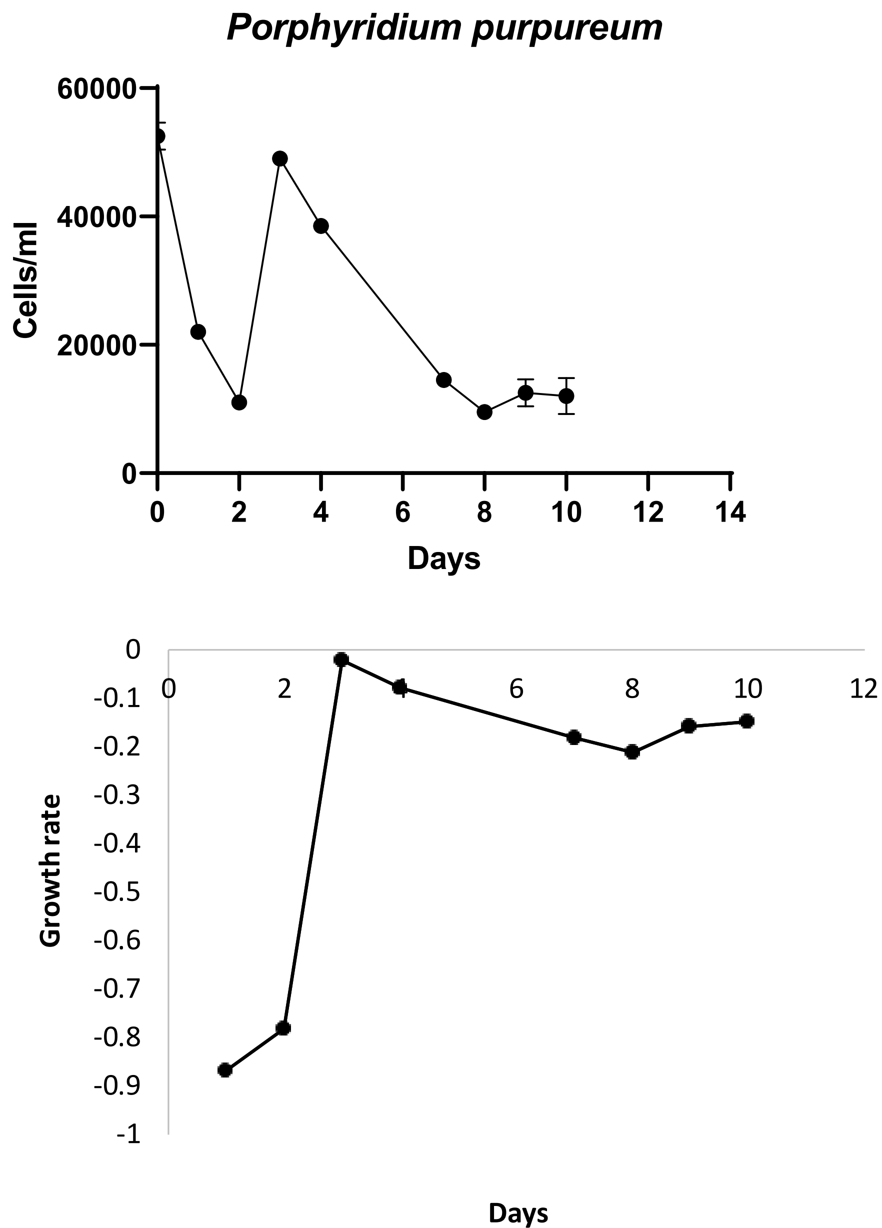

Figure 26. Characteristic growth and time-course profile of growth rate for *Porphyridium purpureum* (NIVA-1/92).

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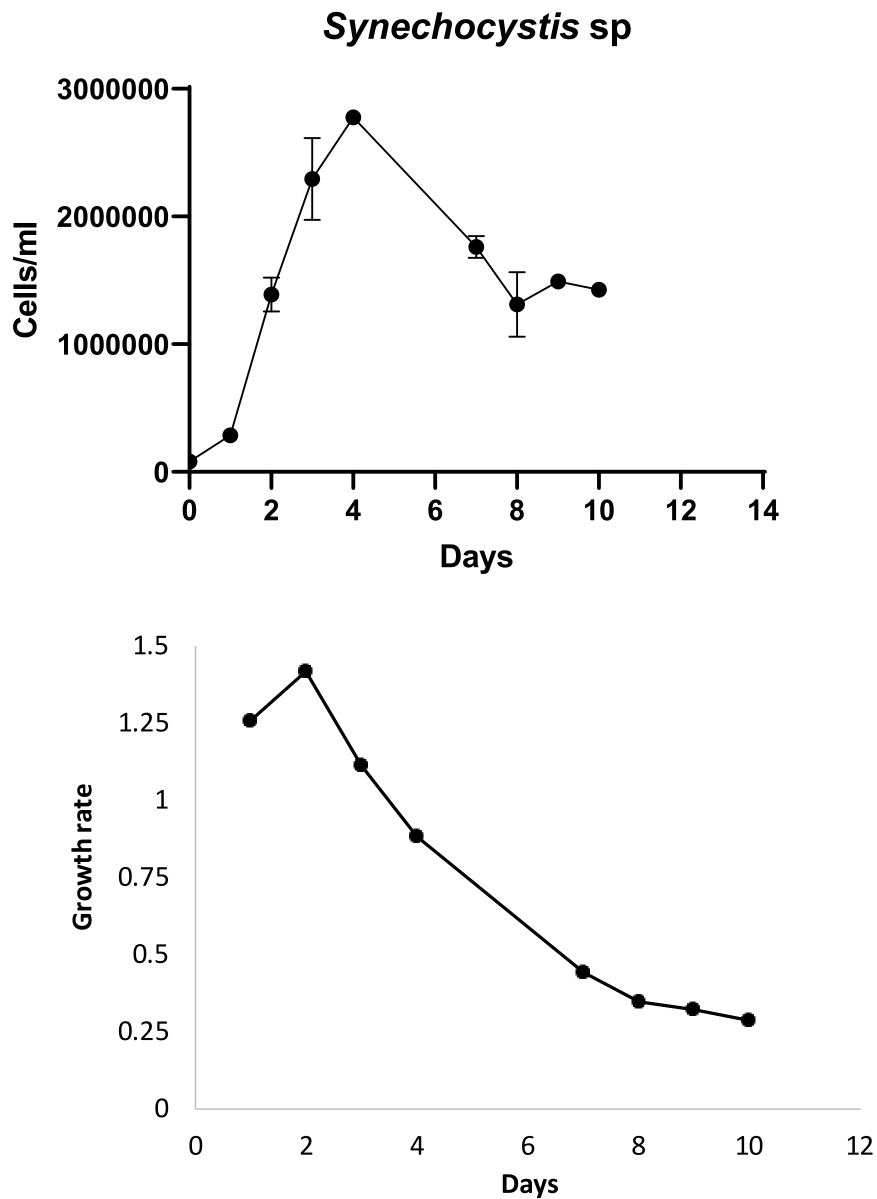

