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Cultivation of *Haematococcus lacustris* for Production of Astaxanthin and Chl *a*.

Utilization of Processed Water from Paper Industry for Microalgae Cultivation.

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Abstract

Microalgae such as *Haematococcus lacustris* are rich sources for pigments such as astaxanthin and chlorophyll, and are attractive organisms of both commercial and scientific interest. Processed water from several industries is often rich in nutrient such as nitrogen and phosphorus and utilization of this type of water for microalgae cultivation could have several benefits. Untreated industrial water may disturb the surrounding environment if not treated. As microalgae are dependent on inorganic nutrients in order to grow, cultivation of microalgae in industrial processed water may give both an increased biomass and production of pigments, along with treated water as a result of reduced content of nutrients.

In this thesis, the possibility of using processed water from paper industry to cultivate the freshwater alga, *H. lacustris* was investigated. A batch culture experiment was conducted over 18 days and the cultures were illuminated with the same light intensity of 120-150 photons μmol $m^{-2}s^{-1}$ and temperature of 22 °C throughout the experiment. The cell density was determined daily, and the cultures with only industrial processed water showed a maximal specific growth rate (μ_{max}) similar to the cultures grown with Kuhl growth medium. The Kuhl-cultures possessed more astaxanthin and chlorophyll *a* compared to the cultures grown with industrial processed water. Different factors are believed to have affected the cultures. For astaxanthin accumulation it is proposed that the illumination intensity and the temperature used in this experiment were too low yet sufficient for cellular growth.

Key words: *Haematococcus lacustris*, astaxanthin, chlorophyll *a* (chl *a*), biomass, microalgae, cultivation, bioreactor, cell density, specific growth rate, fluorescence, Kuhl-medium.

Sammendrag

Mikroalger, som *Haematococcus lacustris*, er kilde til produksjon av pigmenter som astaxanthin og klorofyll *a*. I tillegg regnes disse som attraktive organismer, både kommersielt og i forskningssammenheng. Industrien innehar ofte produksjonsvann som er næringsrikt, og inneholder ofte både nitrogen og fosfor. Dette industrielle prosessvannet kan utnyttes for flere årsaker. Mikroalger er avhengige av uorganiske næringsstoffer for å kunne vokse. Kultivering av mikroalger med bruk av industrielt prosessvann kan dermed gi flere fordeler – økt biomasse ved cellevekst, produksjon av pigmenter, samt vannbehandling ved å redusere mengden næringsstoffer som ellers kunne hatt negativ påvirkning på det omkringliggende miljø.

I denne oppgaven blir muligheten for å bruke industrielt prosessvann fra papirindustri for å dyrke opp ferskvannsalgen *H. lacustris*, undersøkt. Det ble brukt et batch-kultur eksperiment over 18 dager. Kulturene ble belyst med samme intensitet på 120-150 μmol fotoner $m^{-2}s^{-1}$ samt en temperatur på 22 °C gjennom eksperimentet. Celletettheten ble bestemt daglig. Kulturene med kun industrielt prosessvann viste en lik maksimal spesifikk vekstrate (μ_{max}) som kulturene med kun Kuhl vekstmedium. Kuhl-kulturene hadde mer astaxanthin og klorofyll *a* sammenlignet med de kulturene med industrielt prosessvann. Ulike faktorer kan ha påvirket kulturene. For økt andel astaxanthin er det foreslått en høyere lysintensitet og temperatur enn det som ble brukt i dette eksperimentet, men for cellevekst var disse parameterne tilfredsstillende.

Nøkkelord: *Haematococcus lacustris*, astaxanthin, klorofyll *a*, biomasse, mikroalge, kutlivering, bioreaktor, celletetthet, spesifikk vekstrate, fluorescence, Kuhl-medium.

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Abbreviations

BT	Bürker-Türk (counting chamber)
Chl *a*	Chlorophyll *a*
CO_2	Carbon dioxide
Ft	Chlorophyll fluorescence
HPLC	High-Performance Liquid Chromatography
m^{-2} s^{-1}	Square meter per second
N	Nitrogen
NH_4-N	Ammonium-Nitrogen
NO_3-N	Nitrate-Nitrogen
n_x	Number of replicates
P	Phosphorus
PAR	Photosynthetically active radiation
pg	Picogram ($1x10^{-9}$ mg)
PO_4-P	Phosphate-Phosphorus
p	Significant coefficient
Qy	Quantum yield
r	Correlation coefficient
rpm	Rounds per minute
SD	Standard deviation
μ	Specific growth rate
μ_{max}	Maximum specific growth rate
μmol	Micromole
#	"Number of"

1 Introduction and theory

In a world of growing human population, the interest in use of energy carrying organisms for industrial purposes is increasing. The idea is to utilize organisms who produce their own energy thus reducing production of synthetic products. These organisms are called microalgae and they are key elements for use in various industries, including pharmaceutical, cosmetic and food industries (Begum, Yusoff, Banerjee, Khatoon & Shariff, 2016). Microalgae have a large diversity of size, shape, habitat and growth patterns of both prokaryotic and eukaryotic organisms, which makes this assemblage a major source for commercial and scientific interest (Pires, 2015, p: 55-68).

Microalgae are one of the major primary producers on earth, as well as the main producers of important pigments such as chlorophyll *a*, *b* and *c*, β-carotene and astaxanthin. Until recent years, synthetic colourants have been used in different industries. Even though the concentration of different pigments in the cells of microalgae is often too low for an economically feasible pigment production, the interest of naturally produced colourants is increasing (Mulders, Lamers, Martens & Wijffels, 2014).

Processed water obtained from industry is usually rich in nutrients and needs to be treated before released to the environment in order to prevent harmful effects such as eutrophication. Microalgae are dependent on inorganic nutrients such as nitrogen (N) and phosphorus (P) in order to grow. For this cause, microalgae are also a source for waste water treatment (Abdel-Raouf, Al-Homaidan & Ibraheem, 2012).

Pigments such as carotenoids and chlorophylls are used in the food industry, in medicine and cosmetics. Astaxanthin, being a carotenoid, is the major natural colourant used in aquaculture providing the red colour of salmon and crustaceans (Begum et al., 2016).

1.1 Microalgae

Microalgae are microscopic prokaryotes and eukaryotes in a size range of a few to hundreds μm, depending on the species. They are found in both freshwater and marine environment, and are considered of vital importance to their surrounding environment as they act as one of the major photosynthetic group of organisms on earth. Based on these properties, microalgae are named phototrophs because of their ability to generate energy out of light and water along with production of oxygen (Begum et al., 2016). Most of the species are free-living, though some are living in symbiosis with other organisms (Richmond, 2004).

Microalgae can be classified based on their colour. Chlorophytes are named as green algae, though chlorophyll, carotenoids and phycobiliproteins range in colour from yellow, green and brown to red.

Table 1 Number of species, name and type of pigments in the Phylum Chlorophyta (Graham and Wilcox, 2000).

Phylum	**# species**	**Name**	**Pigments**
Chlorophyta	~16 000	Green microalgae	Chlorophyll *a,* and *b*, β-carotene, prasionoxanthin, siphonaxanthin, astaxanthin

A) Growth requirements and patterns

The growth of microalgae can be divided into six major stages: 1) lag phase, 2) acceleration phase, 3) exponential phase, 4) retardation phase, 5) stationary phase and 6) decline phase (Figure 1). Figure 1 illustrates both the growth curve of an algae culture (a) and the corresponding growth rate (b). The growth rate increases at the beginning, from lag-phase and during the acceleration-phase. Through the stationary phase the growth rate is constant while the biomass is still increasing until reached stationary phase. Here the biomass stabilises. At this point the growth rate decreases until reached zero and the biomass remains stable at its maximal values. The declining phase is characterized by a negative growth rate and a decreasing of biomass until culture crash (Bersanti & Gualtieri, 2014, p: 253-254).

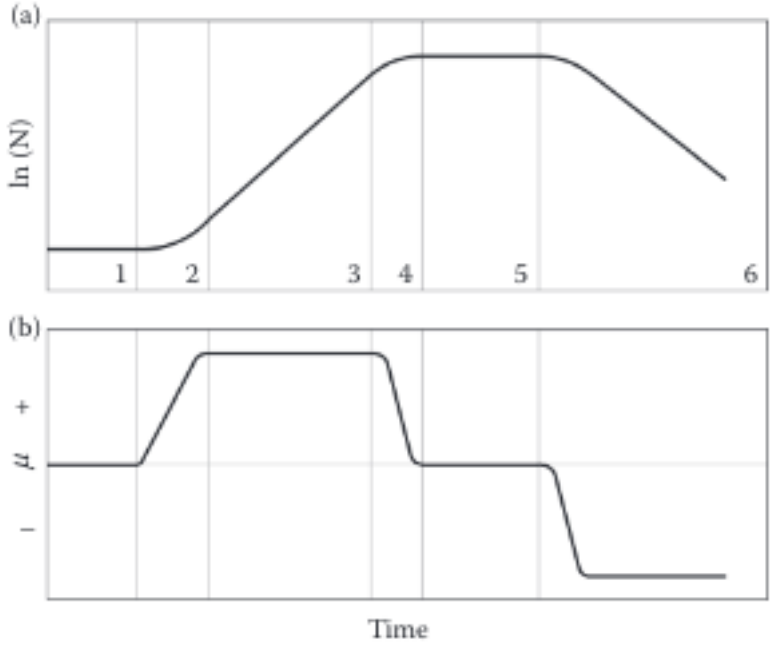


Figure 1 Growth phase of microalgae. (a): general growth curve. (b): corresponding growth rate curve (Bersanti & Gualtieri, 2014, p: 253).

