Thesis

Optimization of Production and Extraction of Proteins from *Ulva lactuca*.

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van hall larenstein
university of applied sciences

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Optimization of production and extraction of proteins from Ulva lactuca.

For Delta Areas and Resources & Food and Dairy Applied Research Centre Van Hall Larenstein

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Preface

This thesis project was commissioned by the Applied Research Centre of Van Hall Larenstein University of Applied sciences, supervised by T. Visser and T. Wijers.

We want to use this preface as an opportunity to thank certain people who really helped us during this internship. We want to thank Roel, Evelien and Aize for always being ready to answer our lab related questions during the extraction process. Reinier Nauta, we thank you for providing us with the *Ulva lactuca* for our experiment, as well as helping us with questions about the cultivation of this seaweed.

Renzo Elias & Rimco Slagter
03-12-2019, Leeuwarden

Summary

This thesis project has been carried out at VHL (Van Hall Larenstein University of Applied Sciences) and consists of three different parts; the first part is an experiment in which during 20 days of cultivation the optimum nitrate concentration for protein production in *Ulva lactuca* is sought. We exposed *U. lactuca* to four different concentrations of nitrate to find an optimum for growth and protein production for 20 days. The different nitrate concentrations were: 0 µmol/L^{-1}, 50 µmol/L^{-1}, 100 µmol/L^{-1} to 200 µmol/L^{-1} that was delivered to the *U. lactuca* in artificial seawater with a salinity of 30 parts per thousand. During the cultivation the temperature has been kept at 15°C. After the cultivation experiment, research has been conducted on the influence of different protein concentrations in the start material on the extraction of protein in both pellet and supernatant. Extractions were conducted in triplicate for both extraction methods, the first extraction method was at room temperature with demineralized water and the second extraction method was one with increased pH and a temperature of 50°C. In this research it has been concluded that protein concentrations in the start material did not significantly influence the extractable amount of protein. The final part of the thesis project is a literature study on the effect of protein extracted from *U. lactuca* on people, planet and profit in the aquaculture sector based on literature. The main points gathered from this study were that *U. lactuca* protein has small impacts as protein alternative in the aquaculture sector in a triple P perspective (people, planet, profit) depending on what cultivation methods are used. Furthermore it became clear that the European market for seaweed has to grow in order for protein from *U. lactuca* to become an alternative protein source in the aquaculture sector. Summarized, the results found provide us with the conclusion that it is not feasible yet for protein extracted from *U. lactuca* to be integrated in the aquaculture sector as an alternative protein source. With more time, research and improvement of production systems, the production of seaweed protein as an alternative source of protein will become a possibility in the future.

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1.0 Introduction

Globally there is a large increase in population, the expectation is that the world population will grow to 8.6 billion in 2030 (United Nations Department of Economic and Social Affairs, 2017). In order to be able to supply enough protein for this increased population more protein production methods are being utilized and developed. One of the sources of protein is fish, wild caught as well as aquaculture production. Aquaculture as a sector has been growing steadily over the last couple decades, it is even expected to surpass fisheries in terms of production in a few years (Food and Agriculture Organization of the United Nations, 2018). To facilitate the growth in aquaculture production, large amounts of proteins are being used as feed. This protein originates from plants, fishmeal and fish oil, the latter two are both produced of wild caught fish. Fishmeal and fish oil are used to supply the required omega 3 fatty acids and essential amino acids needed for fish growth. While aquaculture production has been contributing to the worlds fish supplies, the pressure on ocean stocks of which many are already overfished or exploited needs to be reduced by the aquaculture sector to sustainably grow in the near future (Food and Agriculture Organization of the United Nations, 2018). With the requirements of becoming more sustainable (Science for Environment Policy, 2015), the aquaculture sector has been carrying out research for years on feed efficiency and on alternative sources of protein that can be used in aquaculture.

One alternative for fishmeal usage in fish feed is soybean meal, this is produced mainly in South-America where large plots of agricultural land are used for the production of soybeans, a legume that uses freshwater to grow. Although soybean meal has a high protein content, it cannot be used in large quantities for every fish species. In most species which are carnivorous and situated on top of the food-chain, adverse effects can occur when high amounts of soymeal are included in the diet due to anti-nutritional factors, these factors can interfere with the absorption of other nutrients or minerals (Francis, Makkar, & Becker, 2001; Kaushik & Hemre, 2008). Another issue with the inclusion of soymeal in fish feed is that the protein from soy consists of different essential amino acids compositions, mainly methionine is lacking in the soymeal (Kaushik & Hemre, 2008). Complete replacement of fishmeal with soybean meal is for those reasons not possible, and other protein sources need to be researched to facilitate the growth of the aquaculture sector in a sustainable way. An alternative protein source available for inclusion in fish feed is protein from seaweed. Protein content of seaweeds differ between species, on average the protein content of brown seaweeds is low, moderate for green species, and mainly higher for red seaweed species (Fleurence, Morançais, & Dumay, 2017). Seaweed is one of the most cultured aquatic products and is mainly produced for: inclusion in human consumption, the production of carrageen and agar (Food and Agriculture Organization of the United Nations, 2018).

Seaweed can be produced in a more environmentally friendly process than soybean meal as it does not make use of freshwater to grow. There are possibilities to cultivate the seaweed in a way that it does not compete with space for agriculture. Seaweed grows through uptake of a nitrogen source which could be nitrate or ammonium, these nutrients are freely available in the sea or found in the effluent of fish producing aquaculture systems (Ale, Mikkelsen, & Meyer, 2011). The difficulties with seaweed protein production is that the yield differs per seaweed species per season (Marinho-Soriano, Fonseca, Carneiro, & Moreira, 2006). Therefore, more research is needed on seaweed production eventually aiming for a steady supply on large scale such as soybeans. In this project the green seaweed species *Ulva lactuca* is being researched, the reasoning for cultivating a green seaweed instead of

species of red seaweed is that the cultivation of red seaweeds is not as developed in Europe as with green seaweeds (Walker et al., 2014). *U. lactuca* is chosen for its high growth rate to up to 18,7% per day and protein content (Bruhn et al., 2011). For *U. lactuca* the protein content varies between 8,7%-32,7% of the dry mass (Fleurence et al., 2017; Ortiz, 2006; Shuuluka, Bolton, & Anderson, 2013). This protein content can be increased through the addition of nitrogen. Common forms of nitrogen are ammonium (NH_4^+) and nitrate (NO_3)(Msuya & Neori, 2008; Neori et al., 2003). To use seaweed protein in fish feed it is important to extract the protein from the seaweed, as fish feed manufacturers prefer protein sources that have a protein content of 48-80 percent and low levels of fibre, starch and non-soluble carbohydrates (Pelletier, Klinger, Sims, Yoshioka, & Kittinger, 2018). Furthermore, seaweed have poorer protein digestibility in their raw form, compared with other protein sources. For *U. lactuca* the *in vitro* digestibility is 85,7% ± 1,9% which is comparable with grains, legumes and vegetables (Bleakley & Hayes, 2017). Which means that extraction of the protein is necessary to utilize all of the protein within *U. lactuca*. The products of the extraction are as follows: the supernatant, the fluid and the pellet including the remaining biomass. With extraction methods like osmotic stress, acid-alkaline treatment or polysaccharide degradation it is possible to create an extract (supernatant) containing the protein, or create a residue (pellet) with higher protein concentrations without having high levels of carbohydrates and minerals (Bikker et al., 2016). Depending on what is favourable for the inclusion in fish feed an extraction method can be chosen (Fleurence et al., 2017; Mæhre, Malde, Eilertsen, & Elvevoll, 2014; Walker et al., 2014; Yildirim & Türker, 2009).

This research was carried out at VHL (Van Hall Larenstein University of Applied Sciences) in Leeuwarden. It is a research which uses the information and expertise of previous projects working with *U. lactuca* conducted at VHL. ZEEVIVO for example is a project which was met with national and international interests. In ZEEVIVO, the possibilities of inclusion of seaweed protein in fish feed were researched. Concluding that seaweed protein has possibilities for inclusion in fish feed if the protein is extracted from the material. In this ZEEVIVO project, Tsjippie Visser conducted research on the effect of elevated nitrate concentrations on the protein percentages in *U. lactuca*. She was able to conclude that there was a significant positive effect on the protein content percentages of *U. lactuca* when there was an elevated nitrate concentration in the medium. A critical open question was the search for an optimum nitrate concentration in order to optimize the protein production in *U. lactuca*. This question makes up the first part of this thesis project. Together Tsjippie Visser and Tom Wijers are supervisors of this thesis and both conducted research for ZEEVIVO. Tom Wijers has years of experience with protein production and extraction in algae and macro algae. For ZEEVIVO he conducted research on the effects of different preservation techniques and different extraction methods in several seaweed species. His research found that extraction of protein in *U. lactuca* with a simple extraction method (an extraction on room temperature with demineralized water) was effective to increase the protein purity of the pellet. Furthermore, he found that for *U. lactuca* an extraction with increased pH at 50°C (an extraction on 50°C with 0.2M NaOH) is a great way to improve the amount of protein in the supernatant. A question that remained unanswered was what effect different protein concentrations in the starting biomass of *U. lactuca* have on the extractability of the protein, in both the pellet and supernatant. This question is answered in part two of our thesis project.

With the aim towards more sustainable production of aquaculture fish it is important that all the steps from protein production for fish feed, up towards aquaculture production are done in an environmentally friendly and efficient manner. For alternative protein sources in fish feed it is key that the production process is not limiting. In the third part of the thesis project the effects on the three P's

in the aquaculture sector are researched for protein from *U. lactuca* in particular. The countries that were in the scope of this literature study were the countries surrounding the North Sea: Denmark, Norway, Belgium, England, Scotland, Germany, France & the Netherlands. People are influenced by this project through the creation of new jobs and with the new knowledge on the production of *U. lactuca*. Through optimization of the production process this thesis provides important information for the profit and planet side of cultivation of seaweed proteins.

Problem statement

Direct inclusion of *U. lactuca* in fish feed is currently not possible as the protein concentrations are not high enough to compete with soybean meal or fish meal, furthermore, unprocessed seaweed has lower digestibility *in vitro* than seaweed protein concentrates. An optimum concentration of nutrients for the production of protein in *U. lactuca* has not been described before. Therefore, it is needed to optimize the *U. lactuca* production and protein extraction in order to become an alternative source of protein in the aquaculture sector.

Aim of the research

The aim is to provide more knowledge on the cultivation and extraction of protein from *U. lactuca*. In order to increase the possibilities for *U. lactuca* to be integrated into the aquaculture sector as an alternative protein source in fish feed. Providing a report which researches in part one, a nitrate concentration optimum for growth and protein production in *U. lactuca*. Part two will give an answer on the effect of different starting concentrations of protein in *U. lactuca* on the extraction efficiency. The last part of the thesis consists of an assessment on the effect of protein extracted from *U. lactuca* as an alternative source of protein in the aquaculture sector on people, planet and profit. The answers on the three parts combined provide a conclusion on whether or not protein from *U. lactuca* can be integrated as an alternative protein source, or if more research needs to be carried out in order to make it a successful alternative protein source. In short: the feasibility of *U. lactuca* protein as an alternative protein source.

Research question

Within this research there are several questions regarding optimizing the production, the extraction method and what the impact is of production of protein from *U. lactuca* on people, planet and profit within the aquaculture sector. In the end these answers will provide a conclusion on the following research question:

- What is the feasibility of integrating *Ulva lactuca* as an alternative protein source in the aquaculture sector?

To answer this research question the following sub questions have to be researched:

- What is the optimum nitrate concentration for the growth and production of protein in *Ulva lactuca*?
- How does protein content in *Ulva lactuca* influence both the extractable amount of protein and the remaining biomass (pellet)?
- What is the effect on people, planet and profit of protein extracted from *Ulva lactuca* as an alternative source of protein in aquaculture?

Outline

This thesis consists out of six chapters. In the first chapter the thesis project is explained briefly, here the cause of the project, the need for this project and the project research questions are explained. The second chapter will go over the various materials and methods that are used in this thesis project. It starts with an explanation about the cultivation and moves on towards the extraction project and the literature study in the later parts of chapter 2. Chapter three presents all the findings and results of the projects. The fourth section of this thesis is the discussion. After the discussion the research questions are answered and concluded in chapter 5. In the final chapter of this thesis project we give our recommendations for further research.

2.0 Materials & Methods

As our research was divided into 3 parts, each has a different chapter in this material and methods. The experimental parts of the research were: seaweed cultivation (chapter 2.1) and the evaluation of protein extraction processes (chapter 2.2). Next to the experimental part, the last part of this research was an impact study concerning the possible impacts of seaweed-based proteins on the aquaculture sector (chapter 2.3). The experiments were executed within the building of VHL, the cultivation experiment in the Aquaculture Research Room (ARR) and the protein extraction experiment in the Water Application Centre (WAC).

This thesis research is considered applied research as well-known accepted theories, principles and research were used. In the experimental part, the seaweed cultivation categorizes as quantitative research as it was numerical, non-descriptive and applies statistics but a longitudinal research as well due to the fact that it was a trend study in which the *U. lactuca* was measured over multiple points in time. And the evaluation of the protein extraction processes was also due to its numerical, non-descriptive and applied statistics nature, quantitative research but not longitudinal. Finally, the impact study was unlike the previous parts not quantitative research but qualitative research instead, being a literature study, non-numerical and used reasoning. It was considered descriptive research as well, studying the effect on people, planet and profit of protein extracted from *Ulva lactuca* as an alternative source of protein in aquaculture.