Figure 27. Characteristic growth and time-course profile of growth rate for *Synechocystis sp* (strain A1).

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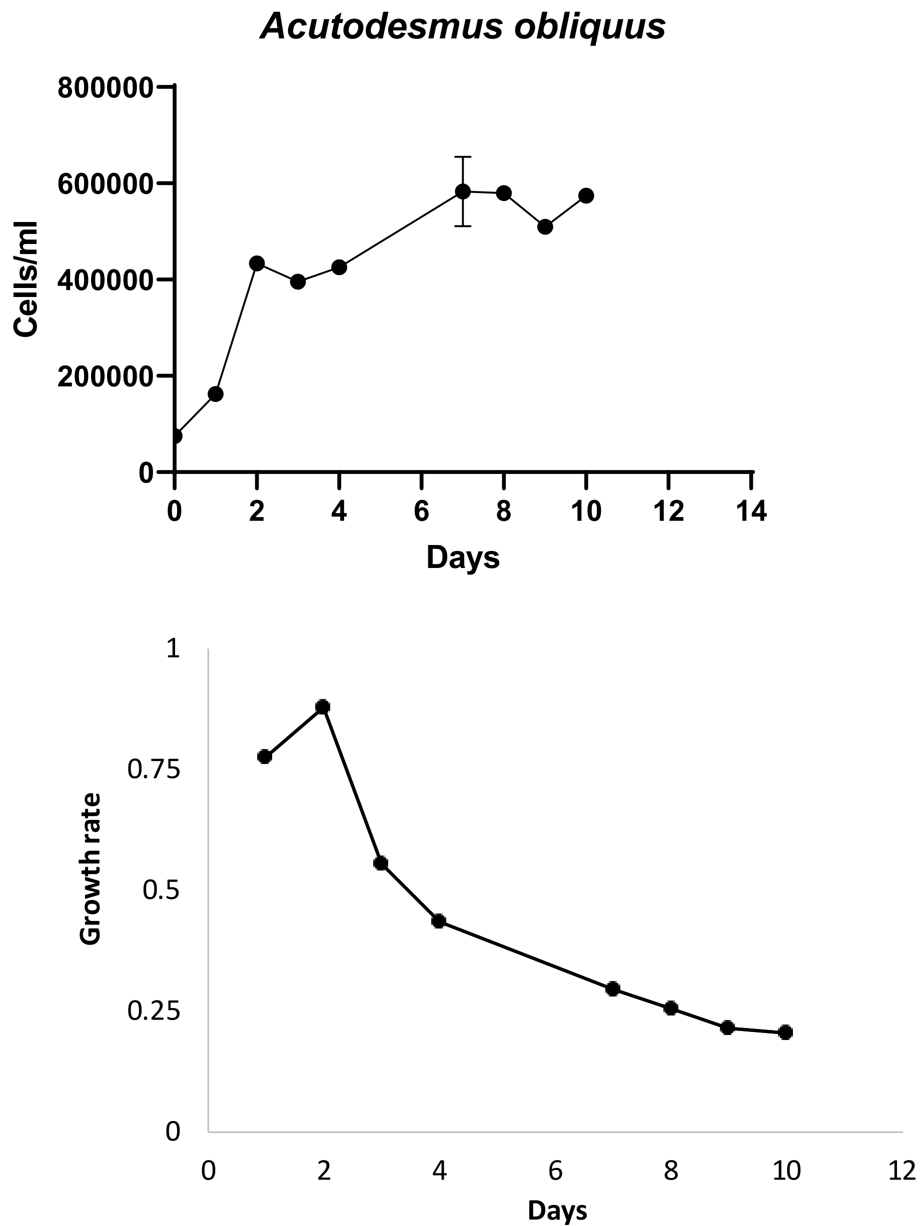

Figure 28. Characteristic growth and time-course profile of growth rate for *Acutodesmus obliquus* (strain A2).