Several factors are needed in order to support algae growth. These factors as CO_2, light, water and nutrients, which also may act as limiting factors for growth (Brennan & Owende, 2010). The term "limiting factor" is known from Von Liebig's "Law of the Minimum", which states that algal growth is not controlled by the total amount of nutrient, rather the concentration of each essential nutrient. Microalgae growth is dependent upon inorganic nutrients such as phosphorus (P), nitrogen (N), vitamins and trace metals (e.g iron, manganese, cobalt). Sufficient ratio between the nutrients is considered essential for an optimal biomass increase (Singh, Gupta, Guldhe, Rawat & Bux, 2015, p: 43-54).

Since microalgae utilize nutrients such as N and P, they are a natural part of the biogeochemical cycles occurring in the marine ecosystem. With help from bacteria, unavailable forms of N (e.g. atmospheric $N_{2(g)}$) are transferred to bioavailable forms as ammonium (NH_4) and nitrate (NO_3) (Ward, 2012, p: 45-46). In cases of N-limiting cultures, studies have shown that low N:P ratio induces accumulation of carotenoids (Borowitzka, Huisman & Osborn, 1991).

P in the marine ecosystem is often found as orthophosphate (PO_4^{3-}) as a result from either weathering of rocks or by decomposition of organic material. Both N and P are known as key

nutrients that may become a limiting nutrient. In a freshwater environment, P is referred to as the main limiting-nutrient. In P-limiting cultures, an algae culture may cease independently of the amount of N, which can further lead to eutrophication (Barsanti & Gueltieri, 2014, s. 202-203).

B) Photobioreactor and batch culture experiments

In general, two main systems may be used for algae cultivation: open cultivation and closed photobioreactor (PBRs). Open cultivation is one of the oldest cultivation methods and typically occurs in ponds or lakes. As this method is difficult to maintain and is less reliant, closed PBRs are often preferred. Bioreactors are designed with internal heat exchange and external illumination with controlled gas exchange (CO_2 and O_2). Due to its high surface:volume ratio the productivity for biomass production increases as a result of optimal light condition. This, along with its low risk of contamination of bacteria and other organisms, allow for a highly productive biomass production (Hosikan, Lim, Halim & Danquah, 2010).

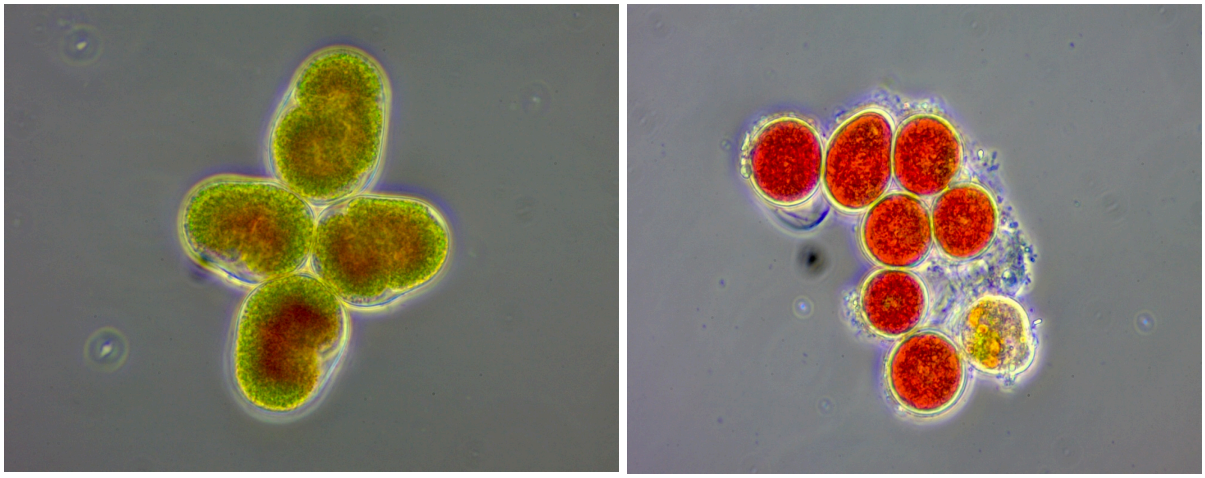
A batch culture is one of the most common methods for cultivation of microalgae as it is considered as simple and with a low cost. The algae culture increases to a given point where the culture stabilises for a short period in a stationary phase before it decreases as a result of deficiency of some vital factors, such as loss of oxygen or exhaustion of some limiting factors (Bersanti & Gueltieri, 2014, s. 254-255). This is the idea with batch culture. Since no external modification of the factors occurs during the cultivation process, a similar growth curve as presented in Figure 1 is expected.

C) Haematococcus lacustris

H. lacustris is a unicellular volvocalean green alga of the class Chlorophyceae (Grünewald et al 1997). This species of microalgae is often used as a source of keto-carotenoids, more specifically for the pigment astaxanthin. *H. lacustris* are grown in two main phases; a green and a red phase. In the green vegetative phase, cell density is increasing due to sufficient nutrients, light and environmental condition. A change in these conditions, either decreasing nutrient content or increasing light, will turn cells from a vegetative state to a red “spore” phase as a protective mechanism (Yong and Lee, 1991). This red phase is also referred to as an encysted

secondary carotenoid accumulation phase, which is shown as a clear red colour along with thickened cell walls as Figure 2 show. The thickened cell wall consists mostly of carbohydrates and accumulates astaxanthin (Sarada, Vidhyavathi, Usha & Ravinshankar, 2006).

For cultivation, *H. lacustris* are preferred grown in bioreactors as it is a species sensitive to large variation in pH and temperature (Chen, Chen & Gong, 1997).



A **B**

*Figure 2 Pictures taken of H. lacustris cells with a light microscope of 400 x magnification **A**: Green vegetative cells with a hint of red colour. **B**: Cells in a red "spore" phase. Photo: Sunniva Tofte*

1.2 Pigments and photosynthesis

Pigments are a major part of the photosynthetic activity of microalgae as they absorb photons and generate movement of electrons in the photosystems. Pigments absorb light of different wavelength and transmit light of different colour. The colour that appears is the colour that is most transmitted, or reflected by the pigment (Bersanti & Gualtieri, 2014, p: 153). In microalgae cultivation, pigments are a key for biomass production along with the commercially interest of colourants in aquaculture industry (Higuera-Ciapara, Felix-Valenzuela & Goyoolea, 2007).

The green colour of microalgae is due to pigments called chlorophyll, which absorb blue-violet and red light, and reflect green light. An absorption spectrum of chlorophyll *a* shows blue-violet and red light is the most effective for photosynthesis while green light is the least effective. This is confirmed by comparing the absorption spectrum to an action spectrum of photosynthesis, as presented in Figure 3 (Reece, Urry, Cain, Wasserman, Minorsky & Jackson, 2011, p: 237).

To quantify photosynthetic efficiency of a cell, different methods may be used. A simple and ubiquitous method is measurements of the chlorophyll fluorescence. As light penetrates a cell and gets absorbed by chlorophyll-pigments, different possible outputs may occur. Either the light energy will be used for photosynthesis to produce oxygen and biomass whereas possible excess energy may be dissipated as heat, or the light energy can be re-emitted as chlorophyll fluorescence (Reece et al., 2011, p: 238). As these are dependent upon each other, an increase in one would give a decrease in the other. By exposing a cell to light with a given wavelength and then measure the amount of light re-emitted at another wavelength, yield of fluorescence can be quantified and the photosynthetic activity may be determined (Maxwell & Johnsen, 2000).

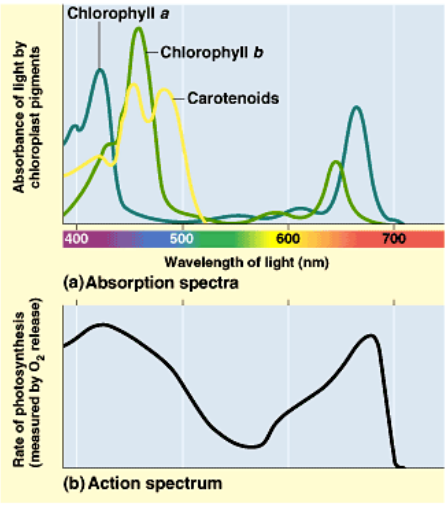


Figure 3 Illustration of the absorption spectra of chl a and b, and carotenoids (a), along with action spectrum of photosynthesis versus wavelength (b). Chl a *is showing highest absorbance in violet-blue and red light, chl* b *shows highest at blue and orange, while the carotenoids have highest effectiveness at blue-green light (Reece et al., 2011, p. 237).*

A) Astaxanthin

Carotenoids are known as an accessory group to photosynthetic activity along with chlorophyll *a* and *b*. Astaxanthin is a keto-carotenoid of the xanthophyll family and is considered to be the main carotenoid pigment found in marine organisms. Due to its characteristic red colour, astaxanthin is often used in aquaculture to provide the pink colour in salmonids and crustaceans (Begum et al., 2016). The natural sources of astaxanthin is increasing, yet it is the synthetic market that still dominates (National Center for Biotechnology Information [NCBI], 2005). Commercially, astaxanthin acts as an important contribution to the nutraceutical market based on its antioxidant activity and protection towards sun damage (Higuera-Ciapara et al., 2007). Presently *Haematococcus pluvialis* is considered as the microalgae with the highest astaxanthin production obtained at 3 % dry weight. Additionally, *H. pluvialis* are able to produce a astaxanthin purity of 95 % compared to 85 % in other species from carotenoids. Production of astaxanthin usually involves a two-staged cultivation: a green vegetative phase for biomass increase and a red "spore" phase for astaxanthin accumulation (Butler, McDougall, Campbell, Stanley & Day, 2017).

