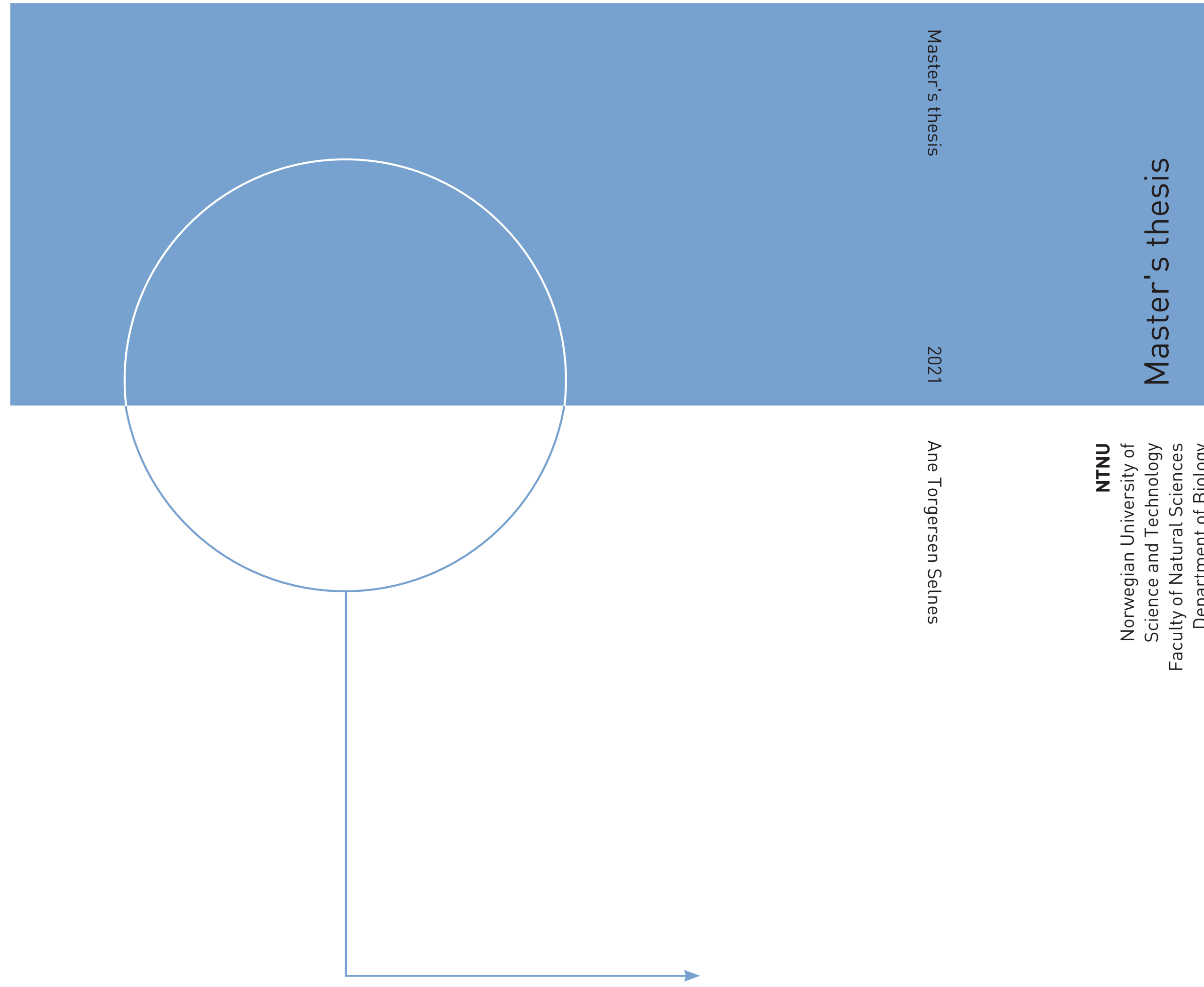
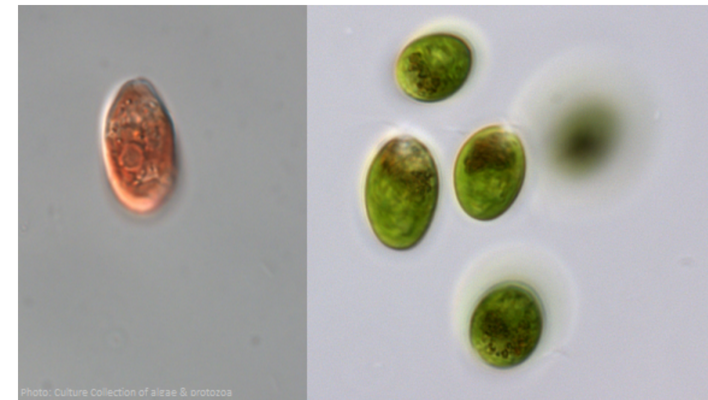


NTNU
Norwegian University of
Science and Technology

Ane Torgersen Selnes

The efficiency of the two microalgae species *Rhodomonas baltica* and *Dunaliella tertiolecta* for removal of dissolved nitrogen and phosphorus from RAS water

June 2021



NTNU
Norwegian University of
Science and Technology

Norwegian University of
Science and Technology

The efficiency of the two microalgae species *Rhodomonas baltica* and *Dunaliella tertiolecta* for removal of dissolved nitrogen and phosphorus from RAS water

Ane Torgersen Selnes

Master's thesis in Natural Science with Teacher Education
Submission date: June 2021
Supervisor: Kjell Inge Reitan
Co-supervisor: Siv Anina Etter

Norwegian University of Science and Technology
Department of Biology

Abstract

The aquaculture industry is the fastest growing food production sector in the world and is expected to contribute even more for the future global food demand. Increasing growth of the industry may give some challenges, and one of the major ones is how to continue a high production while at the same time keeping a sustainable and environmental friendly industry. Recirculation of water in land based facilities have increasingly been implemented, and by use of RAS technology, the total amount of water needed are drastically reduced and the environmental impact on surrounding areas may be even better controlled as the water effluent are thoroughly treated. As an integrated part of this technology, researchers have been looking into the possibilities of using microalgae for the purpose of removal of dissolved inorganic nitrogen and phosphorus from the wastewater. In addition to wastewater removal, the microalgae biomass with its nutritional qualities could possibly be utilized for other purposes, like aquafeed.

The aim of this study was to investigate the ability and potential of using the two microalgae species *Rhodomonas baltica* and *Dunaliella tertiolecta* for the purpose of removing dissolved inorganic nitrogen and phosphorus from imitated RAS wastewater. Whether or not, and to what extend difference in salinity and light intensity have any impact on the removal effectiveness was also investigated. To assess this, the two microalgae species was cultured separately as batch cultures. As well as investigating the removal effectiveness of the two species alone, the potential of carrying out a semicontinuous mixed culture of the two microalgae was also investigated.

The results suggest that both species had a highly effective removal of nitrogen and phosphorus, as almost all the available nitrogen and phosphorus in the RAS water was removed, shown by analysing the water content at the end of the culture period and comparing it to the available amount at start. Neither salinity nor light intensity did seem to have any major effect of the removal ability and effectiveness, although low light intensity did seem to induce a slightly higher removal of phosphorus for *R. baltica*. The overall impression is although that both species did obtain a highly adequate removal. Results from the mixed semicontinuous culturing experiment did also suggest a high removal of nitrogen, when comparing nitrogen content in the daily added medium to the cellular content harvested. The removal efficiency of phosphorus was lower, but there is a chance that not all the cellular phosphorus was detected and hence the actual cellular content might have been higher as well.

Abstract in Norwegian

Akvakulturindustrien er den raskest voksende matsektoren i verden og er forventet å bidra ytterligere til verdens matbehov i framtiden. En økt vekst kan by på noen utfordringer, og en av de største er hvordan man skal kunne opprettholde en høy produksjon og samtidig drive en bærekraftig og miljøvennlig industri. Resirkulering av vann i landbaserte oppdrettsanlegg har blitt mer og mer vanlig, og gjennom RAS-teknologi blir det totale vannforbruket betydelig redusert og miljøpåvirkningen til nærliggende områder kan bli enda bedre kontrollert da produksjonsvann som slippes ut blir godt renset. Som en integrert del av denne teknologien har forskere nå sett på muligheten av å benytte mikroalger for å rense RAS-vann for løst uorganisk nitrogen og fosfor. I tillegg til å rense vannet kan mikroalgenes biomasse benyttes til andre formål, for eksempel som bruk i akvakulturfôr.

Målet med dette studiet var å undersøke muligheten og potensialet til å bruke de to mikroalgeartene *Rhodomonas baltica* og *Dunaliella tertiolecta* for å rense unna uorganisk nitrogen og fosfor fra imitert RAS-vann. Om, og eventuelt i hvilken grad forskjellig salinitet og lysintensitet spiller inn på effektiviteten av rensingen ble også undersøkt. For å finne ut av dette ble de to mikroalgeartene kultivert separat som batchkulturer. I tillegg til å undersøke den enkelte art sin renseevne, ble det også gjennomført et forsøk med en semikontinuerlig kultur av de to artene kombinert.

Resultatene indikerer at begge artene oppnådde en svært effektiv rensing av nitrogen og fosfor, da analysene av vannet ved kultiveringsslutt viste at nesten alt det nitrogenet og fosforet som var tilgjengelig ved start var blitt fjernet. Hverken salinitet eller lysintensitet ser ut til å ha hatt noen vesentlig innvirkning på effektiviteten av rensingen, selv om det kan se ut til at lavt lysintensitet bidro til en litt høyere renseeffektivitet av fosfor for *R. baltica*. Hovedinntrykket er likevel at begge arter oppnådde en tilfredsstillende grad av rensing. Resultatene fra det semikontinuerlige blandingsforsøket viste også høy grad av nitrogenrensing. Dette ble observert når det cellulære innholdet ved høsting ble sammenlignet med den tilgjengelige mengden nitrogen i vannet ved start. Renseeffektiviteten av fosfor var derimot lavere, men det er en mulighet for at ikke alt det cellulære fosforet ble detektert i analysen. Det kan derfor bety at det faktiske fosforinnholdet var høyere enn det resultatene indikerer.

Table of Contents

Abbreviations ..... **ix**

1 Introduction **1**

1.1 Recirculating aquaculture systems and wastewater treatment 2

1.2 Uptake of nitrogen and phosphorus in microalgae 5

1.3 Culturing of microalgae 6

1.4 Microalgae as aquafeed 7

1.5 Aim of study 8

2 Materials and Methods **10**

2.1 Overview and experimental setup 10

2.2 Cultivation system 13

2.3 Culture medium 16

2.4 Cell number registrations of the cultures 17

2.5 Samples of culture media and microalgae cells 17

2.6 Analyses 18

2.7 Calculations 19

2.8 Statistics 22

3 Results **24**

3.1 Biomass development of *R. baltica* and *D. tertiolecta* 24

3.2 Cellular contents 31

3.3 Removal of nitrogen and phosphorus from cultivation medium 40

3.4 Semicontinuous cultures 48

4 Discussion **54**

5 Conclusion **57**

6 Acknowledgement **58**

7 References **59**

Appendix A **67**

Appendix B **68**

Appendix C **70**

Appendix D **72**
Appendix E.. **73**
Appendix F.. **75**
Appendix G **77**
Appendix H **78**
Appendix I.. **82**

Abbreviations

C	Carbon
Chl a	Chlorophyll a
CO_2	Carbon dioxide
DIN	Dissolved inorganic nitrogen
DIP	Dissolved inorganic phosphorus
μ	Specific growth rate
μ_{max}	Maximum specific growth rate
N	Nitrogen
P	Phosphorus
POC	Particulate organic carbon
PON	Particulate organic nitrogen
POP	Particulate organic phosphorus
P1E1	Part 1 – Experiment 1 (*R. baltica* experiment)
P1E2	Part 1 – Experiment 2 (*D. tertiolecta* experiment)
P2	Part 2
RAS	Recirculation Aquaculture Systems
RAS10+/-	RAS culturing medium – 10‰ salinity – high/low light intensity
RAS15+/-	RAS culturing medium - 15‰ salinity – high/low light intensity
RAS34+/-	RAS culturing medium - 34‰ salinity – high/low light intensity
Conway34+/-	Conway culturing medium - 34‰ salinity – high/low light intensity
SD	Standard deviation

1 Introduction

From 1994 to 2019 the global population has increased from 5.7 to 7.7 billion people, and within 2030 it is expected to reach as much as 8.5 billion (UN, 2019). As the population increases, there is, and will be an even higher demand of food in the upcoming years (Alexandratos and Bruinsma, 2012, Olsen, 2011). According to the Food and Agriculture Organization of the United Nations, aquaculture is the fastest growing major food production sector (FAO, 2016). With a higher food demand, the aquaculture industry may contribute even more to cope with this challenge. There is also a concern about how to produce enough food while at the same time keeping a sustainable and environmentally friendly aquaculture industry (Olsen, 2011).

Several production systems are used for cultivation of fish. In land based fish farming, a technology known as recirculation aquaculture systems (RAS) is frequently used, where production water in the fish tanks is treated and recirculated back to the tanks in order to reduce the total amount of water needed (Wik et al., 2009). RAS wastewater has a high content of nitrogenous and phosphorus compounds (originating from the feeding) released directly through feed spill and indirectly through faeces and metabolic products from the fish (Bregnballe, 2015, Wang et al., 2012). For wastewater to be reused in the fish tanks, it is crucial that ammonium and ammonia is removed, as it will create a toxic environment for the fish if not. It is also favourable to remove nitrogenous and phosphorus compounds for the purpose of reducing the risk of eutrophication if water is released to the environment. For those reasons, RAS technology may become very important as a part of keeping a sustainable aquacultural production.

Today, aquacultural salmon feed does consist of ingredients were only 30% originates from marine sources. The remaining consists of a lot of plant based ingredients, and the use of marine sources seems to be more and more succeeded by plant based ingredient as well (Bai et al., 2015, Olsen, 2011, Ytrestøyl et al., 2015). As a result of this, it will be important to substitute some of these traditional ingredients with other ingredients with adequate nutritional value. By choosing ingredients from outside the human food chain as well as moving fish to a lower trophic level, the aquaculture industry may certainly gain a higher production as well as becoming more sustainable (Olsen, 2011).

1.1 Recirculating aquaculture systems and wastewater treatment

Recirculating aquaculture systems (RAS) is a type of aquaculture application that constantly reuse production water in closed systems by removing waste particles and transfer dissolved nutrients to keep a healthy environment for the fish. As a high portion of the water is reused, the demand of new water to the facility is hence reduced. This makes it relatively easy and effective to remove waste nutrients from the water, especially in comparison with traditional aquaculture facilities with a greater volume of wastewater (Bregnballe, 2015). Following RAS wastewater treatment, nutrients might not only be seen as waste, but does also have the possibility to be converted into valuable resources (Su, 2020). An example is the utilization of aquacultural waste for microalgal or seaweed cultivation (Stevčić et al., 2019, Wang et al., 2012).

While there has been a lot of concerns regarding environmental impact from traditionally aquaculture systems, RAS has been developed to offer technological solutions to face environmental challenges and try to turn aquaculture towards a more environmentally friendly industry. At the same time it is an aim to achieve a high production and securing animal welfare (Martins et al., 2010). Even though RAS has a lot of benefits, it is currently not widely used, mainly because the treatment is relatively costly compared to conventional systems (Nie et al., 2020).

The concept of RAS derives from the process of nutrient removal from wastewater, and in the following section there will be given a brief introduction to the different steps of a recirculation system. Different alternatives for nitrogen removal will also be presented.

Nitrogen and phosphorus, as well as carbon are released from the fish in different ways and in different forms (Figure 1). Dissolved inorganic nitrogen and phosphorus (DIN and DIP) are excreted through gills, carbon (in the form of CO_2) through respiration, and larger particles are released as particulate organic carbon, nitrogen and phosphorus (POC, PON and POP) originating from feed spill and faeces (Olsen and Olsen, 2008). Research has found that nitrogen and phosphorus seems to be the most dominating compounds from aquaculture facilities causing pollution to the environment (Herath and Satoh, 2015). Therefore, it is important to remove these nutrients to decrease the risk of emission and eutrophication to the environment, as well as keeping a healthy environment for the fish living in recirculated water (Wik et al., 2009, Martins et al., 2010).

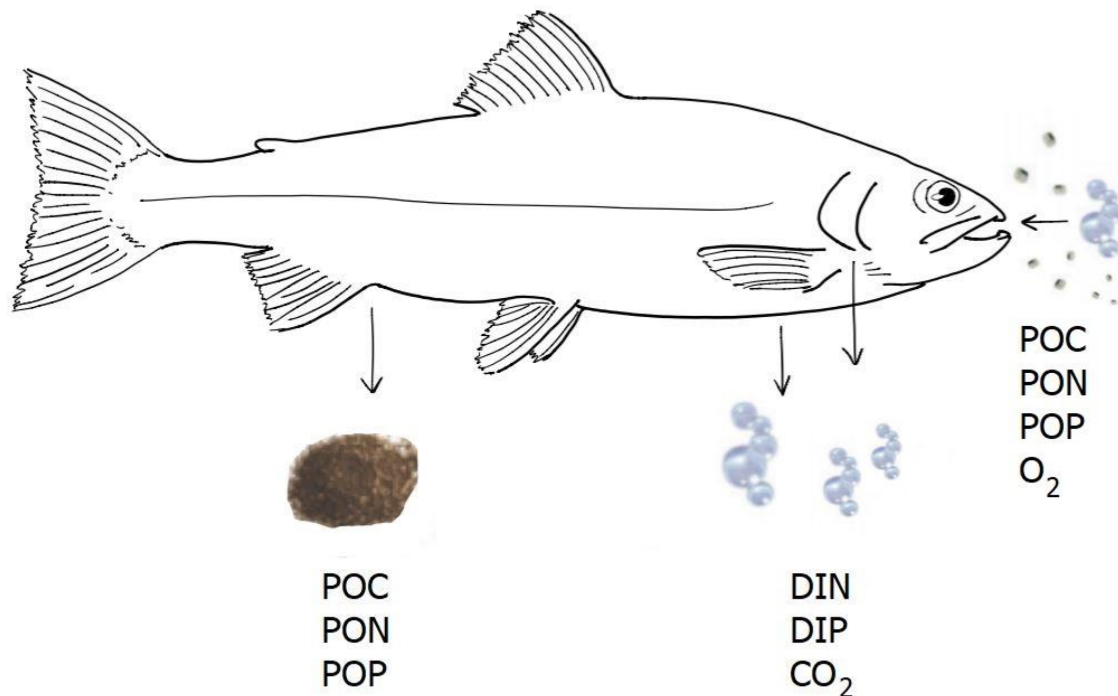
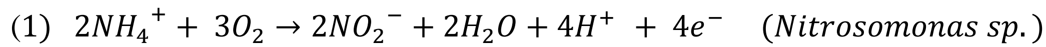
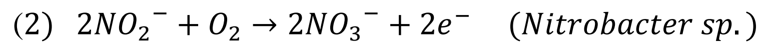


Figure 1: Waste products from the fish. POC, PON and POP are released to the water through feed spill and faeces. DIN and DIP are released through excretion from the gills. Respiration is illustrated as inlet of O_2 and release of CO_2. (Bregnballe, 2015, Olsen and Olsen, 2008).

To get rid of waste products originating from the fish, water is treated mechanically and biologically. Particulate organic compounds are mechanically removed from the wastewater using a drumfilter, while smaller organic compounds and dissolved inorganic compounds go through (Figure 2). High levels of inorganic phosphorus in the form of phosphate are not toxic for the fish, but high levels of nitrogen in the form of ammonia (NH_3) and ammonium (NH_4^+) certainly is. Fish will excrete a mix of ammonia (NH_3) and ammonium, often referred to as total ammonia nitrate (TAN). These two compounds exist in an equilibrium, and at pH lower than 7 most TAN will be in the form of ammonium. At that point, it will be possible for ammonium to undergo nitrification to nitrite (NO_2^-) (1). Nitrite is less toxic, but too high exposure should nevertheless be avoided. Next step of the nitrification process is to convert nitrite to nitrate (NO_3^-) (2). The whole process is carried out in a biofilter by so called nitrifying bacteria, *Nitrosomonas sp*. and *Nitrobacter sp*. respectively (Bregnballe, 2015, Hargreaves, 1998, Locey, 2005).



$$(1)\quad 2NH_4{}^{+} + 3O_2 \rightarrow 2NO_2{}^{-} + 2H_2O + 4H^{+} + 4e^{-} \quad (Nitrosomonas\ sp.)$$



$$(2)\quad 2NO_2{}^{-} + O_2 \rightarrow 2NO_3{}^{-} + 2e^{-} \quad (Nitrobacter\ sp.)$$

Nitrate is not that harmful for the fish, although too high levels should also be avoided since it seems to have a negative effect on growth (Bregnballe, 2015). From this point, there are a couple of alternatives for how to take care of the nitrate. One alternative is to increase the exchange of new water to the system, hence diluting and lowering the level of nitrate to an acceptable level. Since one of the main goals of RAS is to reduce the exchange of water (both for the simple reason of saving water as well as limiting the environmental impact), this alternative might not be the preferred method for nitrate removal. Another alternative is to reduce nitrate to nitrogen gas (N_2) through the process of denitrification carried out by certain strains of bacteria. In gaseous form, nitrogen can then be aerated out of the system. Compared to the procedure of water exchange, the process of denitrification is relatively complicated and time consuming (Bregnballe, 2015, Van Rijn et al., 2006).

A third option is to use microalgae for nitrogen and phosphorus removal instead of denitrifying bacteria. Cultivation of microalgae require light, carbon dioxide, water, and nutrients, where nitrogen and phosphorus are the main ones. Microalgae are relatively flexible making them suitable for growth in different aqueous environments, and due to their fast growth they have the potential of achieving a high biomass yield (Khan et al., 2018, Zullaikah et al., 2019). These qualities have made it interesting to investigate the possibility of incorporation of microalgae in RAS for the purpose of nitrogen and phosphorus removal (Stevčić et al., 2019, Nie et al., 2020). With a potential of nutrient removal as well as the possibility of utilizing the microalgae biomass for other purposes, the use of microalgae for RAS wastewater treatment might seem promising for the future. One of the challenges is although that RAS wastewater are relatively dynamic and biologically diverse, hence making it challenging to develop a standardized method for application of microalgae in RAS treatment (Nie et al., 2020).