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4.2. Summarizing growth characteristics of microalgae

Different culture media have been employed for the different strains, to provide the most suitable environment for them. The recipes employed to prepare the different culture media can be found as annex to this document.

The different microorganisms evaluated in this study have shown very different growth profiles. Both *Dunaliella* species (*D. salina* and *D. tertiolecta*) have quite similar cell densities and growth rate plots. Both managed to grow after inoculation, but their productivity was moderate, reaching a maximum cell concentration of approximately 550.000 (*salina*) and 410.000 cells/mL (*tertiolecta*) after 4 and 2 days of incubation respectively. After this maximum, the cell concentration slightly decreased and kept constant at about 500.000 in the case of *D. salina* whilst in the case of *D. tertiolecta*, a pronounced decay occurred after 2 to 3 days to reach a relatively stable concentration of about 275.000 cells/mL. Two of the *Tetraselmis* strains available at the NORCCA collection were evaluated. Both of them showed a similar growth profile but they didn't manage to form a dense culture in the conditions employed.

A very successful microalgae in terms of growth yield was *Isochrysis galbana*, that in only 4 days after inoculation managed to grow from 100.000 to 1.750.000 cells/mL. Also, *Phaeodactylum tricornutum,* that reached a stable concentration about 3.000.000 after only 4 days and kept this high biomass concentration almost constant till the end of the experiment.

Among the most productive strains in this study, we found *Nannochloropsis occulata* and *Nannochloropsis oceanica.* These strains managed to grow from the inoculating concentration (about 100.000 cells/mL) up to 7.000.000 cells/ml (*N. occulata*) and 6.000.0000 cells/ml (*N. oceanic*a) in 10 and 7 days respectively. The growth rates of both were quite similar, showing an increasing trend from inoculation till day 4 and afterwards decreasing till the end of the experiment.

Also, *Isochrysis galbana* showed an excellent productivity, growing very fast from the inoculation point to reach a maximum and constant concentration the day 4, with about 1.750.000 cells/mL. *Isochrysis sp* also showed a high productivity in the time span from 0 to 4 days, reaching a maximum cell concentration about 1.800.000 cells/mL in the day 4. After this time point, the culture started losing cells and reached a concentration about 1.000.000 cells/mL at the end of the experiment. *Pavlova sp* showed a remarkable growth along the first 4 days, achieving a stable cell concentration around 2.000.000 cells/ml that remained constant till the end of the assay. The cyanobacterium *Synechocystis sp.* started growing extremely fast from the beginning of the experiment and reached its maximum concentration at day 4 (about 2.800.000 cells/mL). This maximum was followed by a decrease in the cell concentration to reach approximately 1.500.000 cells/mL at day 9.

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Other strains such as *Porphyridium aerugineum, Cyanobium sp, Monoraphidium sp, Cryptomonas sp, Chlamydomonas noctigama, Chlamydomonas sp, Chlorococcum* and *Porphyridium purpureum* didn't managed to produce a dense culture under the conditions explored (even though each strain was transferred to suitable culture media and kept under stable growth conditions).

4.3. Tracking the growth of macroalgae

Growth of the macroalgae *U. ohnoi* was measured as the increase in fresh weight biomass per cultivation unit in a specific time interval. Relative growth rate (RGR), expressed as % d^{-1} was calculated using the exponential growth equation with the following equation, where W_t and W_0 are final and initial fresh weight (determined after spin drying in domestic or professional lettuce dryer until no more water came out) respectively and t is time (days):

$$RGR = \frac{\ln W_t - \ln W_0}{t} \ (\times 100\ \%)$$

Indoor growth in photobioreactors was started in 2019. For the purpose of biomass production and its use in different projects, initially 6 PBRs were employed simultaneously and growth was monitored weekly. In 2020, in the new facility, two PBRs were used dedicated to SIMBA. Growth showed considerable variation; periods of stable growth (average growth rates of 10 % d^{-1}, average yield per PBR of 104 g FW) were alternated with weeks of considerable variation (**Figures 29** and **30**).

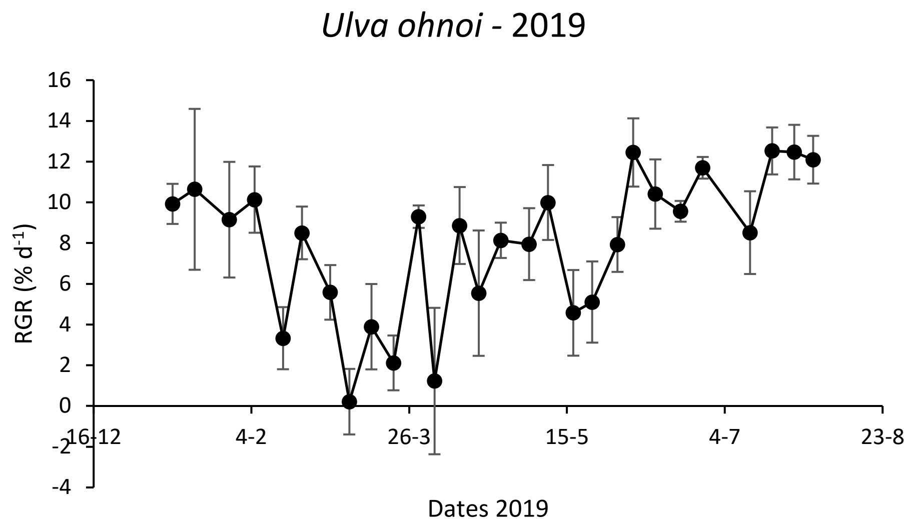

Figure 29. Growth of *Ulva ohnoi* in indoor photobioreactors at CTAQUA in 2019. Error bars indicate ± 1 SD.

