THESIS

Choosing the most suitable micro algae for rearing Artemia salina at VHL with the overarching goal of determining algae suitability for Diadema antillarum larvae

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Thesis

Choosing the most suitable micro algae for rearing Artemia salina at VHL with the overarching goal of determining algae suitability for Diadema antillarum larvae

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Module: LKZ428VNA01, bachelor thesis Coastal and Marine Management

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1 Introduction

Coral ecosystems in the Caribbean have been in decline since the early 1970s (Jackson, Donovan, Cramer, & Lam, 2014). Since then, there has been a reduction in coral coverage of 37% in the 1970s to a mere 14% in 2012 (Jackson et al., 2014). The reasons for this decline are widespread, but most research shows pollution in form of sedimentation caused by human activities on land and sea (Moberg & Folke, 1999; Risk & Edinger, 2011) creating an opportunity for macro algae to expand. These macro algae can compete with coral by overgrowing them causing a decline in the amount of coral present (Lirman, 2001). Further direct human interaction in the form of fisheries and tourism (Jackson et al., 2014), add additional pressure to the coral.

In addition to this pressure created by humans for unknown reason a massive decline occurred of 95-99% of a key species of herbivore that kept the macro algae under control (Carpenter, 1990): the long-spiked sea urchin (*Diadema antillarum)* for the purposes of this report: *Diadema.* This massive decline in sea urchins changed the ecosystem drastically. Macro algae coverage increased by 22 to 439% on reefs in the Caribbean (Carpenter, 1990). Ever since, the sea urchin numbers never fully recovered. In 2015 the total population of *Diadema* was estimated at a mere 15% of its pre-1980 level (Lessios, 2015). Recovery is slow and seems to be limited in some way (Beck, Miller, & Ebersole, 2014).

With the increase in macro algae coverage and no recovery of *Diadema* the health of the coral reefs has been adversely affected (Lirman, 2001). With the importance of coral reefs for local biodiversity this has big negative effects on the ecosystems diversity and stability (Moberg & Folke, 1999). However, it is also a loss for the local people as fishing and tourism around the reefs is a source of many people's livelihoods (UN Environment; ISU; ICRI and Trucost, 2018).

Because of the importance of coral reefs and the lack of recovery of *Diadema,* research has been done on the potential limitations of *Diadema* recovery. (Chiappone, Swanson, Miller, & Smith, 2002; Miller, Kramer, Williams, Johnston, & Szmant, 2009; Prog, Hunte, & Younglao, 1988). The University of Applied Sciences Van Hall Larenstein (VHL) plans to expand on this research by looking into the survival of *Diadema's* larval stage. The larval stage of *Diadema* is a stage in which it is planktonic and carried by currents while feeding on micro algae (figure 1) (Warner, Lyons, & McClay, 2012).

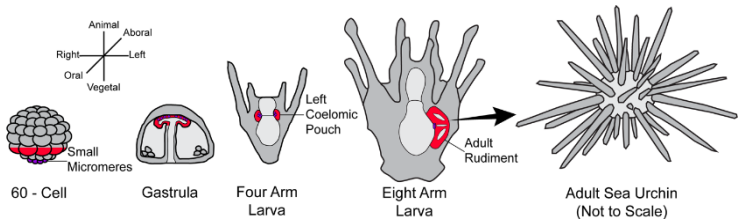

Figure 1: Life stages sea urchin (Warner et al., 2012)

The research into the survival of *Diadema* larvae has the potential to improve recovery in the wild by exploring one of the potential reasons of the slow recovery*:* the low amount of larvae that end up as settled adults (Miller *et al.*, 2009).

For experiments on *Diadema* larvae the larvae themselves will first have to be acquired, and in order to be able to maintain a culture of *Diadema* larvae their primary food source micro algae will also need to be readily available (Carrier, Reitzel, & Heyland, 2017).

Micro algae are single celled algae that are at the bottom of the food chain and provide food for 70% of the world's biomass in addition to being responsible for half the world's photosynthetic activity (Singh & Saxena, 2015). There are a lot of microalgae species, with current scientific estimates ranging from 200.000 to several million species in existence (Norton, Melkonian, & Andersen, 1996).

This wide variety of micro algal species means it is not known exactly what species of microalgae *Diadema* larvae consume. In general, there are four groups of micro algae: cyanobacteria, which are closely related to ordinary bacteria but fall into the category of micro algae due to their ability to photosynthesize; green algae, which are closely related to plants; mesokaryotes, which have aspects of both cyanobacteria as well as the green algae and lastly red algae. All these algae can vary wildly in nutritional value, size and even movement through the water column (Norton et al., 1996).

Being able to provide the right species of microalgae is crucial in keeping sea urchin larvae alive. This is an important starting point for the experiments that eventually might lead to the recovery of *Diadema* and the coral reefs of the Caribbean. This thesis hopes to provide answers to what the right species of micro algae is, how it can be effectively grown at VHL and how the sea urchin larvae might react consuming different types of micro algae. This consumption of the micro algae by *Diadema* larvae cannot be measured directly as the *Diadema* are currently not available to provide the supply of larvae for this project due to time constraints.

For this reason, the question of the potential development and growth of *Diadema* larvae on a certain type of micro algae will be answered using a model organism: *Artemia salina* more commonly known as *Artemia. Artemia* is chosen because it closely resembles the *Diadema* larvae in size (Daintith, 1996). In addition to this they are pelagic and feed on micro algae (Daintith, 1996). *Diadema* larvae and *Artemia* also share a high sensitivity to metals, in particular coper, in the water (Bielmyer, Brix, Capo, & Grosell, 2005; MacRae & Pandey, 1991) . One of the key differences in the living constraints between *Diadema* and *Artemia* is salinity tolerance. *Artemia* are capable of surviving in as much as 180 parts per thousand (ppt) (Browne & Wanigasekera, 2000) of salt to water whereas sea urchin larvae are known to have trouble developing above 37ppt (Sarifudin, Rahman, Yusoff, Arshad, & Tan, 2017) with an ideal of 32ppt. The ideal for *Artemia* ranges from 25-35ppt (Kumar & Babu, 2015).

Temperature sensitivity is also different between the species, *Artemia* are known to live in conditions where temperatures can range anywhere from 6 to 37 °C (Emslie, 2003) whereas the water temperature *Diadema* lives in is 27 °C on average and does not differ more than 3 °C year round (World Sea Temperature, 2019). These parameters mean keeping temperature and salinity in the ideal range for *Diadema* larvae will allow the *Artemia* to grow. This allows *Artemia* to provide a suitable means of testing all the different methods for conducting future *Diadema* experiments such as feeding methods, setting up tanks as well as maintaining water parameters.

While *Artemia* and *Diadema* are different species the author judged their similarities to be sufficient for *Artemia* to be used as model organism due to its size, the fact that both species filter algae from the water column (Smith, Cruz Smith, Cameron, & Urry, 2008; Thach, Elizabeth C., Thompson, Karen J., Morris, 2006) and compatible living conditions. Allowing for an experimental setup very similar to one with actual *Diadema* larvae. During this thesis four micro algae species were tested for numerous traits and practicalities ranging from sustainable growth to potential uptake by the *Artemia*.

To measure the growth and eventual uptake of the micro algae by *Artemia*, first a correlation between different biomass indicators for the micro algae was required. In algae research different types of measurements are used for different purposes (Lu, Yang, Zhu, & Pan, 2017). With optical density being used as a quick and easy measurement, cell counts to get exact measurements of the number of cells in a sample and measurements by weight to determine the amount of biomass. (Lu et al., 2017; Steinberg et al., 2012). To measure the growth and eventual uptake of the micro algae by *Artemia*, a correlation between different biomass indicators for the micro algae has been made between these methods of measuring algae.

The micro algae have been selected according to the criteria that the chosen algae should have traits that make them potentially effective food sources. Two red algae and two green algae were chosen. The first two species are *Rhodomonas salina* (Fig. 2a) and *Rhodomonas baltica* (Fig. 2b), both red algae. These species have been chosen because in literature *Rhodomonas sp.* is often shown as the algae species of choice for feeding sea urchin larvae (Cameron & Schroeter, 1980; Salas-Garza, Carpizo-Ituarte, Parés-Sierra, Martínez-López, & Quintana-Rodríguez, 2005). The third micro algae is a species of green algae called *Nannochloropsis gaditana* (Fig. 2c), this species was chosen due to its high concentration of fatty acids (Boussiba, Vonshak, Cohen, Avissar, & Richmond, 1987), a trait that makes this species a promising candidate as food for the *Diadema* larvae. The fourth micro algae chosen is *Tetraselmis chuii* (Fig. 2d), another green algae with a high lipid content (Alonso, Lago, Vieites, & Espiñeira, 2012).

One of these four algae was selected as a suitable alga to feed *Artemia*. The results obtained over the duration of this thesis will allow future researchers at VHL to have access to a suitable, healthy culture of micro algae to use as food for *Diadema* larvae.

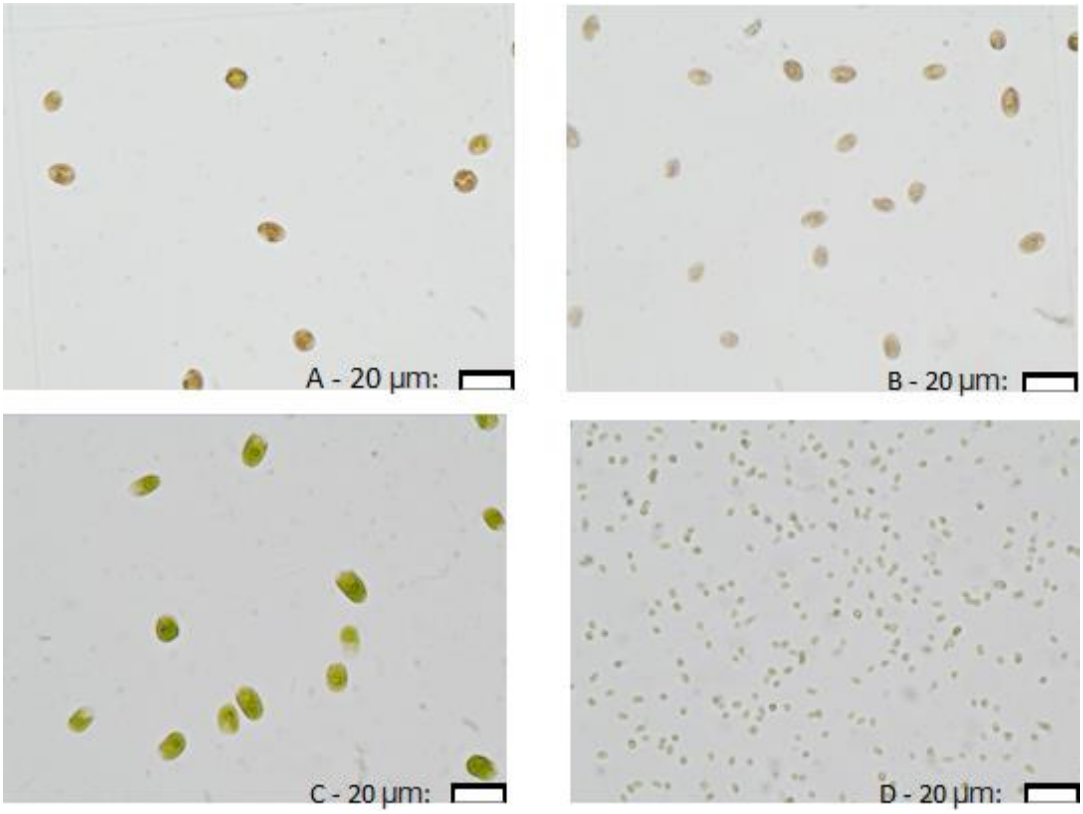

Figure 2: Microalgae species used for this research compared by size. A: Rhodomonas baltica, B: Rhodomonas salina, C: Tetraselmis chuii, D: Nannochrloropsis gaditana.