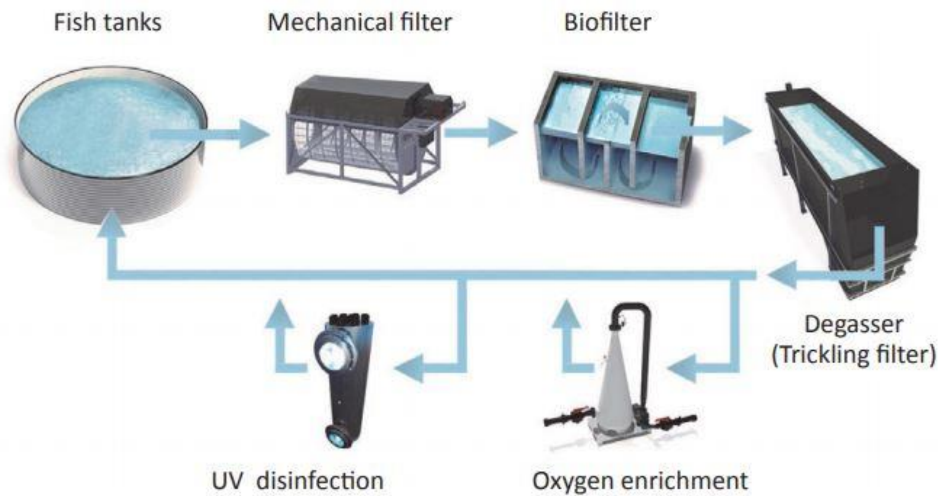


Figure 2: Steps of water treatment of a recirculation system. After mechanical and biological treatment, CO_2 is removed from the wastewater, oxygen is added, the water is disinfected in a UV treatment procedure, and sent back to the fish tanks (Bregnballe, 2015).

1.2 Uptake of nitrogen and phosphorus in microalgae

Microalgae take up carbon in the form of carbon dioxide and fixates it into organic matter through the Calvin cycle. Nitrogen is taken up and transformed into amino acids, proteins, RNA and DNA. Along with nitrogen, phosphorus does also play a critical role when it comes to the building of nucleic acids but it is also essential in the formation of ATP and as a building blocks in cellular membranes (Su, 2020). In other worlds, uptake of these elements is crucial for microalgae to grow. Microalgae in general is not too picky when it comes to uptake of nitrogenous compounds, and they can utilize nitrogen in the form of e.g., nitrate (NO_3^-), nitrite (NO_2) or ammonium (NH_4^+). The preferred one seems although to differ between species (Salbitani and Carfagna, 2021, Arumugam et al., 2013, Ruangsomboon, 2015). Phosphate (PO_4^{3-}) is the preferred form of phosphorus for the microalgae (Dyhrman, 2016).

Evaluating the cellular contents of carbon (C), nitrogen (N) and phosphorus (P) in microalgae, the Redfield ratio is used to describe the balanced saturated cellular ration between the three elements in marine phytoplankton in the ocean, and it is helpful when evaluating nutrient limitations of the microalgae (Anderson and Sarmiento, 1994, Martiny et al., 2014, Goldman et al., 1979, Redfield et al., 1963).

1.3 Culturing of microalgae

Microalgae are frequently cultivated in aquaculture, and there are a lot of examples of research on the possibility of using different microalgae species for the purpose of being fed to commercially valuable aquaculture species (Sørensen et al., 2016, Sørensen et al., 2017, Reitan et al., 1997).

Microalgae can be cultured in batch cultures and semicontinuous cultures. In a batch culture, a relatively low number of cells are added at start, as well as nutrients. After a certain amount of time, the whole culture is harvested. A semicontinuous culture on the other hand is characterized by regular harvesting and addition of nutrients at fixed time intervals during the culturing period (Akerlund et al., 1995, Salgueiro et al., 2018, Forget et al., 2010). A characteristic batch culture growth curve is divided into separate phases. The first phase is the log phase where there the cells adapt to the new environment. The following is an exponential phase where the cell population increase exponentially with a relatively constant generation time. Then there will be a stationary phase/steady state where the growth is basically terminated due to all nutrients being consumed. The last phase is the phase of declining cell population due to death (Prescott et al., 2003). The source of nutrients added at start can be different types of nutrient mediums, for example Conway medium or f/2 medium with different amounts of nitrogen and phosphorus (Gonzalez-Rodriguez and Maestrini, 1984, Walne, 1966, Lananan et al., 2013, Guillard, 1975).

The growth in a microalgae culture can be described by the specific growth rate (μ), that is determined by the culture conditions and the specific microalgae strain. The specific growth rate can be found using a semi log plot of the logarithm of cell number versus time (Equation 2.1) (Moheimani et al., 2013). The maximum specific growth rate (μ_{max}) is where the increase in logarithm of the cell numbers versus time is linear and have the highest slope. The μ_{max} value can be used as a measure giving the maximal growth capacity of the algae culture. When the culture reaches stationary phase, the growth rate will be equal to zero (Molin, 1983, Nokkaew et al., 2012).

In the experiments carried out in this project, the two microalgae species *Rhodomonas baltica* and *Dunaliella tertiolecta* were cultured.

The *R. baltica* species is distributed in coastal areas, and the algae illustrated in Figure 3 were collected from a marine environment around the island of Guernsey (Throndsen, 1997, CCAP, 2021b). The species is characterized by a slightly flattened cell, one or two chloroplasts and

two flagella. The length ranges between 18-30 µm (Throndsen, 1997). The genus of *Rhodomonas* is already found to be useful when it comes to feeding certain species of copepods in the aquaculture industry (Knuckey et al., 2005).

The *Dunaliella* genus consists of 28 different species, with *D. tertiolecta* distributed in coastal, Atlantic areas (González et al., 2009, Throndsen, 1997). As an example, the *D. tertiolecta* algae illustrated in Figure 4 were collected from a brackish environment in the Oslofjord (CCAP, 2021a). The genus is characterised by a radially symmetrical appearance but can have different shaping. The species of *D. tertiolecta* is characterized by a rounded posterior end, yellow-green colour, two relatively long flagella and it contains a lot of small globules as illustrated in Figure 4. The species is smaller than *R. baltica* with an average length of 9-11 µm (Throndsen, 1997). The genus is relatively easily cultured in different types of media, and *D. tertiolecta* in particular is often shown to obtain a rapid growth with a lot of suspension (Butcher, 1959).

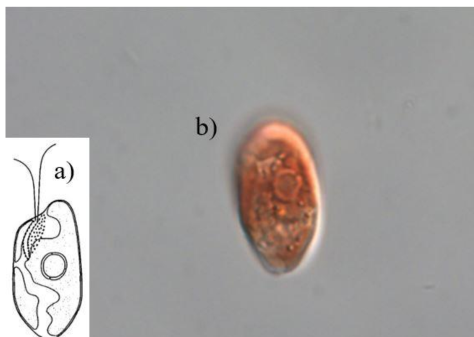


Figure 3: *R. baltica*. Drawing (Throndsen, 1997) (a), and real life image of (CCAP, 2021b) (b).

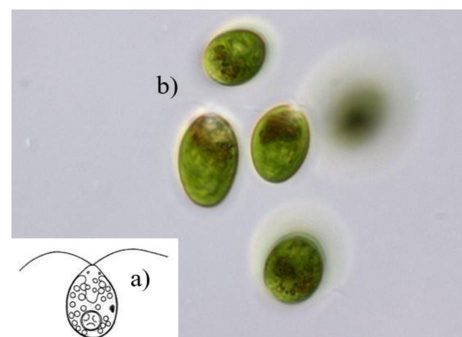


Figure 4: *D. tertiolecta*. Drawing (Throndsen, 1997) (a), and real life image (CCAP, 2021a) (b).

1.4 Microalgae as aquafeed

As already mentioned, aquaculture feed does consist of some amount of ingredients of marine origin, like fish meal and fish oil, but a greater amount is plant based ingredients (Bai et al., 2015, Boyd, 2015, Olsen, 2011, Ytrestøyl et al., 2015). In a world of growing human population and increased food demand, there will be less fish meal and fish oil available on the marked, as well as more competition for plant based ingredients. A possible solution to these challenges is to replace ingredients with nutritious sources from outside the human food chain and move fish to lower tropic levels and hence gain a higher production (Olsen, 2011). Researchers have been

looking into the possibilities of using microalgae as such a replacer (Wang et al., 2019). Microalgae can directly, or indirectly serve as a source of feed for fish larvae in aquacultural production. Indirectly as a source of feed for species like rotifers, whom in the next step can serve as live feed for fish larvae. As a direct source of feed, microalgae can be given to larvae alongside the live feed serving as a source of feed for both larvae and the live feed itself (Reitan et al., 1997). There has also been seen a potential of using defatted microalgal biomass as a replacement for fish meal (Sørensen et al., 2017, Sørensen et al., 2016). This is an interesting field of study, because the high protein content of some microalgae species seems to be a suitable alternative to the traditional ingredients in aquacultural feed, seen from a nutritional point of view (Olsen, 2011).

1.5 Aim of study

In this study, three different culturing experiments were carried out to investigate the ability and potential of using the two microalgae species *R. baltica* and *D. tertiolecta* to remove dissolved inorganic nitrogen and phosphorus from imitated RAS wastewater. To answer this question the two species were first cultivated separately in batch cultures, and afterwards cultivated as a mix in semicontinuous cultures. Following the experimental setup, each of the batch culture experiments was exposed to two different light intensities and three different salinities to create environmental conditions of RAS water (Figure 5). The semicontinuous experiment was exposed to one light intensity only. For all the separated experiments, a culture cultivated in a standard Conway culture medium was used as a control. Note that the Conway cultures were cultured in one salinity only.

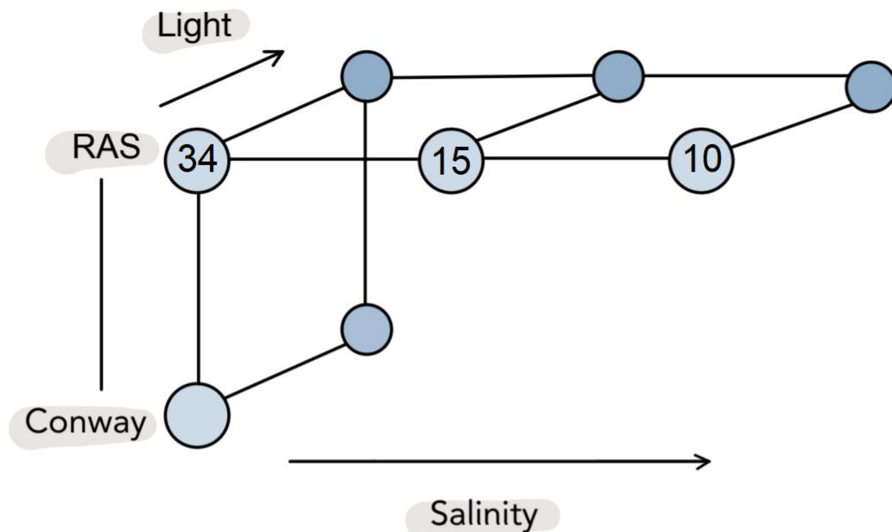


Figure 5: Factorial setup for the batch culture experiments. Presenting two different culture medium (RAS/Conway), two different light intensities and three different salinities. Note that microalgae cultured in Conway medium was exposed to one salinity only. Each treatment was cultured as triplicates.

The following research questions were defined:

1) How effective are the two microalgae *R. baltica* and *D. tertiolecta* to remove dissolved inorganic nitrogen (DIN) and dissolved inorganic phosphorus (DIP) from imitated RAS wastewater?
2) To what extend does different light intensities and salinities influence the removal of DIN and DIP from imitated RAS wastewater?
3) What potential lies in carrying out a semicontinuous mix culture of the two microalgae species?

2 Materials and Methods

The experiments were conducted at NTNU SeaLab in the period between January 26th and February 26th, 2021. The following analysis of cellular and medium content of the sampled material, were conducted at Trondheim Biological Station between March 3rd and March 19th, 2021.

2.1 Overview and experimental setup

The experiments were separated into three different sub experiments (Part1- Experiment 1, Part1 – Experiment 2 and Part2). The culture medium of the different experiments consisted of sea water of a specific salinity (10 ‰, 15 ‰ or 34 ‰), and a nutrient solution (RAS or Conway). Each nutrient and salinity treatment were run at two different light intensities (low and high) for P1, and one light intensity for P2. The algae inoculum was gathered from an already growing culture at SeaLab (*R. baltica* Karsten 1898 (clone NIVA 5/91) and *D. tertiolecta* Butcher 1959 (clone CCAP 19/27)). The setup of benches and flasks are illustrated in Figure 6 and 7.

<u>Part 1 – Experiment 1</u>

Batch cultures of *R. baltica* were carried out from January 26th to February 3rd, giving a total culturing period of eight days. The experiment consisted of eight different treatments and were run in triplicates (n=3) giving a total of twenty-four algae cultures (Table 1). Sampling of cell number (10 mL) was done every day during the culturing period.

<u>Part 1 – Experiment 2</u>

Batch cultures of *D. tertiolecta* were carried out from February 8th to February 18th, giving a total culturing period of ten days. The experiment consisted of eight different treatments and were run in triplicates giving a total of twenty-four algae cultures, similar as in Part 1-Experiment 1 (Table 1). Sampling of cell number (10 mL) was done every day during the culturing period. The daily sampling was divided into four groups because *D. tertiolecta* very easily sticked to the side of the sampling glass.

Figure 6: The two benches of culturing flasks. On the top bench the light intensity was higher than on the lower. The picture is taken during the culturing of *R. baltica*.

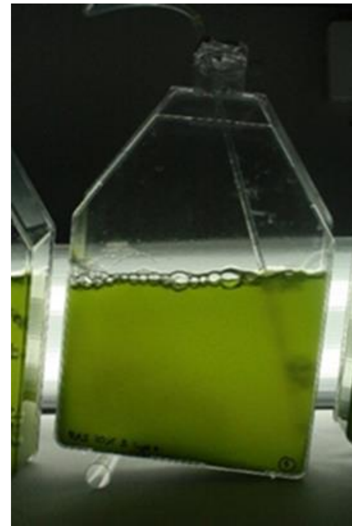


Figure 7: Culturing flask from the culturing experiment of *D. tertiolecta*.

Table 1: Experimental setup for P1, presenting the culturing experiment of *R. baltica* (P1E1) and *D. tertiolecta* (P1E2) respectively. Consisting of two different culture media (RAS and Conway), with three different salinities (10‰, 15‰ and 34‰) and two different light intensities (low and high). Each treatment was run in triplicates.

	Simulated RAS water						Conway	
	10 ‰		15 ‰		34 ‰		34 ‰	
	Low light	High light	Low light	High light	Low light	High light	Low light	High light
R. baltica	3	3	3	3	3	3	3	3
D. tertiolecta	3	3	3	3	3	3	3	3

Part 2

In the third experiment, semicontinuous mix cultures of *R. baltica* and *D. tertiolecta* were carried out. At start, both species had approximately equal cell concentration, as shown in Table 2. The experiment was carried out from February 22nd and February 26th, giving a total culturing period of four days. The experiment set up consisted of four different treatments and were run in triplicates giving a total of twelve algae cultures. The cultures were harvested and added new culture medium regularly at the same time every day during the culturing period. Table 3 show the experimental setup for P2.

Table 2: Exact cell concentration of *R. baltica* and *D. tertiolecta* in the different culturing flasks at start of the experiment.

	R. baltica (cells/mL)	*D. tertiolecta* (cells/mL)
RAS10	521533	642400
RAS15	621633	673600
RAS34	702633	555566
Conway34	614233	575500

Table 3: Experimental setup for P2. Two different culture media (RAS and Conway), three different salinities (10‰, 15‰ and 34‰) and one light intensity (high) were used. Each treatment was run in triplicates.

	Simulated RAS water			Conway
	10 ‰	15 ‰	34 ‰	34 ‰
	High light	High light	High light	High light
R. baltica + *D. tertiolecta*	3	3	3	3

As it was necessary with a higher volume of inoculum, the salinity of the inoculum itself would affect the wanted salinity in each culture medium. Therefore, two pre cultures of *D. tertiolecta* and two pre cultures of *R. baltica* were started on beforehand on a salinity of 10‰ and 34‰ respectively. The algae used in the Part 2 experiment were taken from pre cultures of the same salinity, and a mix between 10‰ and 34‰ were used to obtain the 15‰ culture medium. Hence the wanted salinity and the number of cells in each medium (approximately equal number of

each species) was achieved. Tables presenting the exact volumes of all the different components added to the culturing flasks are accessible in Appendix B.

2.2 Cultivation system

For all the experiments, cuboid culturing flasks (850 mL - VWR International) were used. The flasks were equipped with a pipe supplying each culture with a mix of CO_2 (carbon source) and air. The air bubbling was both to prevent sedimentation of algae and supply of CO_2. Before sampling the flasks were gently stirred. A plastic tube was placed under the left side of the culture flask, trying to force a circular airflow to prevent sedimentation. The top of each flask was covered with aluminium foil to avoid particles from the air entering the flasks. A small opening in the foil served as an air outlet. The flask setup is illustrated in Figure 7 and 8.

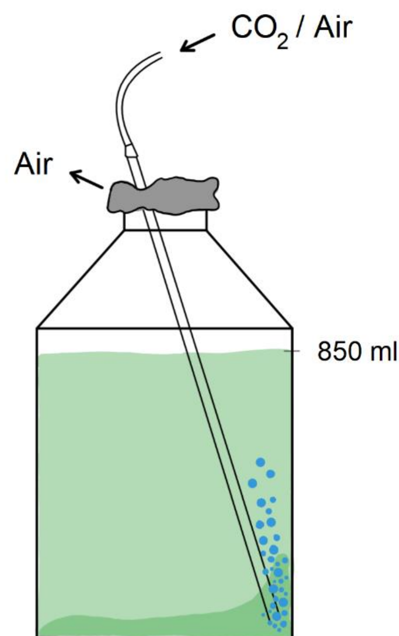


Figure 8: Experimental setup of a culturing flask illustrating inlet of CO_2 and air, outlet of air, stirring pipe and aluminium foil on top.

2.2.1 Light intensity

The flasks were placed on two separate benches, twelve on each. The culture flasks at the upper bench were exposed to high light intensity than on the lower. Each flask was placed 15 cm from the light source. P1 was exposed to both high and low light intensity, and P2 was exposed to high intensity only. A radiometer (QSL-2100) was used to measure exact light intensity.

Several light intensity registrations were done on the two different benches, spread over different places ranging from left to right as illustrated in Table 4. The registrations were carried out 15 cm from the light source.

Table 4: Overview over the light intensities (μE m^{-2} s^{-1}) on the two benches.

	Left				Centre					Right
High light intensity	55	121	141	122	83	80	122	140	122	53
Low light intensity	40	80	92	89	59	59	83	90	80	44

2.2.2 pH and CO_2

Supply of air and CO_2 was carried out through a central input and distributed to each bench through a main tube. Then it was distributed again to all the separate flasks through smaller pipes. A desired inlet of air and CO_2 was manually controlled using two separate controllers in the laboratory. A CO_2 meter (Extech CO250) made it possible to keep track on the input level, and measures was carried out regularly. A pH meter (WTW pH 3210) was used to measure pH of the cultures every day and made it possible to determine if the CO_2 input should be increased, decreased, or kept at the same level. An increase of CO_2 would make the pH decrease, and a decrease of CO_2 would hence make the pH increase. The CO_2 level was kept relatively stable after adjustment, but during P2 there was observed a higher fluctuation and no systematic recordings were done. Although the level was closely followed during the day and mostly kept within the range of 1565 – 2559. Table 5 presents the CO_2 ranges and Table 6 presents the pH ranges during the experimental periods.

Table 5: Overview of the CO2 ranges during the culturing period for each experiment.

Experiment	**CO_2 range (ppm)**
Part 1 – Experiment 1	2010 – 2559
Part 1 – Experiment 2	1565 – 2214
Part 2	

Table 6: Overview of the pH ranges during the culturing period for each experiment.

Experiment	**pH range**
Part 1 – Experiment 1	7.15 – 7.89
Part 1 – Experiment 2	7.02 – 8.13
Part 2	7.61 – 8.12

2.2.3 Temperature

The room temperature in the laboratory was controlled by an air pump, keeping it fairly stable at 20°C. The exact temperatures during the culturing periods were measured using a temperature logger (Testo 174T). The daily room temperature was registered as shown in Table 7.

Table 7: Overview of the room temperature ranges during the culturing period for each experiment. Measuring instrument nr.1 was located closest to the entrance, and nr.2 further into the room.

	Temperature interval (°C)		
	Part 1 Experiment 1	Part 1 – Experiment 2	Part 2
Measuring instrument nr.1	19.0 – 21.3	19.9 – 20.8	19.6 – 20.8
Measuring instrument nr.2	17.5 - 21.9	18.20 - 20.30	18.6 – 20.3

2.3 Culture medium

The culture medium of the different experiments consisted of sea water of a specific salinity (10 ‰, 15 ‰ or 34 ‰) and nutrient solution (RAS or Conway). The treatments consisting of RAS nutrient solution were designated "RAS", and treatments consisting of Conway nutrient solution were designated "Conway" as seen in Table 1 and Table 3.

Salinity

Sea water of 34 ‰ salinity was tappet from a seawater inlet at NTNU SeaLab. The other salinities were made by mixing the normal seawater with fresh water in different proportions.

Nutrient solution

The RAS and Conway nutrient solutions consisted of different amounts of nitrogen and phosphorus. The Conway treatment was used as a control, and the $NaNO_3$ (nitrogen source) and NaH_2PO_4 (phosphorus source) concentration in the RAS and Conway nutrient solution is accessible in Appendix C.

The RAS nutrient solution consisted of nitrogen and phosphorus concentration simulating real RAS water. The concentration of nitrogen and phosphorus (in the form of $NaNO_3$ and NaH_2PO_4) of to the simulated RAS water, was based on data from a CIRIS and Nofitech report, accessible in Appendix G (Jakobsen, 2020). Nofitech AS is a company producing and selling standardized and certificated RAS-solutions to the aquaculture industry.

A complete list of nutrients and calculations of the two culture media are accessible in Appendix C, D and E. Table 8 show the concentration of nitrogen and phosphorus in the two culture media when all ingredients were added to the culturing flask.

Table 8: Concentration (µg/L) of nitrogen and phosphorus in the culture flasks (850 mL) for both types of culture media. The N/P ratios for the two media are also listed.

	N (µg/L)	P (µg/L)	N/P
RAS	47 000	3 741	12.6
Conway	25 000	6 689	3.74

2.4 Cell number registrations of the cultures

Samples for registrations of cell concentrations of the cultures were taken once every day of the culturing period. A sample (10 mL for P1, and 250 mL for P2) was taken from each flask and put in adjoining sample glasses. Before samples were taken, the air pipe was used to whip up possible sedimentation and the flasks were gentely stirred to ensure a homogenous medium.

For P1, the sampling was done approximately within the same hour every day, but for P2 it was very important to do the sampling at the exact same time every day.