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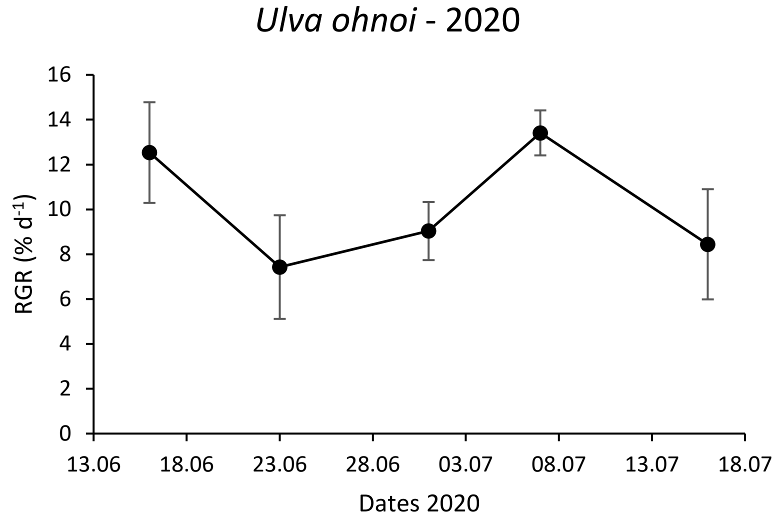

Figure 30. Growth of *Ulva ohnoi* in indoor photobioreactors at CTAQUA in 2020. Error bars indicate ± 1 SD.

Collapses were observed in February and March, most likely due to low day temperatures. Only few data are available for 2020 thus far with the PBRs in the new facility. It appears that same growth rates can be obtained as in 2019, with similar variations. An infection of the algae with the parasitic brown macroalga *Myrionema* cf. *strangulans* Greville was observed to coincide with a drop in growth rates, however, *U. ohnoi* seemed capable to overcome this and the infection could no longer be traced thereafter. The origin of the infection is unclear. Cultures in the PBRs are well separated from the other cultures and the materials used for their maintenance are not used for other cultures. The fact that the infection was detected at the same time in both the PBRs, the outdoor tanks (see below) and the field, suggests that the water has been the source. Although cultivation water is treated and 1 µm filtered before use, it has been observed that bacterial infections can take place. Alternative explications include the existence of “dormant” spores of the parasite that are triggered by certain conditions (high temperatures for instance) or even aerial transport of spores (also see Conclusion).

U. ohnoi thrived at all three salinities tested. Although growth appeared slightly higher at reduced, the differences were insignificant (ANOVA, $p>0.05$, **Figure 31**).

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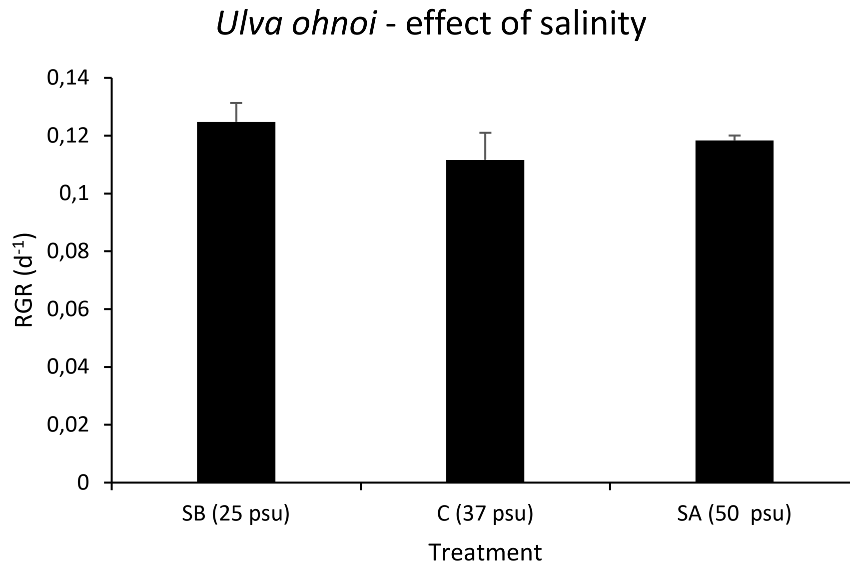

Figure 31. Growth of *Ulva ohnoi* in indoor photobioreactors at three different salinities. Error bars indicate ± 1 SD.

Nitrogen source did have a statistically significant effect on growth rate (ANOVA, $p<0.05$).; algae grown on ammonium achieved approximately 10% higher growth rates compared to algae grown on nitrate (**Figure 32**).