1.1 Problem Statement

There is a need for a stable micro algae culture at Van Hall Larenstein that provides suitable food for the rearing of future *Diadema* larvae. The culturing of these algae can be complicated, and the nutritional needs and uptake of the larvae is uncertain.

1.2. Aim

The aim of this project was to have a stable growth of the right micro algae to feed *Artemia,* with as overarching goal to determine a suitable food source for *Diadema* larvae.

To achieve this aim there are two distinct phases (also see figure 3):

Phase one was setting up the culture of the micro algae and selecting one red and one green algae that remain stable and healthy over the course of 6 weeks.

Phase two was researching the uptake of the chosen algae by *Artemia* and selecting one of the two algae species that shows the best results for permanent culturing.

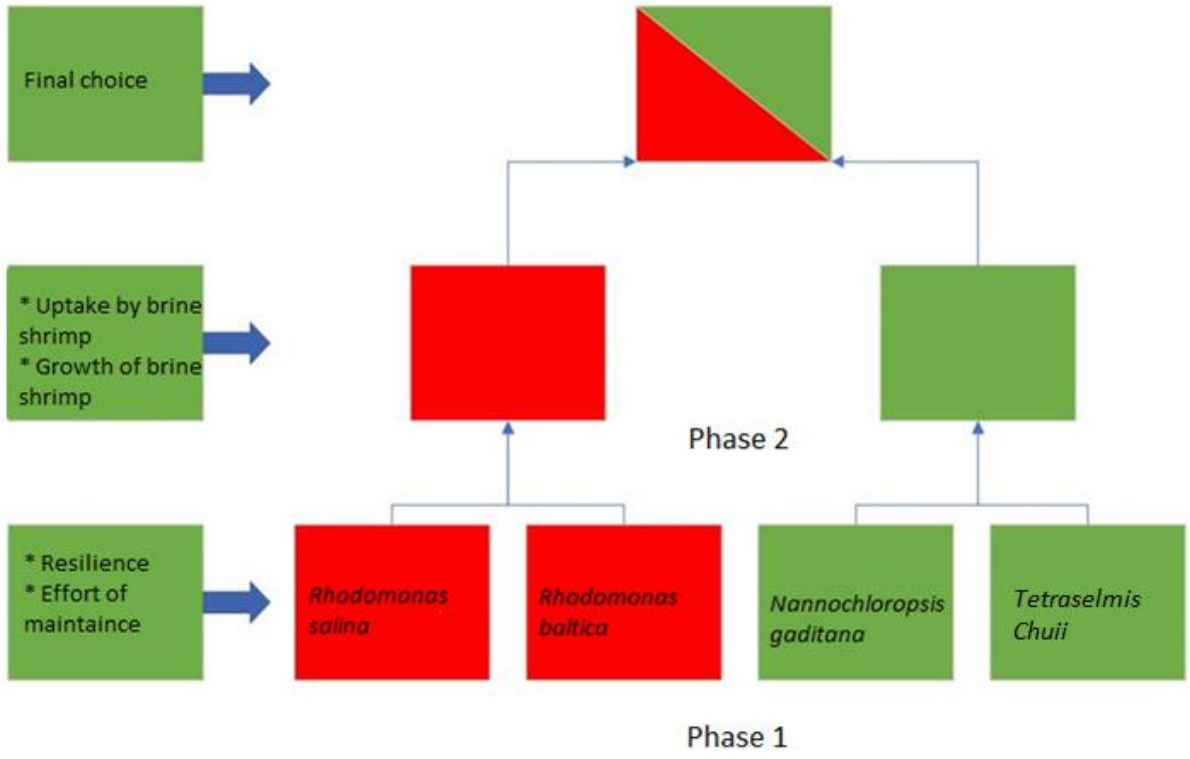

Figure 3: Schematic overview project process: Two red algae and two green algae were tested for suitability of culturing in phase one. In phase two one red and one green algae were tested for suitability as feed source using Artemia, after which one algae was chosen for culturing at VHL.

1.3 Research questions

The main research question of this project was:

Which of the four chosen algae is the most suitable for the rearing of *Artemia*, and ultimately *Diadema antillarum* larvae, at Van Hall Larenstein University of applied sciences?

This question led to several sub questions which could in turn be operationalized to provide an answer to the main question.

Phase 1

How are different biomass values (mg/ml, cell count and optical density) correlated for each of the four algae?

- *What is the relationship between mg/ml, cell count and optical density for: Rhodomonas salina*, *Rhodomonas baltica, Nannochloropsis gaditana and Tetraselmis chuii?*

Which algae species is best maintainable over a period of 6 weeks?

- Does the algae in question have practical issues that makes culturing more difficult for example quicker colony forming, susceptibility to grazer infestation, susceptibility to pollution by other algae?
- Can the growth of the algae be maintained over 6 weeks?

Phase 2

Which algae species provides the best development and survivability for *Artemia*?

- Which of the two best algae species from phase 1 leads to the highest number of living *Artemia* at the end of the experiment?
- Which of the two best algae species lead to most growth in *Artemia*?

1.4 Reading guide

Chapter 2 will contain the project context in which this thesis was performed, meaning it provides background information and gives a clearer idea of the purpose and impact of the research that was performed.

Chapter 3 contains the material and method section of the report in which you can find explanation about how the experiments in this report were conducted and how to reproduce them.

Chapter 4 contains all results obtained from the experiments.

Chapter 5 contains a discussion of the obtained results as well as recommendations on how to improve the results and materials used to obtain them.

Chapter 6 is the conclusion of the report in which a final tally is made of everything that was learned.

2 Project context

Before examining the setup and results of this research, it is important to examine the context in which it has been conducted.

2.1 Stakeholders

The key stakeholder was the Coastal and Marine Management department of Van Hall Larenstein University of Applied Sciences (VHL) for which this research was conducted. They had an interest in both the results of the research as well as a direct need for the products. To ensure their needs were clear meeting were held throughout the thesis with the problem owner to discuss the progress and talk about potential issues.

An initial constraint had been placed on the project at the start by the problem owner in that he wished to see one red alga and one green alga as the final two contenders. In practice this meant that the two green and the two red alga species only needed to be compared with one another when reducing the alga species considered for culturing.

Relevant stakeholders also included future employees or students who will use the micro algae cultures to conduct further research. To ensure that people doing follow up research can maintain the culture as envisioned and use it effectively, the thesis was written in such a way that it is understandable to people with only a starting knowledge of micro algae culturing.

The algae culture was set up with user friendliness in mind, with a focus on solutions that do not require a lot of effort or time. Furthermore, to ensure the final product is easily understood, feedback was requested from students or employees who were likely to be working with the micro algae in the future. This feedback was taken into account when writing the protocols that can be found in the appendices of this thesis.

There were also indirect stakeholders– the residents of the Caribbean. These people were not involved directly but could stand to profit because if this project succeeds, research can be conducted into the survival of *Diadema* larvae. Results from that research might lead to formulating a strategy for the recovery of *Diadema* in the wild. This can increase coral reef recovery and all economic advantages that such a recovery might bring in the tourism, coastal development and fisheries sectors. There is an estimated potential loss of $3.1 billion annually by 2030 if decline of Caribbean coral reef continues and a potential gain of $ 2.5 billion by 2030 if the coral reef health is improved substantially (UN Environment; ISU; ICRI and Trucost, 2018).

2.2 The project scale and position within the policy cycle

The scale of this project was local for the most part, with the direct stakeholders being present in the same building and the initial algae used obtained from the Groningen University. However, there were certain aspects that were international. Since the eventual results attained with help of my initial research could have international impact in the form of assisting coral recovery in the Caribbean's.

When considering the project as part of the policy of coral recovery in the Caribbean's, it falls under the banner of agenda setting in the policy cycle as understood by Knill & Tusun (Knill & Tusun, 2008). Currently efforts of coral recovery in the Caribbean's is often focused on engineering reef structures to speed up coral recovery (Kaufman, 2006). In addition to this there has been a rise in the use of ecological reef restoration where live corals are grown and transplanted (Diego Lirman & Schopmeyer, 2016). However, both these methods do not target the underlying cause of coral decline. Finding limiting factors in the recruitment of *Diadema* might provide an additional policy tool to use in recovering coral through the reintroduction of the species to the ecosystem. This means the outcomes of the project, for which my research facilitates the micro algae, might influence the other projects in the field of coral recovery. For instance, by making coral that is planted or is starting to grow on engineered reef structures more likely to survive due to a decrease in competition with macro algae, thanks to the grazing of *Diadema.*

2.3 People, planet and profit

As mentioned in the introduction of this report, restoring the *Diadema* population in the Caribbean's could have a positive impact on coral reefs in that area (Carpenter, 1990). This means that by providing the algae required to perform tests on *Diadema* larvae this project has the potential to be of positive influence for the people living around the Caribbean reefs and the profit they make (Carpenter, 1990; Moberg & Folke, 1999; UN Environment; ISU; ICRI and Trucost, 2018). By improving the health of the coral reefs, the people stand to gain socially. Not only is there the risk of losing up to $3.1 billion in economic gains by 2030 due to coral reef decline in the region (UN Environment; ISU; ICRI and Trucost, 2018). But coral reefs also provide other services that are vital to the Caribbean's. Services like coastal protection, protecting the land from erosion by tropical storms (Moberg & Folke, 1999). This is especially important as research shows a potential increase in both the intensity and frequency of these storms in the future (Goldenberg, Landsea, Mestas-Nunez, & Gray, 2001; Knutson, Tuleya, & Kurihara, 1998) While these threats impacts all countries in the Caribbean's it is important to note that overseas territories have been found to be more vulnerable to social economical change (Siegel et al., 2019). This means the issue of coral reef protection should be a high priority for the Kingdom of the Netherlands, which consists of three separate nations within the Caribbean as well as three special municipalities tightly bound to the country of the Netherlands.

2.4 Relation to other projects

This was the first project working with micro algae for this purpose at VHL and is essential for all other future projects involving the use of *Diadema* larvae. As a result of this it was important that at the end of the projects duration there was a system capable of producing the right micro algae at the right quantity otherwise follow-up projects would be unable to proceed. With the combination of protocols and this thesis a solid foundation has been build. Making future projects involving micro algae culturing easier to get started.

There was a large amount of expertise present at VHL between the departments of coastal and marine management and environmental sciences with regards to growing both macro and micro algae. This meant I could get feedback on the means of growing the algae.

The room in which the micro algae and larvae are kept was shared with a project growing *Ulva lactuca.* This at times resulted in disagreements about the correct temperature for the room and the limited supply of demi-water. As these problems reared their heads we quickly came to mutually acceptable agreements and it did not impact this project in a significant way.

3 Materials and methods

3.1 Phase 1: culturing the algae

The algae were obtained as sterile cultures from the university of Groningen and were grown in artificial sea water on an f/2 medium, which is a nutrient formula ideal for maintaining microalgae cultures (Guillard, 1975). This nutrient formula was selected to provide the right amount of nitrate, phosphate, trace elements and vitamins for the different micro algae species to grow.