Cell countings of the samples weres done using a Multisizer 3 coulter counter (Beckman Coulter). Each sampling glass was mixed well and then diluted with filtrated seawater at a suitable proportion for the counter to handle (the number of cells/mL should not be greater than 30 000). Three counts for each sample were carried out. Results from the daily counting were filled into the laboratory journal as well as in an excel line graph, making it easy to follow the growth of the cultures from day to day. When the stationary phase was reached, the experiment was terminated.

2.5 Samples of culture media and microalgae cells

Water samples of batch cultures - start

Before adding inoculum to the culture flasks, water samples (5 mL) were taken from each flask for later analysis of nitrogen and phosphorus.

Filtration and water samples of batch cultures - end

When the culturing period was terminated, each culture flask was filtrated. The exact volume filtrated was evaluated ongoing during the filtration process, depending on how dense each culture appeared to be. A graduated cylinder (30 mL) was used for measuring. The filtrate (24 mL) from each filtration was stored in centrifuge tubes, and the filters were put in separatee petri dishes and covered in aluminium foil. The samples were then stored in the freezer (-21℃). Figure 9 illustrates the filtration setup.

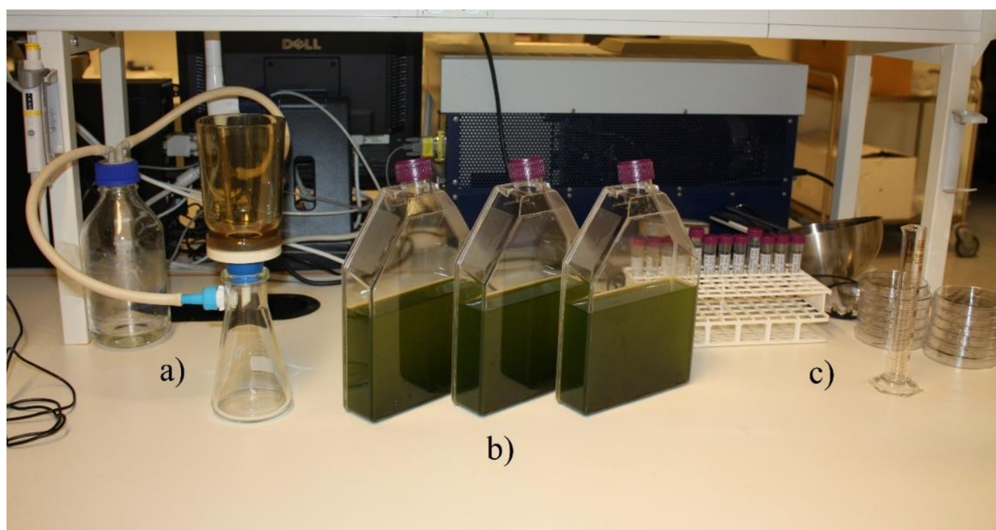


Figure 9: Filtration setup with Büchner flask, filtration funnel and rubber tubing (a), culturing flasks (b), plastic containers and petri dishes for preserving of samples (c).

Filtration and water samples of semicontinuous cultures (Part 2)

Filtrations were carried out in the end of the cultivation period as this would represent the steady state of the cultures. The filtration design and performance were identical as for P1.

2.6 Analyses

2.6.1 Chlorophyll a analysis

Prior to the analysis, two small pieces (factor 20.51 of the total filter) from each filter was cut out and placed in separate glass containers. Each container was then added cold 100% methanol (5 mL for P1E1 and start filters for P1E2, and 10 mL for P1E2 and P2), mixed well and stored in the freezer. Due to dense filters from P1E2 and P2, even smaller filter pieces (factor 61.27 of the total filter) needed to be cut out for the algae to fully dissolve in the methanol. After 18 hours in the freezer, chlorophyll a content in each sample was measured in a fluorometer (Turner designs) following well established methods. Each sample was measured twice, giving a total of four measures for each filter.

2.6.2 Cellular nitrogen and carbon analysis

Small filter pieces (factor 20.51 of the total filter for P1E1 and start filters for P1E2, and factor 61.27 for P1E2 and P2) were cut out, put in separate tin capsules, wrapped into balls, and stored in a heating cabinet overnight. In total, two pieces from each filter were analysed for cellular nitrogen and carbon on an elemental analyser (vario EL cube, Elementar Analysensysteme GmbH, Germany).

2.6.3 Cellular phosphorus analysis

Small filter pieces (factor 20.51 of the total filter) were put in scintillation vials, added aqua pure (10 mL), oxidizing reagent (2 mL) and sulfuric acid 4 M (0.1 mL), giving a total of 0.0121 L liquid added. The samples were then autoclaved (120°C at 1.1 bar), cooled and analysed in an autoanalyzer following Norwegian Standard 6878:2004 for phosphate analysis (SN, 2004) . In total, two pieces from each filter were analysed for cellular phosphorus content. To keep the reading of the sample within the standard curve, some of the samples were diluted with aqua pure.

2.6.4 Culture medium nitrogen and phosphorus analysis

Samples from the filtrated medium were analysed for nitrogen and phosphorus in an autoanalyzer following Norwegian Standard 4745:1991 and 6878:2004 respectively (SN, 1991, SN, 2004). To keep the reading of the sample within the standard curve, some of the samples were diluted with aqua pure.

2.7 Calculations

2.7.1 Growth rate

Specific growth rate (μ) of the cultures for each day was determined according to Equation 2.1 (Andersen, 2005). Time between sampling was measured in hours and multiplied with 24 to calculate per day. Maximum specific growth rate (μ_{max}) was calculated using the same equation in the exponential initial growth phase, when lnN versus time was linear and had the highest slope.

$$\mu = \left(\frac{lnN_1 - lnN_0}{\Delta t}\right) \times 24$$ Equation 2.1

$N_1 = number\ of\ cells/ml\ at\ the\ end\ of\ the\ time\ interval$

$N_0 = number\ of\ cells/ml\ at\ the\ beginning\ of\ the\ time\ interval$

$\Delta t = Length\ of\ time\ interval\ in\ hours\ (t_1 - t_0)$

2.7.2 Cellular content of chlorophyll a

The chlorophyll a content per cell for each experiment was calculated according to Equation 2.2.

$\rho g\ chla/cell =$

$$\left(\left(\left(\frac{(FL - BL) \times f \times E \times 1000}{V \times 1000}\right) \times S\right)/cells\ /L\right) \times 1000000$$ Equation 2.2

$FL = reading\ of\ the\ sample\ (\mu g/L)$

$BL = reading\ of\ the\ blanc\ (100\%\ methanol)$

$f = calibration\ factor\ of\ the\ instrument\ (0.47)$

$E = extraction\ volume\ in\ ml\ (5\ ml\ or\ 10\ ml)$

$V = filtered\ volume\ in\ ml$

$S = factor\ representing\ the\ size\ of$

$the\ smaller\ filter\ piece$ (20.51 or 61.27)

2.7.3 Cellular content of carbon and nitrogen

The result is given in µg/capsule and converted to tissue content per cell according to Equation 2.3.

$$\rho g\ \ C\ or\ N/cell = \left(\left(\left(\frac{FL \times 1000}{V}\right) \times S\right) /\ cells/L\ \right) \times 1000000 \qquad \text{Equation 2.3}$$

$FL = reading\ of\ the\ sample\ (\mu g/capsule)$

$V = filtered\ volume\ in\ ml$

$S = factor\ representing\ the\ size\ of$

$\quad the\ smaller\ filter\ piece$ (20.51 or 61.27)

2.7.4 Cellular content of phosphorus

With respect to the proportion between sample and water (dilution), the reading of the sample needed to be multiplied with a dilution factor. The result is given in µg P/L and converted to tissue content per cell according to Equation 2.4.

Equation 2.4

$$\rho g\ P/cell = \left(\left(\left(\frac{FL \times D \times 0.0121 \times 1000}{V}\right) \times S\right)\ /cells/L\right) \times 1000000$$

$FL = reading\ of\ the\ sample\ (\mu g/L)$

$V = filtered\ volume\ in\ ml$

$D = dilution\ factor$

$S = \ factor\ representing\ the\ size\ of$

$\quad the\ smaller\ filter\ piece\ (20.51\ or\ 61.27)$

2.7.5 Content of nitrogen and phosphorus in medium

With respect to the proportion between sample and water (dilution), the reading of the sample needed to be multiplied with a dilution factor. The results given in µg P or N/L were calculated with respect to dilution according to Equation 2.5.

$$\mu g\ N\ or\ P/L = FL \times D \quad \text{Equation 2.5}$$

$$FL = reading\ of\ the\ sample\ (\mu g/L)$$

$$D = dilution\ factor$$

Equation 2.6 was used to calculate percentage removal of nitrogen and phosphorus from the medium, comparing start and end concentration.

$$r\% = \frac{S_0 - S_1}{S_0} \times 100 \quad \text{Equation 2.6}$$

$$S_0 = concentration\ of\ N\ or\ P\ at\ start$$
$$S_1 = concentration\ of\ N\ or\ P\ at\ the\ end$$

2.8 Statistics

Handling and sorting of raw data material, as well as minor calculations was carried out using Microsoft Excel 2016. SigmaPlot 14.0 was used to make graphs, and SPSS for statistical analyses. The data was assumed normally distributed and homogenous. Testing of significance ($p < 0.05$) between low and high light intensities within separate treatments, as well as the overall comparison of end biomass between the different treatments, was carried out by performing t-tests. It would have been possible to run a two-way ANOVA with simple effects analysis for the purpose of comparing low and high light within separate treatments, but it was considered inconvenient because the Conway treatment did have a smaller sample size than RAS.

Testing of significance between the different RAS salinities at the same light intensity was carried out by performing two one-way ANOVAs combined with a post hoc Tukey's test (one ANOVA for high light intensity, and one for low).

Comparison with respect to different nutrient medium was done by performing a t-test between RAS34 (high light intensity) and Conway34 (high light intensity), as well as between RAS34 (low intensity) and Conway34 (low light intensity).

3 Results

3.1 Biomass development of *R. baltica* and *D. tertiolecta*

3.1.1 Growth curves

The following section presents growth curves for the different cultures illustrating the biomass development for the *R. baltica* and *D. tertiolecta* culturing experiment, respectively. The cell numbers for each graph are accessible in Appendix H.

R. baltica

The overall picture is that use of RAS media resulted in a higher biomass compared to the Conway treatments, when comparing the end cell densities (Figure 10, a). Comparing the RAS treatments alone, RAS15- seems to have gained the highest biomass, although the number is not significantly higher that RAS10- and RAS15+. Comparing high and low light intensity within each treatment, there was no significant difference between RAS10+/-, RAS15+/-, RAS34+/- or Conway34+/-. Although it is no significant difference, it does seem to be a trend suggesting that low light intensity resulted in higher biomass than high light intensity for the RAS treatments (Figure 10, a). Comparing RAS treatments with high light intensity to each other and RAS treatments with low light intensity to each other, no significant difference was found. Comparison between the two media did unveil a significant difference between RAS34+ and Conway34+ ($p = 0.005$) and between RAS34- and Conway34- ($p = 0.010$), with RAS having the highest biomass.

D. tertiolecta

The overall picture is that use of RAS media resulted in a higher biomass compared to the Conway treatment, when comparing the end cell densities (Figure 10, b). Comparing all the RAS treatments alone, RAS15+ seems to have gained the highest biomass, although the number is not significantly higher than RAS34+ and RAS10-. Comparing high and low light intensity within each treatment, there was no significant difference between RAS10+/- and Conway34+/-, but it was a significant difference between RAS15+/- ($p = 0.022$) and RAS34+/- (0.023). Based on the results, it does seem to be a trend suggesting that high light intensity resulted in higher biomass than low light intensity for the RAS treatments (Figure 10, b). Comparing RAS treatments with high light intensity to each other and RAS treatments with low light intensity

to each other, no significant difference was found. There was observed a slight significant difference between RAS34+ and Conway34+ (p = 0.049) and RAS34- and Conway34- (p = 0.006).

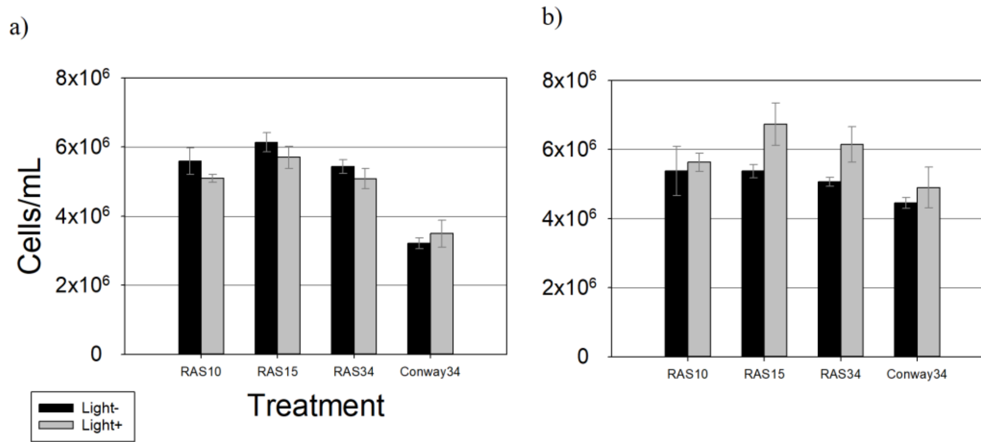


Figure 10: Final biomass for the different treatments of the culturing of *R. baltica* (a) and *D. tertiolecta* (b). Error bars are shown as SD (n=3).

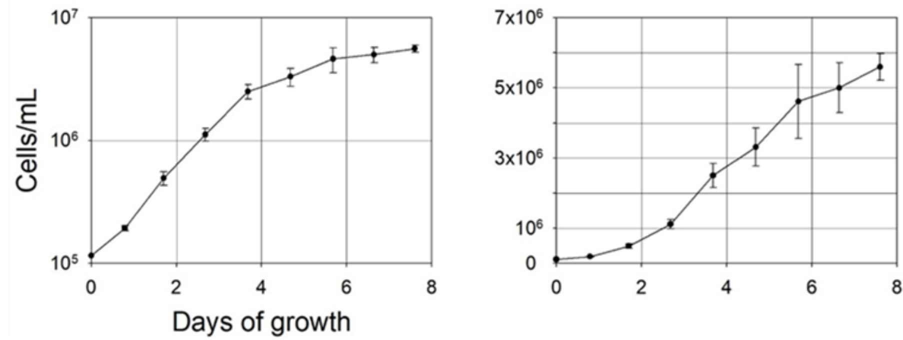


Figure 11: Growth curves of *R. baltica* cultured in RAS 10‰ at low light intensity, shown in logarithmic scale (left panels) and linear scale (right panels). Error bars are shown as SD (n=3).

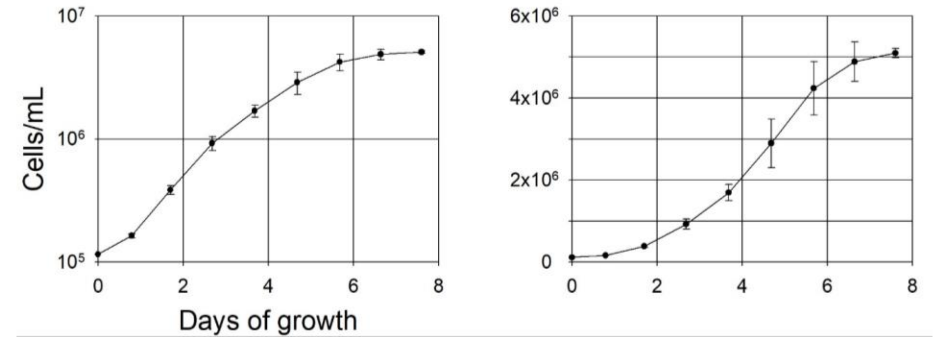


Figure 12: Growth curves of *R. baltica* cultured in RAS 10‰ at high light intensity, shown in logarithmic scale (left panels) and linear scale (right panels). Error bars are shown as SD (n=3).

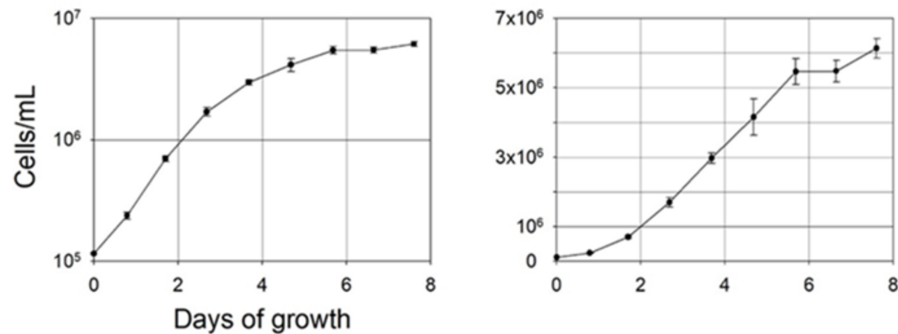


Figure 13: Growth curves of *R. baltica* cultured in RAS 15‰ at low light intensity, shown in logarithmic scale (left panels) and linear scale (right panels). Error bars are shown as SD (n=3).

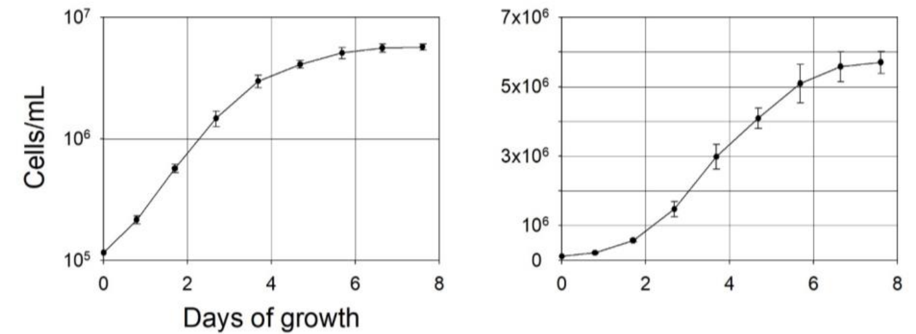


Figure 14: Growth curves for *R. baltica* cultured in RAS 15‰ at high light intensity, shown in logarithmic scale (left panels) and linear scale (right panels). Error bars are shown as SD (n=3).

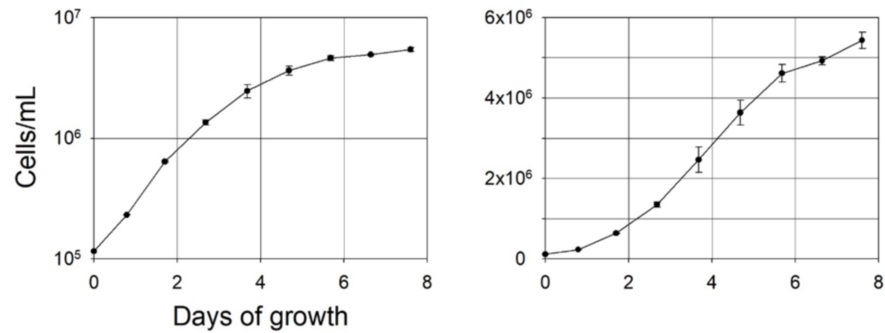


Figure 15: Growth curves of *R. baltica* cultured in RAS 34‰ at low light intensity, shown in logarithmic scale (left panels) and linear scale (right panels). Error bars are shown as SD (n=3).

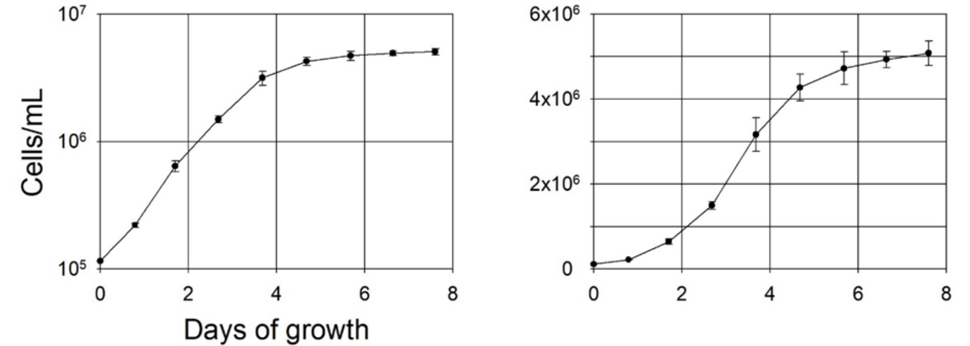


Figure 16: Growth curves of *R. baltica* cultured in RAS 34‰ at high light intensity, shown in logarithmic scale (left panels) and linear scale (right panels). Error bars are shown as SD (n=3).

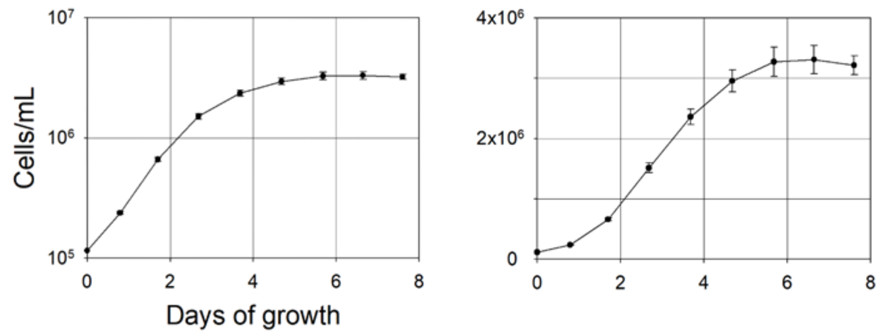


Figure 17: Growth curves of *R. baltica* cultured in Conway 34‰ at low light intensity, shown in logarithmic scale (left panels) and linear scale (right panels). Error bars are shown as SD (n=3).

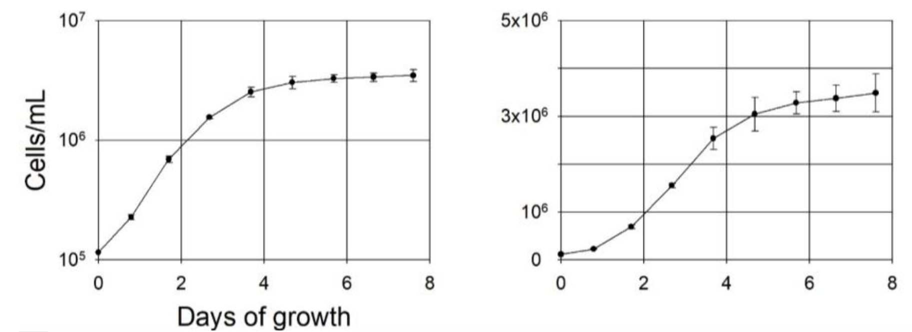


Figure 18: Growth curves of *R. baltica* cultured in Conway 34‰ at high light intensity, shown in logarithmic scale (left panels) and linear scale (right panels). Error bars are shown as SD (n=3).