B) Chlorophyll a

Chlorophyll *a* (Chl *a*) is one of two pigments associated with the chlorophyll group along with chlorophyll *b.* Chlorophyll is an abundant molecule in nature due to its critical role of harvesting light to the photosynthesis. The importance of these molecules is highly reflected in the products of photosynthetic reactions, as these are carbohydrates of vital importance for plants and oxygen being the molecule responsible for all life (Hosikan et al., 2010).

Chl *a* is of major interest concerning primary production and oxygen production as this is the only molecule able to convert energy from sunlight to chemical energy. This molecule participates directly in the light reactions of the photosynthesis along with its accessory pigments as chlorophyll *b* and carotenoids (Reece et al., 2011, 236-237).

In the context of microalgae, chlorophyll is considered to be a highly valuable compound from microalgae biomass extraction. It has been widely used as a colorant due to its stability (Begum et al., 2016). As described for astaxanthin, chl *a* is also of both commercial and industrial interest due to its antimutagenic and antioxidizing properties (Hosikan et al., 2010).

1.3 Aim and hypotheses of study

The aim of this experiment was to examine if processed water from paper industry could be used as a nutrient source for cultivation of *Haematococcus lacustris* for value added production. The thesis is a part of the COMPLETE research project at SINTEF Ocean. The research activity was in collaboration with Biokraft AS, NTNU, NIBIO and Mattilsynet, and its overall aim is to develop new standards for sustainable biogas production.

The experiment was based on two objectives:

(1) Investigate the industrial processed water for cellular growth of *H. lacustris*.
(2) Discuss how well *H. lacustris* produce astaxanthin and chlorophyll *a* in industrial processed water compared to Kuhl as growth medium.

The hypotheses are:

(1) Industrial processed water is suitable as growth medium for *H. lacustris* cultivation.
(2) Cultivation of *H. lacustris* in industrial processed water will produce astaxanthin and chlorophyll *a*.

2 Materials and methods

2.1 Background

The experiment and its preparations were carried out between November 2017 and February 2018 at the laboratories of NTNU Centre of Fisheries and Aquaculture (SeaLab) in Trondheim. A batch-culture experiment was conducted with the fresh water microalga species, *Haematococcus lacustris*. The selected species was obtained from NIVA, Oslo (strain no. 5/91).

The alga was cultivated with use of processed water obtained from paper industry with different addition of Kuhl growth media (Kuhl & Lorentzen 1964). The rate of growth and the amount of astaxanthin and chl *a*, was determined.

Prior to the main experiment, a pilot was performed. This pilot was helpful in order to prepare and determine a suitable construction for the cultivation planned. Both *Rhodomonas baltica* and *Haematococcus lacustris* were used. Since *R. baltica* is a marine alga and the industrial processed water is freshwater, *H. lacustris* was selected for the main experiment, it being a freshwater alga.

2.2 Industrial processed water

The industrial processed water was provided by Norske Skog in Skogn and was taken out after active sedimentation towards the end of the production process. The water had a light-brown colour and some visible particles, and was stored in darkness at 2 °C at the laboratories of SINTEF Ocean before use in the experiment. Due to the risk of *Legionella*-contamination, specific legionella-analysis was conducted at the beginning of the experiments. This analysis was performed by Marianne Aas at SINTEF Ocean. A 0.5 L sample was concentrated 200x with a 0.45 µm sterile filter and DNA-extracted. This extract was further analysed using Real-time PCR with specific primers and probes for *Legionella* spp. and *Legionella pneumophila* (Stølhaug and Bergh 2006). The results showed no detection of any genetic materials from *Legionella* spp. Until these test results were approved, all work in the experiment was conducted with gloves and sufficient hand hygiene.

Prior to the experiments the chemical composition of the industrial processed water was analysed by the Scandinavian Biogas Fuels AB, Stockholm. Several substances were analysed but only selected ones were reported based on the interest in this thesis. The selected substances are presented in Table 2. See appendix IV for further information.

Table 2 Composition of selected chemical compounds in the industrial processed water from the Eurofins analysis report, see Appendix IV.

Element	**Result (mg L^{-1})**
Inorganic N*	1.4
Phosphate (PO_4)	0.68

*Inorganic nitrogen including NH_4-N, NO_3-N and NO_2-N.

2.3 Cultivation experiment

A) Experimental set up

The cultivation experiments were run with three replicates with only industrial processed water (named "Processed water"), three replicates with 1L industrial processed water with addition of 25 % growth media (named "Enriched processed"), and two replicates with 1L distilled water with Kuhl-growth medium only (named "Kuhl"), see Figure 4.

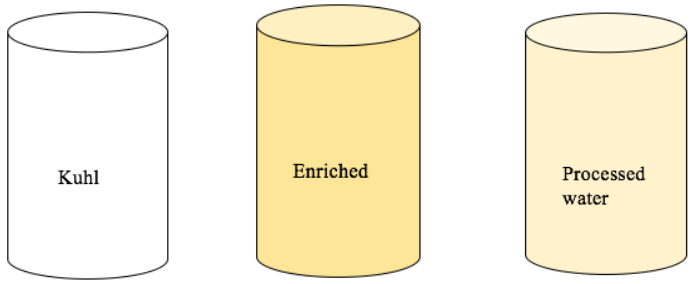


Figure 4 Kuhl-cultures with 1L distilled water and Kuhl-media added. Enriched processed-cultures with 1L processed water and 25 % added Kuhl-media. Processed water with 1L processed water only.

In total eight cylinders were used for this experiment. The algae were cultivated in batch-cultures with 1 L glass cylinders illuminated continuously with white artificial light of 120-150 µmol photons $m^{-2}s^{-1}$ (LED, Evolys AS). Photosynthetically active radiation (PAR) was controlled by using a Quantum Flux (Apogee Instrument). As there was one LED-light per four cylinders which was manually controlled, the two control replicates were placed on each LED-light, as presented in Figure 5 and 6.

Temperature was kept constant at 22 °C with double cylinder walls and continually circulating water. The cells were grown and aerated with bubbling air added CO_2 with a concentration of 0.3 %. The addition of air combined with a magnetic stirrer gave an even mixing of water. pH was measured daily by a standard pH-meter (pH/mV Portable, Mettler Toledo AS).

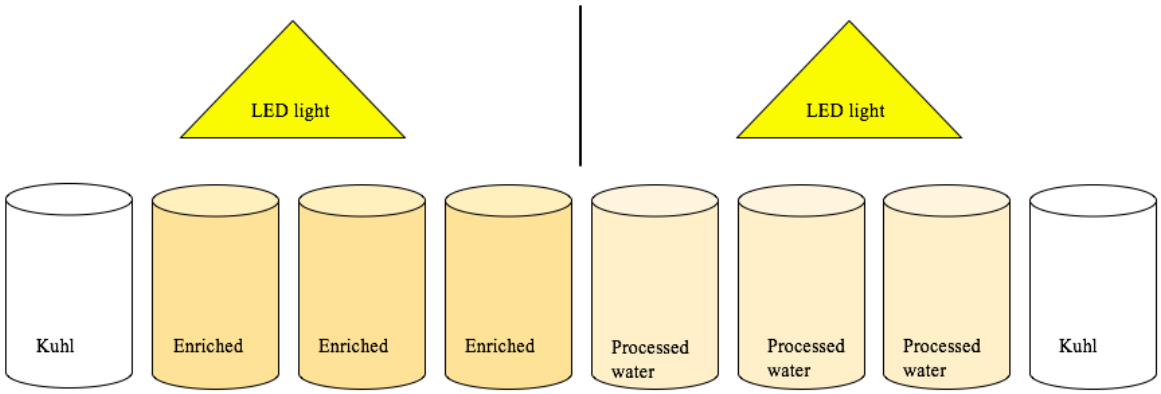


Figure 5 Illustration of the system: two replicates called Kuhl (clear), three processed water and 25 % Kuhl-media addition called Enriched processed (yellow) and three with the processed water only (light yellow). The bright yellow triangles illustrate the LED-lights.