Algal cultures were kept in 1 litre bottles (figure 4). With air bubbling through to supply CO_2 and ensure mixing. The pH of the algae was kept around 8 by controlling the airflow. The air was passed through a vessel holding demi water to increase the moisture content of the air, reducing evaporation. This air was directed through a VWR sterile air filter (0.2 µm) attached on each bottle to reduce aerial pollutants (see figure 5).

Figure 4: 1-liter bottles filled with algae

The bottles were placed in front of 6 TL lights covering the length of the setup providing 80.43 µmol/m^2 in a standard fluorescent light spectrum with peaks at 546 nanometres and 611 nanometres.

In phase one a total of 8 bottles were maintained: each species was cultured in duplicate (n=2). These cultures were maintained for 6 weeks.

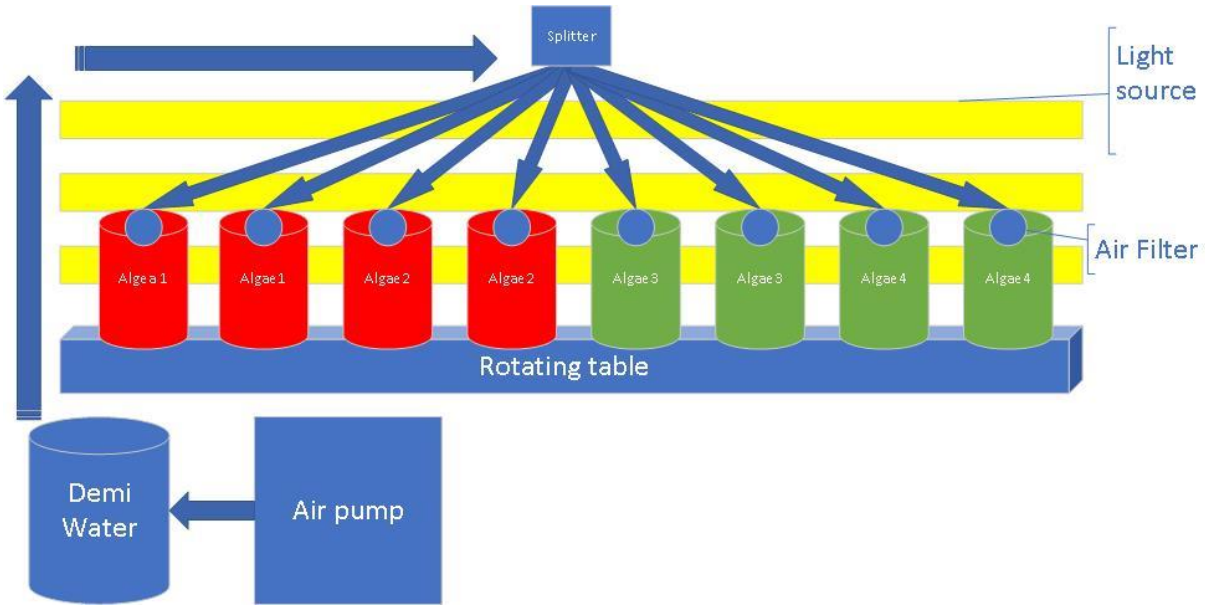

Figure 5: Schematic overview algae growth setup. Pressurised air is first hydrated through demi water and then divided over the 8 bottles, first passing an air filter of 0.2µm to minimize the risk of contamination.

3.1.1 Measurement correlations

Optical density

Optical density (OD) is an easy measurement of algae culture density. An optical density measurement is done by sending out a pulse of light at a certain wavelength and measuring the amount of light going into a solution compared to the light going out. This is an indication for the number of algae present and is used because it is easier to measure than obtaining mg/ml values or cell count numbers. You do however need mg/ml and cell counts to know what the OD value corresponds to. (see figure 7 for overview of up and downsides of each required measurement).

In this thesis OD measurements were done at OD 720. 720 refers to measuring the light absorption of the solution at a wavelength of 720nm. 720nm was chosen as it is the highest available wavelength available on the device used for this research, and lower wavelength lead to more inaccuracy in density measurements as pigments of the algae can interfere with results (Griffiths, Garcin, van Hille, & Harrison, 2011). This was done by using the Aquapen AP 100, selecting the OD720 option and inserting a cuvette with 3ml algae solution into the device (figure 6).

Figure 6: Picture showing PAM meter (on the left) and 3ml cuvettes filled with algae solution.

Mg/ml

The mg/ml allowed the most accurate measurement of the total amount of algae biomass present in the system and allowed adding the same weight of different algae species when doing *Artemia* tests. This is essential due to the size difference between algae species with *Tetraselmis chuii* growing up to 14 µm compared to *Nannochloropsis gaditana* growing to a maximum of 4 µm (figure 2 C & D). The mg/ml was determined by filtering a 30ml sample of the algae solution over a 0.2 µm filter and measuring the algae without water content, resulting in a measurement of mg of dry weight per ml of algae. (for detailed guide to dry weight measurements please refer to appendix II: protocol dry weight measurement (provided by problem owner and adapted for this project).

Cell count

The cell count per ml is what is used in literature when measuring the amount of algae needed to feed larvae cultures (Lu et al., 2017; Basch, 1996; Supan, 2014), meaning it was essential to know in order to determine what amount of different algae to feed the *Artemia sp.*. The measurement was used to determine the number of algae present in the water when doing the feed experiments. The cell count per ml was measured using a haemocytometer. For an accurate guide to cell counting using a haemocytometer, please refer to: (Lavens & Sorgeloos, 1996a). For a detailed protocol of this measurement refer to appendix II: Cell count.

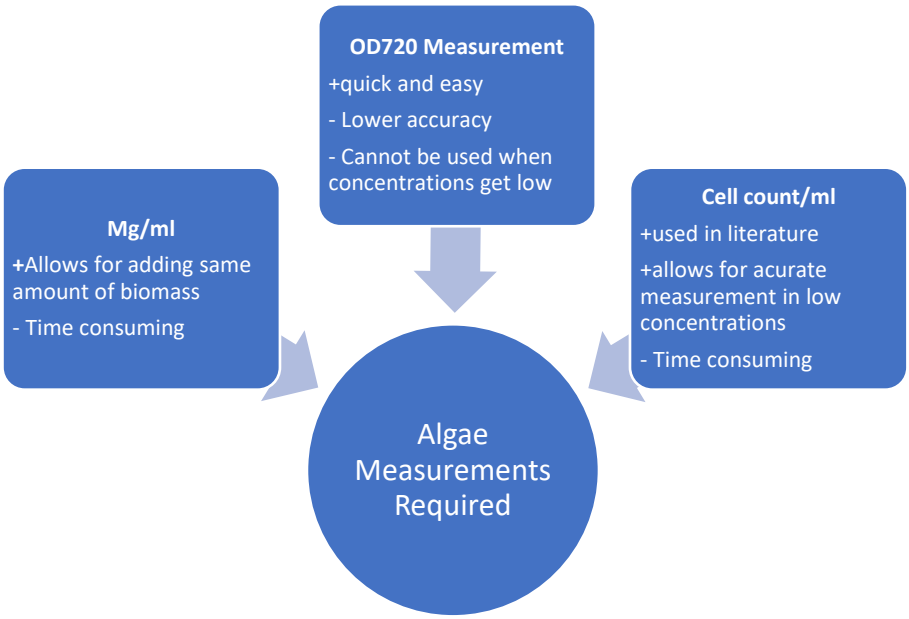

Figure 7: The pros and cons of the different typed of algae measurements required for this research.

Data collection

The data required for developing these measurements has been gathered by taking daily measurements during weekdays of; cell count, mg/ml and optical density, while the algae were being maintained.

Data analysis

The data has been analysed using Excel 2018. Scatterplots have been used to visualize possible correlations between cell concentration (cell count), dry weight (mg/ml) and optical density. The correlations have been tested using a Pearson's correlation test. In all analyses, the level of significance has been considered at $\alpha = 0.05$.

3.1.2 Long term algae maintainability

Two factors were taken into consideration for long term maintenance: health and ease of maintenance. Health was measured to ensure potential problems in the algae cultures were noticed. The ease of maintaining the culture is an important factor to keep the workload for maintaining the algae culture low. In addition, density was measured in order to effectively maintain the algae.

Density

While not part of the measurement criteria, an important part of maintaining algae was ensuring they remained in an exponential growth phase. This is a phase in which the algae are actively splitting, which reduces risk of a collapse of the algae culture (Lavens & Sorgeloos, 1996b) (see figure 8). To this end the algae were diluted 2-3 times weekly depending on the speed of growth. Their density being monitored with the previously explained OD measurement.

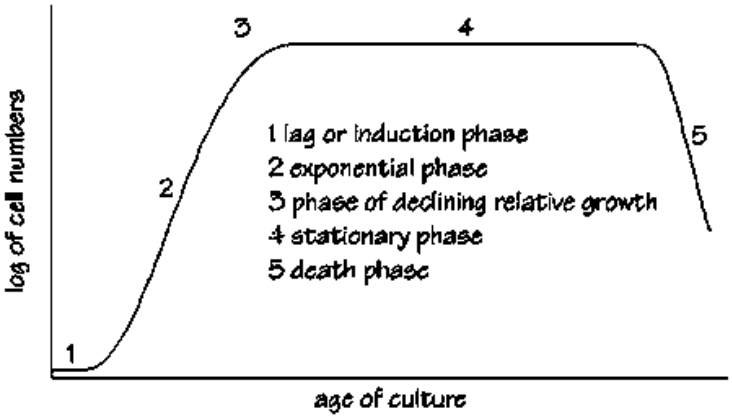

Figure 8: The growth phases of micro algae (Lavens & Sorgeloos, 1996b)

Health measurements

To ensure the health of the algae a daily PAM (Pulse-Amplitude-Modulation) measurement was done. PAM measures the effectiveness of the photosynthesis reaction of the algae. These measurements were done with the Aquapen AP 100. For a healthy micro alga, the results of the PAM were expected to be between 0.65 and 0.50. To measure PAM a cuvette with 3 ml of algae that has been kept dark for 1 minute was inserted it in the device and the QY option was pressed. (for a detailed description of how to take a PAM measurement refer to appendix II: Aquapen ap100 measurements).

Ease of culturing

A check for contamination introduced in the algae was performed by conducting bi-weekly examinations under the microscope on Monday and Friday. In these examinations a single drop of algae solution was examined under a microscope. Three factors were counted: number of colonies formed, other algae species and zooplankton grazing on the algae. For a detailed protocol of ease of culturing please refer to appendix II: health of culture.

Nutrients

The nutrient concentration was measured using Quantofix™ Nitrate and Nitrite measuring strips and were based on the amount of NO_3 present in the water, with the attempted concentration kept at 100mg/l, this concentration was chosen by doubling the concentration of the whole f/2 micro algae growth medium (Guillard, 1975) and was meant to stimulate rapid growth in the algae. This concentration was measured on the same days as the algae were reduced in density through dilution.

Data analysis

The results obtained were examined in Excel to compare between algae species. The results were then combined in a table to create an overview of the most suitable algae by examining four factors: Colony formation, other algae, grazer infestation and PAM instability (Tab 1.). One red and one green alga were chosen for further experimentation with *Artemia*. This was done by giving each measured factor of algae suitability a score ranging from 3 to 0.

Four measurements were preformed over a period of two weeks. The data was scored according to table 1 and combined into a final total score over all 4 measurements for each algae.

Table 1: The four measurements used for creating the algea suitability score. The more prevelant a trait is in a sample of algae the less points will be awarded to that algae based on that trait.