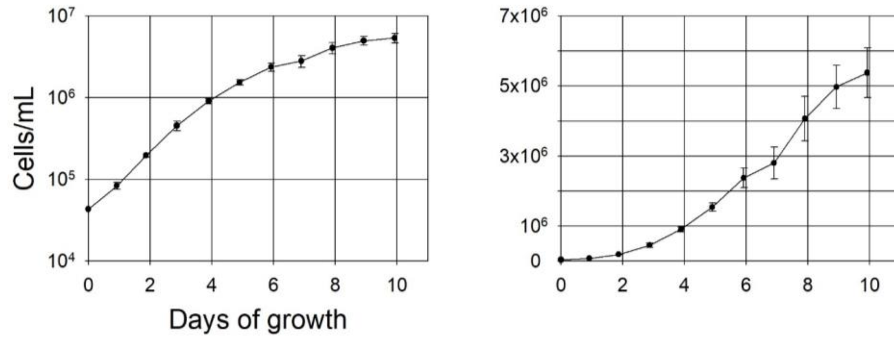


Figure 19: Growth curves of *D. tertiolecta* cultured in RAS 10‰ at low light intensity, shown in logarithmic scale (left panels) and linear scale (right panels). Error bars are shown as SD (n=3).

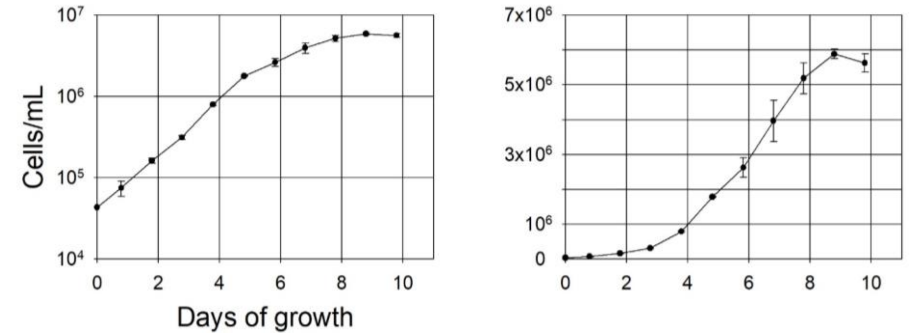


Figure 20: Growth curves of *D. tertiolecta* cultured in RAS 10‰ at high light intensity, shown in logarithmic scale (left panels) and linear scale (right panels). Error bars are shown as SD (n=3).

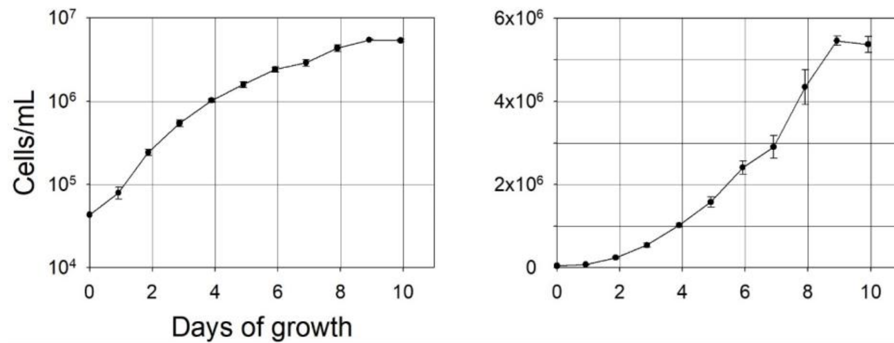


Figure 21: Growth curves of *D. tertiolecta* cultured in RAS 15‰ at low light intensity, shown in logarithmic scale (left panels) and linear scale (right panels). Error bars are shown as SD (n=3).

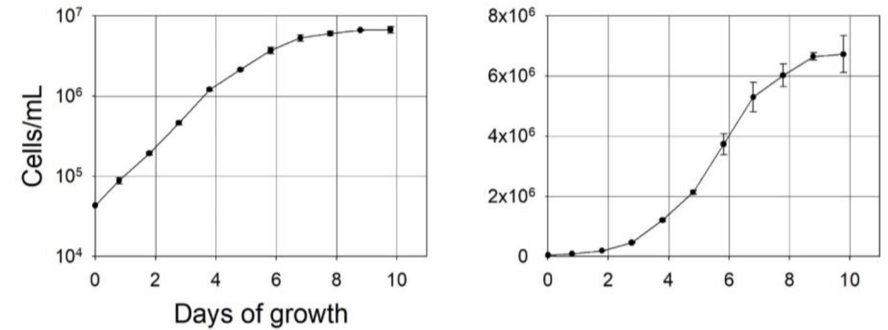


Figure 22: Growth curves of *D. tertiolecta* cultured in RAS 10‰ at high light intensity, shown in logarithmic scale (left panels) and linear scale (right panels). Error bars are shown as SD (n=3).

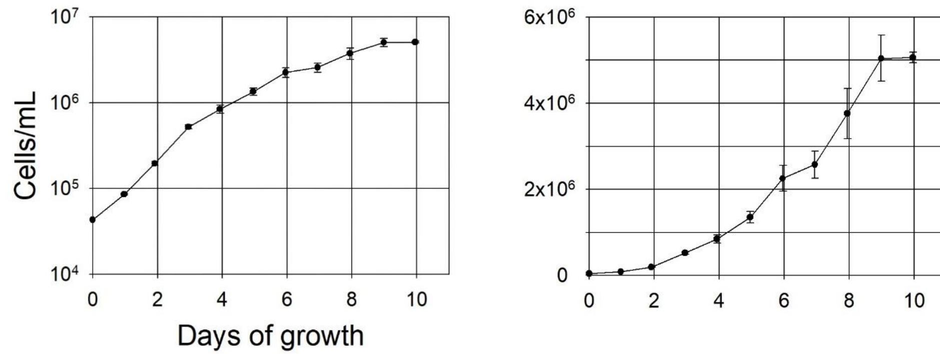


Figure 23: Growth curves of *D. tertiolecta* cultured in RAS 34‰ at low light intensity, shown in logarithmic scale (left panels) and linear scale (right panels). Error bars are shown as SD (n=3).

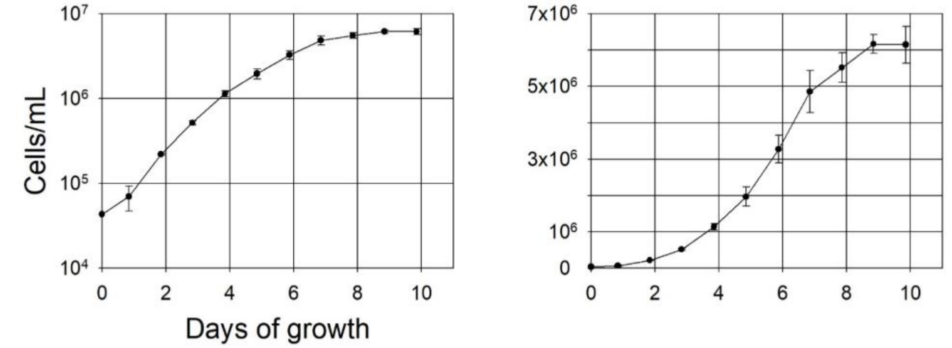


Figure 24: Growth curves of *D. tertiolecta* cultured in RAS 34‰ at high light intensity, shown in logarithmic scale (left panels) and linear scale (right panels). Error bars are shown as SD (n=3).

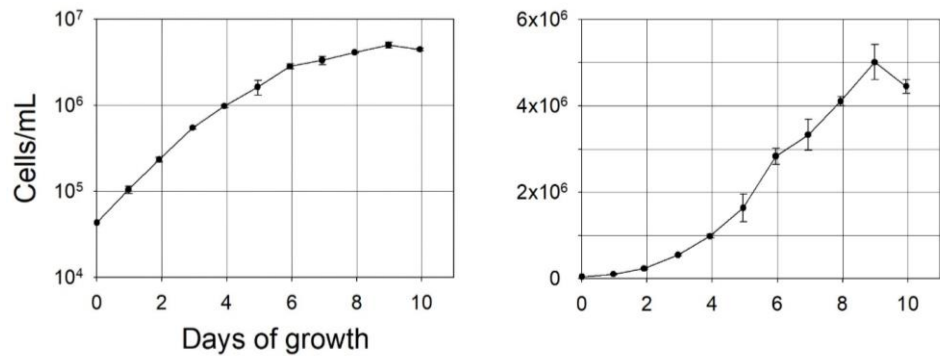


Figure 25: Growth curves of *D. tertiolecta* cultured in Conway 34‰ at low light intensity, shown in logarithmic scale (left panels) and linear scale (right panels). Error bars are shown as SD (n=3).

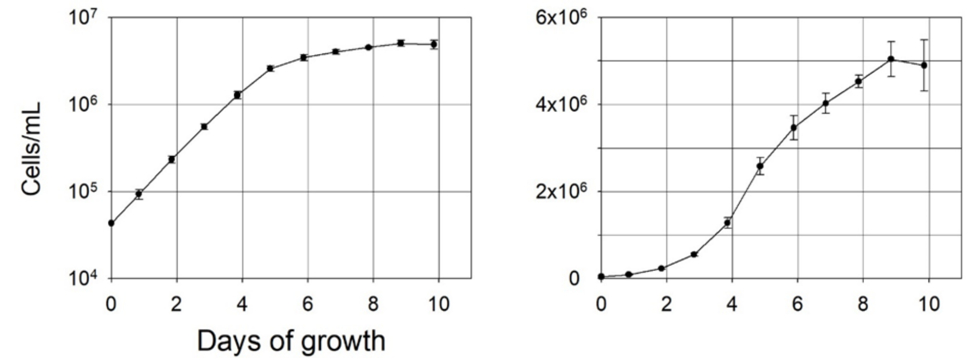


Figure 26: Growth curves of *D. tertiolecta* cultured in Conway 34‰ at high light intensity, shown in logarithmic scale (left panels) and linear scale (right panels). Error bars are shown as SD (n=3).

3.1.2 Maximum specific growth rate

A *R. baltica*

The daily specific growth rate for each treatment is accessible in Appendix I. No significant differences between high and low light intensity within the separate treatments was found, except from Conway34 with high and low light intensities ($p = 0.037$) where the treatment with high light showed a slightly higher μ_{max} value. Comparing RAS treatments with high light intensity to each other and RAS treatments with low light intensity to each other, no significant difference was found. There was found no significant difference between RAS34 and Conway34 with same light intensity either. The overall impression of the graph gives no indication of any trends of differences.

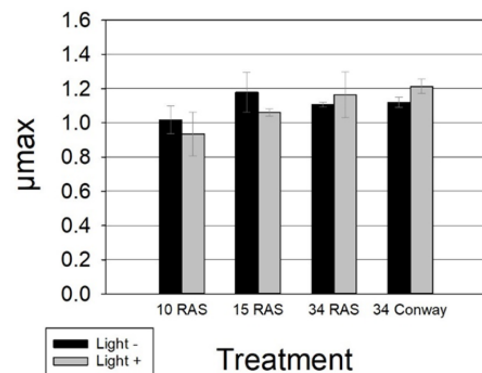


Figure 27: Maximum specific growth rate (μ_{max}) for each treatment during the cultivation experiment of *R. baltica*. The two light intensities are presented as separate bars. Error bars are shown as SD (n=3).

B *D. tertiolecta*

The daily specific growth rate for each treatment is accessible in Appendix I. No significant difference between high and low light intensity within the separate treatments was found. Comparing RAS treatments with high light intensity to each other and RAS treatments with low light intensity to each other, no significant difference was found. There was found no significant difference between RAS34 and Conway34 with same light intensity either. The overall impression of the graph gives no indication of any trends of differences.

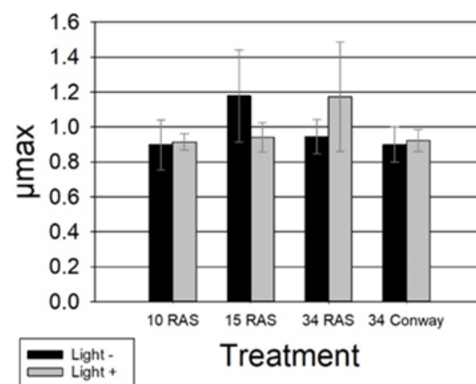


Figure 28: Maximum specific growth rate (μ_{max}) for each treatment during the culturing experiment of *D. tertiolecta*. The two light intensities are presented as separate bars. Error bars are shown as SD (n=3).

3.2 Cellular contents

3.2.1 Chlorophyll a contents

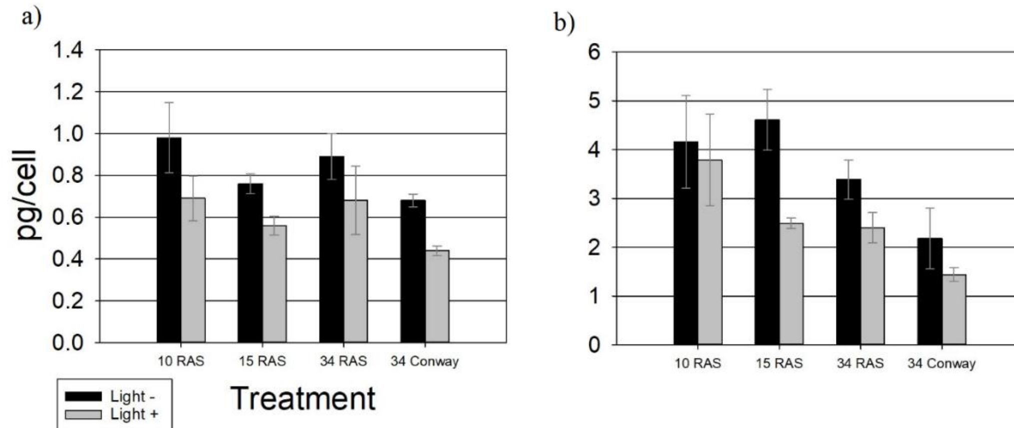


Figure 29: Cellular chlorophyll a content (picogram/cell) at the end of the culturing period for *R. baltica* (a) and *D. tertiolecta* (b). The two light intensities are presented as separate bars. Error bars are shown as SD (n=3). Notice the different scaling on the y axis.

R. baltica

A significant differences was found between high and low light intensity for RAS15+/- ($p = 0.005$) and Conway34+/- ($p = 0.000422$), with the lowest light intensity giving the highest content. This is easily observed on the graph as well. The graph gives an overall impression that there is a higher content of chlorophyll a in the algae cultivated on lower light intensity, even though there is no significant difference between high and low light intensity for RAS10 and RAS34. Comparing all RAS treatments with high light intensity to each other and all RAS treatments with low light intensity to each other, no significant difference was found. Comparison between the two media did unveil a significant difference between RAS34- and Conway34- ($p = 0.035$), but no difference between RAS34+ and Conway34-. The graph does although give the impression of a trend showing a difference between the two medium with respect to chlorophyll a content.

D. tertiolecta

A significant differences was found between high and low light intensity for RAS15+/- ($p = 0.004$) and RAS34+/- ($p = 0.027$), with the lowest light intensity giving the highest content. This is easily observed on the graph as well. The graph gives an overall impression that there is a higher content of chlorophyll a in the algae cultivated on lower light intensity, even though

there is no significant difference between high and low light intensity for RAS10 and high and low light intensity for Conway34. Comparing RAS treatments with high light intensity to each other and RAS treatments with low light intensity to each other, no significant difference was found. Although the graph gives an impression of RAS10+ ranging over RAS15+ and RAS34+. Comparison between the two media did unveil a significant difference between RAS34+ and Conway34+ (p = 0.008), as well as between RAS34- and Conway34- (p = 0.048) with RAS34 giving the highest content for both.

3.2.2. Carbon contents

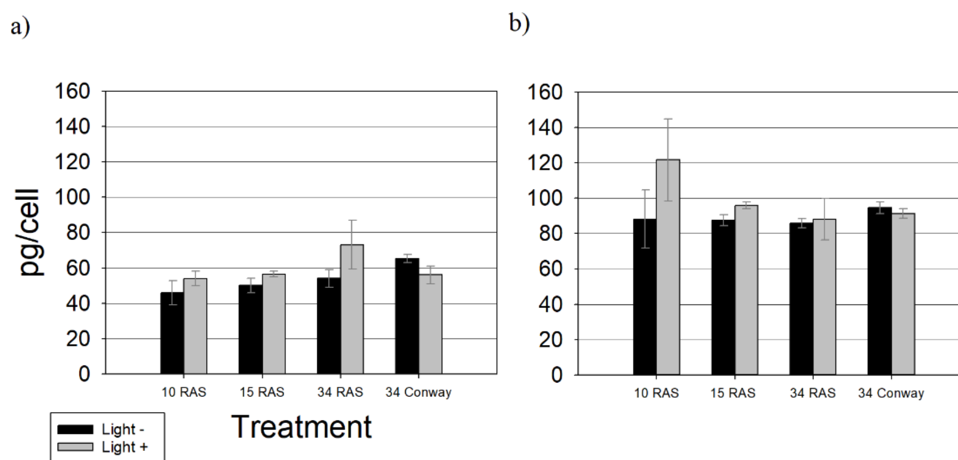


Figure 30: Cellular carbon content (picogram/cell) at the end of the culturing period for *R. baltica* (a) and *D. tertiolecta* (b). The two light intensities are presented as separate bars. Error bars are shown as SD (n=3).

<u>*R. baltica*</u>

No significant difference was found between high and low light intensity within the separate treatments, except from a minor difference between Conway34+/- (p = 0.045), with the lowest light intensity giving the highest content. The graph does although give an impression of a trend for the RAS treatments, leaving the suggestion that the highest light intensity gives the highest content. Comparing RAS treatments with high light intensity to each other and RAS treatments with low light intensity to each other, no significant difference was found although the graph gives do give an impression of RAS34+ ranging above RAS10+ and RAS15+. Comparison between the two media did unveil a significant difference between RAS34- and Conway34- (p = 0.023).

D. tertiolecta

No significant difference was found between high and low light intensity within the different treatments, except from RAS15 (p = 0.017) with the highest light intensity giving the highest content. The graph does although give an impression of a trend, leaving the suggestion that the highest light intensity gives the highest content. Comparing RAS treatments with high light intensity to each other and RAS treatments with low light intensity to each other, no significant difference was found although the graph does give an impression of RAS10+ ranging above RAS15+ and RAS34+. Comparison between the two media did unveil a significant difference between RAS34- and Conway34- (p = 0.021) with Conway34 giving the highest content.

3.2.3 Nitrogen contents

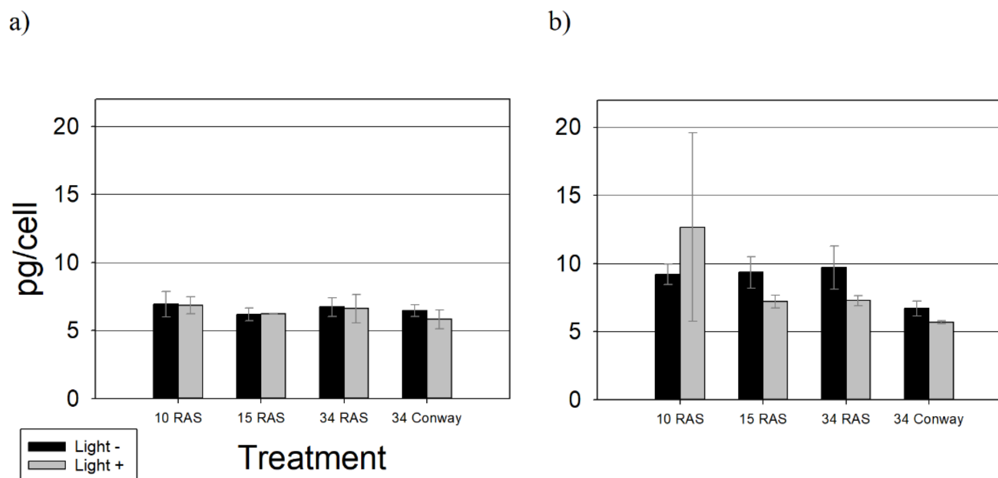


Figure 31: Cellular nitrogen content (picogram/cell) at the end of the culturing period for *R. baltica* (a) and *D. tertiolecta* (b). The two light intensities are presented as separate bars. Error bars are shown as SD (n=3).

R. baltica

No significant difference was found between high and low light intensity within the separate treatments, and it does not seem to be any trends either. Comparing RAS treatments with high light intensity to each other and RAS treatments with low light intensity to each other, no significant difference was found. Comparison between the two media did not unveil any significant differences, neither for high nor low light intensity. The overall impression of the graph is that there are only minor differences, and no clear trends.

D. tertiolecta

No significant difference was found between high and low light intensity within the separate treatments, except from Conway34+/- (p = 0.033) and a minor difference between RAS15+/- (p = 0.040), with the lowest light intensity giving the highest content. Comparing RAS treatments with high light intensity to each other and RAS treatments with low light intensity to each other, no significant difference was found. The graph gives an impression of RAS10+ ranging high above all the other treatments. Comparison between the two media did unveil a significant difference between RAS34+ and Conway34+ (p = 0.002), as well as between RAS34- and Conway34- (p = 0.036) with RAS34 giving the highest content for both. The overall impression is that the algae cultured in Conway medium did gain a lower nitrogen content.

3.2.4 Phosphorus contents

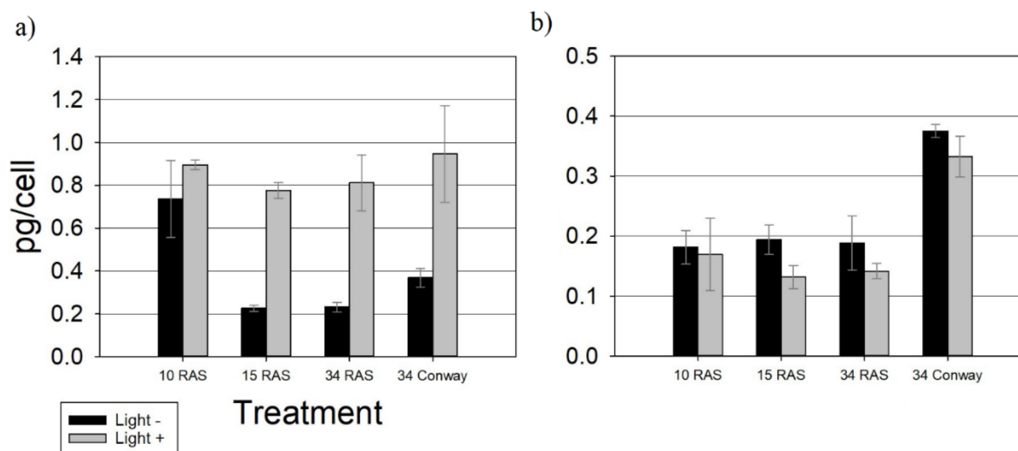


Figure 32: Cellular phosphorus content (picogram/cell) at the end of the culturing period for *R. baltica* (a) and *D. tertiolecta* (b). The two light intensities are presented as separate bars. Error bars are shown as SD (n=3). Notice the different scaling on the y axis.