2.1 The cultivation of *U. lactuca* with different nitrate concentrations

The *U. lactuca* used in this research originated from the cultivation tanks of the Royal Netherlands Institute for Sea Research (NIOZ), Texel. The *U. lactuca* was originally collected from the coastlines of the island of Texel in the summer of 2013. After transportation from NIOZ to VHL, the *U. lactuca* was placed in a holding tank containing artificial seawater (ASW) with a temperature of 16°C for acclimatization. The holding tank had a salinity of 30 ppm, matching the salinity of the natural sea water (NSW) in the NIOZ cultivation systems. Once placed in the holding tank the *U. lactuca* was starved for homogenization purposes. Starvation is a process where the *U. lactuca* was placed in nutrient depleted salt water in order to deplete the vacuoles from nutrients. This resolved the differences between the specimen. After the starvation process the experiment was carried out.

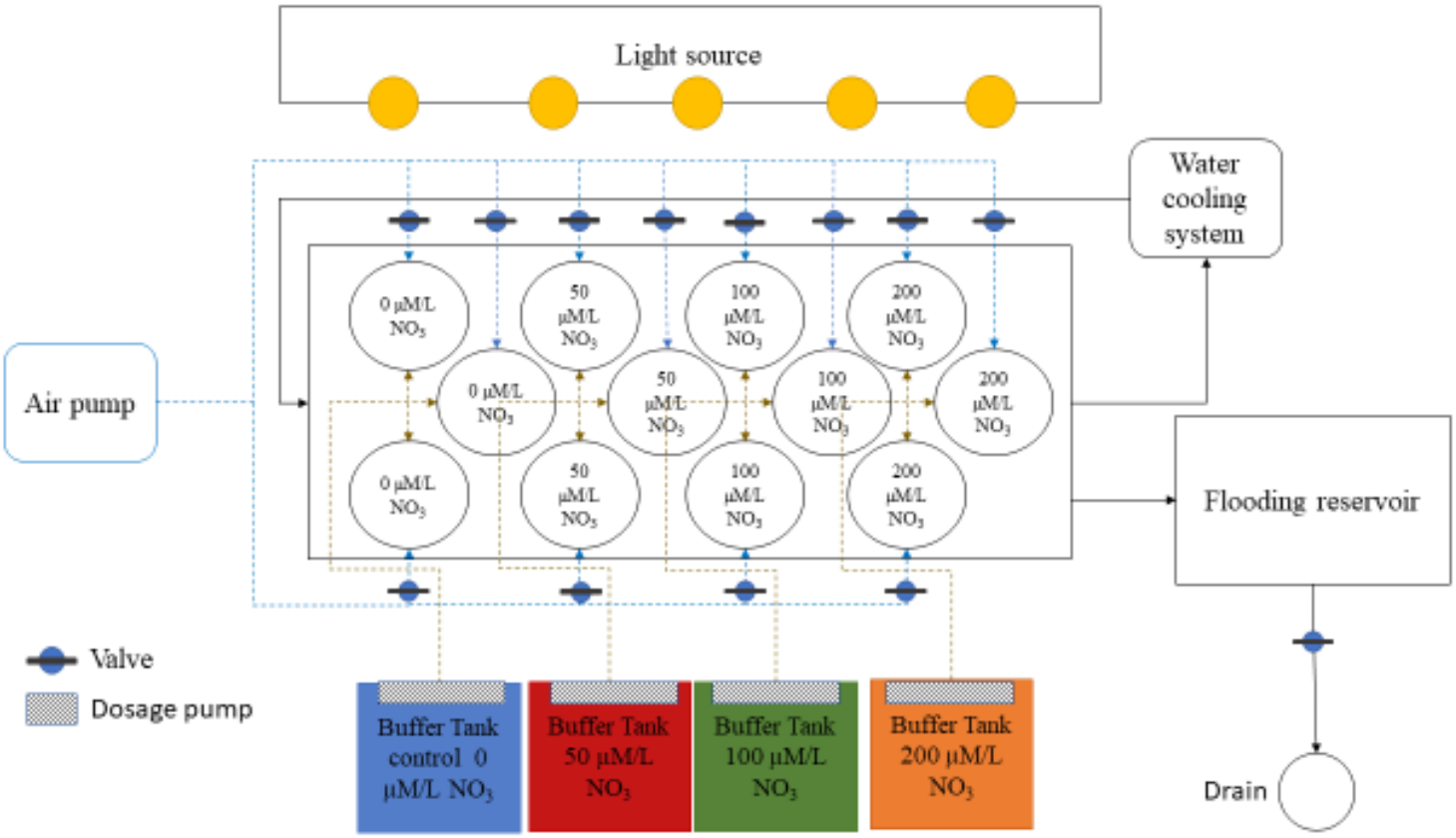


FIGURE 1: EXPERIMENTAL SET -UP SCHEME FOR CULTIVATING U. LACTUCA. INCLUDING THE LAYOUT OF THE BUFFER TANKS WITH THE DIFFERENT NITRATE CONCENTRATIONS IN THEIR PLACE WITHIN THE SET-UP AND A LEGEND DEPICTING THE DOSAGE PUMPS AND VALVES.

Experimental setup

The experimental set-up (figure 1) consisted of a holding tank measuring 200X100X50 cm (Length X Width X Height) with 12 buckets all containing 50 gr. *U. lactuca*. Surrounding the buckets was 228 litres of ASW used for temperature regulation. The buckets had a diameter of 30 cm, a height of 32 cm and were filled with 20 litres of medium (composition is discussed further in this chapter). And were labelled and equally divided into 4 groups. Each group was given different concentrations NO_3 (with phosphate added in N:P 32:1), ranging from 0 µmol/L^{-1}, 50 µmol/L^{-1}, 100 µmol/L^{-1} to 200 µmol/L^{-1} delivered to the specific group of buckets through dosing pumps (Jecod DP4s 4-channel). The different concentrations were chosen as a result of the studies done by ZEEVIVO and summarised by A. Zwiers (2018). A. Zwiers (2018) concluded that raised levels of nitrate increased protein production in *U. lactuca* by either growth and/or protein content. A large increase in protein content was found between the concentrations NO_3 10 µmol/L^{-1} and 100 µmol/L^{-1}, but only a small increase between the regimes of 100 µmol/L^{-1} and 150 µmol/L^{-1}. From this previous research, it was concluded that no optimum was found (Zwiers, 2018). This fuelled the decision to add 200 µmol/L^{-1} in the set-up as a variable, searching for the concentration where the growth would stop increasing, and thus finding the optimum. The exact parameters that were worked with in this experiment can be found in table 1.

Temperature regulation and water circulation

The buckets in this experiment are kept at 15°C regulated by the water surrounding the buckets. 15°C was chosen because it has been reported as optimum temperature for growth (Duke, Litaker, & Ramus, 1989; Nielsen et al., 2011). To improve gas and nutrient exchange between the *U. lactuca* and the medium, a single air-pump with a 12-way splitter including independent valves was used to provide each bucket the same amount of water circulation. *U. lactuca* is a seaweed which does not attach itself to substrate of any kind but instead drifts freely in the water column. The water circulation provides a homogenous distribution of the *U. lactuca* in the buckets and homogenous irradiance negating the effects of prolonged self-shading.

Lighting

The lighting is provided by 6 AquaRay GroBeam 1500 Natural Daylight LED Aquarium Lighting Tiles by Tropical Marine Centre© (Full specifications are in appendix IV). One tile at 400mm in the air provides the system with a PAR of 148 µmol/sec/m^2. The study of Fortes & Lüning (1980) found the highest specific growth rate at 150 µmol/sec/m^2. The lighting schedule during the cultivation experiment was kept at 16 h light a day which is for the *U. lactuca* the optimum. 16 h light a day is where daylight saturation occurs which is coupled to growth inhibition if exceeded (Fortes & Lüning, 1980; Nielsen et al., 2011). The only light sensor (LI-COR LI-190R Quantum Sensor) in the facility used for light measurements was wrongly calibrated and impossible to re-calibrate correctly. Instead of being able to check the actual values of PAR emitted from the light-source, we could only use the sensor to investigate the lighting difference between the regimes of from 0 µmol/L^{-1}, 50 µmol/L^{-1}, 100 µmol/L^{-1} to 200 µmol/L^{-1}. As seen below in figure 2 the difference between regimes does not differ.

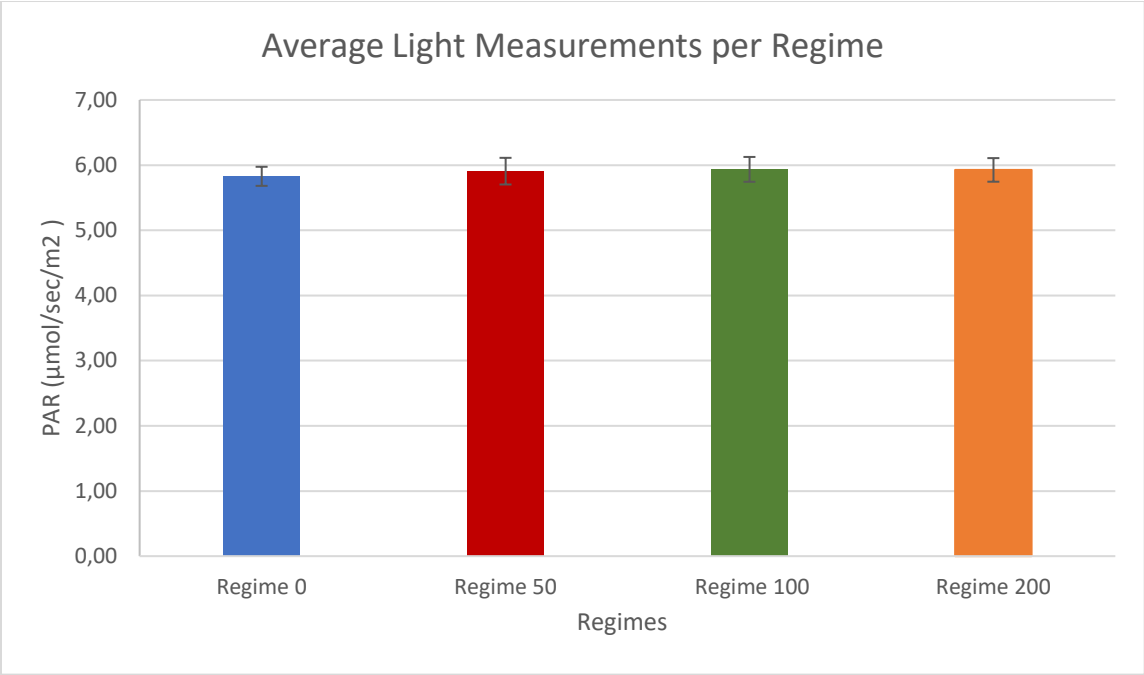


FIGURE 2: THE MEAN LIGHT MEASUREMENTS IN PHOTOSYNTHETICALLY ACTIVE RADIATION (PAR) (µMOL/SEC/M2) OF THE LIGHTING ABOVE THE CULTIVATION SETUP.

Medium composition

Four buffer tanks were set up with artificial seawater made from Instant Ocean salts, Aquarium Systems with a concentration of 30 gram per litre, this resulted in salt water with a salinity of 30 parts per thousand (ppt). This salt water provided the trace elements and metals, micronutrients and major cations & anions for the *U. lactuca.* The elemental composition by Atkinson & Bingman (1999) can be found in appendix III. In this medium nitrate and phosphate (N:P 32:1) were added to affect the growth of the *U. lactuca*, both are important for the growth and metabolism in macro-algae. Next to the nitrate and phosphor sources, vitamins from the f/2 medium designed by Guillard (1975) were added to the buffer tank. The three vitamins added were; thiamine HCl (vit. B_1), 2.96 x 10^{-7} mol/L^{-1} , biotin (vit. H) 2.05 x 10^{-9} mol/L^{-1} and cyanocobalamin (vit. B_{12}) 3.69 x 10^{-10} mol/L^{-1}.

The addition of nutrients

The experimental set-up was carried out with a stocking density of 50 grams WW of *U. lactuca* in each bucket (2.4 g/l), the reasoning behind the 50 grams was that this provided enough material for the extraction experiments as well as enough material for a protein content analysis as the relative growth rate of *U. lactuca* is the highest at a stocking density of 1 kg FW/m^{-2}. Higher stocking densities could also result in a less homogeneous result, as self-shading becomes a factor that limits the growth in material on the bottom (Bruhn et al., 2011; Neori, Cohen, & Gordin, 1991). In order to calculate the amount of nitrate that the *U. lactuca* needed during the experiment it was important to calculate the surface area. Lubsch & Timmermans (2018) found that weight and surface area were highly correlated. For fresh weight the weight goes up by 0.013 g. per cm^2. This means that every 100 cm^2 has a weight of 1.3 grams. For this experiment 50 grams of *U. lactuca* per bucket was needed, to calculate the surface area of the 50 grams the equation was as following: $\frac{(100\ cm^2 \times 50\ g.)}{1{,}3\ g.}$ This resulted in a surface area of 3846 cm^2 for 50 grams of *U. lactuca*. After the starvation in the holding tank, the *U. lactuca* was divided over the buckets in the experimental set-up. Here the *U. lactuca* went through the surge uptake state, in which the macro-algae depict a rapid increase in nutrient uptake until the nutrient reservoirs were filled. To calculate the nitrate needed for the surge the following equation by Lubsch & Timmermans (2018) was used: $cm^2 \times ({}^{\mu mol}/_{cm^2}\ per\ day)$ which gives the µmol of nitrate needed for the surge uptake per day. Lubsch & Timmermans found that the nitrate uptake in the surge phase was 12.54 ± 1.9 µmol/cm^2 per day. To be safe with the calculations we used the maximum µmol/cm^2 per day instead of using the average. This resulted in the following calculation: $3846cm^2 \times 14{,}44\ {}^{\mu mol}/_{cm^2}\ per\ day =$ 55,539 µmol of nitrate per day for the surge uptake. After the surge uptake, the steady state uptake took place, in this steady state the uptake of nutrients matches the actual nutrient assimilation. The steady state uptake was found to be around 20% of the surge uptake in the study of Lubsch & Timmermans (2018) which results in a steady state uptake of around 2.26 ± 0.86 µmol/cm^2 per day

Next to nitrate it was important to add phosphate (PO_4) to the medium for the *U. lactuca*. The amount of phosphate added was in the ratio N:P 32:1 to prevent phosphate being a limiting factor on the growth. On the third day, after two days of experiencing surge uptake the medium was replaced to get rid of build-up NO_3 which might were present as the calculations of the volume and concentration NO_3 were based on the maximum uptake of 14.44 µmol/cm^2 per day.