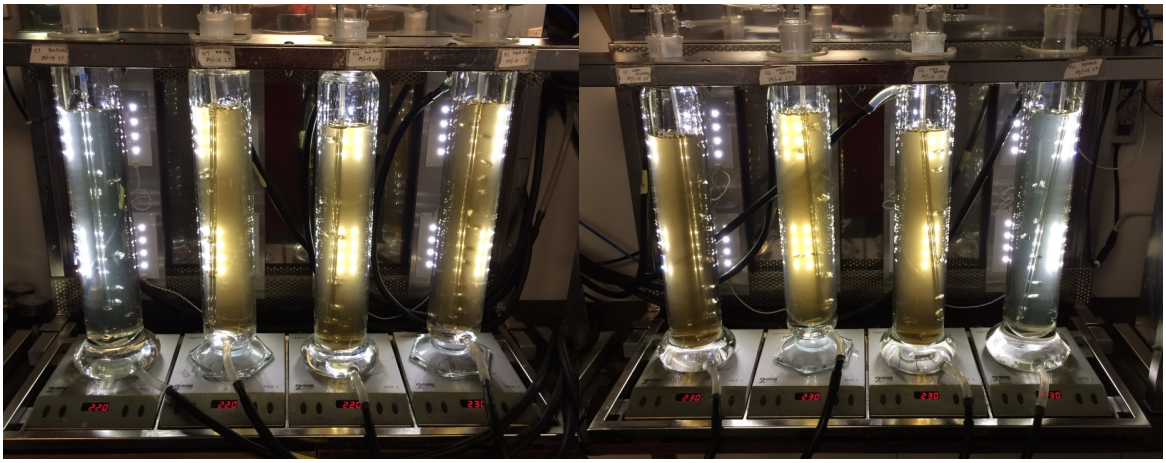


Figure 6 Set-up of the experiment. Eight glass cylinders connected to a constant air- and water flow placed on a magnetic field. Identical set-up as illustrated in Figure 5. Photo: Sunniva Tofte.

B) Algae species

In this experiment, *H. lacustris* was chosen as a suitable algal species based on two main reasons. First, as *H. lacustris* is a freshwater alga along with the industrial processed water also being freshwater, no pre-treatment as seawater dilution was necessary. The second reason was the interest and curiosity of using a species of *Haematococcus*, not being *H. pluvialis* which are known as a promising species astaxanthin production (Göksan, Ak & Kilic, 2011) and is more used in literature compared to *H. lacustris*. Though studies of *H. pluvialis* was used for further discussion later on in this thesis.

2.4 Analyses of culture parameters

A) Growth parameters – cell density and specific growth rate

The number of cells in the cultures was quantified with use of microscope with 100x magnification and is in this situation described as cell density. Samples were taken every day, fixated by using 50 µL Lugol's solution in 2 mL-samples and transferred to a Bürker-Türk (BT) counting chamber. Samples were mixed well when adding Lugol's solution and before counting to get an even mixing. BT-chambers have four squares of 1 mm^2 each and cells lying in the grid system were counted, as Figure 7 illustrates. As the cell density was lower at the beginning, a larger area was counted. When number of cells increased, cells lying within the four squares named A1-A4 were counted. An average value from eight replicates was used for calculation. Cells per mL were calculated using Formula 1 where *n* equals the number of cells counted and *z* is the number of squares.

$$Cell\ density\ (cells\ mL^{-1}) = n * \frac{10^4}{z} \quad [1]$$

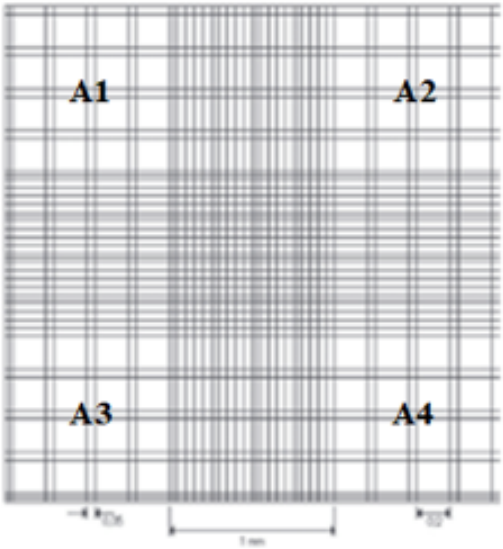


Figure 7 Illustration of a Bürker-Türk counting chamber. At the beginning when the cell density typically is low the whole grid system gets counted. Whenever the density increases, it is possible to reduce the amount of counting and only count the cells within A1-A4.

The specific growth rate (μ) was in this experiment determined based on the cell density in each culture, see Formula 2. μ is the specific growth rate, N_t is the number of cells at time *t,* while N_0 is the number of cells at t_0. The maximal specific growth rate (μ_{max}) was calculated from each culture in the early exponential phase where the number of cells was linearly correlated to time.

$$\mu = \frac{\ln(\frac{N_t}{N_0})}{t} \quad [2]$$

B) Fluorescence and quantum yield

Instantaneous chlorophyll fluorescence (Ft) was measured daily (AquaPen-C AP-C 100, Photon Systems Instruments). Chlorophyll fluorescence is the measured light-emission from an excited to a non-excited state. As light penetrates a surface, the light-energy could either be used in photosynthesis or as excess energy such as heat or re-emitted light as chlorophyll fluorescence (Maxwell & Johnson, 2000). This measurement gives information about changes in the photochemical effectivity and heat dissipation and therefore can be used as a growth parameter for microalgae. In this project Ft will be used as a proxy to cell density measurements and values are given as relative units (r.u).

Similar for the Ft-measurements, quantum yield (Qy) was determined daily (AquaPen-C AP-C 100, Photon Systems Instruments). Qy is given as a ratio between the number of photons emitted and those absorbed, see Formula 3. This ratio is given as a value between 0 and 1 and is used to determine the photo-physiological condition of the cells. Qy will in this project be used as a characteristic for the photo-physiological condition.

$$\text{Qy} = \frac{\#\ photons\ emitted}{\#\ photons\ absorbed} \quad [3]$$

C) Turbidity

Turbidity is expressed as the optical properties of a given media and was measured daily (AquaFluor HandHeld Fluorometer and Turbidimeter, Turner Design). As light penetrates through a unit of water, light gets distorted due to scattering and absorption of different particles, including biological particles. This measurement gives an indication of the concentration of suspended particles in a water sample. Such measurements could be affected by discolouring of the water. As for this experiment and the discolouring of the industrial processed water, one needs to take into account the possibility of an interference with the results, see section 2.2. Turbidity was measured as a proxy for cell density.

D) Nutrient analysis

For nutrient analysis, concentrations of nitrate (NO_3-N), ammonium (NH_4-N) and phosphate (PO_4-P) were determined. For PO_4-P measurements two methods were used, though the preparation before analysis was identical. 20 mL samples were collected on day 1, day 7 and day 10. These samples were filtered with a 0.45 μm-filter, marked and stored frozen at – 20 °C until analysis.

The first method for PO4-P analysis was performed by Kjersti Andresen at Trondhjem Biological Station (TBS). Due to visible particles after the samples were defrosted, the samples (approx. 4 mL) were re-filtered with 0.45 μm-filter before adding them to small plastic chambers. The samples were analysed with a Flow Solution-system (Flow Solution IV, OI Analyser). This instrument uses a multichannel peristatic pump with chemical reagents, and determines concentrations of a chemical element based on colorimetric analysis. The samples react with chemicals to produce a coloured mixture and concentrations are calculated based on its light absorbance (Gordon, Jennings, Ross & Krest, 1994).

The other method, along with NO_3-N and NH_4-N analysis, selected samples were sent to Eurofins, Trondheim. The samples were pre-treated as described for PO4-P analysis, and stored frozen at -20 °C until analysis at Eurofins.

An additional PO_4-P value was determined based on the information from the analysis report of the industrial processed water and the Kuhl-media used, see Appendix III and IV. This shows the initial concentration of PO_4-P before start of experiment.

2.6 Pigment analysis

A) Pigment determination from HPLC- analysis

Chl *a* and astaxanthin were measured using the principle of chromatography with an HPLC-instrument (Agilent 1100 Series, Agilent Technologies). This analysis was performed by Kjersti Andresen at TBS. Samples of approx. 40 mL of each replicate were collected, filtered onto Whatman GF/C glassfiber-filters by a vacuum-filter, kept away from direct light and frozen at -20 °C until analysis. Filtered samples were transferred to small glass cylinders and added 6 mL methanol as extraction solvent. With results from the chromatography analysis, the number of pigments was calculated based on a response factor (Rsf_λ) and measurement of absorption at 440 nm. The calculation is shown in Formula 4 where V_e equals the volume of extraction, V_i is the volume of injection on HPLC and V_f is the filtered volume.

$$\text{Pigments (µg/L)} = (area_\lambda * Rsf_\lambda) * \frac{V_e * 1000}{(V_i * V_f)} \quad [4]$$

B) Spectrophotometric determination of astaxanthin and chl a

The amount of astaxanthin and chl *a* was determined spectrophotometrically from harvested samples collected the last day of the experiment. Samples were taken from the Kuhl-culture and the Enriched processed-culture. No samples from Processed water culture was taken out for this method.

Samples were collected and centrifuged at 6000 rpm for 6 minutes (Centrifuge 5804 R, Eppendorf^{TM}) in 250 mL tubes to concentrate the dry matter. The dry mass was further transferred to 15 mL tubes and centrifuged with the same intensity as described before, freeze-

dried and stored in a deep freezer of – 80 °C until analysis. The analysis was performed at the chemistry laboratories of NTNU Gløshaugen with a method based on the study of Sarada, Vidhyavathi, Usha & Ravinshankar (2006) and calculations described in Lichtenhaler and Bushmann (2001). From each sample, two parallels were analysed. 10 mg biomass was weighed out and transferred to a new 15 mL tube. The samples were added 5 drops of 4 M HCl and heat-treated in a water bath of 70 °C for 10 minutes. The pre-treated biomass was further washed with 2 mL of distilled water, and treated with 8 mL of 100 % acetone for 1 hr. The addition of acetone was done in a ventilation cabinet. The biomass was then centrifuged at 5000 rpm for 10 min, and the absorbance of the extract was measured at 470, 480, 645 and 661.5 nm.