R. baltica

A significant difference was found between high and low light intensity for RAS15+/- (p = 0.000018), RAS34+/- (p = 0.002) and Conway34+/- (p = 0.012) with the highest light intensity giving the highest phosphorus content for all of them. Both the graph and the statistical results gives an overall suggestion of high light intensity giving a higher phosphorus content. Comparing RAS10- and RAS15-, there was a significant difference (p = 0.002) as well as

between RAS10- and RAS34- (p = 0.003). Comparison between the two media did unveil a significant difference between RAS34- and Conway34- (p = 0.002).

D. tertiolecta

No significant difference was found between high and low light intensity within the different treatments, except from RAS15+/- (p = 0.025) with the lowest light intensity giving the highest phosphorus content. Although, the graph gives an overall impression that a lower light intensity gives a higher phosphorus content. Comparing RAS treatments with high light intensity to each other and RAS treatments with low light intensity to each other, no significant difference was found. Comparison between the two media did unveil a significant difference between RAS34+ and Conway34+ (p = 0.001), as well as between RAS34- and Conway34- (p = 0.002), with Conway34 giving the highest content for both. The overall impression of this graph is that the Conway treatment regardless of high or low light intensity, range above the RAS treatments.

3.2.5 Ratios between cellular contents

Chlorophyll a/carbon

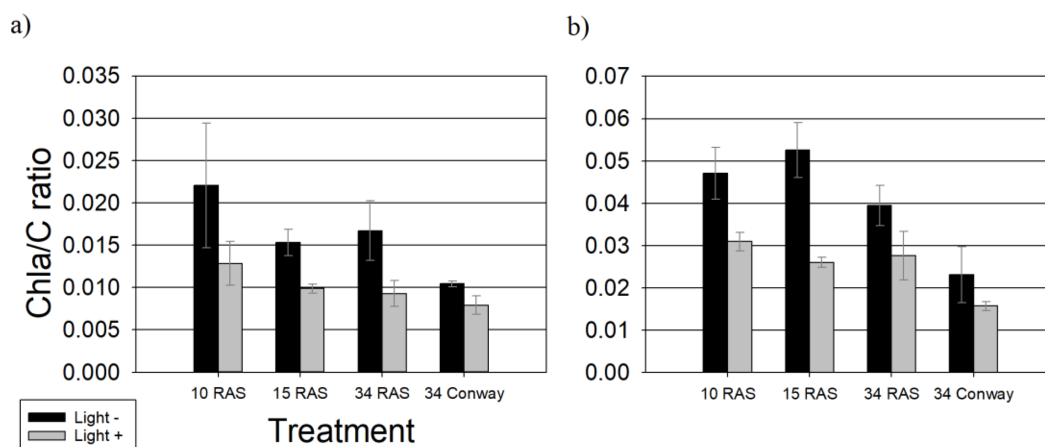


Figure 33: Chlorophyll a/carbon ratio (mass) for the different treatments at the end of the culturing period for *R. baltica* (a) and *D. tertiolecta* (b). The two light intensities are presented as separate bars. Error bars are shown as SD (n=3). Notice the different scaling on the y axis.

R. baltica

A significant difference was found between high and low light intensity within the separate treatments comparing RAS15+/- (p = 0.004), RAS34+/- (p = 0.029) and Conway34+/- (p =

0.020), with the lowest light intensity giving the highest ratio. This leaves the suggestion that low light intensity resulted in higher ratio than high light intensity, as it seems to be a trend. Comparing RAS treatments with high light intensity to each other and RAS treatments with low light intensity to each other, no significant difference was found although the graph does give an impression of RAS10- range above RAS15- and RAS34-. Comparison between the two media did unveil a significant difference between RAS34- and Conway34- ($p = 0.037$), and the graph gives an overall impression of Conway having a lower ratio than RAS.

D. tertiolecta

A significant difference was found between high and low light intensity within the different treatments comparing RAS10+/- (0 = 0.012) and RAS15+/- ($p = 0.002$), with the lowest light intensity giving the highest ratio. As for *R. baltica*, the graph and these statistical results leaves the suggestion that low light intensity resulted in a higher ratio than high light intensity, as it seems to be a trend. Comparing RAS treatments with high light intensity to each other and RAS treatments with low light intensity to each other, no significant difference was found, although there seems to be some difference between the low light treatment. Comparison between the two media did unveil a significant difference between RAS34+ and Conway34+ ($p = 0.024$), as well as between RAS34- and Conway34- ($p = 0.026$), with RAS34 giving the highest ratio for both. The graph gives an overall impression of Conway having a lower ratio than RAS.

Nitrogen/carbon

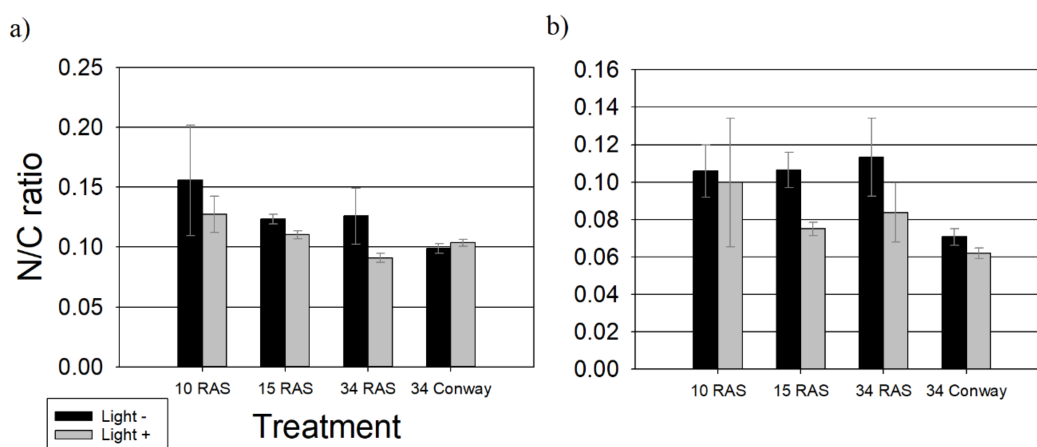


Figure 34: Nitrogen/carbon ratio (mass) for the different treatments at the end of the culturing period for *R. baltica* (a) and *D. tertiolecta* (b). The two light intensities are presented as separate bars. Error bars are shown as SD (n=3). Notice the different scaling on the y axis.

R. baltica

No significant difference was found between high and low light intensity within the separate treatments, except RAS15+/- ($p = 0.011$), with the lowest light intensity giving the highest ratio. Although it looks like an overall trend that low light intensity gives a higher ratio than high light intensity. Comparing RAS treatments with high light intensity to each other, there was found a difference between RAS10+ and RAS34+ ($p = 0.007$). Comparison between the two media did unveil a significant difference between RAS34+ and Conway34+ ($p = 0.010$), but it does also seem to be a difference between RAS34- and Conway34- although not significant.

D. tertiolecta

A significant difference was found between high and low light intensity for RAS15+/- ($p = 0.006$) and Conway34+/- ($p = 0.045$), with the lowest light intensity giving the highest ratio for both. As for *R. baltica*, it looks like an overall trend that low light intensity gives a higher ratio than high light intensity. Comparing RAS treatments with high light intensity to each other and RAS treatments with low light intensity to each other, no significant difference was found, although the graph does give an impression of RAS10+ range above RAS15+ and RAS34+. Comparison between the two media did unveil a significant difference between RAS34- and Conway34- ($p = 0.025$), but it does also seem to be a difference between RAS34+ and Conway34+ although not significant.

Nitrogen/phosphorus

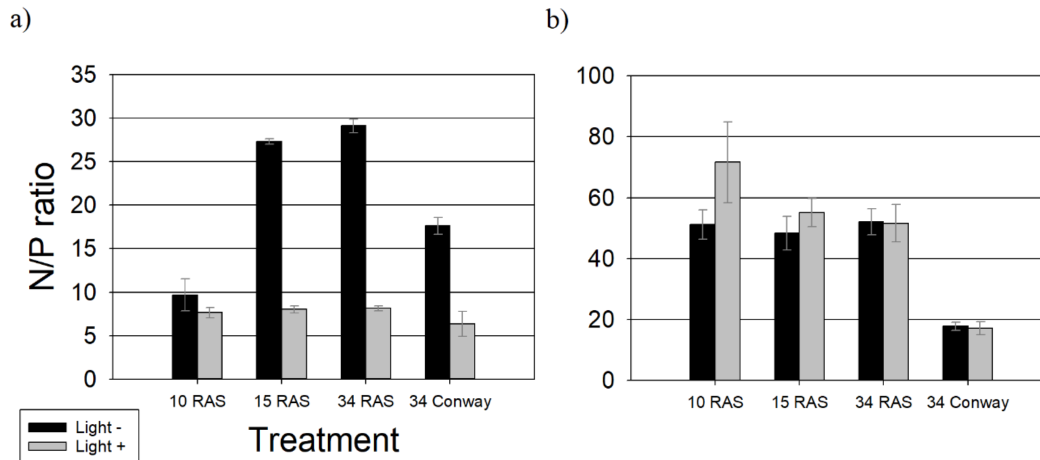


Figure 35: Nitrogen/phosphorus ratio (mass) for the different treatments at the end of the culturing period for *R. baltica* (a) and *D. tertiolecta* (b). The two light intensities are presented as separate bars. Error bars are shown as SD (n=3). Notice the different scaling on the y axis.

R. baltica

A significant difference was found between high and low light intensity within all the separate treatments, except from RAS10+/-. For RAS15+/- ($p = 0.0032$), RAS34+/- ($p = 0.000002$) and Conway34+/- ($p = 0.000347$), there is no doubt that it is the lowest light intensity that gives the highest ratio when looking at the graph. Comparing all RAS treatments exposed to low light intensity, there was a significant difference between RAS10+ and RAS15+ ($p = 0.000004$) and RAS10+ and RAS34+ ($p = 0.000002$). There was also a difference between RAS34- and Conway34- ($p = 0.000092$).

D. tertiolecta

No significant difference was found between high and low light intensity within each treatment, although it seems like a trend that high light intensity gives a slightly higher ratio. Comparing RAS treatments with high light intensity to each other and RAS treatments with low light intensity to each other, no significant difference was found. There was a significant difference between RAS34+ and Conway34+ ($p = 0.001$), as well as between RAS34- and Conway34- ($p = 0.000177$) with RAS34 giving the highest ratio for both.

Phosphorus/carbon

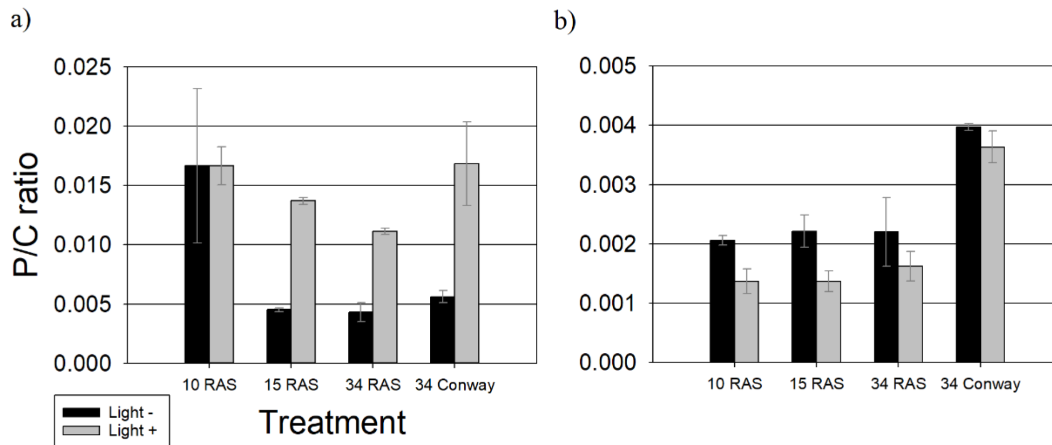


Figure 36: Phosphorus/carbon ratio (mass)for the different treatments at the end of the culturing period for *R. baltica* (a) and *D. tertiolecta* (b). The two light intensities are presented as separate bars. Error bars are shown as SD (n=3). Notice the different scaling on the y axis.

<u>*R. baltica*</u>

A significant difference was found between high and low light intensity within all the separate treatments, except from RAS10+/-. For RAS15+/- (p = 0.000001), RAS34+/- (p = 0.000156) and Conway34+/- (p = 0.005) respectively. There is no doubt that it is the highest light intensity that gives the highest ratio when looking at the graph. Comparing all RAS treatments exposed to high light intensity, there was a significant difference between all of them, RAS10+ and RAS15+ (p = 0.022), RAS10+ and RAS34+ (p = 0.001) and RAS15+ and RAS34+ (p = 0.038). Comparing RAS treatments exposed to low light intensity, there was a significant difference between RAS10- and RAS15- (p = 0.018) as well as RAS10- and RAS34- (p = 0.017). Comparing RAS34+ and Conway34+, there was a significant difference (p = 0.049).

<u>*D. tertiolecta*</u>

A significant difference was found between RAS10+/- (p = 0.006) and RAS15+/- (p = 0.010), with the lowest light intensity giving the highest ratio for both. The overall picture of the graphs show an overall trend suggesting that the lower light intensity giving a higher ratio. Comparing RAS treatments with high light intensity to each other and RAS treatments with low light intensity to each other, no significant difference was found. There was a significant difference between RAS34+ and Conway34+ (p = 0.001), as well as between RAS34- and Conway34- (p = 0.006), with Conway34 giving the highest ratio for both.

3.3 Removal of nitrogen and phosphorus from cultivation medium

3.3.1 Nitrogen removal

The percentage nitrogen removal calculations are based on the content of nitrogen in the medium at start and in the end of the cultivation.

A *R. baltica*

<u>Percentage removal from medium</u>

No significant difference was found between high and low light intensity within the separate treatments, except from Conway34+/- (p = 0.006) where the treatment with low light intensity showed a significant higher value and is easily observed on the graph as well. Comparing RAS treatments with high light intensity to each other and RAS treatments with low light intensity to each other, no significant difference was found. There was a distinct difference between RAS34+ and Conway34+ (p = 0.006), as well as a minor difference between RAS34- and Conway34- (p = 0.045), with RAS34- having the highest percentage removal. The overall picture is although that all RAS treatments have managed to remove a lot of the nitrogen available.

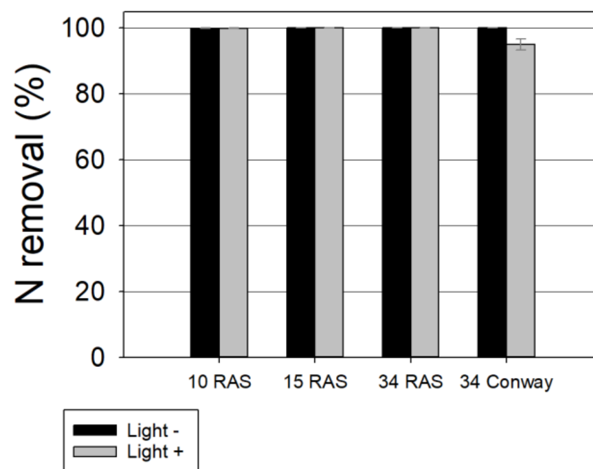


Figure 37: Percentage nitrogen removal from medium for each treatment. The two light intensities are presented as separate bars. Error bars are shown as SD (n=3).

Cellular content vs. medium content

The following table (Table 9) presents the content of nitrogen in medium at start (µg/L), and cellular content of nitrogen in the end (µg/L). The content is given with standard deviation.

Table 9: Nitrogen content in medium at start and cellular nitrogen content in the end for the different treatments for the *R. baltica* experiment. Error is shown as SD (n=3).

	N content in medium at start (µg/L)	SD	Cellular N content in the end (µg/L)	SD
RAS10+	43 711.7	3785.7	34 889.2	2395.8
RAS15+	44 908.7	5996.7	35 549.5	1844.2
RAS34+	42 018.7	5471.6	33 816.1	6751.3
Conway34+	22 796.9	1914.9	20 148.7	397.1
RAS10-	44 339.9	2548.9	38 658.6	2464.8
RAS15-	39 681.9	2542.2	37 845.6	1359.9
RAS34-	38 201.9	3871.0	36 664.4	4671.4
Conway34-	23 959.5	2075.2	20 724.0	445.4

B *D. tertiolecta*

Percentage removal from medium

No significant difference was found between RAS10+/- and RAS15+/-, but a difference was found within the different light intensities for RAS34+/- ($p = 0.014$) and Conway34+/- ($p = 0.039$), with the highest light intensity giving the highest percentage nitrogen removal. Comparing RAS treatments with high light intensity to each other and RAS treatments with low light intensity to each other, no significant difference was found. There was a significant difference between RAS34+ and Conway34+ ($p = 0.024$), as well as between RAS34- and Conway34- ($p = 0.014$), with RAS34 giving the highest percent removal for both. The overall picture is although that all RAS treatments as well as the Conway treatments have managed to remove a lot of the nitrogen available, and that the significant differences are so minor that they may not be considered differences at all.

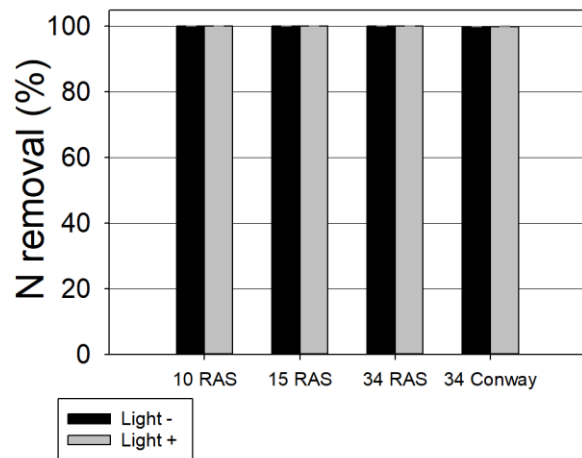


Figure 38: Percentage nitrogen removal from medium for each treatment. The two light intensities are presented as separate bars. Error bars are shown as SD (n=3).

Cellular content vs. medium content

The following table (Table 10) presents the content of nitrogen in medium at start (µg/L), and cellular content of nitrogen in the end (µg/L). Error is given as SD (n=3).

Table 10: Nitrogen content in medium at start and cellular nitrogen content in the end for the different treatments for the *D. tertiolecta* experiment. Error is shown as SD (n=3).

	N content in medium at start (µg/L)	SD	Cellular N content in the end (µg/L)	SD
RAS10+	45496.7	2749.5	49258.3	2943.8
RAS15+	50385.2	5308.6	48299.0	4426.6
RAS34+	48763.6	2631.8	44732.7	5969.2
Conway34+	27361.0	714.3	27748.2	3697.2
RAS10-	49228.3	6291.7	49203.2	3922.8
RAS15-	46748.3	3834.6	50038.1	4433.1
RAS34-	43403.0	2924.2	48997.3	7007.0
Conway34-	22334.7	820.0	29711.0	1427.8

3.3.2 Phosphorus removal

The percentage phosphorus removal calculations are based on the content of phosphorus in the medium at start and in the end of the cultivation.

A *R. baltica*

<u>Percentage removal from medium</u>

A significant difference was found between high and low light intensity for RAS10+/- ($p = 0.003$), RAS34+/- ($p = 0.016$) and Conway34+/- ($p = 0.001$), with the lowest light intensity giving the highest percentage removal for all of them. Comparing RAS treatments with high light intensity to each other and RAS treatments with low light intensity to each other, no significant difference was found. A distinct significant difference was found between RAS34+ and Conway34+ ($p = 0.000236$) as well as between RAS34- and Conway34- ($p = 0.000008$). The overall picture is that all RAS treatments have managed to remove a lot of the phosphorus available, while the Conway treatments have not.

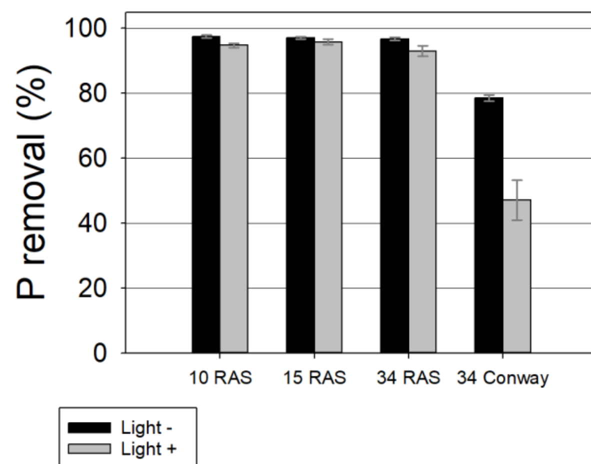


Figure 39: Percentage phosphorus removal from medium for each treatment. The two light intensities are presented as separate bars. Error bars are shown as SD (n=3).

<u>Cellular content vs. medium content</u>

The following table (Table 11) presents the content of phosphorus in medium at start (µg/L), and cellular content of phosphorus in the end (µg/L).

Table 11: Phosphorus content in medium at start and cellular phosphorus content in the end for the different treatments for the *R. baltica* experiment. Error is shown as SD (n=3).

	P content in medium at start (µg/L)	SD	Cellular P content in the end (µg/L)	SD
RAS10+	3 357.4	119.9	4 561.2	71.9
RAS15+	3 146.1	8.1	4 222.8	99.7
RAS34+	2 933.5	57.7	4 139.0	829.4
Conway34+	5 332.6	397.7	3 738.0	245.2
RAS10-	3 268.2	80.8	4 090.5	844.7
RAS15-	3 084.9	305.9	1 386.5	34.8
RAS34-	2 953.6	120.3	1 260.6	159.9
Conway34-	5 401.0	126.8	1 179.2	87.2

B *D. tertiolecta*

Percentage removal from medium

No significant difference was found between high and low light intensity within the different treatments, except from RAS34+/- (p = 0.005) with the lowest light intensity giving the highest percentage removal. Although this difference is hardy recognizable, and its validity must be questioned. Comparing RAS treatments with high light intensity to each other, a significant difference was found between RAS10+ and RAS34+ (p = 0.019), as well as between RAS15+ and RAS34+ (p = 0.024). Comparing the RAS treatments with low light intensity to each other, a significant difference was found between RAS10- and RAS34- (p = 0.002) and RAS15- and RAS34- (p = 0.005). There was a significant difference between RAS34+ and Conway34+ (p =0.025), as well as between RAS34- and Conway34- (p = 0.020), with Conway34 giving the highest percentage removal for both. The overall picture of this graph is although that all of the treatments, either RAS or Conway have gained a high percentage removal, ant that the significant differences are so minor that they may not be considered differences at all.