Algea suitability grading:	None = 3	Low = 2	Medium = 1	High = 0
1. Collony formation	None	Low - 10<% sample	Medium - 10-40% sample	High - 40>% sample
2. Other algae	None	Low - 10<% sample	Medium - 10-40% sample	High - 40>% sample
3. Grazer infestation	None	Low - 10<% sample	Medium - 10-40% sample	High - 40>% sample
4. PAM instability	0.01<	Low - 0.02 - 0.05	Medium - 0.05 - 0.20	High - 0.20>

3.2 Phase 2: Method of conducting *Artemia* experiments

What algae provided the best development and survivability for Artemia

A comparison was made between the two algae that performed best in phase 1 and how *Artemia* reacted to those algae. Uptake and development were tested by feeding the *Artemia* with the two different algae in duplicate.

Experimental setup

The tanks used for phase two were four Kreisel tanks for the two algae (n=2) with an internal volume of 13.7 litre. These are round tanks meant to enable a continuous flow of water without damaging animals inside. The Kreisel tanks were placed in an aquarium and aerated using a pump (see figure 9). The water in the tanks was kept at a salinity of 33g/l using artificial sea salt, lighting was on a 12-hour light/ 12-hour dark cycle. The algae concentration in the tanks were set at 30000 cells/ml per day for *Rhodomonas salina and* 20000 cells/ml per day for *Tetraselmis chuii*. These cell numbers were chosen by starting with a value of 30000 cells/ml for *Rhodomonas salina* (Mcbride, 2005). Then using the data obtained in phase one of this research the correct amount of *Tetraselmis chuii* based on the weight was vs cell count was determined. The *Artemia* was kept at a salinity of 33ppt and a temperature maintained at 27 °C (comparable with ideal *Diadema* living conditions) to rule out a future change of salinity or temperature changing the uptake potential of the algae. The *Artemia* was set at an initial density of 2 per ml to be comparable with sea urchin larvae cultures (Mcbride, 2005)

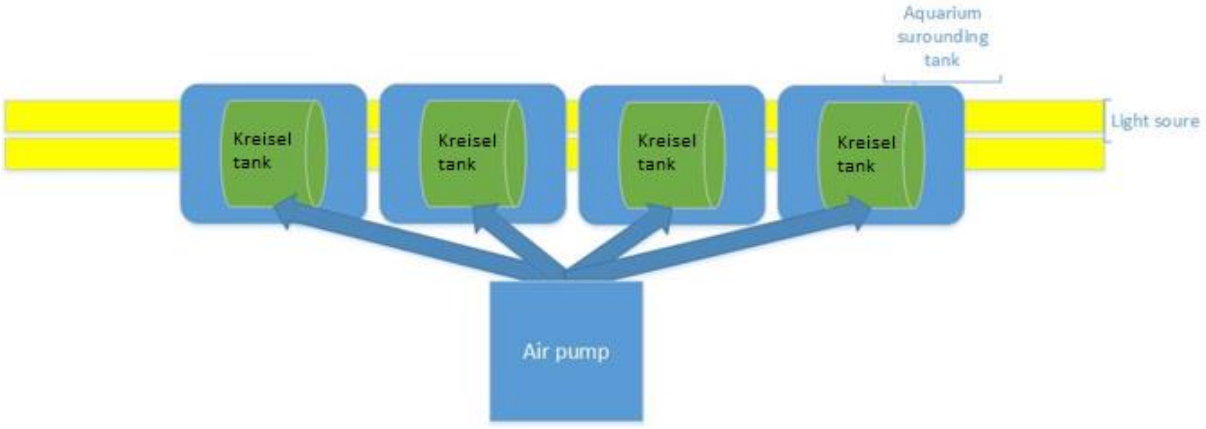

Figure 9: experimental setup larvae tests: pressurized air is pumped to aerate four Kreisel tanks submerged in aquaria.

The density of *Artemia* was then measured by taking 10 samples from each tank with a 10 ml syringe and counting the average amount of *Artemia* per ml. Growth was measured to establish how well *Artemia* can develop on a diet of the different algae. The growth of the *Artemia* was measured by looking at the average length of two *Artemia* from each tank every workday, using a microscope equipped with a camera capable of measuring. For a detailed description of these measurements refer to appendix III.

Data analysis

All data gathered was analyzed with Excel to compare the mean size of the *Artemia* fed with the two different micro algae species. An independent-samples t-test was used (two-tailed) with significance level $\alpha = 0.05$. The mean total number of *Artemia* per ml for both algae was also compared using an independent-samples t-test.

4 Results

4.1 Biomass measurement correlations

The research has shown a clear correlation between the optical density, cell count as well as dry weight measured for all four species as shown in figure 10-12. All correlations are significant at p= <0.05 over the range that was measured. This was determined using a R critical value table by Del Siegle (Siegle, 2015).

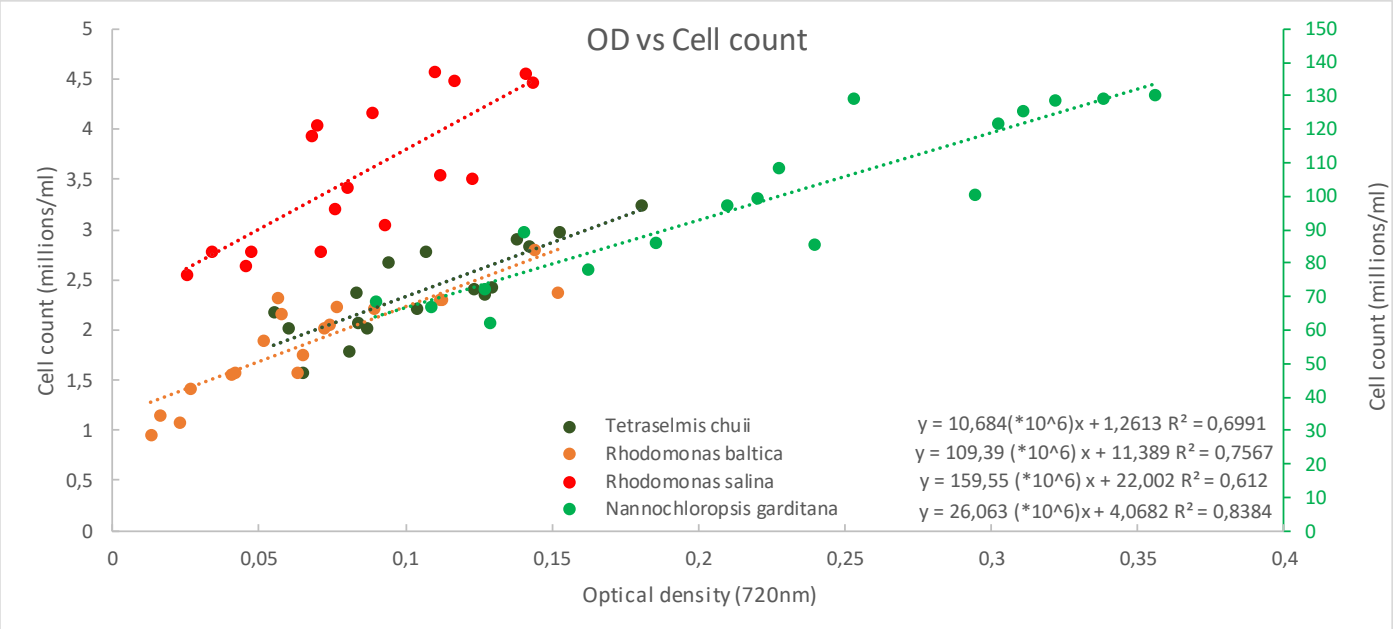

Figure 10: Optical density (720nm) on the X axis correlated with cell count per ml on the left Y axis for Tetraselmis chuii, Rhodomonas baltica and Rhodomonas salina plus cell count per ml for Nannochloropsis gaditana on the right Y axis due to higher cell numbers per ml.

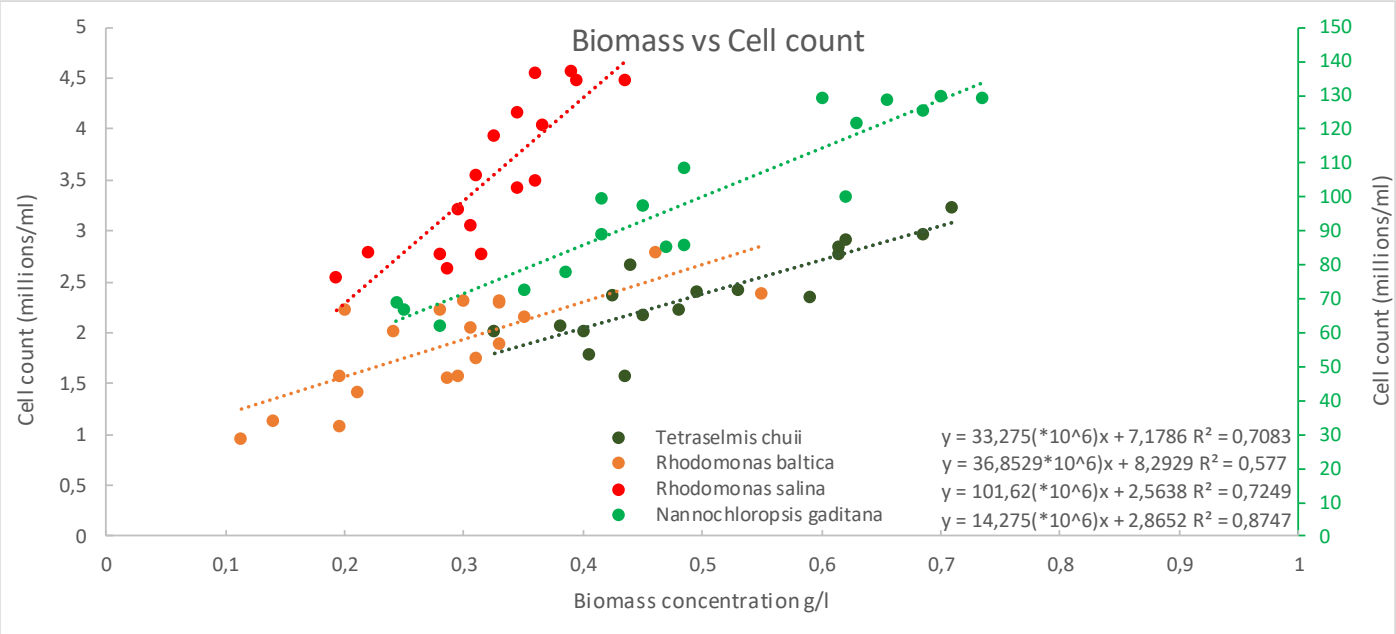

Figure 11: Gram per litre on the X axis correlated with cell count per ml on the left Y axis for Tetraselmis chuii, Rhodomonas baltica and Rhodomonas salina plus cell count per ml for Nannochloropsis gaditana on the right Y axis due to higher cell numbers per ml.