Chl *a* in the three cultures was determined with use of the same method and at the same time as for astaxanthin, shown in section 2.6 B. The amount of chl *a* and chl *b* was calculated based on Formula 5 and 6.

$$\text{Chl } a \text{ (µg/mL)} = (12.25 * A_{661.5nm}) - (2.79 * A_{645nm}) \quad [5]$$

$$\text{Chl } b \text{ (µg/mL)} = (21.50 * A_{645nm}) - (5.10 * A_{661.5nm}) \quad [6]$$

The total carotenoid content was calculated with the use of the Lichentahler equation where A_{nn} shows the absorbance at a given wavelength, see Formula 7. The carotenoid content is in this example the amount of astaxanthin by using the absorbance at 470 nm (Lichtenhaler & Bushmann, 2001).

$$\text{Total carotenoid content (µg/mL)} = \frac{(1000 * A_{470nm}) - (1.82 * Chl\ a) - (85.02 * Chl\ b)}{198} \quad [7]$$

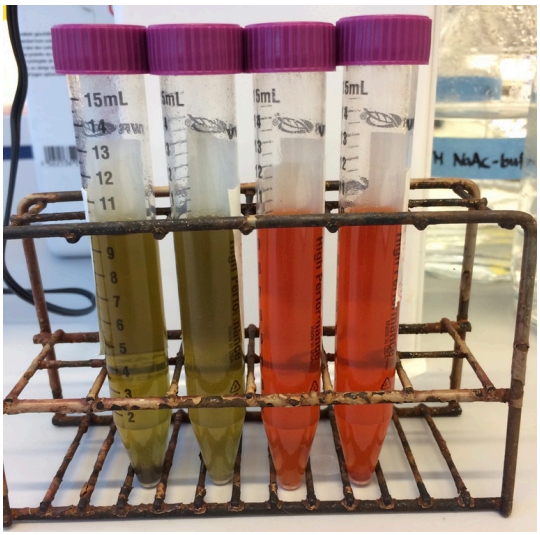


Figure 8 Two replicates of the extract of the Kuhl-cultures (green) and the Enriched processed-cultures (red) before the absorbance was measured. These samples were used for determination of astaxanthin and chl a. Photo: Sunniva Tofte

2.7 Statistics

All experimental data in this thesis were treated by the use of Microsoft Excel 2013 as well as statistical calculations. Determination of standard deviation (SD) was performed to indicate the variation of the mean values. An unpaired two-sample student t-test was calculated to examine significant differences with a level of significance of 0.05 and 0.01.

3 Results

3.1 Cultivation parameters

A) Cell growth and carrying capacity

The batch culture experiment was performed over a time period of 18 days and cell density (cells mL^{-1}) was measured daily. During the cultivation, the cell density increased in all three cultures until it stabilised in a stationary phase, as shown in Appendix I. The stationary phase can be called carrying capacity (CC) for this specific cultivation condition and was determined based on the maximal specific growth rate (μ_{max}). CC was shown to be different in the three cultures with the Processed water-cultures reaching CC first, as shown in Appendix 1. The maximum specific growth rate (μ_{max}) for all three cultures given as μ_{max} day^{-1} and are shown in Table 3.

As a proxy to cell density, measurements of turbidity were done. Results show that the turbidity correlates with the cell density for the Enriched processed-cultures (r = 0.88) and the Processed water-cultures (0.95). This indicates that the number of suspended particles in the two cultures increased along with the cell density, see Figure 9.

Table 3 Cell density (cells mL^{-1}) and μ (day^{-1}) shown as mean±SD (n_{Kuhl} = 2, $n_{Enriched\ processed}$= 3 and $n_{Processed\ water}$ = 3). μ_{max} shown in bold font.

Day (t_n)	Kuhl		Enriched processed		Processed water	
	Cells mL^{-1}	μ (day^{-1})	Cells mL^{-1}	μ (day^{-1})	Cells mL^{-1}	μ (day^{-1})
t_0	5000	0.36 ± 0.5	$1.0x10^4$	-0.07 ± 0.37	$5.0x10^3$	0.75 ± 0.28
t_2	5000 ± 4124	0.59 ± 0.43	9722 ± 3154	$* \mathbf{0.96}$ $\pm \mathbf{0.47}$	$1.1x10^4$ ± 3072	0.52 ± 0.23
t_3	$1.7x10^4$ ± 3535	0.14 ± 0.48	$2.4x10^4$ ± 3469	0.69 ± 0.08	$1.8x10^4$ ± 2373	$* \mathbf{1.06}$ $\pm \mathbf{0.08}$
t_4	$5.1x10^4$ ± 3535	$* \mathbf{1.11}$ $\pm \mathbf{0.14}$	$4.9x10^4$ ± 8785	0.76 ± 0.17	$5.1x10^4$ ± 3047	0.39 ± 0.03
t_8	$6.5x10^4$ $\pm 2.6x10^4$	0.23 ± 0.09	$1.5x10^5$ $\pm 2.3x10^4$	-0.29 ± 0.23	$3.2x10^4 \pm$ $1.0x10^4$	0.007 ± 0.29
t_{11}	$8.0x10^4$ $\pm 3.1x10^4$	0.24 ± 0.02	$1.0x10^5$ $\pm 3.0x10^3$	-0.34 ± 0.17		
t_{17}	$4.3x10^4$ $\pm 3.6x10^4$	0.43 ± 0.04				

* Showing μ_{max} in the different cultures.

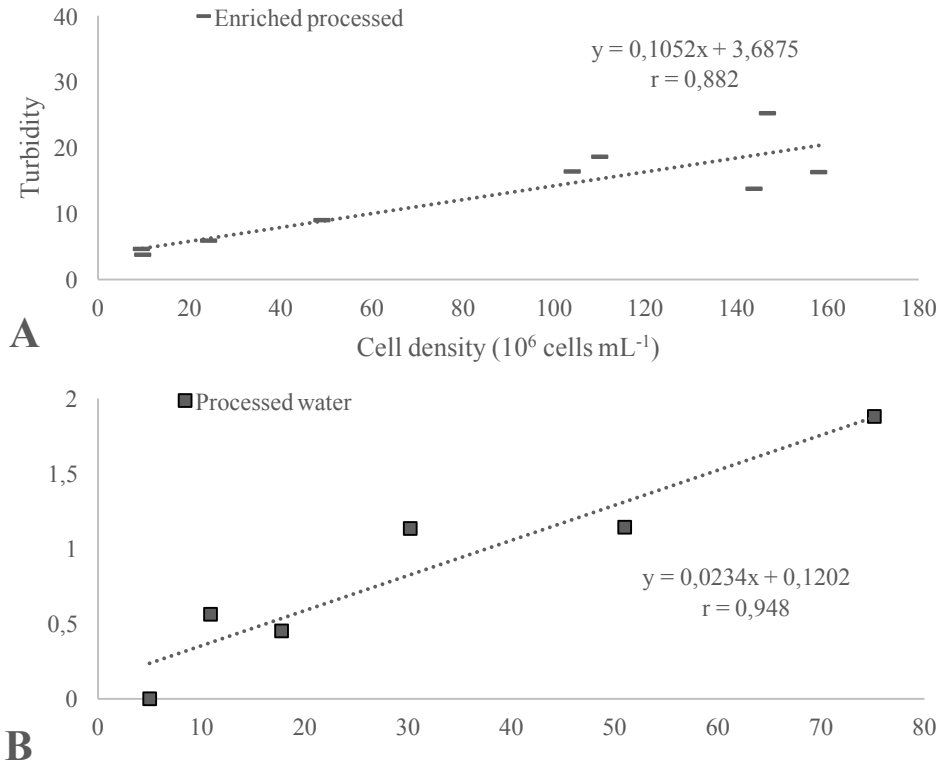


*Figure 9 Relationship between cell density (10^6 cells mL^{-1}) and turbidity. **A**: Enriched processed-cultures. **B**: Processed water-cultures.*

B) Quantum yield (Qy)

Qy was in this experiment used as an indication for photo-physiological cell condition. As Qy is given as a ratio, values are typically between 0 and 1 according to the photosynthetic productivity in a cell culture. Results of Qy in this experiment show the same pattern as cell density for the Kuhl-culture, and some differences in the two other cultures, see Figure 10. All three cultures increase similarly to day 4 when the highest Qy in Kuhl-cultures was measured ($0.68 \pm 0.014, n = 2$). When Qy started to decrease on day 4, the two other cultures were relatively stable as shown in Figure 10. Highest Qy in all three cultures was shown in the culture with Processed water on day 7 with a Qy of $0.77 \pm 0.006, n = 3$. Mean Qy from day 4 when the cultures started to differ is shown in Table 5.