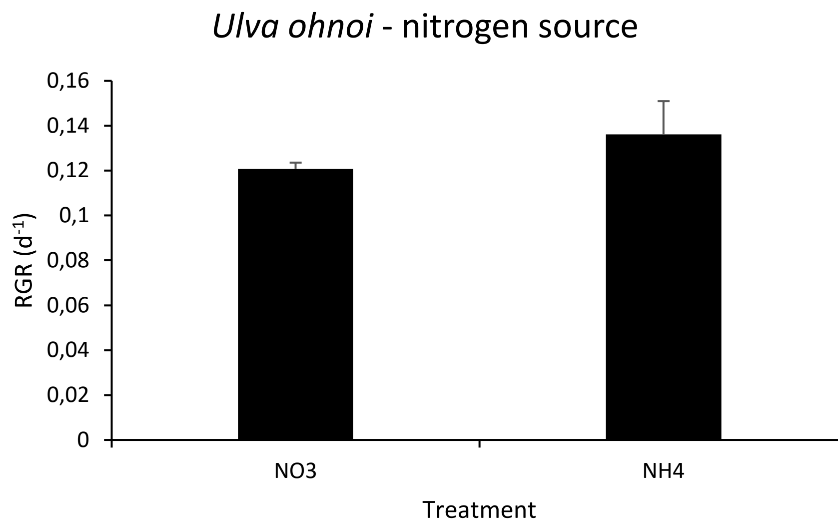

Figure 32. Growth of *Ulva ohnoi* in indoor photobioreactors at two different nitrogen sources (nitrate, NO_3 and ammonium NH_4. Error bars indicate ± 1 SD.

N:P ratio of the cultivation medium on the contrary, did not significantly affect growth rate (ANOVA, $p>0.05$, **Figure 33**). It did affect P content in the seaweeds, which was significantly

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higher at the highest P level (data not shown) so the treatment had an effect, only not on growth rate.

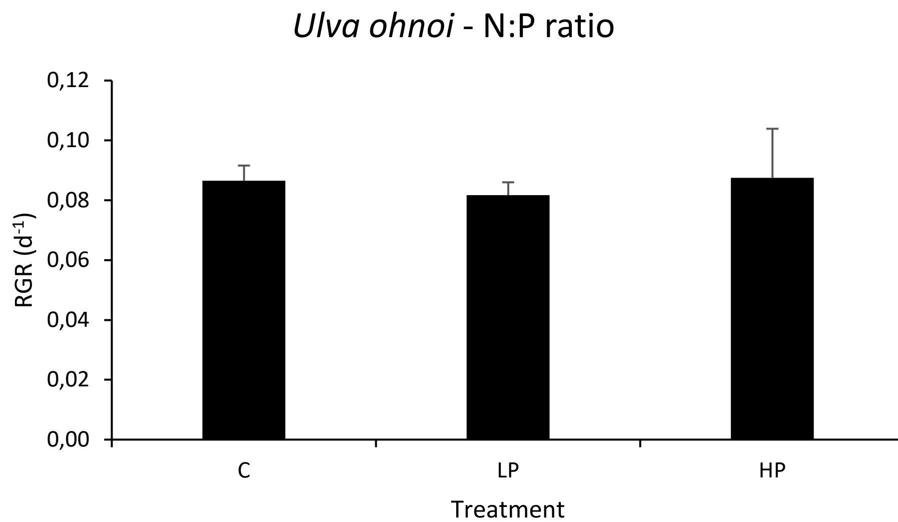

Figure 33. Growth of *Ulva ohnoi* in indoor photobioreactors at three different N:P ratios. C = control, LP = low phosphorous, HP = high phosphorous. Error bars indicate ± 1 SD.

The evolution of *U. ohnoi* growth rates in time in outdoor tanks is shown in **Figure 34**.

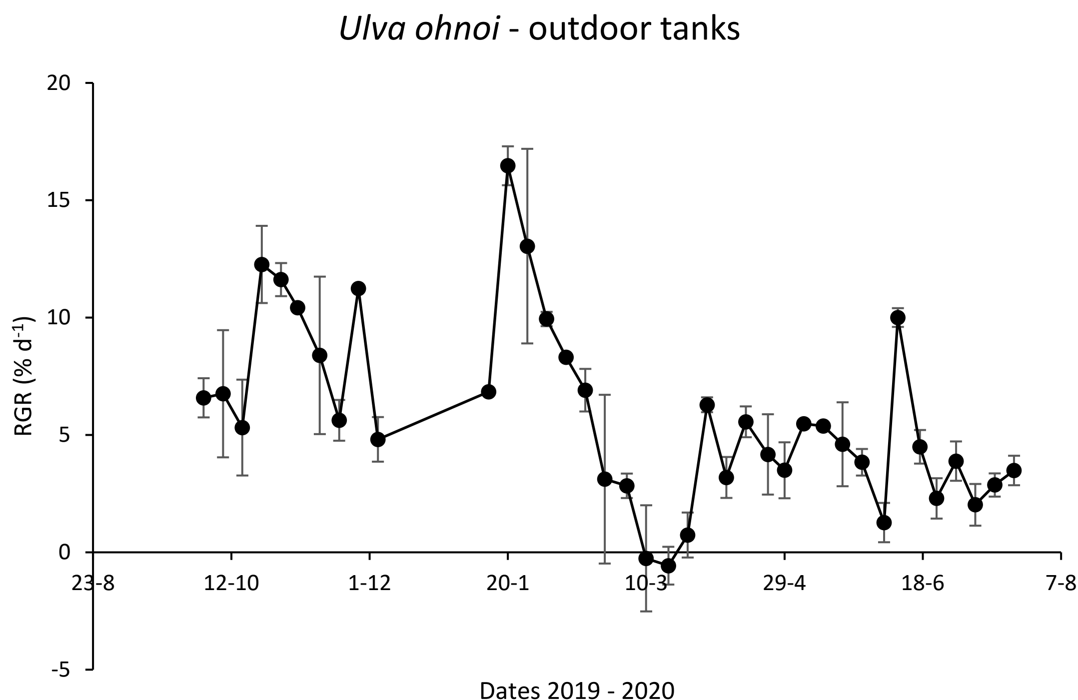

Figure 34. Growth of *Ulva ohnoi* in outdoor tanks between October 2019 and July 2020. Error bars indicate ± 1 SD.