After replacing the medium with medium containing the different regimes, the dosing pumps were connected to the four buffer tanks each contained the growing medium and the previous mentioned regimes of NO_3 and their corresponding amounts of PO_4. The solution in the four buffer tanks was pumped in the buckets with dosing pumps in a maximum rate of 0.4 litre per hour which results in 9.6 litres fresh medium per day per bucket, this provided every bucket with the desired NO_3 concentration as well as a refreshment rate of the medium by 48.13% per day.

TABLE 1: METHOD OVERVIEW CULTIVATION EXPERIMENT, DEPICTING THE PARAMETERS AND THEIR MEASUREMENTS MOMENTS DURING THE CULTIVATION EXPERIMENT OF *U. LACTUCA*.

NO_3 concentration regimes (µM/L)	0, 50, 100, 200
Sample size (n)	12
Volume medium (L)	20
Temperature (^oC)	15
Salinity (‰)	30
Light intensity PAR (µM/m^2/s)	148
Duration experiment (Days)	20
Medium addition & Water change	9,6 l over 24h
Measurements pH, salinity & temperature	Every workday
Growth measurements (Days)	0, 2, 5, 7, 9, 11, 14, 20

2.2 The evaluation of protein extraction in *U. lactuca*

Pre-treatment

After the cultivation experiment of *U. lactuca*, the biomass from each bucket was tested on protein content. After harvesting it was important that the biomass was rinsed with demineralized water to get rid of all the minerals and nutrients not present within the macro algae. When the protein content of the *U. lactuca* from the same regime was similar, the material was pooled and homogenized by cutting the thalli in small, even pieces 5 mm by 5 mm (Appendix I). Subsequently the different batches of *U. lactuca* were stored at minus 20 degrees Celsius.

Protein extraction

Protein can be extracted from macro algae in many ways, in this research we used a method that extracts protein from the sample through an increased pH in combination with an increased temperature. The other method involved osmotic shock as a way to remove excess minerals and nutrients from the material.

There were two extraction methods used, one with frozen material in demineralised water on room temperature. The other with frozen material but in 0.2M NaOH to increase pH at 50°C (Appendix VI). These two extraction methods are both interesting for different reasons: the extraction method with demineralised water on room temperature is an interesting extraction method for when a high protein content in the pellet is favoured, as the demineralised water 'washes' out the minerals in the pellet called protein purification. Previous experiments of Tom Wijers showed an increase in protein purity in the pellet when the simple extraction, demineralized water on room temperature was used. The optimized extraction with a higher temperature and pH showed an increase in protein in the supernatant but the purity of the protein did not increase.

Both extractions were done in triplicate for each of the batches frozen *U. lactuca*, first, the frozen biomass was put in an Erlenmeyer with a ratio of 1:30 (biomass in DW : dissolvent) in this experiment 3 grams of *U. lactuca* in DW was used for the extraction. The second step in the extraction was the biomass sample on a heated rotating table with 140 rpm for 1 hour on the desired extraction temperature. After 1 hour, the samples were transferred to two falcon tubes (50ml each) per sample and placed in the centrifuge. This centrifuge divided the sample with 4500 rpm for 15 minutes into a solid pellet on the bottom of the tubes and a solution on top of the pellet, called the supernatant. For a schematic overview of an extraction see figure 3.

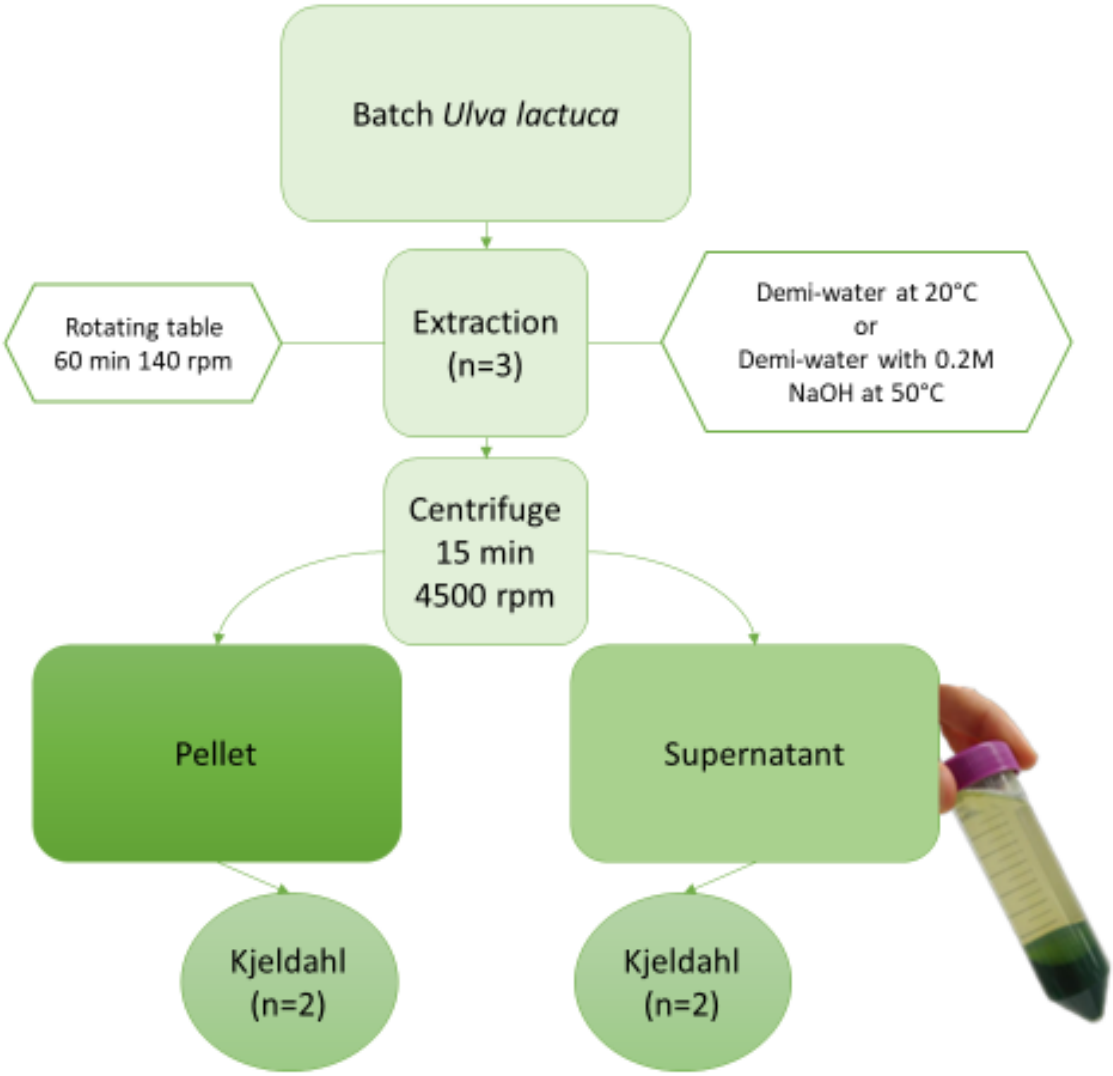


FIGURE 3: SCHEME OF THE EXTRACTION METHOD AND PROTEIN ANALYSIS FROM TOP TO BOTTOM. INCLUDING THE TWO DIFFERENT EXTRACTION METHODS, EITHER DEMINERALIZED WATER AT 20°C OR DEMINERALIZED WATER WITH 0.2 M NAOH AT 50°C.

Dry weight & ash weight

For the extraction experiments frozen and minced biomass of *U. lactuca* was used, but for both the extractions, the biomass to dissolvent ratio of 1:30 was based on dry weight. Therefore, the dry weight of the starting biomass needed to be determined. As an example: if the dry weight content is 10%, 30 grams of frozen biomass results in 3 grams of dry weight biomass. To dissolve this biomass in a ratio of 1:30, 30 grams of frozen biomass is dissolved in 63 ml of dissolvent (90 ml minus the 27 ml present in the biomass) to get to the ratio of 1:30. To determine dry weight the sample was oven-dried at 105°C for at least 16 hours until a constant weight was reached. The material that was left is the dry weight content of the biomass. Ash content was measured to determine the amount of minerals present in the biomass. To determine ash weight, the sample was placed in a muffle furnace at 550 degrees Celsius for four hours removing all organic material (Marinho-Soriano et al., 2006).

2.3 Literature review

For the last sub question a literature review has been carried out on the effects on people, planet and profit (PPP) of protein extracted from *U. lactuca* as an alternative protein source in aquaculture. The scope for this sub question is for the European countries around the North Sea, these include Norway, Denmark, UK, Scotland, The Netherlands, France and Germany. Articles were found on google scholar, journal databases and information retrieved from books. Articles that were included in the literature review are related to the topic and peer reviewed. The articles were only included if the information is obtained lawfully and the information has been reported accurately. Key words used to find the relevant information are: Sustainability, *Ulva lactuca*, protein, carbon footprint, price, economic, soy, fishmeal, fish oil, production, costs, jobs. With the information gathered from literature it was possible to access the feasibility of integrating *U. lactuca* as an alternative protein source in the aquaculture sector.

2.4 Data analysis

2.4.1 What is the feasibility of integrating *Ulva lactuca* as an alternative protein source in the aquaculture sector?

Researching what the feasibility is of integrating *U. lactuca* as an alternative protein source in the aquaculture sector, divided this main question in three sub-questions. The first question was focused on the production process of this seaweed, searching for an optimal regime of nitrate for growth and protein content. The second sub-question looked into protein refining of the *U. lactuca* with different extraction processes is an important step in the process of integrating this seaweed as an alternative protein source, as a high protein content in *U. lactuca* from cultivation alone is not sufficient enough to serve as an alternative protein source. And finally, our last sub-question researches the effects on people, planet and profit that might occur through integration of protein from *U. lactuca* as alternative protein source in the aquaculture. Those three sub-questions combined will give an insight in the feasibility of integrating *U. lactuca* as an alternative protein source in the aquaculture sector.

2.4.2 What is the optimum nitrate concentration for the growth and production of protein in *Ulva lactuca*?

The data from the cultivation experiment was collected in datasheets from Microsoft Excel and was checked first for errors before being analysed with statistics program IBM SPSS. The aim of this part of the research was to investigate which of the four regimes were significantly different. The results of the analysis will determine if there is a NO_3 regime resulting in a significant higher growth and/or protein content. The variables used for analysis can be found below for a clear overview (table 2).

TABLE 2: VARIABLES USED FOR STATISTICAL ANALYSIS OF THE CULTIVATION EXPERIMENT

Regimes NO_3	Time in Days (t)	Wet weight (g)	Protein content (%)	Growth coefficients
0				
50				
100				
200				

To test if the wet weight was related to the regime, a Linear Mixed Model (LMM) was created. Different LMMs were tested to find the most suitable model to analyse the data. The LMM chosen had the lowest Hurvich and Tsai's Criterion (AICC) of 280.640. The syntax of the Model is found in appendix Va. To test what the effect of the four Regimes is on the Protein content an One Way ANOVA and the Post Hoc tests, Bonferroni and Tukey's B were used.

When a significant difference is found among four means, a Post Hoc such as the Tukey's B test identifies subsets which with significant statistical difference and Bonferroni was used to identify which means differ with pairwise comparisons.

2.4.3 How does protein content in *Ulva lactuca* influence the extractable amount of protein?

After the cultivation, the extractions were conducted in triplicate and the Kjeldahl analysis in duplicate (Appendix VII), the results are all collected in Excel datasheets and analysed with the statistical program SPSS. The parameters (table 3) are all tested against the four different regimes with ANOVA searching for a statistical difference between them.

Comparable to the analysis of the previous research question, a Linear Mixed Model was used for the assessment on how the regime influences the difference between the start protein content and the extracted protein content. This model had an AICC of 173,504, the syntax can be found in appendix Vb.

Extraction efficiency refers to the amount of protein that was extracted relative to the amount of protein that was available in the sample. This was calculated through the following equation: Protein from total sample (pellet or supernatant) divided through the total amount of protein found in the 3 grams dry weight *U. lactuca*. This results in an extraction efficiency for pellet and supernatant. The extraction efficiency is analysed with an ANOVA and the Post Hoc tests Tukey B and Bonferroni.