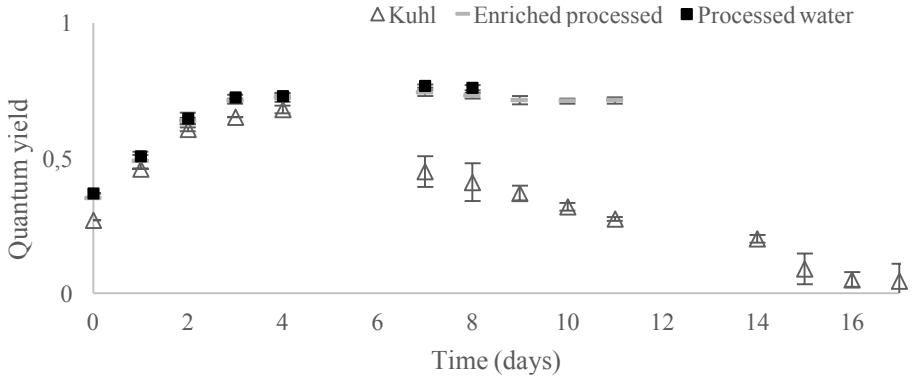


Figure 10 Qy shown as mean±SD, n_{Kuhl} = 2, $n_{Enriched\ processed}$ = 3, $n_{Processed\ water}$ = 3. Qy started between 0.27 and 0.37 for all three cultures.

Table 4 Mean value of Qy from day 4 until end of cultivation in each culture. Results is shown as mean±SD from n replicates.

	Mean Qy
Kuhl	0.29 ± 0.2, n = 13
Enriched processed	0.72 ± 0.01, n = 8
Processed water	0.75 ± 0.02, n = 5

C) Fluorescence (Ft)

Results from Ft-measurements show the same pattern as for cell density, though the Kuhl-culture differs significantly from the two other cultures ($p < 0.05$). The highest Ft was measured in the Kuhl-culture on day 11, while the highest Ft in the Enriched processed-culture was measured on day 9. In the culture with Processed water the highest Ft was measured on day 4, see Figure 11. Figure 12 show that the Ft increases with increased cell density with a r-value of 0.84.

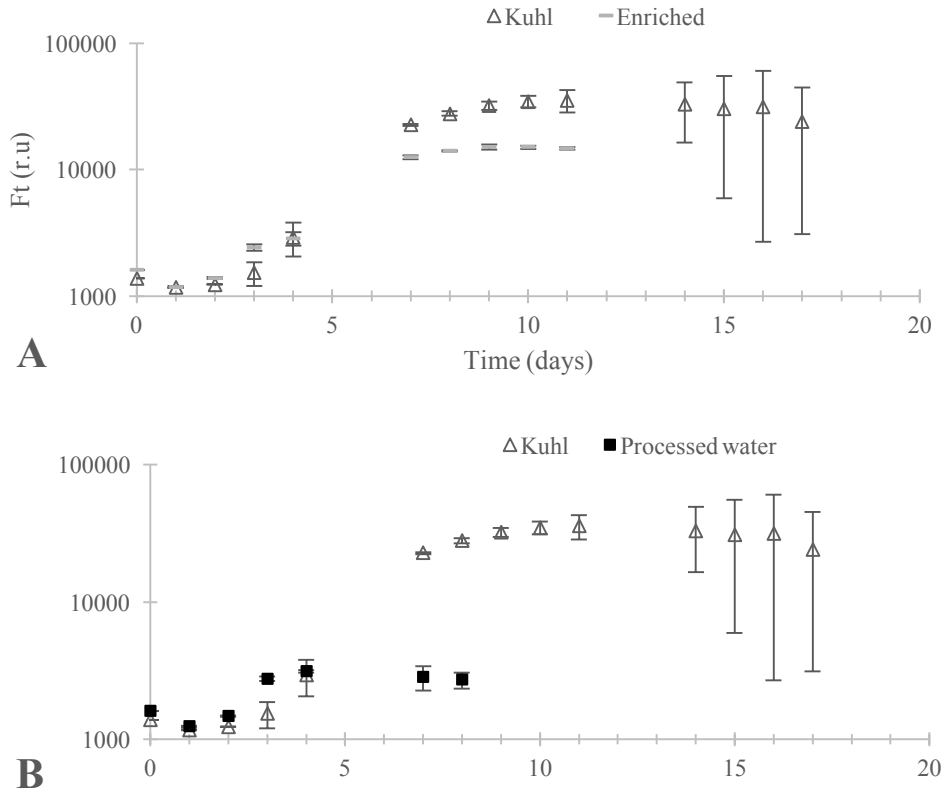


Figure 11 Daily registration of Ft given in relative units (r.u). Results shown as mean Ft ± SD , n_{Kuhl} = 2, $n_{Enriched\ processed}$ = 3, $n_{Processed\ water}$ = 3. Kuhl-cultures shown in both A and B. ***A****: Showing the Kuhl-cultures and the Enriched cultures.* ***B****: Showing the Kuhl-cultures and the cultures with Processed water.*

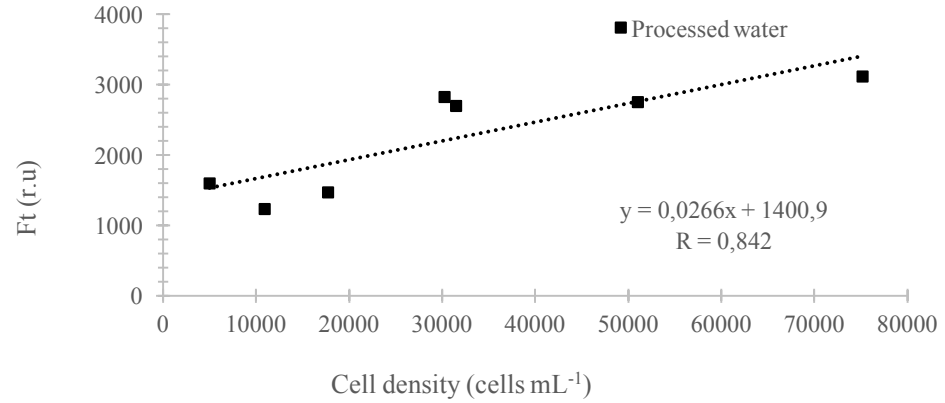


Figure 12 Showing the linearly relationship between cell density (cells mL^{-1}) and Ft in the cultures with Processed water.

D) pH

pH was measured daily during the cultivation. The pH had an average of 7.1 in the Kuhl-culture, and 8.4 in both Enriched and Processed water shown in Figure 13. In addition to the natural CO_2 in air, 0.3 % CO_2 was added to the three cultures. This extra addition was to provide enough inorganic carbon for photosynthesis. The lowest pH was measured in the Kuhl-culture on day 1 with a pH of $6.2 \pm 0.007, n = 2$. The pH in Kuhl increased to a maximum of 8.05 achieved on day 15 and 16. The Enriched processed and the Processed water had an average pH on 8.3 and 8.4, and the highest pH was measured in the Enriched processed of 8.7 on day 8.

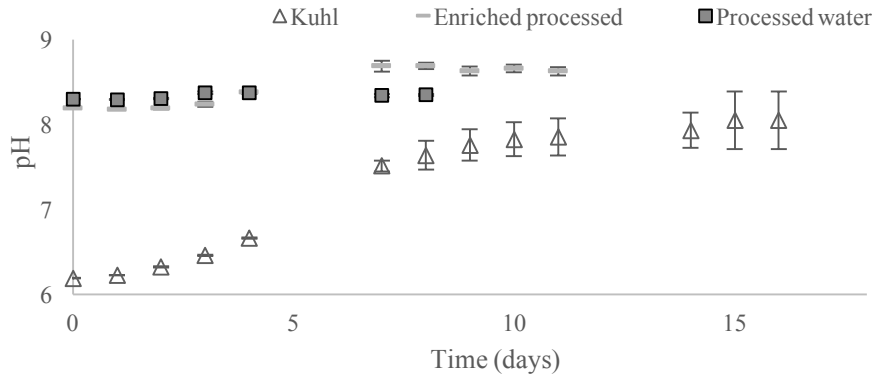


Figure 13 pH-value of the three different cultures during the cultivation process. Values are shown as mean±SD, $n_{Kuhl} = 2$,$n_{Enriched\ process}=3$ and $n_{Processed\ water}=3$.

3.2 Content of nitrogen and phosphorus

The content of ammonium (NH_4-N), nitrate (NO_3-N) and phosphate (PO_4-P) was measured on day 1, 7 and 10. All three cultures show a decrease in all the nutrients mentioned as function of time, as showed in Figure 14. For PO_4-P measurements, two methods were used. When comparing results from these two methods, a significant difference was found in results from day 7 in both the Enriched processed and the Processed water ($p < 0.01$). Only Enriched processed-cultures were measured on day 10 and no significant difference was found in these results ($p > 0.05$). See Appendix II.

The amounts of NH_4-N in both the Enriched processed and Processed water are similar on day 1, both decreasing with time with the Enriched processed-cultures showing the largest range of variance from day 1 till 7. NH_4-N was not measured in the Kuhl-cultures.

The content of NO_3-N in the three cultures at the start of the cultivation was calculated based on the values from the industrial processed water obtained from Norske Skog and the Kuhl-media. Results show a slight decrease of NO_3-N in the Enriched processed, yet a much larger decrease in Processed water as shown in Figure 14.