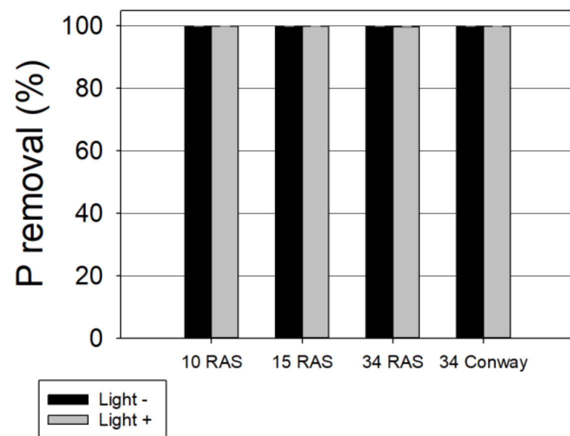


Figure 40: Percentage phosphorus removal from medium for each treatment. The two light intensities are presented as separate bars. Error bars are shown as SD (n=3).

Cellular content vs. medium content

The following table (Table 12) presents the content of phosphorus in medium at start (µg/L), and cellular content of phosphorus in the end (µg/L). The content is given with standard deviation.

Table 12: Phosphorus content in medium at start and cellular phosphorus content in the end for the different treatments for the *D. tertiolecta* experiment. Error is shown as SD (n=3).

	P content in medium at start (µg/L)	SD	Cellular P content in the end (µg/L)	SD
RAS10+	3665.7	79.9	949.3	315.1
RAS15+	3603.3	50.6	884.0	153.0
RAS34+	3637.3	36.1	866.2	47.3
Conway34+	6968.9	128.4	1616.7	17.1
RAS10-	3634.1	39.9	962.1	16.1
RAS15-	3591.6	46.9	1039.9	106.9
RAS34-	3579.1	128.6	950.6	205.8
Conway34-	7059.7	28.7	1670.1	48.5

3.4 Semicontinuous cultures

3.4.1 Biomass development

The cultures reached steady state during the culturing period, and the harvesting carried out on day four represents the steady state data for all semicontinuous cultures.

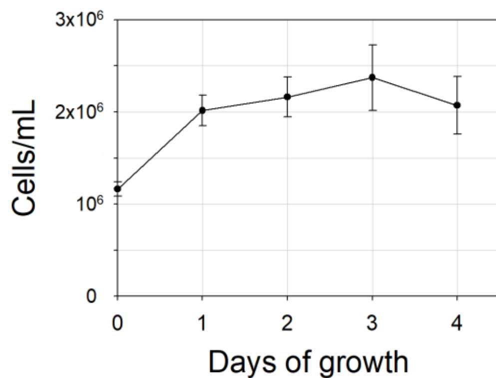


Figure 41: Growth curve of microalga cells (*R. baltica* + *D. tertiolecta*) cultured in RAS 10‰ during the cultivation period, shown in linear scale for RAS10. Error bars are shown as SD (n=3).

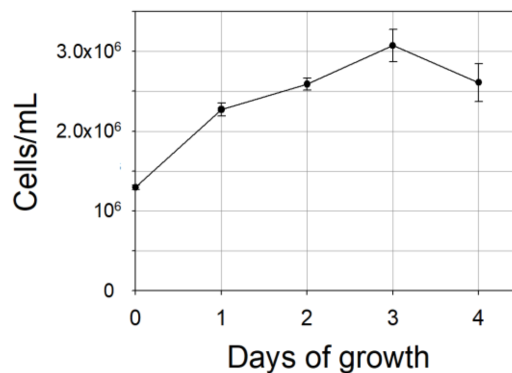


Figure 42: Growth curve of microalga cells (*R. baltica* + *D. tertiolecta*) cultured in RAS 15‰ during the cultivation period, shown in linear scale for RAS15. Error bars are shown as SD (n=3).

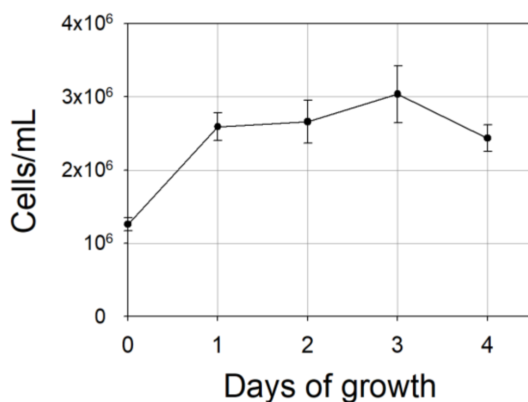


Figure 43: Growth curve of microalga cells (*R. baltica* + *D. tertiolecta*) cultured in RAS 34‰ during the cultivation period, shown in linear scale for RAS34. Error bars are shown as SD (n=3).

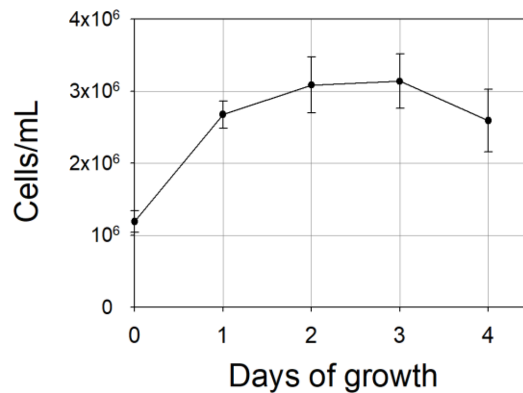


Figure 44: Growth curve of microalga cells (*R. baltica* + *D. tertiolecta*) cultured in Conway 34‰ during the cultivation period, shown in linear scale for Conway34. Error bars are

3.4.2 Species distribution

Using the coulter counter, it was challenging to differentiate between the two species. Even though, it was possible to differentiate between the two species for the RAS34 and Conway34 treatments. This will be presented in the following section. All results are given as triplicates, except for some where it was impossible to distinguish between the two species. All data regarding cell numbers are accessible in Appendix H. The graphs gives an impression of *D. tertiolecta* being the species with the highest biomass in the end, and the bright green colour of the culture itself support this suggestion (Figure 47).

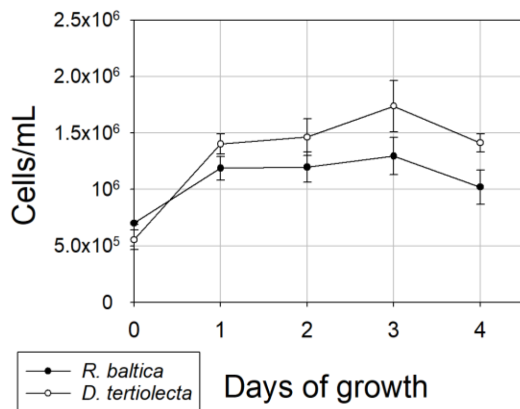


Figure 45: Growth curve of the two microalga species during the cultivation period, shown in linear scale for RAS34. *R. baltica* is marked in black and *D. tertiolecta* in white, and error bars are shown as SD (n=3).

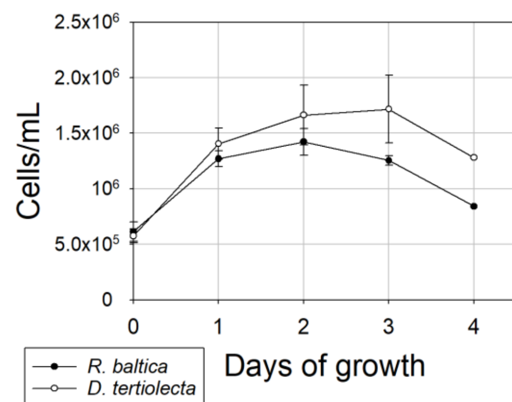


Figure 46: Growth curve of the two microalga species during the cultivation period, shown in linear scale for Conway34. *R. baltica* is marked in black and *D. tertiolecta* in white, and error bars are shown as SD (n=3).

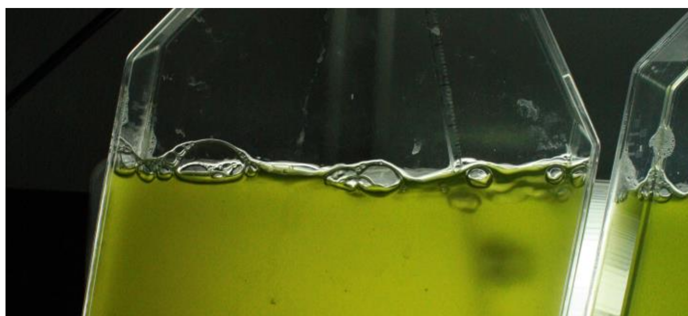


Figure 47: The bright green colour of the cultures at day four of the semicontinuous experiment.

3.4.3 Cellular contents and ratios at steady state

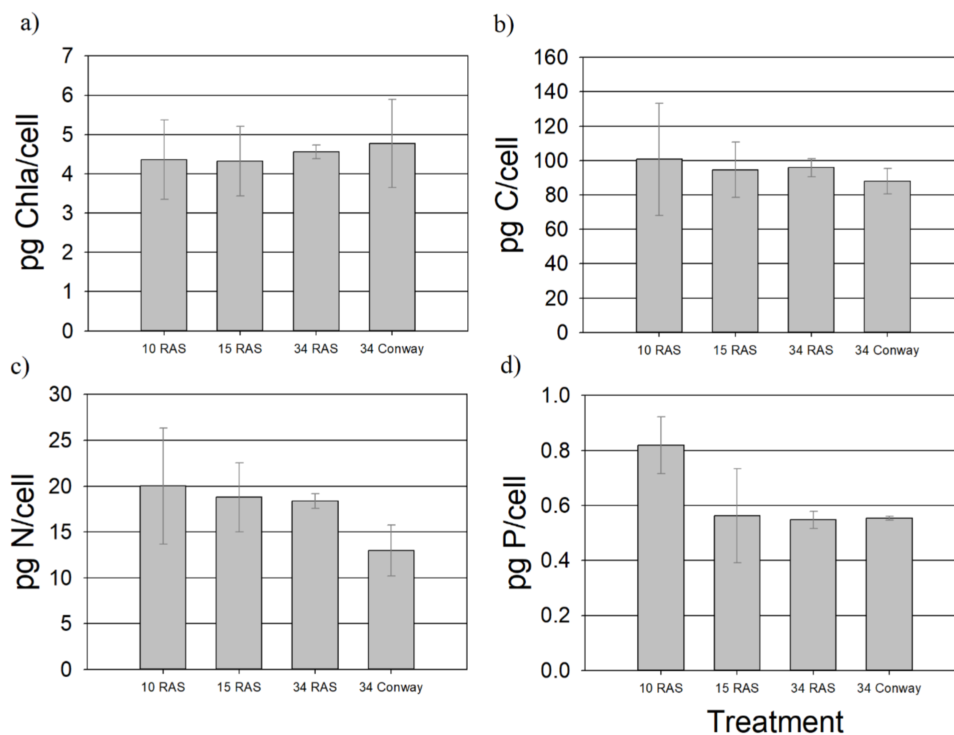


Figure 48: Cellular contents (picogram/cell) for the different treatments at the end of the semicontinuous experiment. Chlorophyll a (a), carbon (b), nitrogen (c) and phosphorus (d). Error bars are shown as SD (n=3). Notice the different scaling on the

Regarding the chlorophyll a content, no significant differences were found when comparing the RAS treatments of different salinities, as well as between RAS34 and Conway34 (Figure 48, a). Regarding the carbon content, no significant difference was found when comparing the RAS treatments of different salinities, as well as between RAS34 and Conway34 (Figure 48, b). Regarding the nitrogen content, no significant difference was found for the use of the RAS treatments with different salinities, but a significant difference between RAS34 and Conway34 was observed ($p = 0.031$), with RAS34 having the highest content (Figure 48, c). Regarding the phosphorus content, no significant difference was found when comparing the RAS treatments of different salinities, although it seems like RAS10 ranges a bit over the other two treatments. No significant difference was observed between RAS34 and Conway34 either (Figure 48, d).

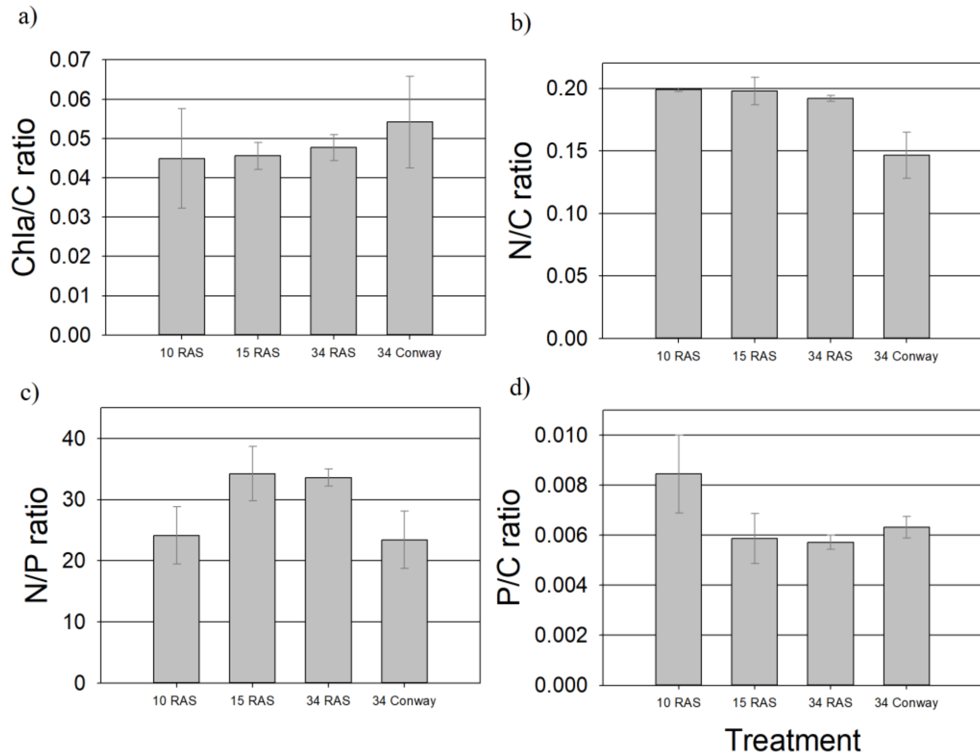


Figure 49: Cellular ratios (mass) between contents for the different treatments at the end of the semicontinuous experiment. Chlorophyll a/carbon (a), nitrogen/carbon (b), nitrogen/phosphorus (c) and phosphorus/carbon (d). Error bars are shown as SD (n=3). Notice the different scaling on the y axis.

Regarding the chlorophyll a/carbon ratio, no significant difference was found comparing the RAS treatments of different salinity. No significant difference was found between RAS34 and Conway34 either, although the graph gives and impression of Conway34 ranging a bit higher (Figure 49, a). Regarding the nitrogen/carbon ratio, no significant difference was found comparing the RAS treatments of different salinity, but it was found a significant difference between RAS34 and Conway34 ($p = 0.013$), with RAS34 having the highest ratio (Figure 49, b). Regarding the nitrogen/phosphorus ratio, a significant difference was found comparing RAS10 and RAS15 ($p = 0.040$), with RAS15 having the highest ratio. A significant difference was also found between RAS34 and Conway34 ($p = 0.023$), with RAS34 having the highest ratio (Figure 49, c). Regarding the phosphorus/carbon ratio, a significant difference was found comparing RAS10 and RAS34 ($p = 0.048$), with RAS10 having the highest ratio. In general. It looks like RAS10 ranges over the other two RAS treatments, although it was not found any

significant difference between RAS10 and RAS15. No significant difference was found between RAS34 and Conway34 (Figure 49, d).

3.4.4 Nitrogen and phosphorus in media vs. final cellular content

The following section presents the comparison of the daily added nitrogen and phosphorus to the cultures, and the cellular content of nitrogen and phosphorus in the volume harvested. Calculations of nitrogen and phosphorus content in the new volume of culture media added are found in Appendix F.

<u>Nitrogen</u>

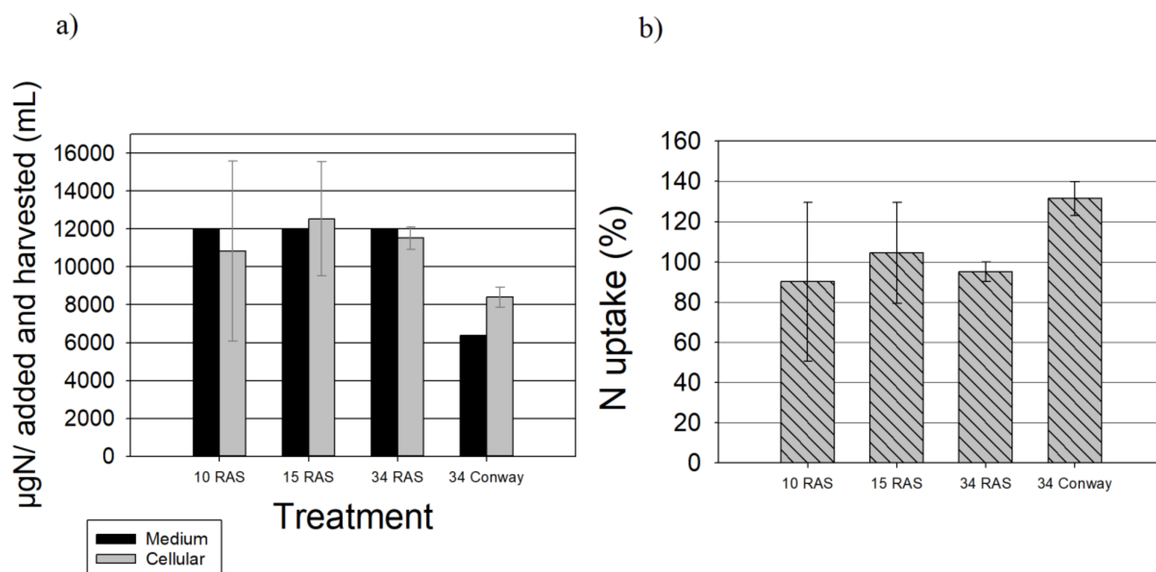


Figure 50: Content of nitrogen in the daily added medium (255.39 mL) and content of nitrogen in the cells harvested of the different treatments (a). Based on these data, a percentage removal graph is presented (b). Error bars are shown as SD (n=3).

No significant difference was found when comparing the different RAS treatments. A significant difference was found between RAS34 and Conway34 ($p = 0.003$), with Conway34 having the highest percentage. Percentage higher than 100% may be due to contamination.

Phosphorus

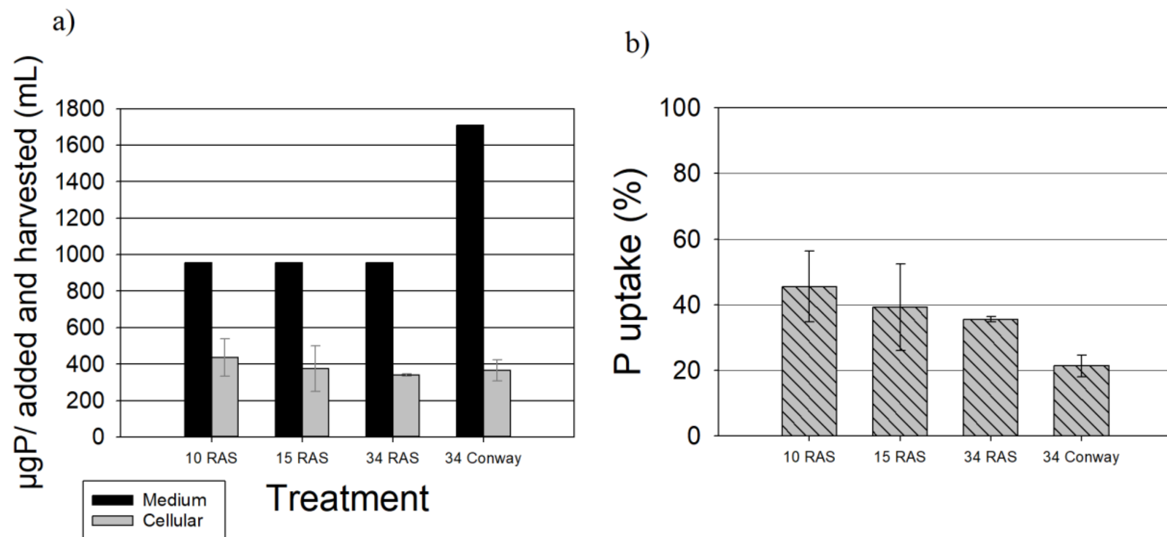


Figure 51: Content of phosphorus in the daily added medium (255.39 mL) and content of phosphorus in the cells harvested of the different treatments (a). Based on these data, a percentage removal graph is presented (b). Error bars are shown as SD (n=3).

No significant difference was found when comparing the different RAS treatments. A significant difference was found between RAS34 and Conway34 ($p = 0.002$), with RAS34 having the highest percentage.

4 Discussion

In this study, the overall aim was to investigate the potential of using the two microalgae species *R. baltica* and *D. tertiolecta* to remove nitrogen and phosphorus from RAS wastewater. The possible effect of different light intensities and salinities was also considered.