TABLE 3: THE VARIABLES USED FOR EXTRACTION ANALYSIS

Regimes NO_3	Extraction type (NaOH/Demineralized)	Product of extraction (Extract/Pellet)	Start Protein content (%)	Extracted Protein content (%)	Difference in protein (Start protein – Extracted protein)
0					
50					
100					
200					

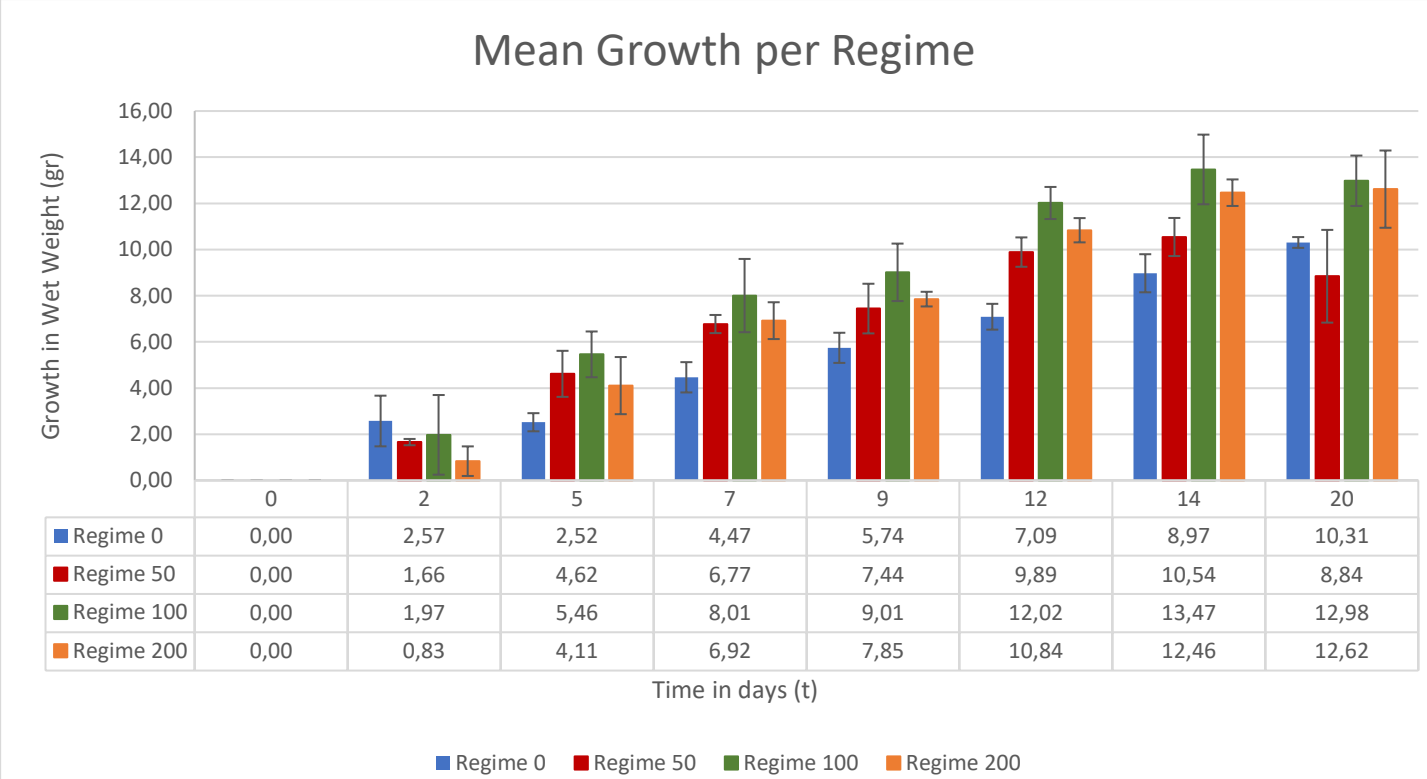
2.4.4 What is the effect on people, planet and profit of protein extracted from *Ulva lactuca* as an alternative source of protein in aquaculture?

This is the only research sub-question answered by a literature study. For the economical part, the production costs of protein extracted from seaweed are researched, furthermore the market for marine ingredients is researched. To give a conclusion on how sustainable protein extracted from *U. lactuca* is, the impacts on the planet are researched. For example: carbon footprint, costs, labour, and impacts of the production of fishmeal and fish oil, soymeal and protein extracted from *U. lactuca* are paralleled to come with a conclusion on how sustainable *U. lactuca* protein is. To give an answer on the effect on people the marine ingredients sector is researched to see if there is room for *U. lactuca* to grow as alternative protein source.

3.0 Results

3.1 Results cultivation of *U. lactuca* with different nitrate concentrations

Searching for the optimum nitrate concentration for the growth and production of protein in *U. lactuca* it was necessary to look for a difference between regimes regarding growth and protein content. For researching the growth of each sample, the weight was measured (Appendix II). The samples (figure 4) all started the experiment with a weight of 50 grams, but on the final day measured a mean weight of and calculated the Standard Error (SE); regime 0= 60.31 SE= 0.233, regime 50= 58.84 SE= 2.008, regime 100= 62.98 SE= 1.091 and regime 200= 62.62 SE= 1.673.



	0	2	5	7	9	12	14	20
Regime 0	0,00	2,57	2,52	4,47	5,74	7,09	8,97	10,31
Regime 50	0,00	1,66	4,62	6,77	7,44	9,89	10,54	8,84
Regime 100	0,00	1,97	5,46	8,01	9,01	12,02	13,47	12,98
Regime 200	0,00	0,83	4,11	6,92	7,85	10,84	12,46	12,62

FIGURE 4: MEAN GROWTH OF EACH REGIME, MEASURED DURING THE EXPERIMENT. THE GROWTH IS MEASURED IN WET-WEIGHT IN GRAMS. COMBINED WITH A TABLE DISPLAYING THE MEAN VALUES OF EACH REGIME FOR EACH DAY. EACH BAR REPRESENTS N=3 AND THE ERROR-BARS THE STANDARD DEVIATION.

Growth VS Regime

Researching the growth of *U. lactuca*, the mean growth-coefficient was calculated for each of the regimes. During the analysis for the growth from t =0 to t= 20 it became clear that the data of the (linear) growth was interrupted by the final measurement done on the last day t= 20 (figure 5). For analysis if the growth was significantly different between the regimes, no significance was found between the calculated growth coefficients of the regimes Sig= 0.157.

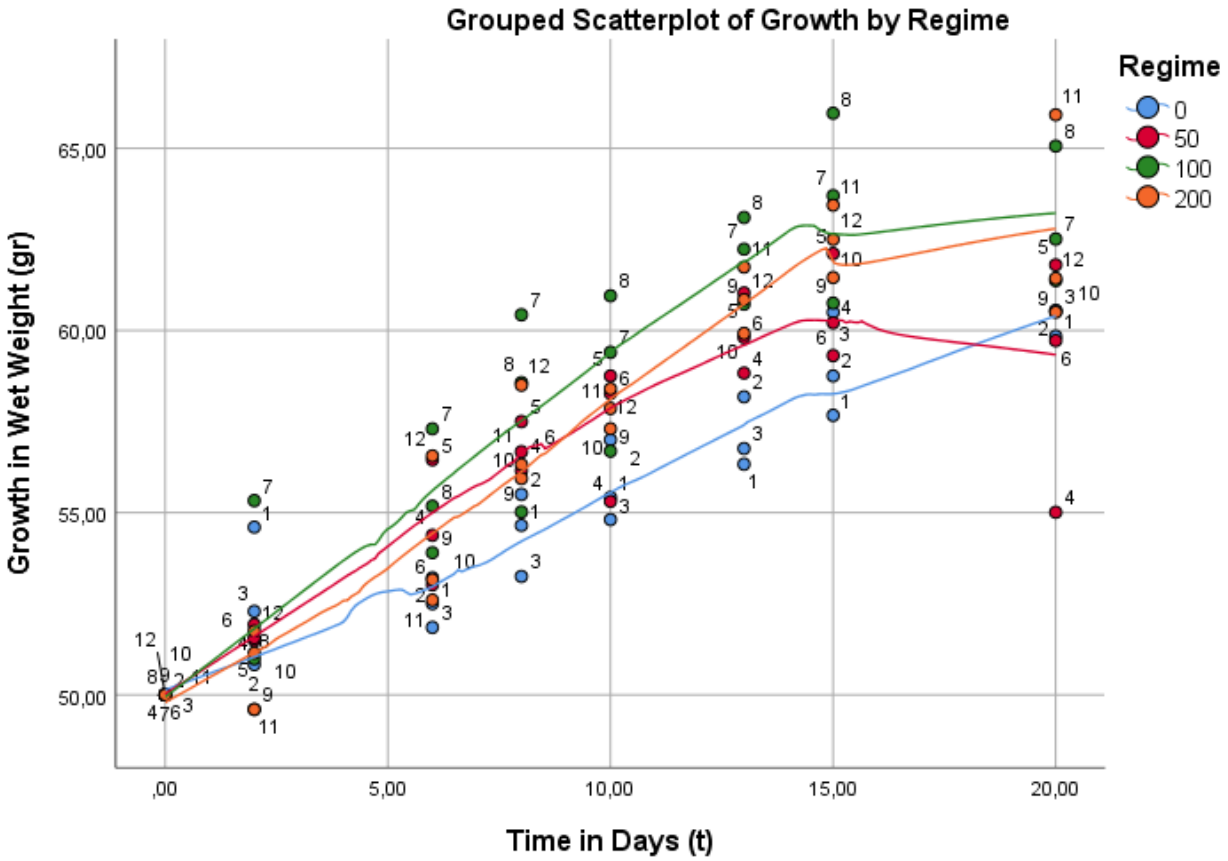


FIGURE 5: A GROUPED SCATTERPLOT OF THE GROWTH MEASURED WITH VALUES OF WET WEIGHT IN GRAMS, LABELED PER SAMPLE AND DIVIDED IN SUBGROUPS BY REGIME. DISPLAYED WITH A LOESS LINE AT 65%

To make the analysis with the growth-coefficients more reliable, the decision was made to analyse the data up until day t=15. Between the measurement points t=15 and t=20 a holiday and a weekend took place and the facility was unavailable during that time. At t=20 it was found that during the check-up of the system the dosing pumps weren't dosing the nutrients properly, the growth of the *U. lactuca* in the buckets has halted, as well as the medium in one of the buckets turned milky (suspected cause was sporulation and disintegration of the *U. lactuca*). As there is no way of precisely telling what happened during the time the facility was closed, is was decided to analyse the data up to t=15 (figure 5). After this decision, the growth coefficients were re-calculated (table 4). The analysis of those coefficients found a significant difference between regimes sig= 0.028. With the post hoc test, Tukey B two subsets were distinguished concluding that the growth, analysed through growth coefficients is significantly higher for regimes which used 100 µmol/L^{-1} and 200 µmol/L^{-1} nitrate than the regime without nitrate addition. The regime with 50 µmol/L^{-1} nitrate does not differ significantly from both the 100 µmol/L^{-1} and the 200 µmol/L^{-1} regimes.

TABLE 4: THE GROWTH COEFFICIENTS CALCULATED WITH THE FULL DATASET (COEFFNON) AND WITHOUT THE MEASUREMENT OF DAY 20(COEFFADJ).

ID number	CoeffNON	CoeffADJ	Regime
1	0,416	0,400	0
2	0,562	0,630	0
3	0,561	0,595	0
4	0,363	0,642	50
5	0,648	0,822	50
6	0,547	0,697	50
7	0,609	0,804	100
8	0,881	1,094	100
9	0,680	0,812	100
10	0,623	0,784	200
11	0,909	0,979	200
12	0,629	0,820	200

Linear Mixed Model Analysis

This Linear Mixed model analyses the both the difference and interaction between regimes and wet weight with days at day t=15 of the experiment. This analysis concluded that only the regimes which significantly differed were 0 µmol/L^{-1} with 100 µmol/L^{-1} (sig=0.0002) and 200 µmol/L^{-1} (sig=0.0052). 50 µmol/L^{-1} did not significantly differ with any of the other regimes. At t=6 none of the regimes differed significantly, but this changes during t=7 where only regimes 0 µmol/L^{-1} and 100 µmol/L^{-1} showed a significant (0.03) difference. Whereas t=10 is the first day both 100 µmol/L^{-1} (sig=0.002) and 200 µmol/L^{-1} (sig=0.031) significantly differed from the regime with 0 µmol/L^{-1}.

Regime VS Protein

To assess if the regime also influences the protein content of the *U. lactuca* after t=20 Days, protein measurements of the samples were done at the start and at the end of the experiment. The mean protein content of the 0 µmol/L^{-1} NO_3 regime = 15,7597% (SE= 0,23898%), 50 µmol/L^{-1}= 17,6704% (SE= 0,62100%), 100 µmol/L^{-1} 18,8100% (SE=0,08106%) and 200 µmol/L^{-1}= 18,3559% (SE= 0,03179%) (figure 6). Regime 0 µmol/L^{-1} NO_3 is found to be significantly different from all other compared regimes; 50 µmol/L^{-1} NO_3 (sig=0.023), 100 µmol/L^{-1} NO_3 (sig=0.0012) and 200 µmol/L^{-1} NO_3 (sig=0.0035).