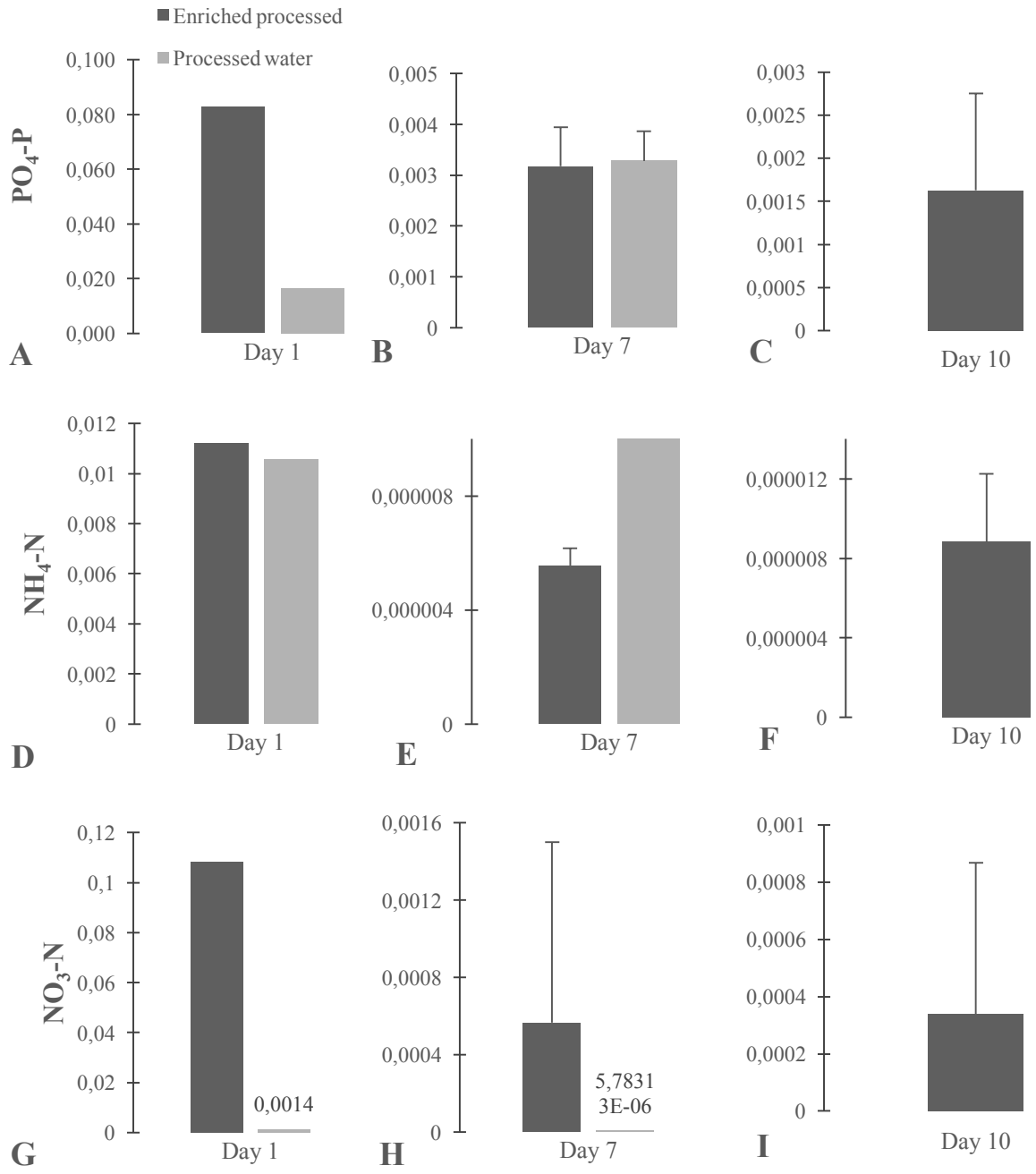


*Figure 14 Amount of PO_4-P, NH_4-N and NO_3-N in the Enriched processed-culture and the culture with Processed water at day 1, 7 and 10 (left to right). Values from the Eurofins-report and shown as µg/cell **A-C**: Amount of PO_4-P. **D-F**: Amount of NH_4-N. **G-I**: Amount of NO_3-N. Results shown as mean ±SD, n = 3. No results from the Processed water-cultures on day 10 as these cultures were terminated on day 8.*

3.3 Determination of astaxanthin and chl *a*

A) Quantity of astaxantin and chl a

The amount of astaxanthin and chl *a* was determined from harvested samples on the last day of cultivation with two different methods. Pigment analysis of the Processed water-cultures was only performed with the HPLC-method. Results are shown as picogram (pg) pigments per cell in Table 5. Significant difference was determined between the two methods used ($p < 0.01$). The HPLC-method shows highest amount of astaxanthin in the Kuhl-cultures, while opposite from the spectrophotometric results. Chl *a* was highest in the Kuhl-cultures for both methods.

Table 5 Amount of astaxanthin and chl a (pg per cell) in the three cultures. Results shown as mean±SD, $n_{Kuhl} = 2$, $n_{Enriched\ processed} = 3$ and $n_{Processed\ water} = 3$.

	Kuhl	**Enriched processed**	**Processed water**
Astaxanthin[1]	$0.035 \pm 1.5x10^{-4}$	$0.0052 \pm 2.3x10^{-5}$	$0.0049 \pm 6.1x10^{-5}$
Astaxanthin[2]	711 ± 10.8	2126 ± 9.67	
Chl *a* [1]	0.062 ± 0.0013	$5.4x10^{-4}$ $\pm 4.1x10^{-6}$	$4.7x10^{-4}$ $\pm 5.3x10^{-6}$
Chl *a* [2]	2539 ± 0.18	332.65 ± 12.52	

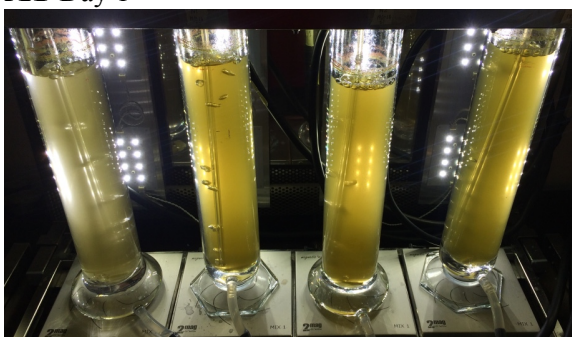
[1]: Pigment content from HPLC. [2]: Pigment content from spectrophotometer.

B) Colour change in the three cultures

The colour of the different cultures varied through the cultivation experiment. At the beginning the Kuhl-cultures were more transparent compared to the other two cultures. The colour of Enriched processed and the Processed water was yellow-brown, similar to the industrial processed water obtained from Norske Skog, described in Section 2.2. The cultures started to change colour on day 4 (Figure 15 B1-2). The Enriched processed got a yellow-green colour, while the cultures of Processed water were slightly red-coloured.

A1 Day 1

A2 Day 1

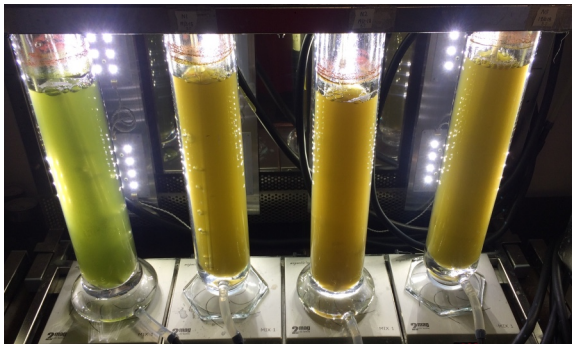


B1 Day 4

B2 Day 4

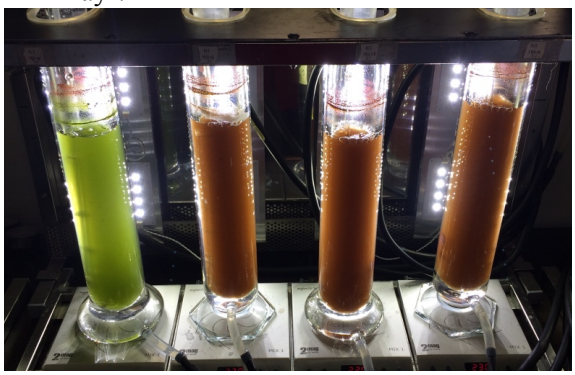
Figure 15 Photos showing the three cultures at the beginning of the cultivation. ***A1-2****: Day 1. The Kuhl-cultures had a transparent colour and the other cultures showing a yellow-brown colour.* ***B1-2****: Day 4. The two Kuhl-cultures showing a more yellow-brown colour. Cultures of Processed water beginning to show a cobber-brown colour. The Kuhl-culture displayed in all images, while the Enriched-culture are shown in A1 and B1 and Processed water shown in A2 and B2.*
Photo: Sunniva Tofte

On day 7 the Kuhl-cultures showed a more distinct green colour, while the cultures with Processed water became clearer red. The Enriched-cultures did not have the same change in colour though the cultures were thickened (Figure 16 A1-2). On day 11 the Enriched processed-cultures showed a more distinct colour-change from yellow-brown to dark red (Figure 16 B1).