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Growth rates appeared to be highly variable. However, it should be noted that especially in autumn of 2019 and in the winter of 2019/20, in general cultivation density was considerably lower (< 700 g FW/tank). Starting March 2020, biomass densities were between 1200 and 1500 g FW/tank to increase weekly yield. The strong decline in growth rates in the beginning of March 2020 coincided with a period of strong rains and cloudy weather. From April on, growth rates stabilized to around 5 % d^{-1}, to decrease again at the beginning of June. In this period, similar to the cultures in the PBRs, an infection of the algae with the parasitical brown macroalga *Myrionema* cf. *strangulans* was detected, causing large brown spots on the blades and part fragmentation. Although the algae seemed to recover from this, growth rates remained lower in early spring, coinciding with a period of very high air temperatures (32 - 38 °C maximum and minimum temperatures ≥ 24 °C). By mid-July, when air temperatures had dropped a few degrees, data suggest a recovery of growth.

5. Conclusion

The methodology selected to track the growth of microalgae and cyanobacteria has been successful and can be further extrapolated to screen larger sets of these microorganisms. Most of the microalgae selected from the Norwegian Culture Collection of Algae (NORCCA) grew well under the conditions that have been chosen based on their natural environment and requirements of the different species. The microorganisms from the set explored in this study that have managed to grow faster and produce a denser culture are: *Isochrysis galbana, Isochrysis sp, Phaeodactylum tricornutum, Pavlova, Synechocystis sp, Nannochloropsis occulata* and *Nannochloropsis oceanica*. All these strains have produced cultures with concentrations above 1.5 million cells/mL. Special potential have shown the two species *Nannochloropsis occulata* and *Nannochloropsis oceanica* that reached 7 million cells/mL (*occulata*) and 6 million cells/mL (*oceanic*a) from a starting concentration about 100.000 cells/mL in just a few days.

U. ohnoi, the selected *Ulva* species, is omnipresent in the warmer waters of the Spanish Atlantic and Mediterranean and most likely also in other parts of the Mediterranean and warmer waters in the rest of the world. It is relatively easy to isolate and maintain in cultivation. *Ulva ohnoi*, in particular, can be considered as an excellent candidate for floating cultivation in southern Europe, as our results show that it has a broad temperature and salinity range for growth as has also been confirmed by others (Coste et al. 2018). Furthermore, it appears that this local variety does not sporulate, which is the often the cause of large biomass loss in mass cultures of other *Ulva* species. This might be a specific property of this particular variety, as sporulation has been observed in this species in populations from Japan (Hiraoka et al. 2003) and India (Prabhu et al. 2019).

Indoor production in PBRs shows great potential for the continuous, year-round production of Ulva biomass. Once established, growth was relatively constant around 10% d-1. Although peak growth rates in the fields are considerably higher (up to 40% d-1; Hernández et al. 1997, Coste et al. 2018), the annual average is quite close to this value, as in the field there are large periods in the year with low or zero growth rate. Temperature appears to be the main factor

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regulating growth in the PBRs; in CTAQUA's new cultivation facility temperature will be more stable, hence it is expected that growth rates will be more stable in 2020.

Tank cultivation also showed potential. Although this has to be explored further, production rates are promising, and tanks offer the potential for production at higher volumes. Here, the only energy input in the cultivation process is the aeration. A lower degree of control can be exerted over the cultivation parameters, so that production will be more susceptible to variation in ambient temperature and light explaining the high variation.

Infections with the parasitic brown macroalga *Myrionema* spp. occur periodically in *Ulva*. They have been described to cause massive depigmentation and fragmentation of *Ulva* thalli (Siniscalchi et al. 2012) and have been associated to large production drops in South African aquaculture systems and are thus considered a threat for *Ulva* cultivation (Bolton et al. 2009). According to the latter, in South Africa the infection appears seasonally at the beginning of the summer, corroborating local observations from south Spain (Malta, pers. obs.). However, very little is known about this disease, its episodic appearance and general difficulty of maintaining a parasite in cultivation complicating the conduction of more thorough studies. Observations mentioned in Bolton et al. (2009) suggest that the parasite is less resistant than *Ulva* to low salinity and high nutrients for instance. Again, our results seem to corroborate this and indicate a capacity of the *Ulva* to recover from the disease. In fact, as growth rates recovered, many thalli were observed with small holes in it and remains of the parasite still on their thalli but otherwise showing a healthy appearance. Nevertheless, this subscribes the importance of general culture hygiene (water filtration, isolation from field strains, etc.) and the essence of having back-up strains under carefully controlled conditions. As the microbiome growing on the blades is analyzed in the framework of the SIMBA project, it would be interesting to see if potential "dormant" presence of this parasite can be detected.

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7. Document Information

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Project website	www.simbaproject.eu		

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Work Package	N°	3	Title	Microbes to produce health and nutritional food and feed

Due date of deliverable:	
Submission date:	28/08/2020
Dissemination level:	Public
Nature of deliverable:	Report

Authors (Partner)	Carlos Escudero (NIVA), Ana Caterina Almeida (NIVA), Erik-Jan Malta (CTAQUA),			
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Version log			
Issue Date	Revision N°	Author	Change
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30/07/2020		NWO-I Henk Bolhuis	Reviewed
30/07/2020		Luke Anne Pihlanto	Checked
31/07/2020	2	NIVA Carlos Escudero	Second version
31/07/2020		Luke Anne Pihlanto	Approved

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8. Appendix

Preparation of the culture media employed in the study

ES-medium (Enriched natural seawater)

The media is prepared in seawater (SW; 34 $^{o}/_{oo}$, GF/C-filtered), topping up with the different salts and extracts indicated in the table below.