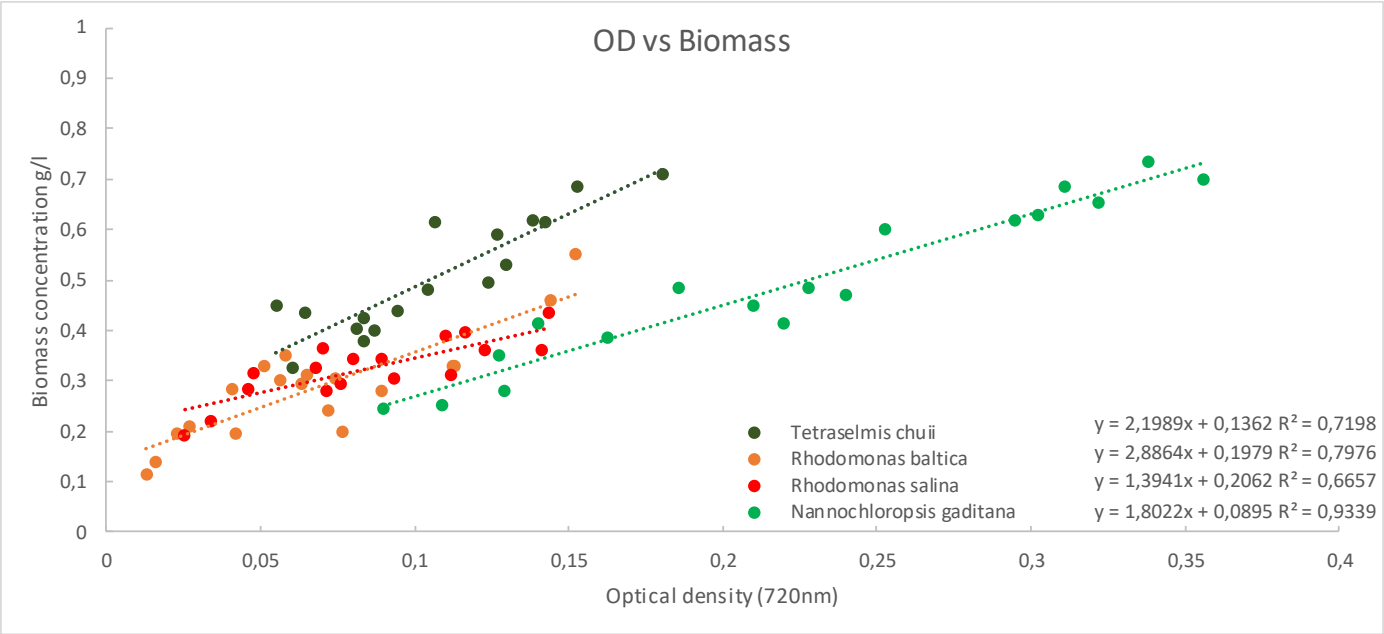

Figure 12: Optical density (720nm) on the X axis correlated with gram per litre on the Y axis for Tetraselmis chuii, Rhodomonas baltica,Rhodomonas salina and Nannochloropsis gaditana

4.2 Algae suitability

The choice was made for *Rhodomonas salina* and *Tetraselmis chuii* to be cultured for phase 2 of the experiments. These algae were chosen over *Rhodomonas baltica* and *Nannchloropsis gaditana* by looking at the results of all measurements. These results are combined as a single score in table 2. In fig 13- 16 the results this table is based on are presented. Rhodomonas *salina* and *Tetraselmis chuii* showed a greater resistance to colony formation compared to their competing algae (fig. 13) and while *Tetraselmis chuii* had a higher infestation by other algae (fig 14) early on, it did not continue rising meaning it was able to outcompete the infestation. This makes *Rhodomonas salina* and *Tetraselmis chuii* the more reliable algae to culture. The grazer infestation data (fig 15) shows only *Tetraselmis chuii* showing resistance. Another measurement taken was the PAM instability. As seen in figure 16 this remained stable for all algae. With both red algae showing a higher average PAM, 0,65 for *Rhodomonas salina* and 0,67 for *Rhodomonas baltica.* In comparison *Tetraselmis chuii* had an average PAM of 0,56 and *Nannocholropsis gaditana* had an average PAM of 0,54.

Table 2: Algae suitability table showing the final combined score from colony formation, other algae, grazer infestation and PAM instability of all 4 algae species for all 4 measurments.

Combined algae suitability score of all measurements	*Rhodomonas baltica*	*Rhodomonas salina*	*Nannochloropsis gaditana*	*Tetraselmis chuii*
Total day 1	9	12	9	8
Total day 5	3	7	10	7
Total day 8	4	7	9	11
Total day 12	8	8	6	8
Final suitability grade:	24	34	34	34

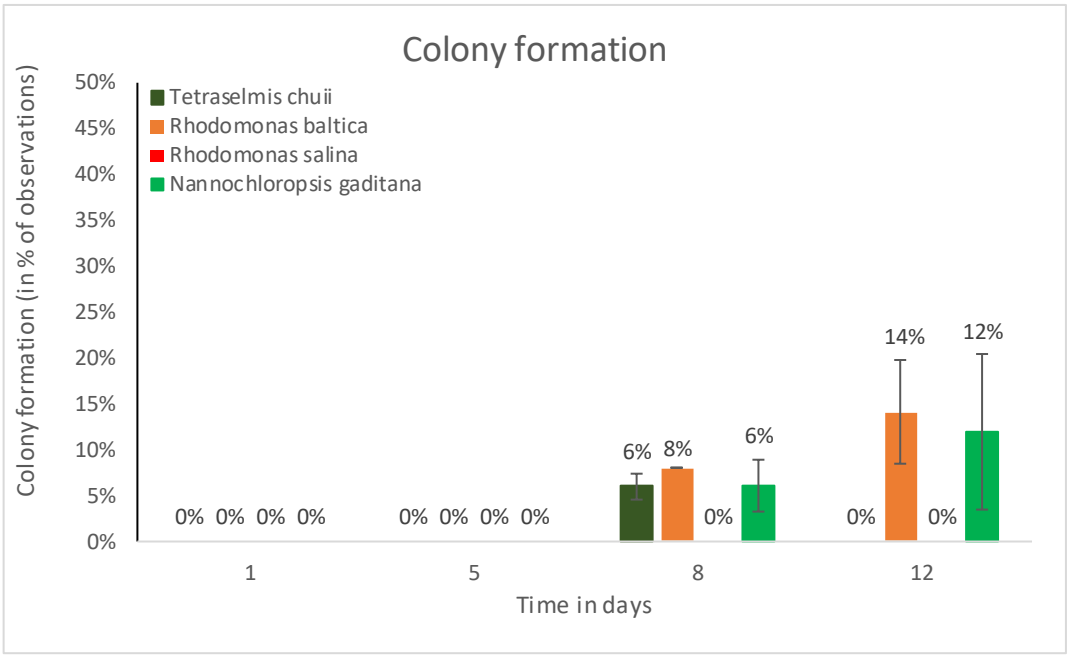

Figure 13: Percentage of observations showing algae colony formation with standard deviation (N=2) for Tetraselmis chuii, Rhodomonas baltica,Rhodomonas salina and Nannochloropsis gaditana

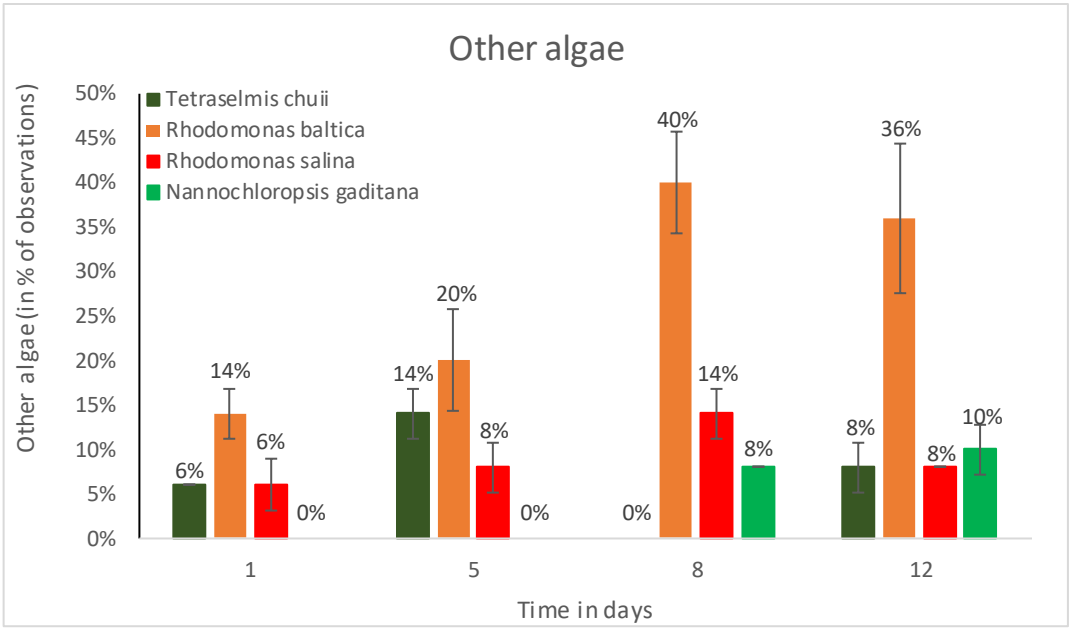

Figure 14: Percentage of observations showing algae other than the target species with standard deviation (N=2) for Tetraselmis chuii, Rhodomonas baltica,Rhodomonas salina and Nannochloropsis gaditana

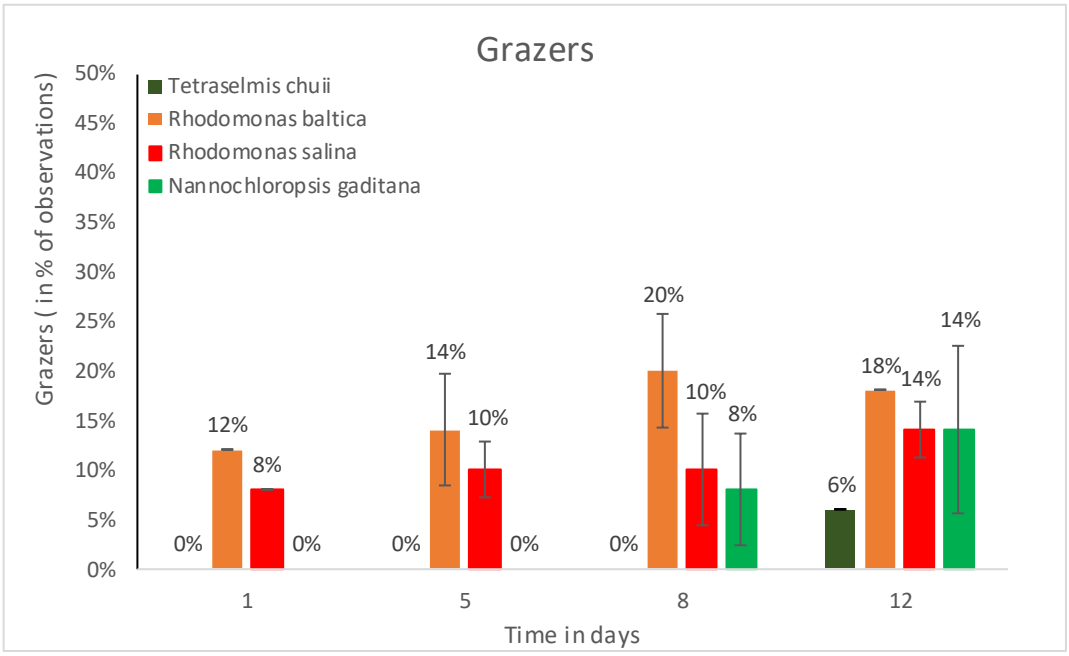

Figure 15: Percentage of observations showing grazer infestation with standard deviation (N=2) for Tetraselmis chuii, Rhodomonas baltica,Rhodomonas salina and Nannochloropsis gaditana