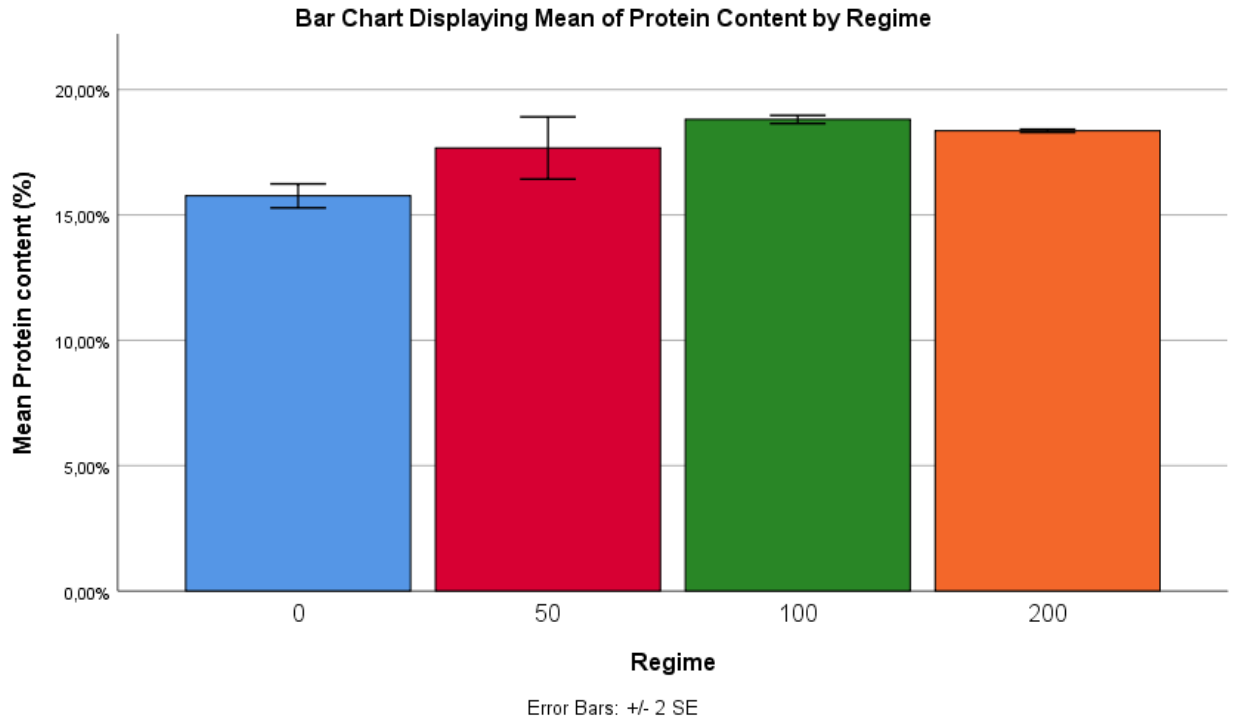


FIGURE 6: AVERAGE PROTEIN CONTENT OF EACH REGIME AFTER THE CULTIVATION PERIOD IN PERCENTAGES. EACH BAR REPRESENTS N=3 AND THE ERROR-BARS REPRESENT THE STANDARD ERROR.

3.2 Results protein content in *U. lactuca* influencing the extractable amount of protein

In the second part of this research, where the protein content of the used *U. lactuca* was the main factor. Protein content was measured with the Kjeldahl method and extraction efficiency was calculated for the products (pellet and supernatant) of both extraction methods.

The predictions made in this thesis concerning how extraction methods would influence the extractable amount of protein corresponded with the results from this research. Where the average protein difference content of the pellet of the NAOH extraction was -3,54 and extract was 7,43, opposite results were found with the demi water extraction(Pellet=6,42, extract=-5,17).

The extract of NaOH and the pellet of demineralized water, did not significantly differ in mean protein difference (sig=0.581). The extract of demi water and the pellet of NaOH were close to be significantly different (sig=0.053) as significant difference is assumed at $P<0.05$.

How treatment influences the difference between the start content and extracted protein content

Comparable to the analysis of the previous research question, a Linear Mixed Model was used for the assessment on how the treatment influences the difference between the start protein content and the extracted protein content. This model had an AICC of 173,504, the syntax can be found in appendix Vb.

Resulting from the analysis of the data done with the LMM, the start content did not significantly influence the extractable amount of protein. Except the extract of Demi water, none of the treatments turned out to have a significant different (negative) growth coefficient (sig=0.0041) (figure 7). But that does not translate in the extract of demineralized water (treatment) having a significant effect on the start content with the extractable amount of protein.

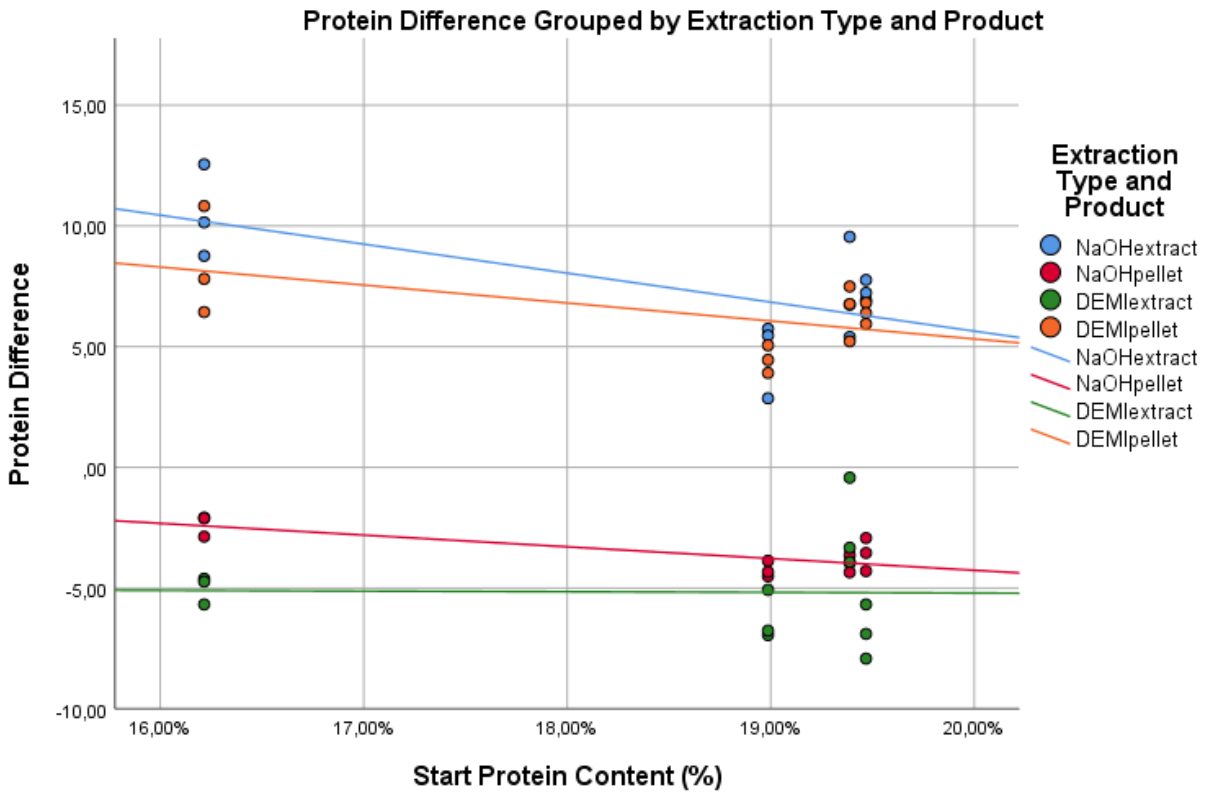


FIGURE 7: A SCATTERPLOT OF THE MEASURED VALUES OF DIFFERENCE IN START PROTEIN CONTENT AND PROTEIN CONTENT WITHIN THE EXTRACTION PRODUCTS, GROUPED BY EXTRACTION TYPE AND THEIR PRODUCTS. DISPLAYED WITH A LINEAR LINE.

For an overview on the protein content categorized by treatment combined with the products of extraction. Which is further subdivided into the sample ID's (figure 8 & figure 9). As for the mean of the four categories, NaOHextract had a mean of 25.940 with a SE= 0.566, NaOHpellet= 14.972 SE= 0.889, DEMIextract= 13.348 SE= 2.433 and DEMIpellet= 24.939 SE= 1.536. Both NaOHpellet and DEMIextract (sig>1.000), and NaOHextract and DEMIpellet (sig=0.192) do not display a significant difference.

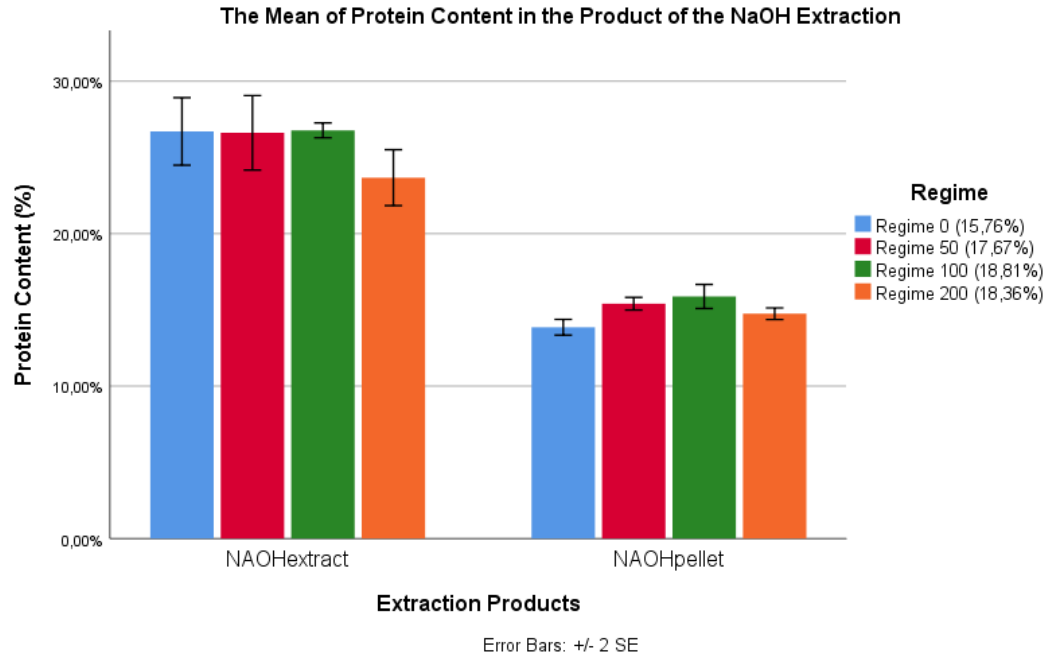


FIGURE 8: THE AVERAGE VALUES OF THE PROTEIN CONCENTRATION ON DRY WEIGHT BASIS OF THE NAOH EXTRACTION PRODUCTS MEASURED IN EACH SAMPLE (N=3). DIVIDED BY REGIME AND THEIR CORRESPONDING START PROTEIN. THE ERROR-BARS REPRESENT THE STANDARD ERROR.

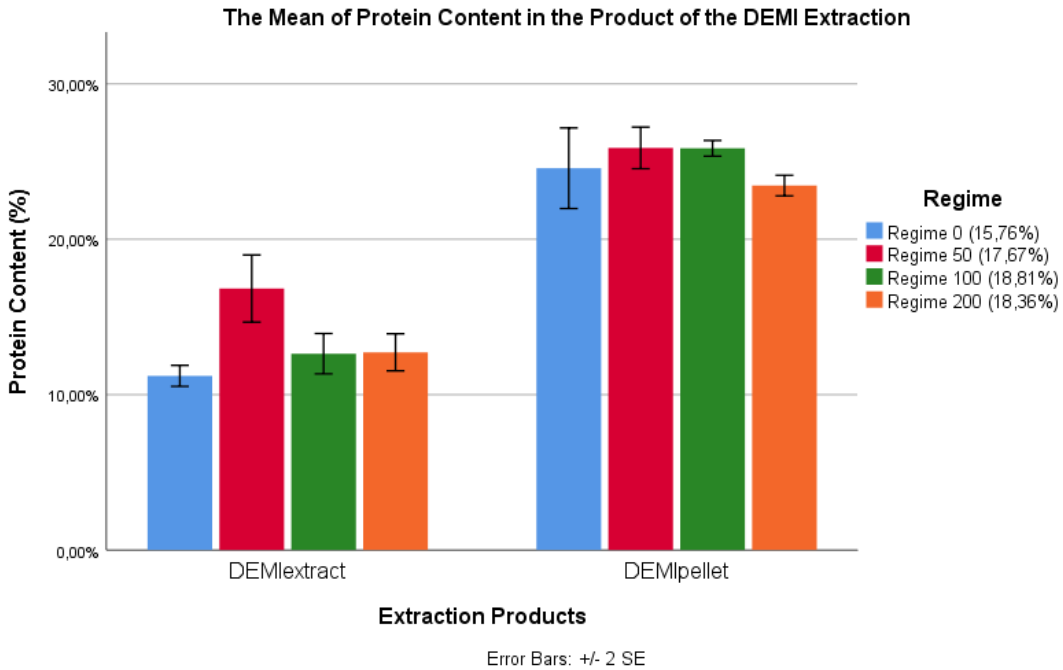


FIGURE 9: THE AVERAGE VALUES OF THE PROTEIN CONCENTRATION ON DRY WEIGHT BASIS OF THE DEMINERALIZED WATER EXTRACTION PRODUCTS MEASURED IN EACH SAMPLE (N=3). DIVIDED BY REGIME AND THEIR CORRESPONDING START PROTEIN. THE ERROR-BARS REPRESENT THE STANDARD ERROR.

The same overview was made with instead of extracted protein, the calculated extraction efficiency of each sample (figure 10 & figure 11). Extraction efficiency works as a safety measure as well, to see if the protein totals add up to about 100 to 110%. The extraction efficiency of the pellet from the demineralized water extraction was significantly higher in regime 0 compared to regime 100 and 200 (sig=0.032). The means of the four categories of products (NaOH pellet and extract, and demineralized water pellet and extract) all significantly differed with a significance of P<0.00001.