Looking at the results, the overall impression was that both species did obtain a high final biomass when using the RAS water. Compared to the use of Conway culture medium, RAS gave a higher biomass of the algae cultures. This information is of interest seen from a commercial point of view, as effective production is always beneficial. Looking at the maximum specific growth rate, both RAS and Conway did have a high and relatively similar growth potential, as none of the values were significant different from each other. A relatively effective growth in Conway was not surprising, as this culture medium is commonly used when culturing microalgae (Gonzalez-Rodriguez and Maestrini, 1984). Looking at biomass and growth capacity (maximum specific growth rate) in respect to differences in salinities, this variable did not seem to be of too high importance for neither one of the species grown in RAS water. This might indicate that both *R. baltica* and *D. tertiolecta* are relatively tolerant to different salinities. According to Chen (2011), *D. tertiolecta* seems to be a species of high salt tolerance, and the fact that *R. baltica* is distributed in both Baltic and Atlantic oceans supports the suggestion of this species being salinity tolerant as well (Throndsen, 1997, Chen et al., 2011). Considering difference in light intensity within each RAS treatment for both species, it is difficult to say if there was a clear difference in the final biomass obtained. Although it does seem to be a trend that for *R. baltica*, low light intensity gave a higher biomass and for *D. tertiolecta,* high light intensity gave a higher biomass. It might be reasonable to consider the higher light intensity the better, and Oostlander et al. (2020) found that an increase of light intensity resulted in an increase of the total biomass production rate under the conditions of no nutrient limitation (Oostlander et al., 2020). A possible explanation for *R. baltica* having a better growth when exposed to low light, is that the lowest light intensity was after all adequate for the microalgae. It is also worth mentioning that the uneven light gradient on each bench might have contributed to a slight difference when considering the importance of light. The maximum specific growth rate on the other hand did not reveal any significant differences between the different light intensities for both species.

When it comes to the effectiveness of nitrogen and phosphorus removal in the RAS water, both species did obtain a high percentage removal for both nitrogen and phosphorus. The result for *D. tertiolecta* was consistent with another study by de Alva et al. (2018), where they found that the microalgae managed to remove more than 94% of the DIN and DIP available in aquacultural wastewater (de Alva et al., 2018). This experiment was carried out in Erlenmeyer flask as batch cultures and are hence comparable to my study. Andreotti et al. did also show a high percentage removal by *D. tertiolecta* (> 96%) although this experiment were carried out in a larger scale cultivated in photobioreactors (Andreotti et al., 2019). Looking at the effect of salinity on the removal in wastewater, salinity was found to have no considerable effect. Again, this might have to do with the two species being relatively salinity tolerant as already stated. Although some of treatments were found significant different when considering salinity, these differences were so minor that it is questionable if they should be considered differences at all. The effect of light intensity on removal on the RAS treatments was hardly noticeable as well, but there was a slight significant difference between high and low light intensity on the phosphorus removal of *R. baltica* giving an impression of a trend toward lower light intensity giving a higher removal. According to Luo et al., effective microalgae nutrient removal efficiency can be achieved under favourable conditions such as light intensity around 150 $\mu mol/m^2$ s, but that the conditions may vary between different microalgae species and the composition of the wastewater itself (Luo et al., 2017). The overall picture of this study does suggest that all treatments and all species did obtain high removal percentage, despite difference in light intensity. It would also have been reasonable to believe that due to the high content of nitrogen compared to phosphorus in the RAS water, not all the nitrogen would have been removed. This was although not the case for this study.

The microalgae cultivated with Conway medium did also gain a high percentage removal, but *R. baltica* seemed to have lower removal of the available phosphorus. This might reveal that the Conway cultures were to some extent nitrogen limited, as when all the available nitrogen were removed, there was still phosphorus left. The nitrogen/phosphorus ratio in start medium was calculated to be 12.6 (weight) for RAS and 3.7 for Conway. This makes the two media interestingly different. In respect to the Redfield ratio, a ratio of greater than 7.2 might suggest a phosphorus limitation in the medium, and a ratio lower than 7.3 might indicate a nitrogen limitation (Redfield et al., 1963). As the nitrogen/phosphorus ratio for Conway was lower than 7.2, a nitrogen limitation might have been the case. 7.2 is given as mass ratio, and not the mol ratio which is the form of Redfield ratio commonly found in literature.

Studying the chlorophyll a/carbon results, there seems to have been a trend tending towards the lower light intensities giving a higher chlorophyll a/carbon ratio for both species. This means that the proportion of chlorophyll a compared to carbon was higher for the low light treatments. This might suggest a slight light limiting, as a microalgae cell would produce more chlorophyll a to amplify the utilization of available light as much as possible. Due to dense filters for the *D. tertiolecta* culturing, not all the chlorophyll a from the filter was fully dissolved. This means that the real chlorophyll a/carbon ratio was most probably even higher as well.

An interesting observation is that even though the percentage medium removal of phosphorus of the different RAS treatments are relatively high for *D. tertiolecta*, the comparison of available phosphorus in the cultivation medium at start and results of the *D. tertiolecta* cellular phosphorus content in the end, does not confirm this. A reasonable thought would be that basically all the available phosphorus would be found as cellular content in the end, based on the percentage removal results. This might indicate a problem with the procedure of gaining the cellular content results, and the only reasonable explanation as I see it might be that the autoclavation procedure of phosphorus before analyzation was somehow incomplete. This may be due to relatively dense filter pieces for the *D. tertiolecta* experiment.

When looking into the results from the mixed semicontinuous culturing experiment, all the cultures had a relatively stable biomass before harvesting, meaning that the cultures, both with use of RAS and Conway media, were in steady state. This means that the composition of the chemical contents of the cultures were stable over time (Kilham, 1978). The interesting thing with this experiment was to see if the cultures managed to remove the available nitrogen and phosphorus added each day. Compared to the available amount of nitrogen and phosphorus in the media added, there was observed a high final cellular content of nitrogen but not that high content of phosphorus. This brings up the issue already mentioned that there is a possibility that not all the cellular phosphorus had been revealed, and that the content may be higher than the results indicate. A study carried out by Pachés et al. (2020) did admittedly reveal that a mixed batch culture of the two microalgae species *Monoraphidium braunii* and *Scenedesmus obliquus* obtained a high ammonium percentage removal (100%), as well as a high biomass productivity with use of effluent from an anaerobic membrane bioreactor. They did also find that in a mixed culture, phosphorus was actually depleted faster than in separate cultures (Pachés et al., 2020). These results make it interesting to look further into the possibility of carrying out comparable studies as semicontinuous cultures as well. Considering the final biomass distribution of *R.*

baltica and *D. tertiolecta*, as well as the bright green colour of the cultures may suggest that *D. tertiolecta* was the dominant species of the two.

5 Conclusion

This study aimed to investigate the potential of using the two microalgae species *R. baltica* and *D. tertiolecta* for removal of DIN and DIP form RAS wastewater. Both species obtained a high percentage removal, and both species did also obtain a high biomass growth in the end of the culturing period. Neither salinity nor light intensity did seem to have any major effect on the removal percentage for the two algae, although *R. baltica* seemed to have gained a slightly higher removal percentage when cultured at low light intensity. The overall impression of the removal results is although that both species cultured in all RAS treatments did a great job removing almost all the available DIN and DIP. Cultured in a mixed semicontinuous culture, the microalgae did also manage to remove almost all the nitrogen, but not that much phosphorus. There might although have been more cellular phosphorus present, but there is a chance that not all of it was available for analysis detection and hence the actual content of phosphorus was higher. Based on the results, it might be reasonable to conclude that RAS wastewater have a promising future for being commercially used as microalgae culture medium. One challenge is although that the RAS wastewater have a dynamic composition and it is therefore challenging to compile a standardized method for treatment with microalgae. Further study should therefore continue investigating the effect of different environmental factors affecting microalgae growth. Hopefully, someone would also further investigate the possibilities of mixing two microalgae species, as well as try to find out whether *R. baltica* and *D. tertiolecta* are the most suitable species to combine in a mixed semicontinuous culture like this. Further research should also investigate the possibilities of culturing *R. baltica* and *D. tertiolecta* with as high nitrogen and phosphorus value as possible. How perfect would it not be to develop an effective integrated removal system and at the same time culture microalgae with high nutritional value? Using these microalgae for aquafeed purposes would hence close the loop and we would be one step closer an even more environmentally friendly aquaculture industry.

6 Acknowledgement

First, I would like to thank my main supervisor Kjell Inge Reitan and my co-supervisor Siv Anina Etter for all guidance and help along the way during this whole process. I am very grateful for all support, good conversations, and the overall opportunity to work with this interesting field of study. I also want to thank Dag Altin and Iurgi Imanol Salaverria-Zabalegui for being very helpful and supportive during labwork. Finally, I want to give a thanks to my friends and family for always being supportive and understanding.

7 References

AKERLUND, T., NORDSTRÖM, K. & BERNANDER, R. 1995. Analysis of cell size and DNA content in exponentially growing and stationary-phase batch cultures of *Escherichia coli*. *Journal of Bacteriology,* 177**,** 6791-6797. https://doi.org/10.1128/jb.177.23.6791-6797.1995

ALEXANDRATOS, N. & BRUINSMA, J. 2012. *World agriculture towards 2030/2050: the 2012 revision,* Rome, Food and Agriculture Organization of the United Nations - Agricultural Development Economics Division.

ANDERSEN, R. A. 2005. *Algal culturing techniques*, Elsevier.

ANDERSON, L. A. & SARMIENTO, J. L. 1994. Redfield ratios of remineralization determined by nutrient data analysis. *Global biogeochemical cycles,* 8**,** 65-80. https://doi.org/10.1029/93GB03318

ANDREOTTI, V., SOLIMENO, A., CHINDRIS, A., MARAZZI, F. & GARCÍA, J. 2019. Growth of *Tetraselmis suecica* and *Dunaliella tertiolecta* in aquaculture wastewater: numerical simulation with the BIO_ALGAE model. *Water, Air, & Soil Pollution,* 230**,** 1-14. https://doi.org/10.1007/s11270-019-4122-0

ARUMUGAM, M., AGARWAL, A., ARYA, M. C. & AHMED, Z. 2013. Influence of nitrogen sources on biomass productivity of microalgae *Scenedesmus bijugatus*. *Bioresource technology,* 131**,** 246-249. https://doi.org/10.1016/j.biortech.2012.12.159

BAI, S. C., KATYA, K. & YUN, H. 2015. Additives in aquafeed: an overview. *In:* DAVIS, D. A. (ed.) *Feed and feeding practices in aquaculture.* Woodhead Publishing.

BOYD, C. E. 2015. Overview of aquaculture feeds: Global impacts of ingredient use. *In:* DAVIS, D. A. (ed.) *Feed and feeding practices in aquaculture.* Woodhead Publishing.

BREGNBALLE, J. 2015. *A Guide to Recirculation Aquaculture*, The Food and Agriculture Organization of the United Nations - EUROFISH International Organisation.

BUTCHER, R. W. 1959. *An introductory account of the smaller algae of British coastal waters Part I. Introduction and Chlorophyceae,* London, Her Majesty's Stationery Office.

CCAP. 2021a. *Dunaliella tertiolecta* [Online]. Culture Collection of algae & protozoa. Available: https://www.ccap.ac.uk/catalogue/strain-19-6B?mfp=search%5Btertiolecta%5D [Accessed 09.06 2021].

CCAP. 2021b. *Rhodomonas baltica* [Online]. Culture Collection of algae & protozoa. Available: https://www.ccap.ac.uk/catalogue/strain-979-9?mfp=search%5Bbaltica%5D [Accessed 09.06 2021].

CHEN, M., TANG, H., MA, H., HOLLAND, T. C., NG, K. S. & SALLEY, S. O. 2011. Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*. *Bioresource technology,* 102**,** 1649-1655. https://doi.org/10.1016/j.biortech.2010.09.062

DE ALVA, M. S., PABELLO, V. M. L., LEDESMA, M. T. O. & GÓMEZ, M. J. C. 2018. Carbon, nitrogen, and phosphorus removal, and lipid production by three saline microalgae grown in synthetic wastewater irradiated with different photon fluxes. *Algal Research,* 34**,** 97-103. https://doi.org/10.1016/j.algal.2018.07.006

DYHRMAN, S. T. 2016. Nutrients and their acquisition: phosphorus physiology in microalgae. *In:* BOROWITZKA, M. A., BEARDALL, J. & RAVEN, J. A. (eds.) *The physiology of microalgae.* 1 ed.: Springer, Cham.

FAO 2016. *The State of World Fisheries and Aquaculture* Rome, Food and Agriculture Organization of the United Nations.

FORGET, N., BELZILE, C., RIOUX, P. & NOZAIS, C. 2010. Teaching the microbial growth curve concept using microalgal cultures and flow cytometry. *Journal of Biological Education,* 44**,** 185-189. https://doi.org/10.1080/00219266.2010.9656220

GOLDMAN, J. C., MCCARTHY, J. J. & PEAVEY, D. G. 1979. Growth rate influence on the chemical composition of phytoplankton in oceanic waters. *Nature,* 279**,** 210-215. https://doi.org/10.1038/279210a0

GONZALEZ-RODRIGUEZ, E. & MAESTRINI, S. Y. 1984. The use of some agricultural fertilizers for the mass production of marine algae. *Aquaculture,* 36**,** 245-256. https://doi.org/10.1016/0044-8486(84)90240-0

GONZÁLEZ, M. A., GÓMEZ, P. I. & POLLE, J. E. W. 2009. Taxonomy and Phylogeny of the Genus *Dunaliella*. *In:* BEN-AMOTZ, A., POLLE, J. E. W. & RAO, D. V. S. (eds.) *The Alga Dunaliella - Biodiversity, Physiology, Genomics and Biotechnology.* 1st ed. Enfield: Science Publishers.

GUILLARD, R. R. 1975. Culture of phytoplankton for feeding marine invertebrates. *Culture of marine invertebrate animals.* Boston: Springer.

HARGREAVES, J. A. 1998. Nitrogen biogeochemistry of aquaculture ponds. *Aquaculture,* 166**,** 181-212. https://doi.org/10.1016/S0044-8486(98)00298-1

HERATH, S. S. & SATOH, S. 2015. Environmental impact of phosphorus and nitrogen from aquaculture. *In:* DAVIS, D. A. (ed.) *Feed and feeding practices in aquaculture.* Woodhead Publishing.

JAKOBSEN, Ø. M. 2020. Kjemisk analyse av prøver fra ERKO Settefisk AS - Periode fra 22. oktober 2019 til 23. januar 2020. Trondheim: CIRis and Nofitech.

KHAN, M. I., SHIN, J. H. & KIM, J. D. 2018. The promising future of microalgae: current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products. *Microbial cell factories,* 17**,** 1-21. https://doi.org/10.1186/s12934-018-0879-x

KILHAM, S. S. 1978. Nutrient kinetics of freshwater planktonic algae using batch and semicontinuous methods: With 6 figures in the text. *Internationale Vereinigung für Theoretische und Angewandte Limnologie: Mitteilungen,* 21**,** 147-157. https://doi.org/10.1080/05384680.1978.11903959

KNUCKEY, R. M., SEMMENS, G. L., MAYER, R. J. & RIMMER, M. A. 2005. Development of an optimal microalgal diet for the culture of the calanoid copepod *Acartia sinjiensis*: effect of algal species and feed concentration on copepod development. *Aquaculture,* 249**,** 339-351. https://doi.org/10.1016/j.aquaculture.2005.02.053

LANANAN, F., JUSOH, A., LAM, S. S. & ENDUT, A. 2013. Effect of Conway Medium and f/2 Medium on the growth of six genera of South China Sea marine microalgae. *Bioresource technology,* 141**,** 75-82. https://doi.org/10.1016/j.biortech.2013.03.006

LOCEY, B. J. 2005. Nitrites. *In:* ANDERSON, B., DE PEYSTER, A., GAD, S., HAKKINEN, P. J., KAMRIN, M., LOCEY, B. J., MEHENDALE, H., POPE, C. & SHUGART, L. (eds.) *Encyclopedia of Toxicology.* Second ed.: Elsevier.

LUO, Y., LE-CLECH, P. & HENDERSON, R. K. 2017. Simultaneous microalgae cultivation and wastewater treatment in submerged membrane photobioreactors: a review. *Algal research,* 24**,** 425-437. https://doi.org/10.1016/j.algal.2016.10.026

MARTINS, C., EDING, E. H., VERDEGEM, M. C., HEINSBROEK, L. T., SCHNEIDER, O., BLANCHETON, J.-P., D'ORBCASTEL, E. R. & VERRETH, J. 2010. New developments in recirculating aquaculture systems in Europe: A perspective on environmental sustainability. *Aquacultural engineering,* 43**,** 83-93. https://doi.org/10.1016/j.aquaeng.2010.09.002

MARTINY, A. C., VRUGT, J. A. & LOMAS, M. W. 2014. Concentrations and ratios of particulate organic carbon, nitrogen, and phosphorus in the global ocean. *Scientific data,* 1**,** 1-7. https://doi.org/10.1038/sdata.2014.48

MOHEIMANI, N. R., BOROWITZKA, M. A., ISDEPSKY, A. & SING, S. F. 2013. Standard methods for measuring growth of algae and their composition. *Algae for biofuels and energy*. Dordrecht: Springer.

MOLIN, G. 1983. Measurement of the maximum specific growth rate in chemostat of *Pseudomonas spp.* with different abilities for biofilm formation. *European journal of applied microbiology and biotechnology,* 18**,** 303-307. https://doi.org/10.1007/BF00500496

NIE, X., MUBASHAR, M., ZHANG, S., QIN, Y. & ZHANG, X. 2020. Current progress, challenges and perspectives in microalgae-based nutrient removal for aquaculture waste: A comprehensive review. *Journal of Cleaner Production***,** 124209. https://doi.org/10.1016/j.jclepro.2020.124209

NOKKAEW, A., PIMPUNCHAT, B., MODCHANG, C., AMORNSAMANKUL, S., TRIAMPO, W. & TRIAMPO, D. 2012. Estimation of Algae Growth Model Parameters by a Double Layer Genetic Algorithm. *WSEAS Transactions on Computers,* 11**,** 377-386. https://www.wseas.org/multimedia/journals/computers/2012/56-122.pdf

OLSEN, Y. 2011. Resources for fish feed in future mariculture. *Aquaculture Environment Interactions,* 1**,** 187-200. https://doi.org/10.3354/aei00019

OLSEN, Y. & OLSEN, L. M. 2008. Environmental Impact of Aquaculture on Coastal Planktonic Ecosystems. *In:* TSUKAMOTO, K., KAWAMURA, T., TAKEUCHI, T. D., BEARD, J. & KAISER, M. J. (eds.) *Fisheries for Global Welfare and Environment - Memorial book of the 5th World Fisheries Congress 2008.* Tokyo: TERRAPUB.

OOSTLANDER, P. C., VAN HOUCKE, J., WIJFFELS, R. H. & BARBOSA, M. J. 2020. Optimization of *Rhodomonas sp.* under continuous cultivation for industrial applications in aquaculture. *Algal Research,* 47**,** 101889. https://doi.org/10.1016/j.algal.2020.101889

PACHÉS, M., MARTÍNEZ-GUIJARRO, R., GONZALEZ-CAMEJO, J., SECO, A. & BARAT, R. 2020. Selecting the most suitable microalgae species to treat the effluent from an anaerobic membrane bioreactor. *Environmental technology,* 41**,** 267-276. https://doi.org/10.1080/09593330.2018.1496148

PRESCOTT, L. M., HARLEY, J. P. & KLEIN, D. A. 2003. *Microbiologie*, De Boeck.

REDFIELD, A., KETCHUM, B. & RICHARDS, F. 1963. The influence of organisms on the composition of seawater. *The sea,* 2**,** 26-77.

REITAN, K. I., RAINUZZO, J. R., ØIE, G. & OLSEN, Y. 1997. A review of the nutritional effects of algae in marine fish larvae. *Aquaculture,* 155**,** 207-221. https://doi.org/10.1016/S0044-8486(97)00118-X

RUANGSOMBOON, S. 2015. Effects of different media and nitrogen sources and levels on growth and lipid of green microalga *Botryococcus braunii* KMITL and its biodiesel properties based on fatty acid composition. *Bioresource technology,* 191**,** 377-384. https://doi.org/10.1016/j.biortech.2015.01.091

SALBITANI, G. & CARFAGNA, S. 2021. Ammonium Utilization in Microalgae: A Sustainable Method for Wastewater Treatment. *Sustainability,* 13**,** 956. https://doi.org/10.3390/su13020956

SALGUEIRO, J. L., PÉREZ, L., MACEIRAS, R., SÁNCHEZ, Á. & CANCELA, Á. 2018. Semicontinuous culture of *Chlorella vulgaris* microalgae for wastewater treatment. *International Journal of Environmental Research,* 12**,** 765-772. https://doi.org/10.1007/s41742-018-0129-4

SN 1991. Water analysis - Determination of the sum of nitrite nitrogen and nitrate nitrogen. Oslo: Standard Norge.

SN 2004. Water quality - Determination of phosphorus - Ammonium molybdate spectrometric method (ISO 6878:2004). Oslo: Standard Norge.

SØRENSEN, M., BERGE, G. M., REITAN, K. I. & RUYTER, B. 2016. Microalga *Phaeodactylum tricornutum* in feed for Atlantic salmon (*Salmo salar*)—Effect on nutrient digestibility, growth and utilization of feed. *Aquaculture,* 460**,** 116-123. https://doi.org/10.1016/j.aquaculture.2016.04.010

SØRENSEN, M., GONG, Y., BJARNASON, F., VASANTH, G. K., DAHLE, D., HUNTLEY, M. & KIRON, V. 2017. *Nannochloropsis oceania*-derived defatted meal as an alternative to fishmeal in Atlantic salmon feeds. *PloS one,* 12**,** 1-20. https://doi.org/10.1371/journal.pone.0179907

STEVČIĆ, Č., PULKKINEN, K. & PIRHONEN, J. 2019. Screening of microalgae and LED grow light spectra for effective removal of dissolved nutrients from cold-water recirculating aquaculture system (RAS) wastewater. *Algal Research,* 44**,** 101681. https://doi.org/10.1016/j.algal.2019.101681

SU, Y. 2020. Revisiting carbon, nitrogen, and phosphorus metabolisms in microalgae for wastewater treatment. *Science of The Total Environment***,** 144590. https://doi.org/10.1016/j.scitotenv.2020.144590

THRONDSEN, J. 1997. The planktonic marine flagellates. *In:* TOMAS, C. R. (ed.) *Identifying marine phytoplankton.* Academic Press.

UN 2019. *World Population Prospects 2019,* New York, United Nations - Department of Economic and Social Affairs.

VAN RIJN, J., TAL, Y. & SCHREIER, H. J. 2006. Denitrification in recirculating systems: theory and applications. *Aquacultural engineering,* 34**,** 364-376. https://doi.org/10.1016/j.aquaeng.2005.04.004

WALNE, P. R. 1966. *Experiments in the large-scale culture of the larvae of Ostrea edulis L,* London, Her Majesty's Stationery Office.

WALNE, P. R. 1974. *Culture of bivalve molluscs*, Fishing News Books Ltd.