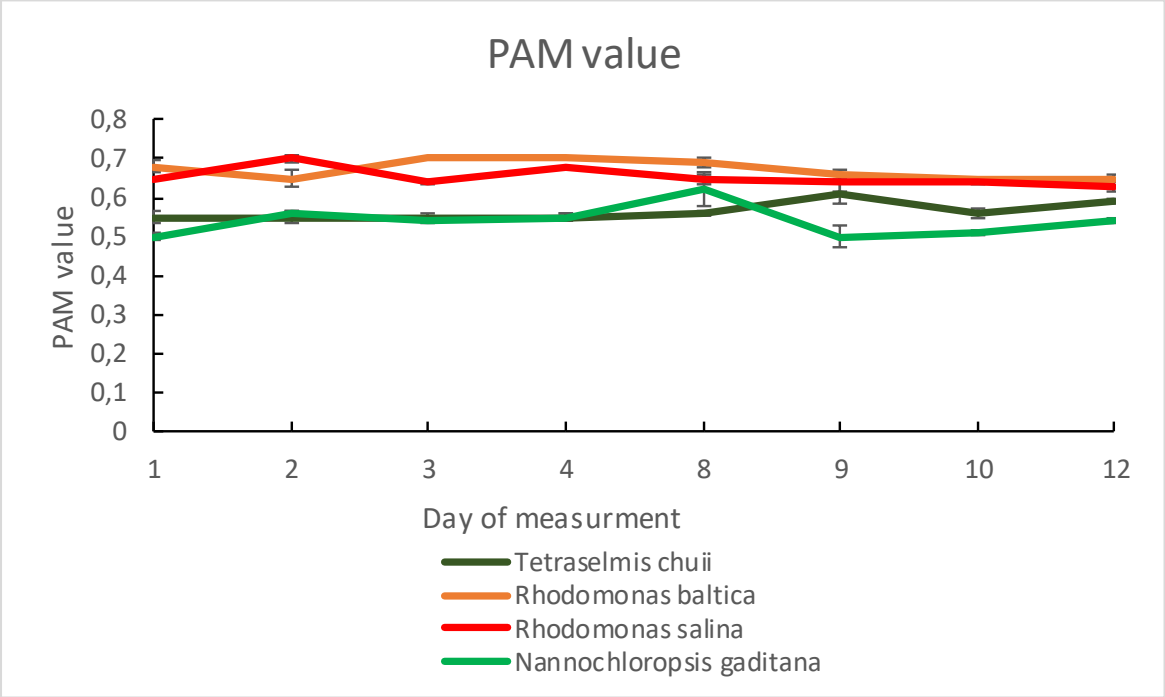

Figure 16: PAM stability with standard deviation (N=2) for Tetraselmis chuii, Rhodomonas baltica, Rhodomonas salina and Nannochlropsis gaditana

4.3 *Artemia* experiment

The results in feeding the *Artemia sp.* with *Rhodomonas salina* and *Tetraselmis chuii* showed a decrease in density from 1.4 *Artemia* per ml to around 0.20 *Artemia* sp. per ml in the first 4 days of measurements (figure 19 & 20) while showing a similar growth in size to approximately from 500 µm to 1500 µm (figure 17 & 18).

Conducting an independent two tailed t-test for both length and density, the length of A*rtemia* fed on *Rhodomonas salina* (M= 2312.55, SD = 1273.6, n = 10) did not significantly differ from the length of *Artemia* fed on *Tetraselmis chuii* (M= 1819,35, SD= 780,31, n=10.),t (18) = 2,1, p = 0,31. The difference in density between *Artemia* fed on *Rhodomonas salina* (M= 0,4865, SD = 0,4470, n = 10) did not significantly differ from *Artemia* fed on *Tetraselmis chuii* (M= 0,457, SD = 0,5370, n = 10), t (18) = 2,10, p = 0,895.

Artemia sp. did however show more growth and a more stable density growing on *Rhodomonas salina* compared to *Tetraselmis* chuii (fig 19 & 20). Which combined with the popularity of *Rhodomonas salina* as an algae species for feeding larvae (Cameron & Schroeter, 1980; Salas-Garza et al., 2005) makes it the overall best algae to use for feeding *Artemia.*

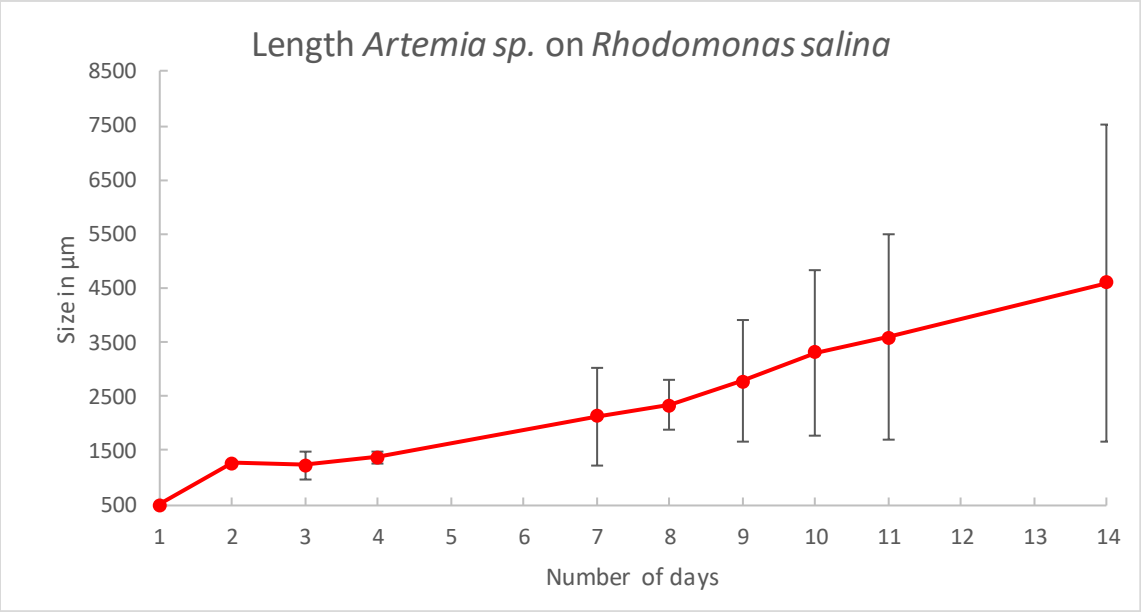

Figure 17: Artemia sp. length with standard deviation (N=2) over 14 days fed by Rhodomonas salina

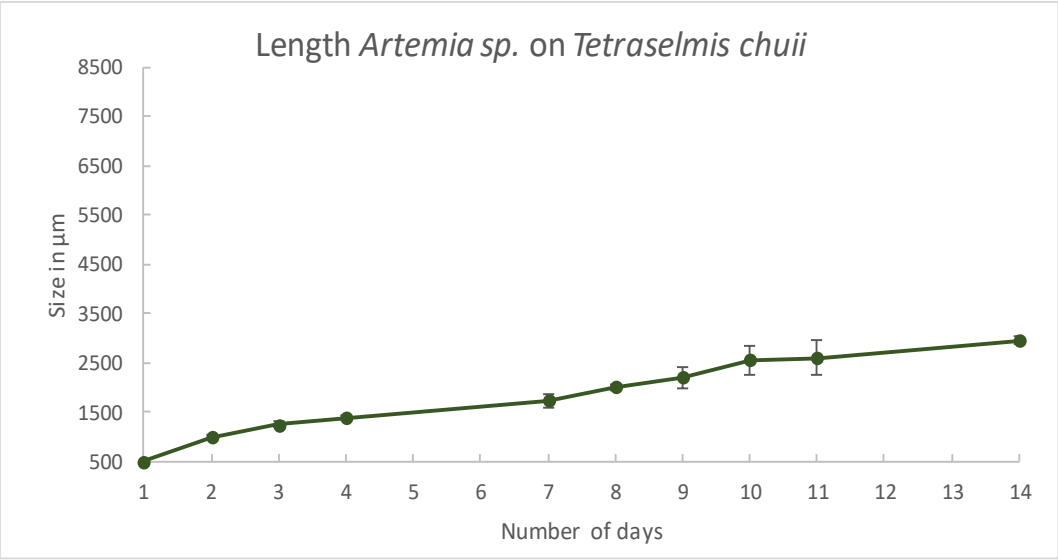

Figure 18: Artemia sp. length with standard deviation (N=2) over 14 days fed by Tetraselmis chuii.

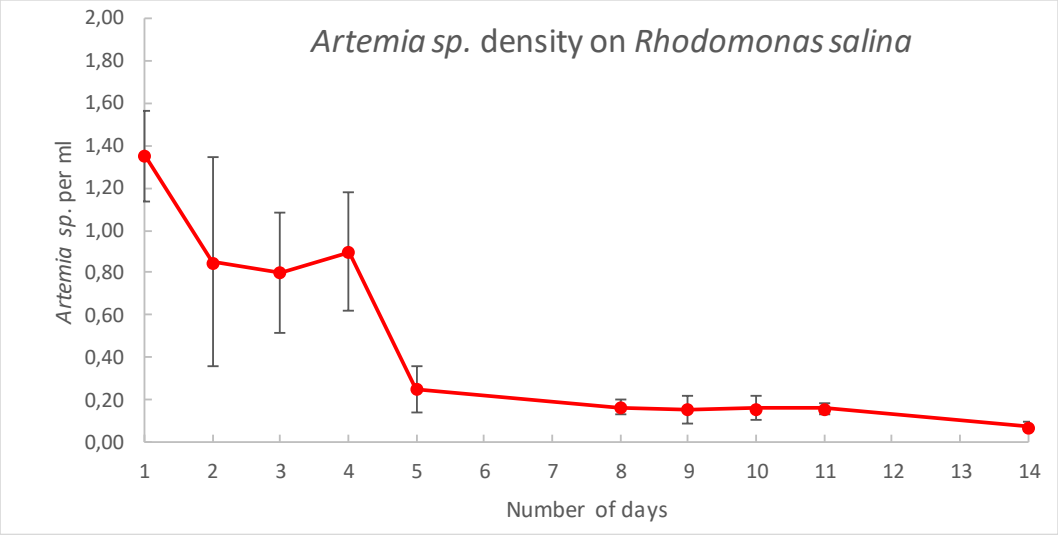

Figure 19: Artemia sp. density with standard deviation (N=2) growing on Rhodomonas salina

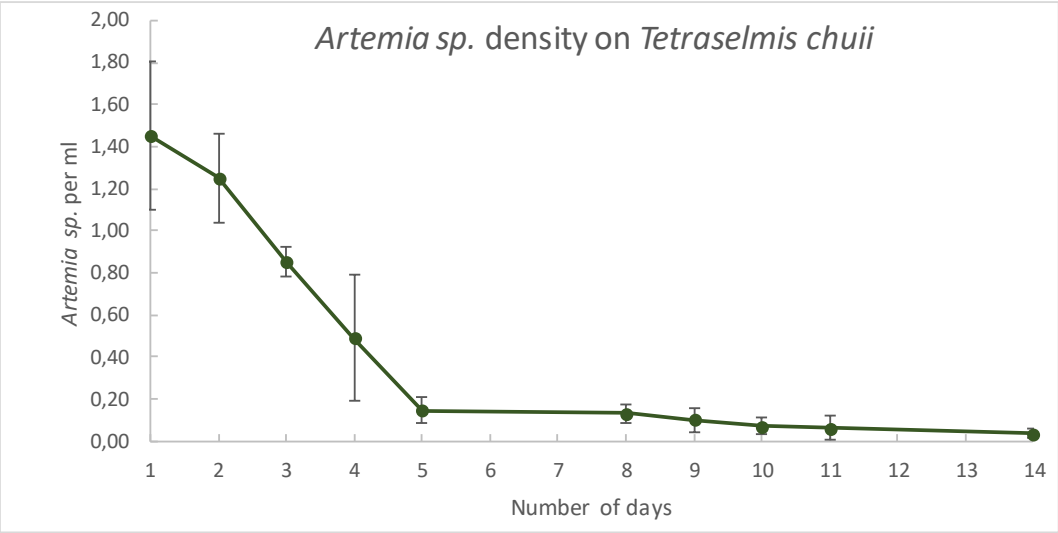

Figure 20: Artemia sp. density with standard deviation (N=2) growing on Tetraselmis chuii

5 Discussion

5.1 Biomass measurements

Discussion

The results obtained from this experiment have answered the question of what the correlation is between optical density, cell count and g/l of the four algae species. This will enable people working with the algae to do an optical density measurement and gain information about the exact cell count and dry weight content of the algae. However, the highest density measured for these experiments was OD 0.356 (720nm). In algae cultures it is possible to get cultures which are denser than this. In these cases, the correlations may no longer be valid. Furthermore, it was found that even with the correlations it was not possible to use the OD measurement in the *Artemia* experiments since at such low density no valid OD measurement could be made.