	Standard solution (in distilled water)	Dose (in SW)
$NaNO_3$	50 g/L	2 ml/L
$Na_2HPO_4 \cdot 12H_2O$	10 g/L	2 ml/L
NaFeEDTA*	1.5 g/L	0,5 ml/L
Vitamins standard solution		2 ml/L
Soil extract**		10 ml/L

* $C_{10}H_{12}FeN_2NaO_8$
** Mix 500 g garden soil and 500 ml distilled water. Autoclave. Allow to sediment (1-3 days), centrifuge at 8000 rpm for 45 minutes and collect the supernatant. Filtrate the extract through glass fiber filter (GF/C) and afterwards through a membrane filter (0.8 µm).
Fill a 10 mL centrifuge tube, autoclave once again and preserve in the refrigerator.

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Z8-medium

Modified Z8-medium (100% Z8)

Chemicals	Concentration	Element	Final element concentration
Na_2CO_3	21 mg/l		
$NaNO_3$	467 mg/l		
K_2HPO_4	31 mg/l	P	5.5 mg/l
		K	14 mg/l
$Ca(NO_3)_2 \cdot 4H_2O$	59 mg/l	Ca	10 mg/l
$MgSO_4 \cdot 7H_2O$	25 mg/l	Mg	2.5 mg/l
		S	3.25 mg/l
$FeCl_3 \cdot 6H_2O$	2.8 mg/l	Fe (III)	580 µg/l
$MnSO_4 \cdot 4H_2O$	223 µg/l	Mn	55 µg/l
H_3BO_3	310 µg/l	B	54 µg/l
KBr	12 µg/l	Br	8 µg/l
KI	8.3 µg/l	I	6.35 µg/l
$ZnSO_4 \cdot 7H_2O$	28.7 µg/l	Zn (II)	6.55 µg/l
$Cd(NO_3)_2 \cdot 4H_2O$	15.4 µg/l	Cd	5.6 µg/l
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	8.8 µg/l	Mo (VI)	4.85 µg/l
$CuSO_4 \cdot 5H_2O$	12.5 µg/l	Cu	3.2 µg/l
$Co(NO_3)_2 \cdot 6H_2O$	14.6 µg/l	Co	2.95 µg/l
$NiSO_4(NH_4)_2SO_4 \cdot 6H_2O$	19.8 µg/l	Ni	2.95 µg/l
$Al_2(SO_4)_3K_2SO_4 \cdot 24H_2O$	47.4 µg/l	Al	3.38 µg/l
$Na_2WO_4 \cdot 2H_2O$	3.3 µg/l	W (VI)	1.85 µg/l
$Cr(NO_3)_3 \cdot 9H_2O$	4.1 µg/l	Cr	0.53 µg/l
V_2O_5	0.89 µg/l	V	0.5 µg/l
EDTA	3.7 mg/l		

pH after autoclaving: 6.5 - 7.7

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Preparation of the stock solutions for Z8 I, II, and III:

Solution Z8 I:

$NaNO_3$	46.7 g
$Ca(NO_3)_2 \cdot 4H_2O$	5.9 g
$MgSO_4 \cdot 7H_2O$	2.5 g
Distilled water	1000 ml

Autoclave. Store in glass bottle, maximum 2 years.

Solution Z8 II:

K_2HPO_4	3.1 g
Na_2CO_3	2.1 g
Distilled water	1000 ml

Autoclave. Store in glass bottle, maximum 2 years

Solution Z8 III:

Fe-solution:

$FeCl_3 \cdot 6H_2O$	2.8 g
0.1 N HCl	100 ml

EDTA-solution:

$Na_2EDTA \cdot 2H_2O$	3.9 g
0.1 N NaOH	100 ml

Mix 10 ml Fe-solution with ca. 900 ml distilled water in a 1000 ml volumetric flask, add 9.5 ml EDTA-solution, and fill with distilled water. Autoclave. Keeping the solution in a dark and cool environment (EDTA is sensitive to light) preserve its integrity for a maximum of 3 months.

Trace metal solution

Prepare single solutions of the substances below in distilled water.

1.	$Na_2WO_4 \cdot 2H_2O$	0.330	g/l
2.	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.880	g/l
3.	KBr	1.20	g/l
4.	KI	0.830	g/l
5.	$ZnSO_4 \cdot 7H_2O$	2.87	g/l
6.	$Cd(NO_3)_2 \cdot 4H_2O$	1.54	g/l
7.	$Co(NO_3)_2 \cdot 6H_2O$	1.46	g/l

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8.	$CuSO_4 \cdot 5H_2O$	1.25	g/l
9.	$NiSO_4(NH_4)_2SO_4 \cdot 6H_2O$	1.98	g/l
10.	$Cr(NO_3)_3 \cdot 9H_2O$	0.410	g/l
11.	V_2O_5	0.089	g/l
12.	$Al_2(SO_4)_3K_2SO_4 \cdot 24H_2O$	4.74	g/l

Additionally, prepare a solution of:

13.	H_3BO_3	3.10	g
	$MnSO_4 \cdot 4H_2O$	2.23	g (= 1.69 g $MnSO_4 \cdot H_2O$)
	Distilled water	1000	ml

Each of the 13 solutions is autoclaved and stored in a glass bottle, in a dark and cool environment. Maximum 20 years.