WANG, X., FOSSE, H. K., LI, K., CHAUTON, M. S., VADSTEIN, O. & REITAN, K. I. 2019. Influence of nitrogen limitation on lipid accumulation and EPA and DHA content in four marine microalgae for possible use in aquafeed. *Frontiers in Marine Science,* 6**,** 1-10. https://doi.org/10.3389/fmars.2019.00095

WANG, X., OLSEN, L. M., REITAN, K. I. & OLSEN, Y. 2012. Discharge of nutrient wastes from salmon farms: environmental effects, and potential for integrated multi-trophic aquaculture. *Aquaculture Environment Interactions,* 2**,** 267-283 https://doi.org/10.3354/aei00044

WIK, T. E. I., LINDÉN, B. T. & WRAMNER, P. I. 2009. Integrated dynamic aquaculture and wastewater treatment modelling for recirculating aquaculture systems. *Aquaculture,* 287**,** 361-370. https://doi.org/10.1016/j.aquaculture.2008.10.056

YTRESTØYL, T., AAS, T. S. & ÅSGÅRD, T. 2015. Utilisation of feed resources in production of Atlantic salmon (*Salmo salar*) in Norway. *Aquaculture,* 448**,** 365-374. https://doi.org/10.1016/j.aquaculture.2015.06.023

ZULLAIKAH, S., UTOMO, A. T., YASMIN, M., ONG, L. K. & JU, Y. H. 2019. Ecofuel conversion technology of inedible lipid feedstocks to renewable fuel. *Advances in Eco-Fuels for a Sustainable Environment.* Elsevier.

Appendix A

Redfield ratio in mass

C:N:P → 106:16:1 (mol)

$m = n \times Mm$

$m = mass\ (g)$

$n = mol$

$Mn = molar\ mass\ (\frac{g}{mol})$

C

$m = 106 \times 12.0107 = 1273.1342\ g$

N

$m = 16 \times 14.0067 = 224.1072\ g$

P

$m = 1 \times 30.973762 = 30.973762\ g$

Redfield ratio in mass → **1273:224:31**

$$\frac{\boldsymbol{N}}{\boldsymbol{C}} = \frac{224}{1273} = 0.17596$$

$$\frac{\boldsymbol{P}}{\boldsymbol{C}} = \frac{31}{1273} = 0.02435$$

$$\frac{\boldsymbol{N}}{\boldsymbol{P}} = \frac{224}{31} = 7.2353$$

Appendix B

Volume of components added to each culture flask for P2

Volumes added to culture flask 1-3 (RAS10)

Component	Volume (mL)	
Sea water (10‰)	103	
Nutrient solution (RAS)	1.3	
Algae inoculum	***R. baltica***	***D. tertiolecta***
	327 (from 10‰)	420 (from 10‰)
Total	851.3	

Volumes added to culture flask 4-6 (RAS15)

Component	Volume (mL)	
Freshwater	203	
Nutrient solution (RAS)	1.3	
Algae inoculum	***R. baltica***	***D. tertiolecta***
	227 (from 34‰)	420 (from 10‰)
Total	851.3	

Volumes added to culture flask 7-9 (RAS34)

Component	Volume (mL)	
Seawater (34‰)	172 mL	
Nutrient solution (RAS)	1.3	
Algae inoculum	***R. baltica***	***D. tertiolecta***
	227 (from 34‰)	451 (from 34‰)
Total	851.3	

Volumes added to culture flask 10-12 (Conway34)

Component	Volume (mL)	
Seawater (34‰)	172	
Nutrient solution (Conway)	1.3	
Algae inoculum	***R. baltica***	***D. tertiolecta***
	227 (from 34‰)	451 (from 34‰)
Total	851.3	

Appendix C

Calculations of amount NaH_2PO_4 and $NaNO_3$ added to prepared Conway and RAS nutrient medium

Molar mass

$NaNO_3 = 23.0\,g/mol + 14.0\,g/mol + (16.0\,g/mol \times 3) = 85.0\,g/mol = 16.5\%\,N$

$NaH_2PO_4 = 23.0\,g/mol + (1.0\,g/mol \times 2) + 31.0\,g/mol + (16.0\,g/mol \times 4) = 120.0\,g/mol$

$\rightarrow 25.8\%P$

Conway

$\mathbf{100\,gNaNO_3/L}$ *added to medium*

$\mathbf{16.95\,gNaH_2PO_4/L}$ *added to medium*

(Walne, 1974)

RAS

To imitate real RAS water, the following amount of N and P in medium are desired (Jakobsen, 2020)

$47.00\,mgN/L$

$3.73\,mgP/L$

$$NaNO_3(in\ medium) = \left(\frac{47.00\,mgN/L}{16.50\%}\right) \times 100\% = 284.80\,mg/L$$

$\rightarrow 1.30ml\ medium\ added\ to\ the\ total\ volume\ of\ 851.30\ ml = 1.52ml\ to\ 1000ml$

$$\rightarrow \frac{284.80mg}{1.52ml} = \mathbf{187.40\,gNaNO_3/L}\ added\ to\ medium$$

$$NaH_2PO_4\ (in\ medium) = \left(\frac{3.73\ mgP/L}{25.80\%}\right) \times 100\% = 14.50\ mg/L$$

$$\rightarrow 1.30ml\ medium\ added\ to\ the\ total\ volume\ of\ 851.30ml = 1.52ml\ to\ 1000ml$$

$$\rightarrow \frac{14.50mg}{1.52ml} = \mathbf{9.50\ \ gNaH_2PO_4/L}\ added\ to\ medium$$

Appendix D

Preparation of nutrients to modified Conway nutrient medium without NaH_2PO_4 and $NaNO_3$

Date: 20.01.2021 *Prepared by: Dag Altin*

	Amount (mg/g/mL/L)	Exact amount
Major nutrients		
$FeCl_3 \times 6H_2O$	1.30 g	1.3017 g
$MnCl_2 \times 4H_2O$	0.360 g	0.3607 g
H_3BO_3	33.60 g	33.6021 g
$Na_2EDTA \times 2H_2O$	45.00 g	45.0039 g
Vitamins		
Thiamine-HCl	0.10 g	0.1009 g
Cyanocobalamine Stock (5.0 mL 1mg/mL = 5.0 mg)	5.00 mg	5.00 mL
Trace Metal Stock (50% strength)	2.0 mL	2.0 mL
De-ionized water	1.00 L	1.00 L

Trace Metal Stock (50% strength) Prepared: 20.01.2021

	Amount (g/mL)	Exact amount
$ZnCl_2$	2.6250 g	2.6293 g
$CoCl_2 \times 6H_2O$	2.5000 g	2.5044 g
$(NH_4)_6Mo_7O_{24} \times 4H_2O$	1.1250 g	1.1215 g
$CuSO_4 \times 5H_2O$	2.5000 g	2.5015 g
De-ionized water	250 mL	250 mL

Cyanocobalamine Stock Prepared: 04.02.2020 (frozen)

	Amount (g/mL)	Exact amount
Cyanocobalamine (vit. B_{12})	0.2500 g	0.2509 g
De-ionized water	250 mL	250 mL

(Andersen, 2005)

Appendix E

Calculations of N and P concentration in 851.3 mL (culturing flask) for Conway and RAS nutrient medium respectively

Conway

$\boldsymbol{NaNO_3}$

$100.00\ g/L \rightarrow aiming\ to\ calculate\ the\ amount\ of\ N\ in\ 100.00\ g\ NaNO_3$:

$$100.00\ g/L \times \frac{14.00\ g/mol}{85.00\ g/mol} = 16.47 g/L$$

$Adding\ 1.30\ ml\ to\ 850.00\ ml$:

$$\left(\left(16.47\ g/L \times \frac{1.30\ ml}{1000.00}\right)/851.30\ ml\right) \times 1000.00 = 0.025\ gN/L = \mathbf{25\ 000.00\ \mu gN/L}$$

$\boldsymbol{NaH_2PO_4}$

$16.95\ g/L \rightarrow aiming\ to\ calculate\ the\ amount\ of\ P\ in\ 16.95\ g/L\ \ NaH_2PO_4$:

$$16.95\ g/L \times \frac{31.00\ g/mol}{120.00\ g/mol} = 4.38\ g/L$$

$Adding\ 1.30\ ml\ to\ 850.00\ ml$:

$$\left(\left(4.38\ g/L \times \frac{1.30\ ml}{1000.00}\right)/851.30\right) \times 1000.00 = 6.69 \times 10^{-3} gP/L = \mathbf{6\ 688.59\ \mu gP/L}$$

$\boldsymbol{NaNO_3}$

$187.40\ g/L \rightarrow aiming\ to\ calculate\ the\ amount\ of\ N\ in\ 187.40\ g\ NaNo_3$:

$$187.40\ g/L \times \frac{14.00\ g/mol}{85.00\ g/mol} = 30.87 g/L$$

$Adding\ 1.30\ ml\ to\ 850.00\ ml$:

$$\left(\left(30.87\ g/L \times \frac{1.30\ ml}{1000.00}\right)/851.30\right) \times 1000.00 = 0.047\ gN/L = \mathbf{47\ 000.00\ \mu gN/L}$$

$\boldsymbol{NaH_2PO_4}$

$9.50\ g/L \rightarrow aiming\ to\ calculate\ the\ amount\ of\ P\ in\ 9.50\ g\ NaH_2PO_4$:

$$9.50\ g/L \times \frac{31.00\ g/mol}{120.00\ g/mol} = 2.45\ g/L$$

$Adding\ 1.30\ ml\ to\ 850.00\ ml$:

$$\left(\left(2.45 \frac{g}{L} \times \frac{1.30\ ml}{1000.00}\right)/851.30\right) \times 1000.00 = 3.74 \times 10^{-3}\ gP/L = \mathbf{3\ 741.34\ \mu gP/L}$$

Appendix F

Calculations of exact amount N and P (µg) in 255.39 mL of culture medium (P2)

Conway

$\boldsymbol{NaNO_3}$

$$\frac{25\,000.00\,\mu gN/L}{1000.00\,ml} = 25.00\,\mu gN/ml$$

$$25.00\,\mu gN \times 255.39 = \mathbf{6\,384.75}\,\boldsymbol{\mu gN}$$

$\boldsymbol{NaH_2PO_4}$

$$\frac{6\,688.59\,\mu gP/L}{1000.00\,ml} = 6.69\,\mu gP/ml$$

$$6.69\,\mu gP \times 255.39 = \mathbf{1\,708.56}\,\boldsymbol{\mu gP}$$

$\boldsymbol{NaNO_3}$

$$\frac{47\,000.00\,\mu gN/L}{1000.00\,ml} = 47.00\,\mu gN/ml$$

$$47.00\,\mu gN \times 255.39 = \mathbf{12\,003.33\,\mu gN}$$

$\boldsymbol{NaH_2PO_4}$

$$\frac{3\,741.34\,\mu gP/L}{1000.00\,ml} = 3.74\,\mu gP/ml$$

$$3.74\,\mu gP \times 255.39 = \mathbf{955.16\,\mu gP}$$

Appendix G

RAS wastewater data (mg/L) from CIRIS and Nofitech report

		22.10.2019	18.11.2019	02.12.2019	18.12.2019	30.12.2019	13.01.2020	28.01.2020	Average
NO_3^- (N)	IC	69	37	64	35	34	45	55	
	Optic	65	33	59	34	30	42	49	
	Average	67	35	62	35	32	44	52	**47**
PO4- (P)	IC	4.2	2.7	4	3.8	3.1	5	4	
	ICP-MS	3.6	2.3	4.8	3	2.4	5.1	4.2	
	Average	3.90	2.50	4.40	3.40	2.75	5.05	4.10	**3.73**
N/P weight		17.2	14.0	14.0	10.1	11.6	8.6	12.7	12.5

Appendix H

Daily cell numbers (CN) with standard deviation (SD) for *R. baltica* batch culture

RAS10-	**0**	**0.79**	**1.7**	**2.68**	**3.68**	**4.68**	**5.68**	**6.67**	**7.64**
CN	115 800	193 506	493 960	1 117 680	2 504 906	3 310 256	4 612 533	5 000 133	5 597 066
SD	0.00	9954.10	60794.43	133349.49	340366.54	543559.60	1050541.68	707797.52	384044.23
RAS10+									
CN	115 800	163 713	386 573	926 186	1 696 640	2 896 794	4 239 200	4 885 466	5 095 733
SD	0.00	5991.00	32568.00	120816.00	195758.00	591209.00	650946.00	482533.00	112960.00
RAS15-									
CN	115800	237533	699333	1698933	2971146	4152350	5466266	5480800	6136000
SD	0.00	14904.29	35068.90	138894.41	145846.15	520805.46	373158.91	310655.82	278927.09
RAS15+									
CN	115 800	215 766	570 493	1 474 266	2 982 453	4 090 918	5 090 193	5 575 600	5 701 866
SD	0.00	16493.06	43398.07	215720.62	360175.65	296903.25	556123.99	433718.99	319542.01
RAS34-									
CN	115 800	231 740	638 520	1 351 493	2 468 853	3 637 500	4 613 466	4 927 333	5 436 133
SD	0.00	904.21	9744.99	62648.55	311504.19	313235.82	218513.65	102547.03	202264.12
RAS34+									
CN	115 800	220 813	643 640	1 495 440	3 164 266	4 272 756	4 722 266	4 932 933	5 082 933
SD	0.00	8677.43	62815.71	91089.44	395537.26	317296.04	383913.60	194521.91	288688.71
Conway34-									
CN	115 800	237 586	660 826	1 513 146	2 359 413	2 954 594	3 273 066	3 310 933	3 215 200
SD	0.00	6901.37	27145.31	82413.57	130752.93	181524.14	239637.17	234662.77	154413.99
Conway34+									
CN	115 800	227 860	693 066	1 556 053	2 540 266	3 045 512	3 281 200	3 375 866	3 489 200
SD	0.00	11652.23	43267.82	45578.54	235619.35	350746.04	231755.73	276967.68	392946.61

Daily cell numbers (CN) with standard deviation (SD) for *D. tertiolecta* batch culture

RAS10-	**0**	**0.92**	**1.87**	**2.87**	**3.89**	**4.90**	**5.92**	**6.90**	**7.90**	**8.92**	**9.92**
CN	43 270	83 836	196 393	454 573	908 213	1 544 337	2 376 388	2 804 700	4 069 230	4 974 145	5 378 266
SD	0.00	8143.66	8727.80	61791.93	63328.29	116953.18	280115.43	457671.81	633018.66	613813.63	710383.03
RAS10+	**0**	**0.79**	**1.79**	**2.77**	**3.79**	**4.81**	**5.82**	**6.80**	**7.79**	**8.79**	**9.79**
CN	43 270	75 139	162 696	313 073	796 186	1 787 179	2 626 335	3 967 094	5 187 606	5 884 722	5 627 733
SD	0.00	16001.09	12482.02	18786.42	20633.54	14102.56	284916.53	592803.26	444372.31	135376.87	260726.78
RAS15-	**0**	**0.92**	**1.87**	**2.87**	**3.89**	**4.90**	**5.92**	**6.90**	**7.90**	**8.92**	**9.92**
CN	43 270	79 560	242 216	544 893	1 023 973	1 581 517	2 410 576	2 903 952	4 343 910	5 457 585	5 371 066
SD	0.00	12713.41	20642.04	48704.86	52889.28	125278.73	162432.26	276977.67	415041.70	110221.15	191684.88
RAS15+	**0**	**0.79**	**1.79**	**2.77**	**3.79**	**4.81**	**5.82**	**6.80**	**7.79**	**8.79**	**9.79**
CN	43 270	88 663	192 876	462 753	1 209 280	2 136 324	3 733 974	5 300 854	6 024 252	6 649 145	6 725 600
SD	0.00	8003.58	9264.18	21303.56	50360.05	64363.72	349619.00	492494.03	376887.14	116970.20	615259.69
RAS34-	**0**	**0.97**	**1.91**	**2.95**	**3.93**	**4.95**	**5.95**	**6.94**	**7.94**	**8.98**	**9.95**
CN	43 270	85 870	195 800	523 520	846 853	1 351 602	2 254 754	2 578 311	3 761 965	5 043 162	5 064 666
SD	0.00	1351.04	11530.08	28077.81	93832.78	133688.72	297749.61	315453.72	584361.97	533670.82	123041.67
RAS34+	**0**	**0.84**	**1.84**	**2.83**	**3.85**	**4.85**	**5.87**	**6.85**	**7.85**	**8.84**	**9.85**
CN	43 270	70 256	219 783	518 386	1 140 453	1 967 628	3 276 816	4 857 905	5 521 581	6 166 346	6 146 933
SD	0.00	22970.96	4924.39	28342.72	89589.01	261141.79	383034.42	578644.54	398793.73	257204.70	508303.90
Conway34-	**0**	**0.97**	**1.91**	**2.95**	**3.93**	**4.95**	**5.95**	**6.94**	**7.94**	**8.98**	**9.95**
CN	43 270	104 756	233 773	549 000	983 253	1 636 591	2 833 814	3 330 128	4 105 448	5 012 286	4 452 266
SD	0.00	10674.63	14516.99	19836.25	44792.17	318963.26	185380.86	360673.06	105098.89	406315.88	158222.29
Conway34+	**0**	**0.84**	**1.84**	**2.83**	**3.85**	**4.85**	**5.87**	**6.85**	**7.85**	**8.84**	**9.85**
CN	43 270	93 430	234 410	554 620	1 280 186	2 583 173	3 464 155	4 027 884	4 528 311	5 042 948	4 898 666
SD	0.00	11756.42	20947.20	38563.67	126016.79	194745.96	281359.53	228341.26	145814.08	404145.19	587953.24

Daily cell numbers (CN) with standard deviation (SD) for *R. baltica* + *D. tertiolecta* semicontinuous culture

RAS10	**0**	**1**	**2**	**3**	**4**
CN	1 163 933	2 014 633	2 162 400	2 372 733	2 071 600
SD	78257.67	165115.61	217063.52	356588.52	313118.44
RAS15					
CN	1 295 233	2 271 966	2 588 533	3 072 400	2 609 000
SD	27313.43	79400.02	75558.61	201976.53	238336.99
RAS34					
CN	1 258 200	2 591 866	2 661 000	3 034 733	2 433 933
SD	90213.25	186821.21	293360.55	388803.36	180270.50
Conway34					
CN	1 189 733	2 675 833	3 087 700	3 138 333	2 592 066
SD	149442.71	189226.70	389682.88	377736.65	434181.64

Daily cell numbers (CN) with standard deviation (SD) for *R. baltica* in semicontinuous culture

RAS34	**0**	**1**	**2**	**3**	**4**
CN	702 633	1 188 366	1 197 233	1 296 600	1 021 266
SD	12191.12	103848.75	132979.64	162943.30	152732.10
Conway34					
CN	614 233	1 269 933	1 423 366	1 255 000*	841 200**
SD	85780.73	72644.98	120477.90	40446.51	0.00

* = one triplicate is missing ** = two triplicates are missing

Daily cell numbers with standard deviation (SD) for *D. tertiolecta* semicontinuous culture

RAS34	**0**	**1**	**2**	**3**	**4**
CN	555566	1403500	1463766	1738133	1412666
SD	88472.05	89567.68	163257.29	226638.24	81496.09
Conway34					
CN	575500	1405900	1664333	1716900*	1281200**
SD	63728.25	141896.55	270887.01	304763.02	0.00

* = one triplicate is missing ** = two triplicates is missing

Appendix I

Specific growth rates

Daily specific growth rate (μ) for each treatment during the *R. baltica* culturing experiment. Maximum specific growth rate (μmax) is marked in bold font. Error is given as SD (n=3).

	Light	1	SD	2	SD	3	SD	4	SD	5	SD	6	SD	7	SD	8	SD
RAS10	-	0.65	0.07	**1.02**	0.08	0.83	0.02	0.81	0.04	0.28	0.05	0.32	0.10	0.09	0.11	0.12	0.09
	+	0.44	0.05	**0.94**	0.13	0.89	0.14	0.61	0.05	0.52	0.12	0.39	0.06	0.15	0.12	0.05	0.08
RAS15	-	0.91	0.08	**1.18**	0.12	0.91	0.11	0.56	0.13	0.33	0.13	0.28	0.09	0.00	0.01	0.12	0.10
	+	0.78	0.09	**1.06**	0.02	0.96	0.09	0.71	0.09	0.32	0.14	0.22	0.10	0.09	0.13	0.02	0.13
RAS34	-	0.88	0.00	**1.11**	0.01	0.77	0.04	0.60	0.09	0.39	0.09	0.24	0.04	0.07	0.04	0.10	0.06
	+	0.82	0.05	**1.16**	0.13	0.86	0.08	0.75	0.10	0.30	0.06	0.10	0.07	0.05	0.07	0.03	0.04
Conway34	-	0.91	0.04	**1.12**	0.03	0.85	0.02	0.44	0.04	0.23	0.01	0.10	0.11	0.01	0.03	-0.03	0.07
	+	0.85	0.06	**1.21**	0.04	0.83	0.05	0.49	0.08	0.18	0.02	0.08	0.05	0.03	0.08	0.03	0.15

Daily specific growth rate (μ) for each treatment during the *D. tertiolecta* culturing experiment. Maximum specific growth rate (μmax) is marked in bold font. Error is given as SD (n=3).

	Light	1	SD	2	SD	3	SD	4	SD	5	SD	6	SD	7	SD	8	SD	9	SD	10	SD
RAS10	-	0.71	0.10	**0.90**	0.14	0.83	0.09	0.68	0.19	0.53	0.01	0.42	0.06	0.16	0.05	0.37	0.01	0.20	0.04	0.08	0.01
	+	0.68	0.26	0.79	0.16	0.67	0.13	**0.92**	0.05	0.79	0.02	0.38	0.10	0.42	0.04	0.28	0.07	0.13	0.10	-0.05	0.07
RAS15	-	0.65	0.18	**1.18**	0.26	0.81	0.17	0.62	0.04	0.43	0.05	0.41	0.05	0.19	0.06	0.40	0.07	0.23	0.10	-0.02	0.05
	+	0.90	0.11	0.78	0.09	0.89	0.08	**0.94**	0.08	0.56	0.01	0.55	0.07	0.36	0.06	0.13	0.06	0.10	0.05	0.01	0.09
RAS34	-	0.70	0.02	0.88	0.05	**0.94**	0.10	0.49	0.06	0.46	0.01	0.51	0.13	0.14	0.09	0.37	0.04	0.29	0.06	0.01	0.09
	+	0.53	0.37	**1.17**	0.31	0.87	0.08	0.77	0.09	0.54	0.06	0.50	0.02	0.40	0.05	0.13	0.07	0.11	0.06	0.00	0.08
Conway34	-	**0.90**	0.10	0.86	0.11	0.82	0.04	0.59	0.03	0.49	0.15	0.56	0.17	0.16	0.10	0.21	0.08	0.19	0.06	-0.12	0.05
	+	0.91	0.15	**0.92**	0.06	0.87	0.03	0.82	0.05	0.70	0.03	0.29	0.12	0.16	0.03	0.12	0.05	0.11	0.07	-0.03	0.06