The limited scope in which the OD measurements can be used means that cell counts will still be required for measuring algae at low density in larvae tanks. It also means that that the correlations made in this project are most applicable to quickly and easily measuring the density of the algae cultures themselves and get an accurate estimate cell count and dry weight per ml.

In another study with similar parameters to this study the correlations between optical density, cell count and g/l found correlated stronger at R^2 0,99 (Lu et al., 2017). This is likely due to a different methodology where all algae were taken from the same batch and diluted to different densities before being measured in triplicate. For this experiment algae were taken from an active culture being diluted and grown continuously, meaning it is possible the algae were in different growth phases for different measurements, potentially affecting results (Piorreck & Pohl, 1984).

Recommendations

Due to the limited range measured over these experiments it is recommended that the results obtained should not be used when measuring densities below or above the range applied in this research.

5.2 Algae suitability

Discussion

The most suitable micro algae chosen for cultivation are *Rhodomonas salina* and *Tetraselmis chuii*. Results for the green algae were tied with *Nannochloropsis gaditana,* the choice for *Tetraselmis chuii* was made due to the fact it is more similar in size to *Rhodomonas salina.* In addition to this there was a higher resilience measured with regards to infestation by other algae and grazers for *Tetraselmis chuii* compared with *Nannochloropsis gaditana.* Their identical score comes from the wide range of measurements having the same score as showed in table 1 which could in future research be avoided by making the scoring more precise.

The chosen algae show low infestation by other algae and grazers and a stable PAM value meaning they have shown to remain healthy over a long period of time. The actual measurements of the algae cultures were done in two weeks following 4 weeks of growth. This is different from this initial research setup which assumed 6 weeks of full observation. The reason for this was a slower than anticipated progression in approval of the project. However, because they were observed to grow steadily with regular dilutions for 4 weeks the results were still suitable for the purpose of choosing a suitable alga for long term culturing.

After these 6 weeks multiple culture collapses have been observed. This was due to the airflow into the system being repeatedly cut off as filters for the air were filling up and new once were becoming scarce.

The speed of growth measured for *Rhodomonas salina* and *Tetraselmis chuii* also produces enough to supply algae for larvae cultures, with a doubling time of roughly two days for *Rhodomonas salina* between OD 0,05 – 0,1 over the course of the 6 weeks it was maintained. This corresponds to an increase of 3 to 4 million cells per ml in two days. At this density using a 1 l culture bottle 500ml of algae can be harvested twice weekly for a total of 4e+9 cells per week. Calculated for 30000 cells per liter this means a one-liter bottle of algae maintained at this density can supply algae for a total of 133 liter of water per week. With one larvae tank having a volume of 13.8 liter this means that if the larvae eat the full supplied 30000 cells per ml every day, two liters of culture would be required to maintain four larvae tanks.

With regards to the PAM the green algae had a lower overall PAM than the red algae. This does not mean that the red algae are more healthy however because PAM values can differ based on species (Beardall, Young, & Roberts, 2001), algae movement and algae colour (Schreiber, Klughammer, & Kolbowski, 2012) which is why the instability was measured.

This research has provided suitable conditions to culture *Rhodomonas salina*, while the ideal culturing conditions are still being researched (Guevara, Arredondo-Vega, Palacios, Saéz, & Gómez, 2016; Vu et al., 2016). For the application at VHL filtered air, 80.4 µmol/m^2 in a standard fluorescent light spectrum and f/2 nutrient medium times two provided successful growth, meaning it is fully viable to continue growing it.

Recommendation

A worthwhile change in growing the algae would be to attempt growing *Rhodomonas salina* on higher concentration of nutrients up to 20 times f/2 (without additional trace metals) as this has recently shown (HZ University of applied sciences, 2019) to lead to more dense cultures of *Rhodomonas salina* which would decrease the final volume that needs to be fed.

Research should be conducted into the potential of growing the algae without the air filters that were used in this research because of the potential for airflow getting cut off and cultures collapsing. Leaving potential larvae without a food source.

5.3 Artemia experiments

Discussion

The research provided the necessary proof of concept that keeping and maintaining a live culture of micro algae for feeding purposes is possible and that it can be implemented as a long-term strategy at VHL. This means that even though the results comparing *Artemia* growth and survivability between *Rhodomonas salina* and *Tetraselmis chuii* proved to be insignificant there is still value to be obtained from the experiment.

The setup of the experiments has shown that that there is a need to filter the water used in the tanks rearing larvae as either due to normal excretion of the *Artemia* or nutrients still present in the algae water nitrite levels rose quickly. It is unlikely that this directly impacted the *Artemia* culture as they can resist nitrite levels of up to 320 mg/L (Daintith, 1996) and levels in the tank did not exceed 60mg/L. However, for more sensitive animals like *Diadema* larvae these fluctuations could prove problematic.

A major decline was seen in both *Rhodomonas salina* and *Tetraselmis chuii* fed *Artemia* density in the first week of the experiment. But several assumptions can be made for which further research could be done. It is possible the current was too strong and kept the young larvae not strong enough to swim pressed to the sides of the Kreisel tank. It is also a possibility that the food supply provided did not mix properly in the water or spilled out of the tank though the filters on the side.

The results showed a wide margin of error with regards to the size of larvae grown on *Rhodomonas salina*. This was due a large difference in growth between the two tanks. The reason for this is not known as the tanks were kept in the same condition. The only possible difference was the distance algae had to travel to the tanks. In the *Rhodomonas salina* tank with lower growth, the algae had to move further. Being pumped from a bottle of algae through tubing to reach the tank. It is possible that this increased distance led to more algae dying within the tubing, but this has not been further examined.

Recommendations

It is recommended that if the setup used in this experiment is used, the flow of the Kreisel tanks is examined to ensure a stable circular flow is maintained. Furthermore, more data on the growth of the *Artemia* on *Rhodomonas salina* would help bolster the results obtained in this study.

It is also suggested that in future research more larvae per tank are measured. This should be done to obtain more trustworthy data as this research has shown that large fluctuations in size between tanks can exist which lead to untrustworthy data if not enough measurements are done.

Another recommendation to ensure a more solid research setup is to have algae fed into the Kreisel tank go straight from the aired feeding bottle to the tank and not have algae left behind. This is because algae left behind showed sighs of dying. Potentially due to prolonged lack of aeration which means that as the algae were fed it is possible, they had already died. If this problem cannot be solved fully due to technical limitations feeding all algae in one go would partially resolve this issue though the effects of such an uneven feeding regime is not examined in this research.

6 Conclusion

This thesis has succeeded in providing the tools and information needed for all future experiment involving micro algae culturing and subsequently using these micro algae for feeding purposes at VHL. It has provided the information needed to start and maintain healthy growth and made recommendations to improve the systems used in this thesis further. In addition to this it has fully answered the research question of what the correlation is between optical density, cell count and biomass measurements. Allowing future researchers to save time on having to conduct al these measurements themselves, instead allowing them to simply perform an OD measurement and find the corresponding density or biomass for their algae culture.

It has provided a choice for the most suitable micro algae to culture, with *Rhodomonas salina* being chosen as the best species from a selection of four. This choice was made based on results obtained during rearing *Artemia* using both *Rhodomonas salina and Tetraselmis chuii.* While these results on their own did not show any significant differences the fact that they proved to be similar in effectiveness in this experiment, combined with the popularity of *Rhodomonas sp.* as an alga for feeding larvae of all types made it the clear choice.

In this thesis multiple protocols were established over the course of the experiments that will allow future students and researchers to easily follow instructions when seeking to set up their own micro algae cultures or perform measurements on micro algae.

In conclusion, this thesis and supplementary protocols have succeeded in provided the groundwork for the *Diadema antillarum* project as was the initial goal.

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Appendices

I. Algae culture setup

Culture setup materials

Lighting

- 24H electricity timer
- A light source

Growing vessels:

- 1 litre bottle per culture
- Input / output cap (see figure 2)
- VWR sterile air filter (0.2 µm)
- Tubing for airflow

Other devices

- Air pump
- container with input & output cap
- splitter for air distribution (see figure 1)

Culture setup described

The algae culture will be kept in 1 litre bottles with air bubbling through to supply CO_2 and ensure mixing. Air will need to be provided with an air pump. The air needs to be passed through a vessel holding demi water to reduce evaporation. Then it needs to be fed into a splitter (see figure 1) which allows you to adjust the amount of airflow going into each bottle. Once the air is through the splitter it needs to go through an VWR sterile air filter (0.2 µm) attached on the bottle to reduce outside aerial pollutants. On top of each bottle needs to be a cap with an input and an output. The input needs to allow for attaching the air filter and allow for feeding a tube with air to the bottom of the bottle. The output needs to be small enough to not allow outside pollutants to enter the bottle while the airflow is active. See figure 2 for an example of this.

The bottles need to be in front of a light source, with the algae's this manual is based on this light source consisted of 6 TL lights covering the length of the setup being on a 12-hour light / 12-hour dark cycle. See figure 3 for a complete schematic overview of the system.