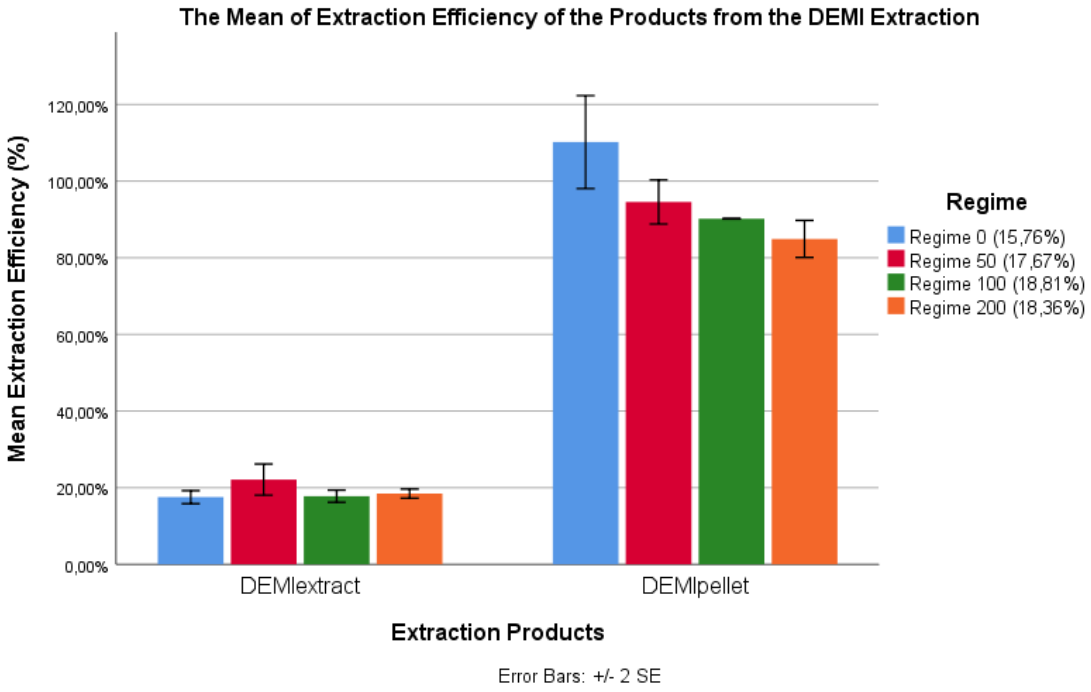


FIGURE 10: THE MEAN EXTRACTION EFFICIENCY OF THE PRODUCTS FROM THE DEMINERALIZED WATER EXTRACTION. DIVIDED BY REGIME AND THEIR CORRESPONDING START PROTEIN. THE BARS REPRESENT N=3, AND THE ERROR-BARS REPRESENT THE STANDARD ERROR.

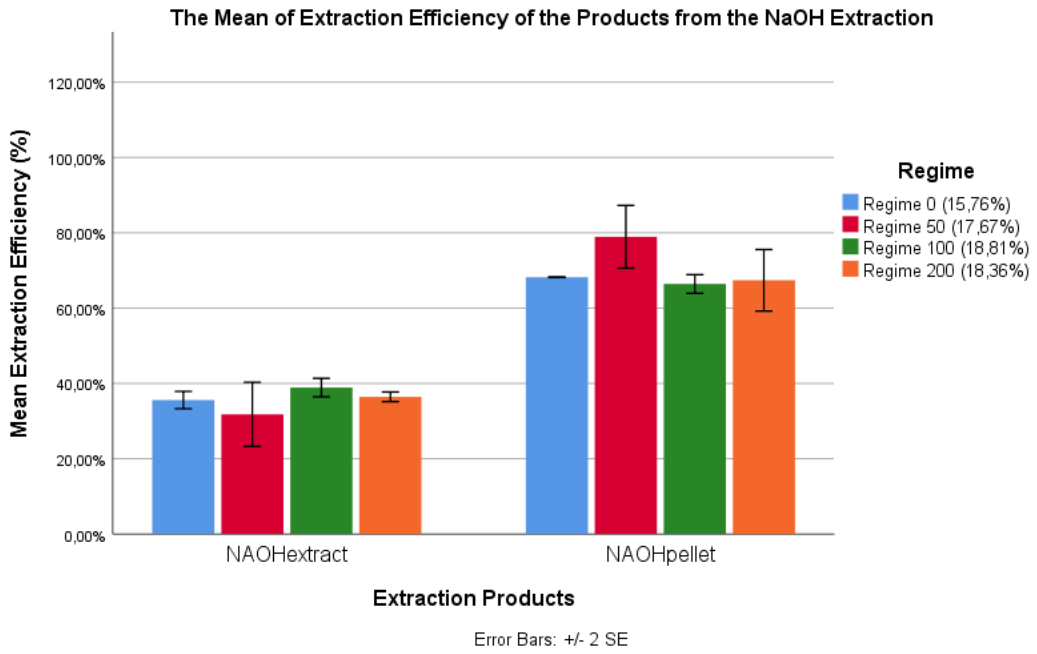


FIGURE 11: THE MEAN EXTRACTION EFFICIENCY OF THE PRODUCTS FROM THE NAOH EXTRACTION. DIVIDED BY REGIME AND THEIR CORRESPONDING START PROTEIN. THE BARS REPRESENT N=3, AND THE ERROR-BARS REPRESENT THE STANDARD ERROR.

3.3 Results effects of *U. lactuca* protein on the aquaculture sector

To measure the impacts of protein extracted from *U. lactuca* on the aquaculture sector it was necessary to do a literature study in which the following aspects were important: jobs in the marine ingredients sector, footprint, environmental effects and costs & revenues. The scope of countries that were investigated were the European countries surrounding the North Sea: Denmark, Norway, Belgium, England, Scotland, Germany, France & the Netherlands.

Marine ingredients for aquaculture

Fishmeal and fish oil can be described as marine ingredients, ingredients used to produce fish feed derived from whole fish and process trimmings. These ingredients provide the fish with omega 3 fatty acids and proteins with the right amino acid profiles. Fishmeal is unique when compared with terrestrial animal and plant protein sources as it is not only a source for high quality animal proteins and amino acids, it is also a great source for essential minerals and vitamins. Worldwide the annual production of fishmeal and fish oil has been stable around 6 to 7 million tons of fishmeal per year when no El Niño occurred. The total production of fishmeal is an estimation as only catch data is aggregated, this is measured in wet weight. The total production of fishmeal and fish oil is calculated using a wet weight to fishmeal ratio of 22,5% and a fish oil ratio of 5%. This means that for 1 kg fishmeal, 4,44 kg fish is needed and for 1 kg fish oil 20 kg fish is used (Shepherd & Jackson, 2013; Tacon & Metian, 2008). The majority of this fishmeal is produced in South America where countries like Peru and Chile produce 84 percent of their fishmeal from whole fish, mainly fishing on the largest single reduction species in the world, the Peruvian anchoveta (Cashion, Le Manach, Zeller, & Pauly, 2017; Seafish, 2016).

People working in Europe's marine ingredients production

Meanwhile, Europe produces 15,1 percent of the world's fishmeal supply where Norway and Denmark lead in terms of production. In Europe 8132 to 8950 people work in the marine ingredients sector working in 29 different companies that are members of the International Fishmeal and Fish Oil organisation (IFFO) (appendix VIII) (IFFO, 2019). Most of these people work in Norway, Denmark and the UK. In these countries 9 companies produce the bulk of European fishmeal and fish oil. In these companies 4748 to 5291 people work on full time basis. In these countries the largest companies are Pelagia, BioMar group and the P/F Havsbrún despite being in the Faroe Islands, it counts as a Danish company. Other members of this organisation either produce small amounts of fishmeal and fish oil or are in the business of producing compound food and researching the possibilities of alternative ingredients. Skretting, located in Norway is the world's largest producer of compound food for aquaculture and has conducted many researches on alternative ingredients for inclusion in fish feed. This research is needed as fishmeal production remains static over the years. Catch numbers for human consumption stay stable while the surplus, that is used for fishmeal production vary a lot. In years where catch totals are low, caused by El Niño for example, the aquaculture industry faces supply shortages (Asche, Oglend, & Tveteras, 2013).

The need for alternative ingredients in fish feed

Over the last few decades fishmeal inclusion in compound feeds has been declining, for many aquaculture species fishmeal is only used in small percentages of their diet. Other protein meal sources make up the majority of the feed. This is effect is caused by the increasing prices of fishmeal and the growth in the industry which comes with an increased demand for fishmeal. In 2000 a ton of fishmeal cost 300 USD while the price at 3 June 2019 comes in at 1620 to 1650 USD per tonne (FAO, 2019b). To support the growth in the aquaculture sector other protein sources like soybean meal, rapeseed oil, insect meal or seaweed proteins usage in compound feeds increased. Soybean meal is worldwide available and is cultivated in large quantities. With protein concentrations of 45 to 50 percent it can function as a reliable and realistic alternative to fishmeal in fish feed. Soybean meal however cannot be included in very high concentrations or completely replace fishmeal as adverse effects can occur in crustaceans and many finfish. Problems like poor feed intake, digestive and immune function disorders can be caused by antinutritional factors that are present in soybean meal (Asche et al., 2013; Shepherd & Jackson, 2013). Besides those antinutritional factors, a lack of methionine, an essential amino acid which a deficiency of causes cataracts (clouding of the lens, loss of vision)(Pelletier et al., 2018) in the soybean meal makes fully replacing fishmeal with soybean meal impossible. For the aquaculture sector to keep growing more alternative sources of protein are needed.

The increased demand for marine ingredients causes an increase of research towards suitable and viable substitute proteins that can be used in fish feed. For this research an answer to the questions around protein supply is searched in the green seaweed species *U. lactuca.* The effects of *U. lactuca* protein on the aquaculture sector are researched in a triple P perspective.

Seaweed protein

Seaweed protein is seen as a legitimate option as protein substitute for soybean meal as the marine proteins from seaweeds have an amino acid profile more like fishmeal than soybean meal or other vegetable protein sources. Seaweed possesses less anti-nutritional minerals and vitamins than other terrestrial vegetable protein sources. It is even reported that seaweed diet supplementation can increase growth rate and provide diverse benefits, namely acting as a prebiotic (Barbier et al., 2019). European production of seaweed has remained stable at around 350,000 tons FW until 2000. Since 2000 the production decreased to a total of 243,014 tons fresh weight seaweed in 2017 as can be seen in tables 5 and 6 (FAO, 2019a) meanwhile production in the rest of the world is only increasing, at the moment Europe only produces 1 percent of the global seaweed production. Currently less than 1 percent of Europe's seaweed production comes from aquaculture production. The rest of the production in Europe comes from wild harvesting the seaweeds. To reverse the downwards trend in Europe it is key that a few issues are resolved. For example, there needs to be a stable and sustainable access to raw materials. The largest issue is the knowledge gap that needs to be filled before the aquaculture production of Europe can grow and become a more realistic replacement for today's conventional raw feed materials such as soybean meal. Knowledge and expertise sharing between developed and less-developed regions can overcome this hurdle (Barbier et al., 2019).

TABLE 5: EUROPEAN SEAWEED PRODUCTION IN AQUACULTURE IN 2017. MEASURED IN TONNES OF FRESH WEIGHT(FAO, 2019a)

Europe	Inland waters	Freshwater	Aquatic plants	Aquatic plants	
			Green seaweeds	Green seaweeds	4
			Aquatic plants	Aquatic plants	156
		Sub-total Freshwater			156
	Sub-total Inland waters				156
	Marine areas	Brackishwater	Aquatic plants	Aquatic plants	
			Seaweeds nei	Algae	5
			Aquatic plants	Aquatic plants	5
		Sub-total Brackishwater			5
		Marine	Aquatic plants	Aquatic plants	
			Brown seaweeds	Brown seaweeds	1 717
			Green seaweeds	Green seaweeds	1
			Red seaweeds	Red seaweeds	0
			Seaweeds nei	Algae	32 *F*
			Aquatic plants	Aquatic plants	1 750
		Sub-total Marine			1 750
	Sub-total Marine areas				1 755
Total Europe					1 912
Grand total					1 912

TABLE 6: EUROPEAN PRODUCTION OF HARVESTED MARINE SEAWEEDS IN TONNES OF FRESH WEIGHT (FAO, 2019a)

Land Area	Ocean Area	Species	Scientific name	2017
Denmark	Marine areas	Aquatic plants	Aquatic plants	10
France	Marine areas	Aquatic plants	Aquatic plants	39 072
Ireland	Marine areas	Aquatic plants	Aquatic plants	29 541 *F*
Italy	Marine areas	Aquatic plants	Aquatic plants	1 200 *F*
Norway	Marine areas	Aquatic plants	Aquatic plants	164 969
Portugal	Marine areas	Aquatic plants	Aquatic plants	2 887
Spain	Marine areas	Aquatic plants	Aquatic plants	3 424
Grand total				241 102

Environmental impacts U. lactuca production

Currently the market for *U. lactuca* in Europe is estimated at several thousand tons dry weight for the food market alone, wild growing biomass is not able to satisfy the market. Therefore, cultivation of *U. lactuca* for food and ingredients is foreseeing a large growth in the coming years. Seaweeds can absorb nutrients like phosphates, Co_2 and ammonium and heavy metal ions from waterbodies that are polluted. Therefore, they have potential and can be used as wastewater treatment or used in combination of aquaculture to remove excess nutrients (Barbier et al., 2019). When used in combination with aquaculture the seaweeds are more likely to absorb ammonium than nitrate in the water as ammonium costs less ATP to assimilate compared to nitrate (Jansen et al., 2019). Not all the seaweed species are successfully produced in open water, marine cultivation of species like *U. lactuca* is due to its size more likely to be successfully produced in raceways and ponds on land. Waves easily break the thalli up in small pieces as the thalli of *U. lactuca* are only 2 cell layers thick. Literature studies indicated that ecosystem interactions such as biodiversity can be influenced with marine production of seaweed species. However empirical data is largely lacking as production of seaweed is still in its

infancy in Europe. Environmental impacts are limited for production when the algae are produced in a land-based basin as the species doesn't interfere with other organisms. Nutrients can be provided more efficiently and the quality of the product can be safeguarded more carefully. Effects that could influence the environment around such production systems are: the release of the nutrient depleted water back into the ecosystem and potential risks with genetic diversity loss due to cultivated strains competing with native strains, if cultivated specimen are released in the environment (Van den Burg, Dagevos, & Helmes, 2019). If we compare protein derived from *U. lactuca* with original protein sources such as fishmeal and soybean meal the impacts are limited. The impacts however are for the production of seaweed based on literature while for soybean meal and fishmeal there are already empirical evidence. Seaweed production compared with fishmeal and soybean meal requires less inputs for cultivation than the other two products. The processing however requires lots of energy to dry and then extract the protein from the seaweed. Based on a literature study of Pelletier, Klinger, Sims, Yoshioka, & Kittinger (2018), who described and paralleled the impacts of soybean meal from America and Brazil with krill meal and fishmeal from trimmings and Peruvian anchoveta meal. In this study they concluded that based on resource use and emissions Peruvian anchoveta meal and soybean meal are produced in an efficient way and that soybean meal can substitute other protein feed inputs in order to reduce the resource use and emissions. When we want to compare the impacts of seaweed protein with the established marine ingredients it heavily depends on what species of seaweed is being cultured, if it is marine cultured or cultured on land in raceway ponds. Furthermore, it is the processing used to extract the protein from the seaweed that is very important in figuring out the environmental impacts and the overall footprint.