Prepare the trace metal solution by adding 10 ml of the solutions 1-12 and 100 ml of solution 13 to ca. 700 ml distilled water. Adjust to 1000 ml with distilled water. Autoclave. The solution is stored in a dark flask, cool and for a maximum of 2 years.

Preparation of 100% Z8-medium

Bubble ca. 800 ml distilled water with CO_2 gas for approx. 10 minutes. *

Add: 10 ml Z8 I - solution
10 ml Z8 II - solution
10 ml Z8 III – solution
1 ml trace metals solution

Adjust the volume to 1000 ml with distilled water.
Sterilize at 121 °C for 15 min.

* CO_2-aeration is required to avoid precipitations in the medium. If the medium is prepared at a lower strength (e.g. 20%) the CO_2-bubbling step is not required.
The right preparation of this solution will provide a pH in the range 6.5–7.7 for a 100% Z8-medium.

SW (Seawater)-medium

This media is prepared directly from marine water, that is previously filtered and afterwards sterilized in an autoclave at 121 ºC for 30 minutes.

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MV-medium

MV is the abbreviation of Marine medium with Vitamins (Christensen, 1982). The salinity is indicated by a number after the abbreviation MV (eg. MV30 = MV-medium based on 30 ‰ filtered sea water).

1. P II trace metal stock solution

$Na_2EDTA \cdot 2H_2O$	1000 mg
H_3BO_3	1144 mg
$MnCl_2 \cdot 4H_2O$	144 mg
$FeCl_3 \cdot 6H_2O$	49 mg
$ZnCl_2$ (0.1 g/100 mL)	10 mL
$CoCl_2 \cdot 6H_2O$ (0.1 % g/100mL)	4 mL
Distilled water	to 1000 mL

The P II trace metal stock solution is divided in 250-mL lots, autoclaved and stored refrigerated.

2. ESI stock solution

Distilled water	1000 mL
Tris(hydroxymethyl)-aminomethane	5000 mg
$NaNO_3$	3500 mg
Na_2-ß-glycerophosphate•$5H_2O$	500 mg
Na_2EDTA•$2H_2O$	251 mg
$FeCl_3$•$6H_2O$	121 mg
KI (0.1 g/100 mL)	1.3 mL
P II trace metal stock solution	250 mL

The solution is adjusted to pH 7.
After adjusting the pH, the ESI stock solution is dispensed in 20-mL lots, autoclaved and stored refrigerated.

3. Vitamin stock solution

Thiamine Hydrochloride (Vitamin B1)	200 mg
0.01 % Biotin	5 mL
0.01 % Cyanocobalamine (Vitamin B12)	5 mL
Distilled water	to 1000 mL

If the vitamin stock solution is to be autoclaved, pH must first be adjusted to 5. The vitamin stock solution is dispensed in appropriate lots and stored frozen.

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4. Final preparation of MV-medium

a) To 1L of seawater (filtered and adjusted to the desired salinity) add 20 mL ESI Stock Solution
b) The ESI-enriched sea water is pasteurized at 73°C and cooled as quickly as possible to avoid precipitation.
c) One mL of the vitamin stock solution is added to the cooled enriched sea water. It is sometimes desirable to sterilize the vitamin stock solution by autoclaving and to add the vitamins aseptically to the enriched sea water. Alternatively add unsterilized vitamin stock solution through a 0.2-µm sterile membrane filter mounted on a disposable syringe.
d) The medium is then dispensed aseptically to sterile culture tubes and stored refrigerated. Notice: We now routinely add the ESI stock solution and vitamins to the sea water and autoclave rather than pasteurize the mixture.

20% Z8 + SW

- 200 ml Z8
- 800 ml distilled water

20% Z8 + SW + Vit

- 1 ml Vitamin solution
- 200 ml Z8
- 800 ml filtered seawater

f/2-medium without Si

f/2 is a general medium for microalgae that with the addition can be made suitable for diatom cultivation as well (Guillard & Ryther 1962). It is also commonly used for cultivation of green macroalgae (Malta et al., 1999).

1. Trace metal stock solution

$Na_2EDTA \cdot 2H_2O$	4.16 g
$FeCl_3 \cdot 6H_2O$	3.15 g
$MnCl_2 \cdot 4H_2O$	0.18 g
$ZnSO_4 \cdot 7H_2O$	0.022 g
$CoCl_2 \cdot 6H_2O$	0.01 g
$CuSO_4 \cdot 5H_2O$	0.01 g
$Na_2MoO_4 \cdot 2H_2O$	0.006 g
Distilled water	to 1000 mL

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Stable for > 6 months in the refrigerator.

2. Vitamin stock solution

Thiamine Hydrochloride (Vitamin B1)	10 mg
Biotin	100 µL (from a stock of 5 mg in 10mL)
Cyanocobalamine (Vitamin B12)	100 µL (from a stock of 5 mg in 10mL)
Distilled water	to 100 mL

The vitamin stock solution is dispensed in appropriate lots and stored frozen.

3. Nutrient stock solution

Distilled water	1000 mL
$NaNO_3$	150 g
$NaH_2PO_4 \cdot 2H_2O$	6.9 g

Stable for > 6 months in the refrigerator.

4. Final preparation of f/2-medium

To 1L of seawater (filtered and autoclaved) add the following filter-sterilized components:

Trace metal stock solution	1 mL
Vitamin stock solution	1 mL
Nutrient stock solution	0.5 mL

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