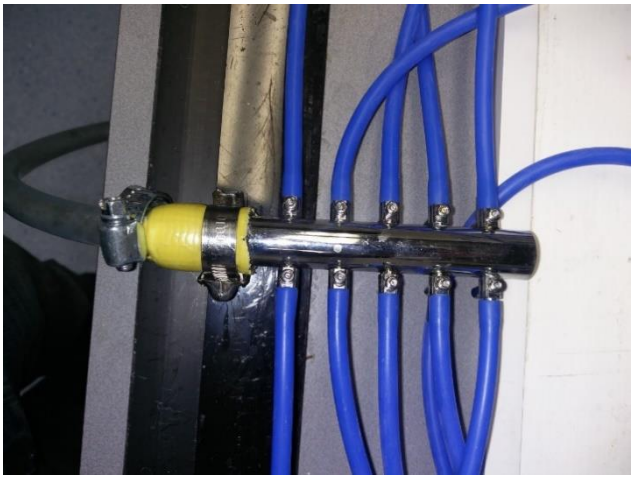

Figure 1: air being split to different culture vessels

Figure 2: culture vessel with air filter and input / output cap

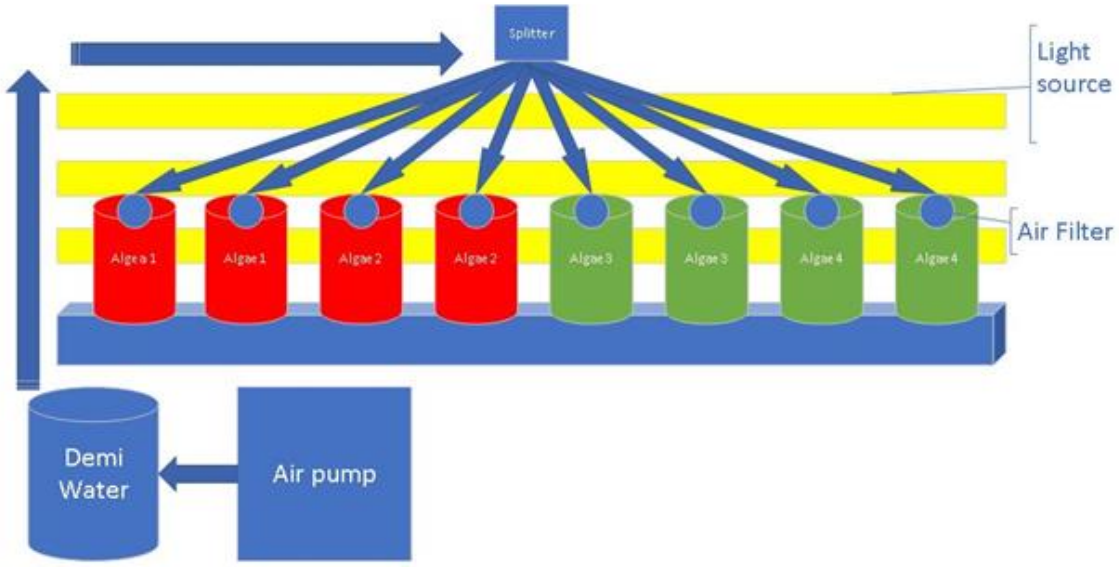

Figure 3: a schematic overview of the culture setup using 8 different bottles

II. Algae culture measurements

While maintaining algae different measurements can be done to determine the amount of alga present, the amount of nutrients available or the photosynthesis effectiveness of the algae. This section will explain how to obtain your algae sample from the culture vessel and how to conduct all different measurements.

Obtaining the sample

To obtain an algae sample from the culture without risking contamination

1. Remove the air filter from a bottle and place it on a clean surface.
2. Take a clean 60ml syringe and insert it in place of the air input.
3. Withdraw the required amount of alga.
4. Reinsert the air filter.

Aquapen ap100 measurements

Two of the most common measurements are both done using the Aquapen ap100, these are the Optical density measurement and the PAM measurement. A detailed guide to installing and using the Aquapen ap100 is found at
https://www.psi.cz/download/document/manuals/aquapen/AquaPen_Manual.pdf

PAM and optical density materials

- Sample of algae
- Cuvette with cap
- Aquapen ap100

PAM measurement

To take a PAM measurement, first ensure you have a clean, dry cuvette without any visible scratching. Any imperfections on the outside of the cuvette can impact results.

1. Shake your algae sample.
2. Add 3ml of your sample to the cuvette and close it with a cap.
3. Place the cuvette in the dark for 1 minute (the box the cuvettes come in is ideal for this purpose).
4. Remove the cuvette and quickly shake until you are sure the algae solution is homogenous
5. Place it in the Aquapen ap-100 and either manually or with the online controls select the QY measurement.

Optical density

To take an optical density measurement you can use the same cuvette as you used for the PAM measurement or make a new one following the same criteria, when using the same cuvette ensure you always do the PAM measurement first.

1. Shake your algae sample.
2. Add 3ml of your sample to the cuvette and close it with a cap.
3. Remove the cuvette and quickly shake until you are sure the algae solution is homogenous
4. Place cuvette in the Aquapen ap-100 and either manually or with the online controls select the OD720 measurement.

Cell count

Cell count materials

- 10 ml of algae sample
- Bürker and turk haemacytometer
- Photomicroscope
- 200 ml volumetric flask
- 50 ml Erlenmeyer
- Acetic acid
- Pasteur pipet
- Cell counter
- Pipet device set to 20 µl
- Gloves
- Glasses
- Lab coat

Method

1. Dilute 10 ml of your algae samples if needed – for bigger algae species this is usually not required, for *Nannochloropsis* a dilution of 20 times is a necessity. To dilute a sample, add prepared sea water to the algae in a 200 ml volumetric flask until the desired dilution is met.
2. Put on gloves, glasses and lab coat before working with acid.
3. Put 10 ml of your sample in a small Erlenmeyer. If you are working with moving cells such as *Rhodomonas baltica, Rhodomonas saline* or *Tetraselmis chuii,* add 20 µl of acetic acid to fixate them.
4. Proceed to shake the sample to ensure the solution is homogenous and to break up any cells that may have grouped together due to the introduction of the acid.
   This is especially important when working with the species of *Rhodomonas.*
5. Press cover glass onto the counting chamber with the provided clamps until Newton diffraction rings appear.
6. Fill both slides of the counting chamber under the cover-glass with a single smooth flow of your sample using a Pasteur pipet
7. Count 25 squares in both chambers, count cells which touch the top and left of the squares but not those which touch the bottom or right.
   For *Rhodomonas baltica, Rhodomonas saline* or *Tetraselmis chuii* a magnification of 20 can be used however for *Nannochloropsis* a magnification of 40 is suggested to ensure you do not miss any cells.
   In addition to a higher magnification the size of *Nannochloropsis* means it can be on multiple heights under the glass even when pressed down according to instruction. To ensure you don't miss any cells it is therefore suggested to adjust the microscope slightly for each square you count to ensure all cells are counted.
8. Finally, you can calculate the number of cells per ml by taking the average number of cells counted per chamber and multiplying by 10.000. If the sample was diluted, divide accordingly.

Dry weight measurement

Dry weight materials

- 20ml of algae sample
- Vacuum pump and tubing
- Filters (Milipore, glasfiber 1µm retention, 4,7 cm diameter)
- Gloves
- Suction flask
- Büchner funnel (5 cm inner diameter)
- Pincers
- Aluminum disks
- Two 50 ml volumetric flask
- (0.5 M Ammonium-bicarbonate water)
- Exicator
- Drying oven at 105°C
- Scale
- Pipet balloon kit
- Wash bottle with demi water

Method

1. Dry filters for at least 16 hours in a drying oven, let cool to room temperature in an exicator.
2. Put on gloves, weigh 1 aluminium disk, then weigh one filter on that disk
3. Place the filter in a Büchner funnel over a suction flask, secure the filter by adding some demineralized water under suction.
4. Shake your algae sample and empty in a small Erlenmeyer
5. Turn on vacuum pump, pour 20 ml culture over the filter using a pipette balloon – ensure that the pressure of the vacuum pump never exceeds 200 bar.
6. When all water has passed through the filter: add ~10 ml of 0.5M Ammonium-bicarbonate water to flush out the salt.
7. When all water has passed through the filter again: turn off vacuum pump
8. Carefully, remove filter from funnel with a pincer and place in the previously weighed aluminium disk.
9. Place the aluminium disk in oven at 105°C for at least 16 hours.
10. Weight the filter with aluminium disk
11. Subtract the weight of the filter plus aluminium disk from the full weight, divide by 20 to reach mg/ml.

Health of culture

Health of culture materials

- Haemacytometer
- 10 ml of algae sample
- Syringe with small tube attached
- Pasteur pipet
- 50 ml erlenmeyer

Microscope observations

1. Put 10 ml of algae sample in a small erlenmeyer.
2. Proceed to shake the sample to ensure the solution is homogenous
3. Press cover glass onto the counting chamber with the provided clamps until Newton diffraction rings appear.
4. Fill both slides of the counting chamber under the cover-glass with a single smooth flow of your sample using a pasteur pipet
5. Count 25 squares in both chambers:

    - The percentage of colony forming is counted by marking every square in which a colony being defined as 4 or more cells fused together has formed as 2% colony forming.
    - The infestation by other algae can be measured in a similar manner but looking at how many squares have algae that are of a different species than the species measured.
    - The infestation by grazers in the same manner.
      Note that as grazers move, they might move from one square to another as you count, try to keep track of this and not count them double if possible.

III. *Artemia* experiments

Artemia culture setup

Artemia culture setup materials

- 1.5 l coned bottle
- Air pump
- Salt
- 1.5 g *Artemia* eggs
- Air tubing
- Heating element
- Syringe
- Lighting

Artemia culture setup

1. Make 1.5 l of full-strength sea water (33g/l)
2. Place the water in the bottle
3. Add a heating element set to 25 degrees
4. Put a light source near the water surface
5. Wait for the water to warm
6. Add aeration
7. Add 1.5 g of *Artemia* to the water
8. Wait 24 hours
9. Turn off aeration
10. Wait for the shells to float to the surface and extract them with a syringe
11. Harvest or grow out hatched *Artemia* from here

Artemia grow out setup

Artemia grow out setup materials

- Fresh starved* micro algae in a bottle
- Air pump & tubing
- Programable dosing pump
- Kreisel tank(s)
- Flow pump
- Aquarium with enough volume to fit your tank(s)
- Heating elements

Artemia grow out setup

1. Place the Kreisel tank(s) in the aquarium and fill with saltwater (33g/l) till it covers the mesh opening in the Kreisel tank completely.
2. Warm the water to 26 degrees Celsius.
3. Setup the air pump.
4. Place an aeration tube into the water of the aquarium as well as a flow pump to ensure water circulation.
5. Put an aeration tube into the bottle of algae used for feeding
6. Hook the algae up to the Kreisel tank via the dosing pump and program how much algae you wish to feed**.
7. Add the Artemia approximately 12 hours after hatching.

Artemia grow out maintenance

The setup described above does not contain any way to filter the water. Its recommended to replace 50% of the volume daily with saltwater at 26 degrees Celsius. While draining the old water you should rub over the mesh on the side of the Kreisel tanks to remove any stuck particles and allow water to flow in and out of the Kreisel tank though the mesh.

* starved micro algae refer to algae that no longer contain any nutrients which would allow them to continue growing

**(For growing *Artemia* in this setup with *Rhodomonas salina* 30000 cells per ml at an *Artemia* density of 0.2 per ml is recommended. To measure how quickly the *Artemia* are consuming the algae take hourly measurements for the first ~4 hours that the *Artemia* are in the system. For detailed instructions on how to measure the cell count of algae in the water please refer to section 3.3 – cell counts)

Artemia grow out measurements

***Artemia* measurement materials**

- Air displacement micropipette with 1 ml volume
- 10 ml syringe
- 10 cm diameter see through petri dish
- 10 ml Erlenmeyer
- Lap counter

Method

1. Take note of the extraction points in your tank. It is recommended to take 10 total extractions per tank. Five at a depth of approximately 4 cm, five at the centre of the tank. Four measurements each corner 3 cm from the tank wall, one in the centre of the tank.
2. Extract artemia, either 1 ml with micropipette or 10 ml with syringe depending on density and of the artemia. Artemia must be able to enter the measuring device freely. With less dense cultures (less than 1 per ml) it is recommended to use the syringe for a larger sample size.
3. Empty your extraction device into a clear petri dish and count the artemia per sample.
4. Divide your total of counted artemia first by the volume of the individual samples you took, and then by the number of samples you took to get the artemia per ml.