Effects on profit

Previously it was mentioned that Europe only produced around 1 percent of the world's production of seaweed. Most of the seaweed that is being produced has direct human consumption as destination. Seaweed as food is not ingrained in the culture of Europeans as much as it is in Asian countries. In Europe seaweeds largest consumers can be found in the nutraceutical and cosmetic markets as these pay the highest prices for seaweed biomass (Barbier et al., 2019). The seaweed market in Europe is still growing, mostly due to European and national policy initiatives to stimulate aquaculture (Van den Burg et al., 2019). It has been estimated that the annual growth rate of this market will be 10 percent annually (BIM report 2014). A case study of van den Burg, van Duijn, Bartelings, van Krimpen, & Poelman (2016) analysed the possibilities for offshore production of seaweed. In this study was assumed that half of the product was used for the production of hydrocolloids and the other half would be used for the production of animal feed. Results were that for the seaweed production to be profitable in the North Sea the price and biomass totals would need to be increased with 300%. Based on the current information on costs and benefits this would result in a price of US $1,747 per metric ton of dry mass. Which means that only the cultivation part of the seaweed protein production would already cost more than a metric ton of prime Peruvian anchoveta meal. After the cultivation of the seaweed it would be necessary to process and extract the protein from the seaweed, which would make seaweed protein even more expensive. For European seaweed to compete with Asian and Chinese seaweed it would mean that producers need to claim and prove the claims of being more sustainable to ensure additional value. Currently production costs are too high for seaweed to be cultured for protein only in the North Sea compared with seaweed producers outside of the EU. Cost of labour is too high which means that production and harvesting would need to be automated to reduce costs (Van den Burg et al., 2019).

4.0 Discussion

The ideal set-up of this research was that the cultivation experiment provides four different batches of *U. lactuca* with different protein levels. So that during the extraction experiment the extractable amount in relationship with the start protein levels in the material could be studied. But the outcome of the cultivation experiment resulted in regime with 0 µmol/L^{-1} only significant differentiated with both 100 µmol/L^{-1} and 200 µmol/L^{-1}. Creating only two actual sub-sets which significantly differed from one another.

The main discussion points regarding the experimental setup were the lighting and the dosing pumps tasked with the input of nutrients and fresh sea water. The facility where the experimental setup was located, did not have a correct light-sensor which is able to measure in PAR. As the only sensor used gave readings below the double digits (after calibration as well) at a distance of 400 mm of the source, those readings were incorrect as the specifications of the new lighting states a PAR of 148 µmol/sec/m^2 at that distance. Due to a lack of budget, a new light-sensor could not be attained. Instead, we used the current sensor to at least measure the difference in lighting between the buckets which contained the samples during the cultivation. The dosing pumps were according to the manufacturer able to go without problems for at least a year, but at day t=15 the pumps had to be recalibrated as the dosages were slightly off. These inaccuracies in the addition of nutrients and new seawater may have influence on the cultivation process of the samples. But as the inaccuracies were not measurable and all the dosing pumps were recalibrated, this was not taken into account during the analysis of the results.

During the cultivation process growth rates of *U. lactuca* did not match the numbers found in literature. In Bruhn et al., (2010) was found that the biomass was able to grow with 10% per day at a stocking density of 1 kg FW m^{-2} with a NO_3 concentration of 30 µmol/L^{-1}. Phosphorus was below 1 µmol/L^{-1} during this experiment. These parameters are slightly below the parameters that we used for the second group where we used 50 µmol/L^{-1} nitrate and 1,56 µmol/L^{-1} phosphate. In our study however, we found a specific growth rate of 3% per day at a stocking density of 707 gr. FW m^{-2}. The difference in growth rate can be caused by the fact that the material has been starved for too long. Instead of 10 days of starvation the material is starved for 92 days waiting for the experiment to start.

Sample number 2.1 of the cultivation experiment was noticed disintegrating at t=20, meaning that the water which contained the sample was turbid with organic material of the *U. lactuca* and the thalli were falling apart. The wet weight of this sample dropped from 60.21 gr to 55.01 gr within five days, a decline of -8.6%. This is the reason why the error-bar of regime 50 µmol/L^{-1} in the wet-weight chart (figure 5) is larger than the other regimes. And the reason why we removed this sample from the extraction experiment as this sample cannot be considered representative for this regime.

As seen in the results, section 3.2 figure 9, the extraction with the demineralized water, causes for the calculated extraction efficiency to be over-estimated. This phenomenon was also seen in the experiments of the supervisor of this research, T. Wijers.

When tested with the LMM if the Wet Weight of the *U. lactuca* and the regimes have an interaction and difference, it was found that the first significant difference between the regimes was during t=7 where regime 100 significantly differed from regime 0. The samples of regime 100 also had the highest mean protein content, albeit not significantly different from regime 50 and 200. This contradicts the studies number III and IV documented by A. Zwiers (2018). Where study III found a higher protein

concentration and growth for regime 200 than regime 100 but was not able to test for significance due to not enough data. Study IV worked with the regimes of 100 and 150 and only found a higher significant difference in the protein content of regime 150 compared to regime 100 and not in growth. The difference in results between those studies and our research could be due to the addition of PO_4 which is in this research kept at a N:P 32:1 ratio. This was to prevent phosphate being the limited factor in both lack or an excess form. The studies kept the phosphate levels at a constant 3 $\mu mol/L^{-1}$ for all regimes. Difference in lighting might also be a factor cannot be checked as the light values during the cultivation is not described in the studies.

Van Hall Larenstein, university of applied sciences facilitated this research and the experiment. As VHL is a university of applied sciences and not a facility dedicated to research, measurements could not be taken during holidays and weekends. This led to irregular measurement days which must be taken into account during the statistical analysis of the data but lowers the statistical power of the model. The lack of continuous access into the facility also prevents regular check-ups during the experiment, and thus increasing the chance of malfunctions in the setup.

In the demineralized water extraction, the products did not significantly differ in protein content. But a significant higher extraction efficiency was calculated with the pellet of regime 0. Meaning that with a lower protein content (15,76%) in the start material, a demineralized water extraction had a higher protein efficiency resulting in no significant difference with protein content in the pellet in regards to regimes which had higher protein content in the starting material (17,67%, 18,81% and 18,36%). No literature was found with the same or contradicting results.

Most of the studies use filtered saline water (Frost-Christensen & Sand-Jensen, 1990; Gao, 2016; Lubsch & Timmermans, 2018; Msuya & Neori, 2008; Steffensen, 1976; Vermaat & Sand-Jensen, 1987) for the cultivation of the *U. lactuca* as well as the experiment done by our supervisor T. Visser. The use of ASW and the difference with filtered seawater on the effects of *U. lactuca* has not been documented. This also could have led to difference in growth rate between this experiment and the growth rate stated in the studies where filtered sea water was used.

Most of the information gathered for the literature study is based on theories and other literature. A lack of empirical evidence and results of how *U. lactuca* protein influences the aquaculture sector could have resulted in unrealistic findings. Most of the studies combined green, brown and red seaweeds and only described the impacts of production, not the impacts of processing. Many of the studies did not report the results for *U. lactuca* specifically, meaning that results could be different for *U. lactuca* when cultivation for protein production is tried on an industrial scale.

5.0 Conclusion

What is the optimum nitrate concentration for the growth and production of protein in Ulva lactuca?

Concluding from the results, no significant difference was found in mean weight between the 100 and 200 µmol/L^{-1} regimes during the measurements of the experiment, which would indicate that in a 15 day period no significant difference in growth will occur in a setup with a 100 and 200 µmol/L^{-1} nitrate with 32-1 ratio phosphate added. So, to answer if the optimum nitrate concentration with adjusted Phosphate levels (to prevent that being the limiting factor) is found for the growth in *U. lactuca*. As been described in the problem statement of this thesis project an optimum concentration of nitrate for the production of protein in *U. lactuca* has not been described in literature before. The results of this experiment can help with the optimization of *U. lactuca* cultivation. At t=7 the growth of the regime supplied with 100 µmol/L^{-1} nitrate was significantly different from the regime supplied with 0 µmol/L^{-1} , this was the first regime to grow significantly quicker than the other regimes. As the overall growth is not significantly higher in the sample supplied with 200 µmol/L^{-1} nitrate, but the protein content of regime 100 is higher than regime 200 albeit not significantly. It is concluded that the optimum is around the 100 µmol/L^{-1} as doubling the amount of available NO_3 and PO_4 (regime 200) did not significantly differ in the results. With the mean growth coefficients per regime calculated, and analysing those values only found a significant difference (sig= 0.028) between regimes 100 and 200 µmol/L^{-1} and the regime without nitrate addition. When looking at the concentration of protein within the samples, the 0 µmol/L^{-1} regime is found to be significantly different from all other compared treatments; 50 µmol/L^{-1} (sig=0.023), 100 µmol/L^{-1} (sig=0.0012) and 200 µmol/L^{-1} (sig=0.0035). The differences in protein concentration for the regimes supplied with nitrate were so minimal that for protein production 100 µmol/L^{-1} nitrate was found the most optimal.

How does protein content in Ulva lactuca influence both the extractable amount of protein and the remaining biomass(pellet)?

According to the results of the LMM analysis, the protein content in the start-material did not significantly influence the extractable amount of protein. The protein content between NaOHpellet and DEMIextract (sig>1.000), and NaOHextract and DEMI pellet (sig=0.192) did not display a significant difference either. Researching the extraction efficiency, the means of the four categories (NaOHpellet, DEMIextract, NaOHextract and DEMIpellet) all significantly differed with a significance of P<0.00001.

In conclusion of this experiment, even though the start-material did not influence the extractable amount of protein and NaOHpellet, DEMIextract, NaOHextract and DEMIpellet did not significantly differentiate regarding the protein content either. They did differ significantly when extraction efficiency was compared. Where both NaOHpellet, NaOHextract displayed a higher extraction efficiency than their demineralized counterparts. This was expected as the NAOH method has an elevated pH making the proteins during the extraction more soluble, potentially increasing extraction efficiency.

What is the effect on people, planet and profit of protein extracted from Ulva lactuca as an alternative source of protein in aquaculture?

The production of protein from *U. lactuca* as alternative source of protein in aquaculture has rather small impacts on people, planet and profit. It is expected that in the following years the seaweed production in Europe will grow with at least 10 percent annually. Therefore, many people would be able to work in this growing sector. Currently almost 9000 people are working in the marine ingredients sector, with many people working at companies that are interested in finding new suitable marine proteins. Combined with European interest in increasing the aquaculture production, it is certain that the sector will grow in the years to come. Environmental impacts of seaweed production are uncertain at the moment as many seaweed species are grown in different ways. For marine grown seaweeds it is important that seaweed strains are derived from local seaweeds to make sure that these do not compete with each other and to make sure that genetic diversity is not impacted. The future for *U. lactuca* production on the other hand will probably be in land-based production systems that minimize the environmental impacts, there is however more usage of electricity which would increase the carbon footprint of this production.

The production of seaweeds and *U. lactuca* specifically has a lot of hurdles to overcome to be able to compete with traditional sources of protein, marine as well as terrestrial. Production in Europe would be too expensive at the moment for protein from seaweed to be profitable. It would not be possible to compete with seaweed production from outside of Europe as production costs would be too high in Europe, the way Europe can compete with these countries is through certification and value adding processes. Furthermore, it is important to import knowledge and expertise to optimize production systems and value chains as production of seaweed is still in its infancy in Europe while in other parts of the world this has been cultivated for many years.

What is the feasibility of integrating Ulva lactuca as an alternative protein source in the aquaculture sector?

To answer the research question all three parts of the study are combined. For the cultivation of *U. lactuca* we found that there is an optimum nitrate concentration at 100 µmol/L^{-1} for protein production. Protein extraction was not influenced by the protein content of the start material in this research. The end product that has been cultivated did not have the protein content necessary for inclusion in fish feed. With the extraction it was not possible to reach a protein content in either pellet or supernatant of 48 percent. We think however, that *U. lactuca* protein production for fish feed is possible if a higher start protein concentration can be reached in the start material. Currently production of *U. lactuca* as protein source for fish feed is not feasible in Europe as labour is too expensive and production is more likely to happen on land than marine production. *U. lactuca* production would be more feasible if certification and added value is ensured so the product can compete with production from outside Europe.

6.0 Recommendations

For cultivation experiments, it is recommended to use filtered seawater as most of the studies cultivating *U. lactuca* have experience with this method. And the cost of producing ASW and storing is higher than filtering NSW.

During the cultivation is recommended to do protein measurements of all the samples on a regular interval next to the growth measurements. This will provide an insight in the protein production during the cultivation as both the increase in weight in a sample and the increase in protein content are researched. Nutrient measurements of the medium is recommended as well to track the nutrient intake of the *U. lactuca* when exposed to different concentrations of NO_3 and PO_4.

For the design and build of an experimental setup, the budget should be known. This will allow for better planning and execution of the experiment as some of the measurements costs money and will be left aside if the budget is unknown.

It is also recommended that a new light-sensor is bought for actual validation of the lighting above the experimental set-up.

For proper measurements, it is recommended that the facility where the cultivation would take place is available every day, including weekends and holidays. This will prevent failures in the set-up and experiment itself as check-ups can be done on daily basis. Measurements can also be done daily or with regular intervals, increasing the statistical power of models in statistic programs while analysing the data.

For further research if different protein concentration affect the extractable amount of protein, an extraction experiment should be carried out with both a larger range of protein content in the samples used in the extractions and more samples with a statistically different protein content. This will improve the results of this research.

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Appendix I

Harvesting and homogenizing of the *Ulva lactuca*

Materials:

- *Ulva lactuca*
- Knife
- Cutting board
- Freezer proof storage bags.

Methods:

1. Take the *U. lactuca* out of the buckets and rinse the seaweed thoroughly with demi water to get rid of the excessive salts.
2. Use the knife to cut the seaweed into small patches of roughly 5 by 5 millimetre, this is to ensure that there is no visible difference between thalli.
3. Code the storage bags with bucket number, nitrate concentration and extraction method.
4. Store the bag with the material in the freezer on minus 20 degrees Celsius.

Appendix II

Weighting the Ulva lactuca

Materials:

- Scale
- Paper towels
- Salad spinner
- Photo camera
- Colander
- Small fishnet
- Bucket

Protocol:

1. Collect the *Ulva lactuca* from the bucket in the system, with the fish net. One bucket at the time.
2. Put the collected material in the colander to remove most of the excess water.
3. When most of the water is drained from the colander the *Ulva lactuca* can be placed in the salad spinner.
4. Use the salad spinner for 20 spins, one spin per second in a clockward motion. Open the salad spinner and remove the water from the reservoir. Close the top part of the salad spinner, the *Ulva lactuca* is now rotated for 20 times with the same speed, but the motion is now counter clockwise.
5. Remove the material from the salad spinner and place it gently on the fresh paper towels. The *Ulva lactuca* is spread evenly on the paper towel.
6. A picture is taken to see if there is a visual change in the growth period.
7. Place a second layer of paper towels on the exposed side of the *Ulva lactuca* and press on the top with light pressure to dry the material.
8. Remove the top layer of paper towels and rotate the thali on the bottom layer of paper towels to dry the material thoroughly.
9. Put the material in a dry bucket on the scale and weight the total material.
10. The *Ulva lactuca* can be placed in their own bucket in the system.

Appendix III

TABLE 1: ELEMENTAL COMPOSITION, INSTANT OCEAN SALTS, SALINITY 29.65 PPT, AQUARIUM SYSTEMS©*(ATKINSON & BINGMAN, 1999)*.

Major Cations (mmol kg^{-1})		Major Anions (mmol kg^{-1})		Nutrients (µmol kg^{-1})		Before equilibration with air		After equilibration with air, pCO_2 = 35µatm	
Na^{+}	462	Cl^{-}	521	PO_4:P	0.05	TCO_2	1.90	TCO_2	1.99
K^{+}	9.4	SO_4^{-2}	23	NO_3:N	1.00	$CO_2(x10^{-3})$	8	$CO_2(x10^{-3})$	11
Mg^{+2}	52	TCO_2	1.90	NH_4:N	10.2	HCO_3^{-1}	1.65	HCO_3^{-1}	1.78
Ca^{+2}	9.4	TB	0.44	SiO_3:Si	4.2	CO_3^{-2}	0.24	CO_3^{-2}	0.19
Sr^{+2}	0.19			DOP:P	0.1			CA	2.17
				DON:N	2.9			BA	0.10
				TOC:C	50			TA	2.27
				pH	8.25			pH	8.21
				TA	2.3			Saturation (Ca x CO_3) Aragonite 0.89 x 10^{-6} at 25°C	1.71
Sum	594	Sum	569						

TABLE 2:TRACE ELEMENTS IN INSTANT OCEAN SALTS, AQUARIUM SYSTEMS©*(ATKINSON & BINGMAN, 1999)*.

Trace Elements	Li	Si	Mo	Ba	V	Ni	Cr	Al	Cu	Zn	Mn	Fe	Cd	Pb	Co	Ag	Ti
µmol kg^{-1}	54	16	1.8	0.85	2.9	1.7	7.5	240	1.8	0.50	1.2	0.24	0.24	2.1	1.3	2.3	0.67

Appendix IV

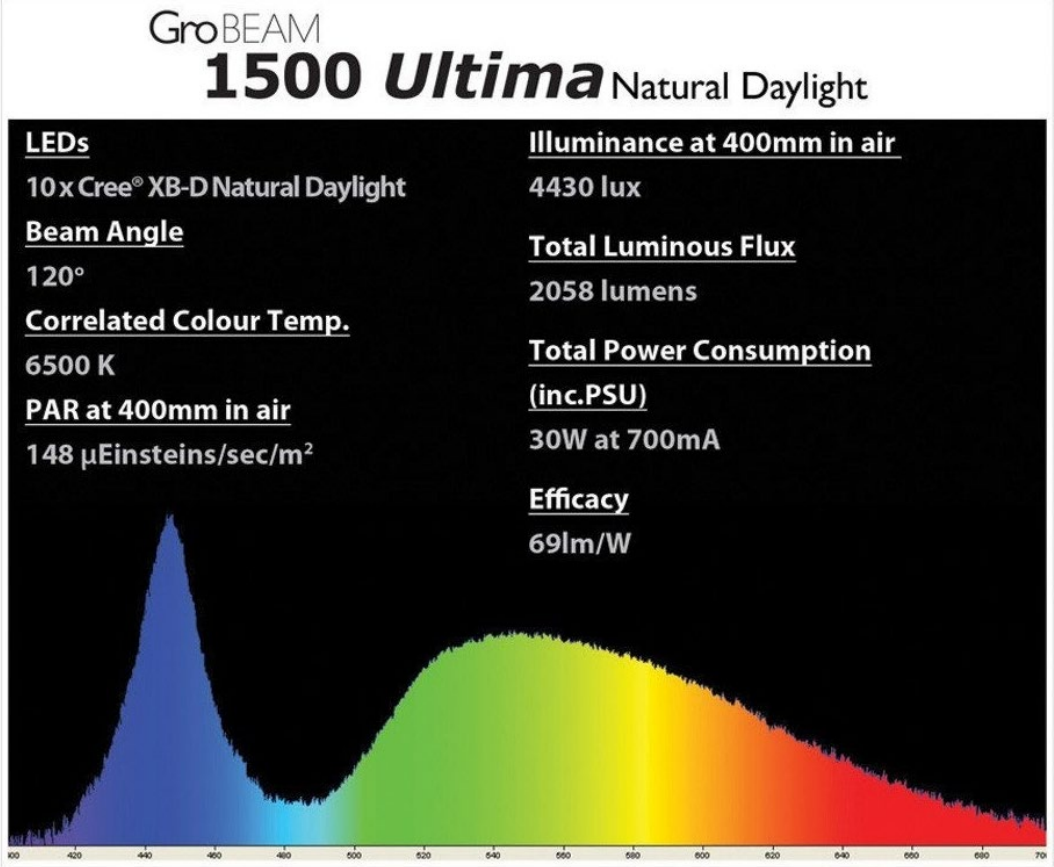

FIGURE 1: THE SPECIFICATIONS AND SPECTRUM OF AQUARAY GROBEAM 1500 NATURAL DAYLIGHT LED AQUARIUM LIGHTING TILES BY TROPICAL MARINE CENTRE©

Appendix V

Appendix Va

```
MIXED Versprotein BY NAOHDEMI WITH startProtein
  /CRITERIA=CIN(95) MXITER(100) MXSTEP(10) SCORING(1) SINGULAR(0.000000000001) HCONVERGE(0,
    ABSOLUTE) LCONVERGE(0, ABSOLUTE) PCONVERGE(0.000001, ABSOLUTE)
  /FIXED=startProtein NAOHDEMI NAOHDEMI*startProtein | SSTYPE(3)
  /METHOD=REML
  /PRINT=SOLUTION
  /RANDOM=INTERCEPT | SUBJECT(Regime) COVTYPE(VC)
  /EMMEANS=TABLES(NAOHDEMI) COMPARE ADJ(BONFERRONI).
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FIGURE 2: THE SYNTAX OF THE MODEL USED IN THE STATISTICAL PACKAGE FOR SOCIAL SCIENCES (SPSS). CONTAINING THE SETTINGS AND VARIABLES USED IN THE ANALYSIS.

Appendix Vb

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DATASET ACTIVATE DataSet2.
MIXED Wetweight BY Treatment WITH Days
  /CRITERIA=CIN(95) MXITER(100) MXSTEP(10) SCORING(1)
SINGULAR(0.000000000001) HCONVERGE(0,
    ABSOLUTE) LCONVERGE(0, ABSOLUTE) PCONVERGE(0.000001, ABSOLUTE)
  /FIXED=Treatment Days Treatment*Days | SSTYPE(3)
  /METHOD=REML
  /REPEATED=Days | SUBJECT(IDnumber) COVTYPE(AR1)
  /EMMEANS=TABLES(Treatment) WITH (Days = 15) COMPARE ADJ(BONFERRONI).
```

FIGURE 3: THE SYNTAX OF THE MODEL USED TO ANALYZE THE DATA IN SPSS. CONTAINING THE SETTINGS AND VARIABLES USED IN THE ANALYSIS.

Appendix VI

Protocol Protein Extraction

Materials

- Scale
- Magnetic stirrer with heating
- Magnetic stirring bar
- pH meter
- Measuring cylinder of 100ml
- Demineralized water
- Centrifuge
- Watch glass plate
- 3 gram Seaweed
- 250ml Erlenmeyer flask
- 1 M NaOH
- 50ml falcon tubes

Protocol

1. Measure 90ml of demineralized water into a measuring cylinder and pour this in a 250ml Erlenmeyer flask .
2. Bring the medium to the preferred temperature, add 3 gram dry weight seaweed and start stirring with the Turrax mixer.
3. Bring the pH to the desired scale with 0,2 M NaOH and put het watch glass plate on the Erlenmeyer flask to prevent evaporation.
4. Stir the Erlenmeyer constantly while maintaining the pH and temperature for one hour.
5. Take the Erlenmeyer of the stirrer and remove the magnetic stirring bar with a magnet.
6. Divide the content over three 50ml falcon tubes and centrifuge for 15 minutes with 4500 rpm.
7. Pour the supernatant in the a measuring cylinder and measure the amount.
8. Weigh the falcon tubes with the pellets still inside and put all the measurements in a Microsoft Excel datasheet.

Appendix VII

Protocol protein measurement using Kjeldahl

Materials

- Seaweed supernatant
- Seaweed pellet
- Kjeldahl tubes
- Kjeldahl destruction block
- Kjeldahl distillator
- Demineralized water
- BSA stock solution
- Erlenmeyer 300ml
- Catalysator tablets
- 98% H_2SO_4
- 30% H_2O_2
- H_3BO_3
- 1 M NaOH
- 0.1 M HCl
- Pipette 10 ml

Protocol

1. Fill the Kjeldahl tubes with the either samples and take one clear and one positive control in in the shape of BSA with.
2. Add 15 ml 98% H_2SO_4 and one catalysator tablet.
3. Add 2 ml 30% H_2O_2 to the sample, with a maximum of 3 times and wait between each time until the sample has reacted completely.
4. Put the samples in the destruction block and start the destruction for 1 hour on 420°C.
5. Let the samples cool down for 15 minutes after the destruction and start the distillation with the next settings:

H_2O	75 mL
H_3BO_3	40 mL
NaOH	50 mL
Time	3 minutes
Steam power	100%

6. Finally, perform a back-titration with 0.1 M HCl until the blue colored liquid changes to a red color. Note the amount of added HCl which is necessary for calculating the amount of protein.

Appendix VIII

List of European companies partnered with IFFO used for the literature study

Country	Jobs	Fishmeal	Fish oil	Fish feed	Development
Denmark					
Alfa Laval Copenhagen A/S, Oil & Protein Technology	-	-	-	-	x
Aller Aqua A/S	201-500	-	-	x	-
BioMar group	1000	-	-	x	-
Danish Dairy & Agricultural Suppliers Ltd	2 to 10	x	x	-	-
Haarslev Industries A/S	1100	-	-	-	x
JS Proputec A/S	11 to 50	-	-	-	x
P/F Havsbrún	1000	x	x	x	-
TripleNine	250	x	x	-	-
FF Skagen	130	x	x	-	x
Total:	3695 to 4040	4	4	3	4
France					
Ch. Daudruy van Cauwenberghe & Fils	11 to 50	-	x	-	-
Copalis	80	x	-	-	-
OLVEA Fish Oils	11 to 50	-	x	-	-
Polaris	11 to 50	-	x	-	-
SPF (Diana Aqua)	140	x	x	-	-
Total:	253 to 370	2	4	0	0
Germany					
BASF SE	-	-	-	-	x
Koester Marine Proteins GmbH	10 to 19	x	x	-	-
K-Pro GmbH	51 to 200	x	-	x	-
Total:	61 to 219	2	1	1	1
Netherlands					
Alltech Coppens	130	-	-	x	x
Demeter B.V.	9	x	-	x	-
IQI B.V.	26	x	x	-	-
Total:	165	2	1	2	1
Norway					
Aker BioMarine Antartic AS	282	x	x	x	x
BLT Berg LipidTech AS	11 to 50	-	x	-	-
Pelagia Feed	209	x	x	-	-
Cargill Aqua Nutrition	350	-	-	x	x
Nutrimar A.S	35	x	x	-	-
Scanbio Marine	51 to 200	x	x	-	-
Skretting	2905	-	-	x	x
Nordsildmel	10	x	x	-	-
Total:	3853 to 4051	5	6	3	3
UK					
United fish industries	105	x	x	-	-
Total:	105	1	1	-	-
Grand total: 29 companies	8132 to 8950	16	17	9	9