A1 Day 7

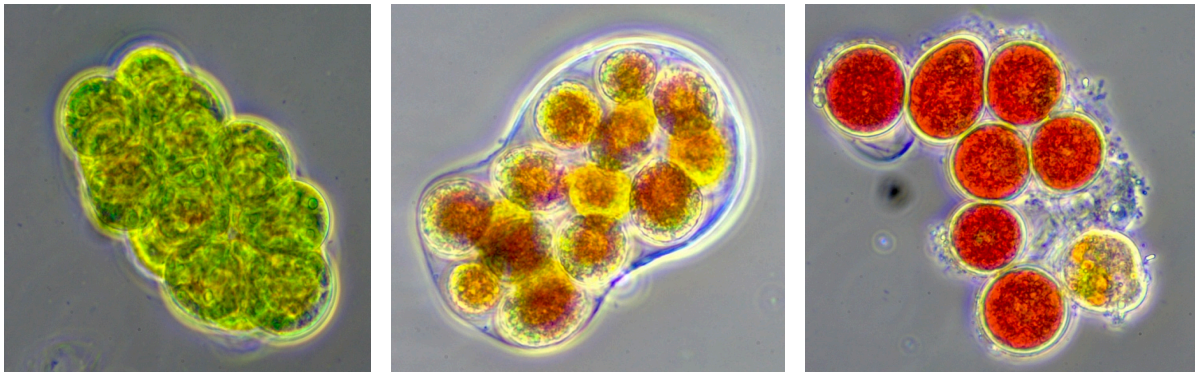
A2 Day 7



B1 Day 11

B2 Day 11

Figure 16 Photos of the setup from day 7 to day 11. ***A1-2****: Cultures from day 7. The two Kuhl-cultures showing a distinct green colour, the Enriched-cultures a yellow-brown colour while the Processed water-cultures were more red-coloured.* ***B1-2****: Day 11. Enriched cultures showing a more noticeable red colour. Processed water were terminated on day 8 and is therefore not shown in this image. The Kuhl-cultures displayed in all images, while the Enriched-cultures are shown in A1 and B1 and Processed water shown in A2 and B2. Photo: Sunniva Tofte*



A **B** **C**

*Figure 17 Photos of H.lacustris cells taken with 400x magnification microscope during the cultivation process. **A**: Cells from Kuhl-cultures on day 1 showing a typically cluster formation. **B**: Cells from Kuhl-cultures on day 4. The characteristic red colour beginning to appear. **C**: Clear red carotenoid colour along with a thickened cell wall from the Processed water cultures towards the end of cultivation. Photo: Sunniva Tofte*

4 Discussion

The aim of this study was to investigate the possibility of using industrial processed water as growth media for the freshwater microalgae *H. lacustris* for pigment production.

4.1 Cultivation parameters

A) Cell density and growth rate

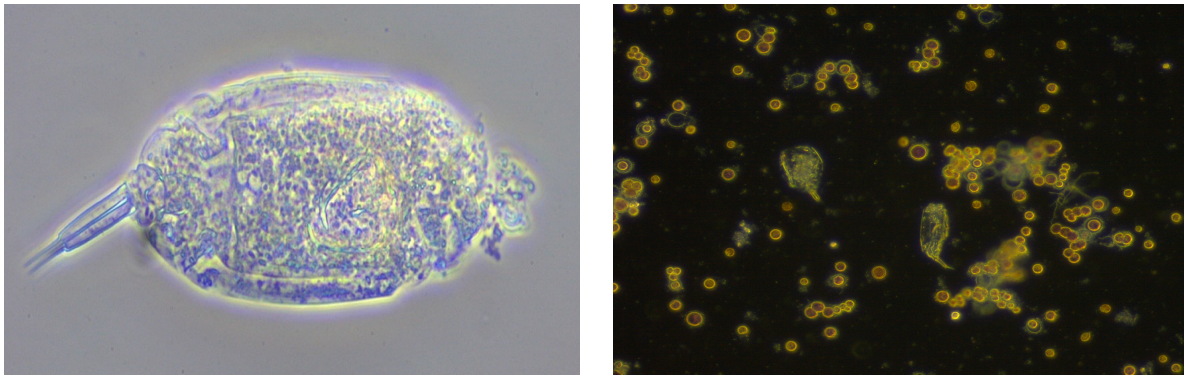
The three cultures used in this experiment showed similar growth pattern as the general growth curve shown in Figure 1. However, some different patterns between the three cultures were present. Figure 15 and 16 shows that the Kuhl-cultures never reached a clear red phase, which indicates that the cultures were kept in the vegetative phase during the experiment. Yet, the Qy in the Kuhl-cultures decreases after day 4, while Qy in the two other cultures was stable throughout the experiment, as shown in Table 4. This indicates that the photosynthetic condition in the Kuhl-cultures were more affected and probably were in worse condition compared to the other two. A prediction of a more optimal condition in the Kuhl-cultures, given being in the vegetative phase (Fabregas, Dominguez, Regueiro, Maseda & Otero, 2000), is therefore in conflict with the Qy determined. The processed water-cultures however, went straight to the red phase without being in a clear vegetative phase, as shown in Figure 15 and 16. According to Fabregas et al. (2000) this could indicate a poor condition in the cultures. Yet, the cell density of the Processed water-cultures shows similar results to the Kuhl-cultures throughout the experiment, which denotes that cell density alone is not enough to explain the different colouring of the cultures.

Results show that all three cultures reached early exponential phase between day 3 and 4. The highest μ_{max} was found in the Kuhl-cultures on day 4 (1.1 ± 0.1 day^{-1}). Little variation was found in the Enriched processed-cultures (1.0 ± 0.4 day^{-1}) and the Processed water-cultures (1.1 ± 0.1 day^{-1}) compared to the Kuhl-cultures. However, μ determined on the last day of cultivation varies more, as seen in Table 3. When the Processed water-cultures were harvested on day 8, the μ was only 0.7 % of μ_{max}, while the Enriched processed-cultures showing a negative μ the last two days of cultivation. Negative μ states a declining growth (Bersanti & Gualtieri, 2014, p: 254). These low growth rates towards the end of the cultivation supports the cell density results, and since this was a batch culture experiment and no nutrients was added

negative μ is expected. However, according to the cell density the method for quantification of cells could be an error. Since the cell density was determined by manually counting in a BT-chamber, it is likely to expect a number of cells not being counted. *H. lacustris* tend to form clusters, as shown in Figure 17, which makes the quantification method more challenging.

The linear relationship between the cell density of the cultures with Processed water showed a correlation with the Ft-results (r = 0.84). This supports that Ft may be used as a proxy for cell density. Since Ft is defined as the excess light energy not being used in the photosystems for photosynthesis, it is likely to expect a decreasing Ft with increasing cell density. In this experiment the opposite occurred, which could be explained by a lower photosynthetic activity. However, the Ft measurements were done with samples from the entire culture, not each individual cell, so the results presented in this thesis is not enough information to determine whether the photosynthetic condition was sufficient or not.

An algae culture should be free from other species as these could negatively interfere on the cell density. In this experiment individuals from the phyla Rotifers were found when studying the samples in the microscope, as Figure 18 show. Species of Rotifers are zooplanktonic and range in size from 50 μm to > 2mm. The species are said to be cosmopolitan and are likely to be found nearly everywhere (Scmidt-Rhaesa, 2015, s. 396-397). One of the Rotifers found in this experiment was measured a size of 103 μm. It was confirmed by Norske Skog that individuals of Rotifers are present in the industrial processed water obtained. No further classification was performed. As this zooplankton was discovered in all three cultures of microalgae, it is possible that this would affect the cell density results as well as calculation of growth rates. The Rotifers could have fed on microalgae, which indicate possible higher growth rate than observed.



A **B**

*Figure 18 Picture taken of individuals of Rotifers found in all three cultures. **A**: Close up of one individual of a Rotifer, 400x magnification. **B**: Overview from a sample from the Enriched processed-cultures taken with 100x magnification. Here shown two individuals centred in the picture with individuals of H. lacustris lying around. Photo: Sunniva Tofte*

B) pH, temperature and light

The amount of CO_2 added to the cultures was held constant (0.3 %), and the pH was measured to be between 6.2 and 8.7 in the three cultures through the experiment, see Figure 13. The pH is of major concern in these types of experiments due to its possible fatal consequences of large deviations either increasing or decreasing (Bersanti & Gualtieri, 2014, p: 227). The largest range of variance was recorded in the Kuhl-cultures, along with having the lowest initial pH-value. This indicates that the pH of the industrial processed water was higher than the distilled water used for the Kuhl-cultures. Given the variation in the Kuhl-cultures, it is possible to predict that the pH determined had a larger influence in these cultures compared to the two others. A study of Sarada, Tripathi and Ravishankar (2002) recorded maximal cellular growth and highest astaxanthin production of *H. pluvialis* at pH of 7 compared to pH of 6, 8 and 9. For this experiment however, the highest cell density was shown in the Enriched processed-cultures, while the highest astaxantin content was found in the Kuhl-cultures. This indicates that a pH of 8 stimulate cellular growth and pH of 7 is optimal for astaxanthin production of *H. lacustris*.

The temperature was held constant at 22 °C and controlled with a circulating water system. According to Olaizola (2000), an optimal temperature for pigment production from *H. pluvialis* is 25 °C. For accumulation of astaxanthin in the red-coloured phase in the same species, an

optimal temperature of 27 °C is proposed (Panis & Carreon, 2016). As the temperature used in this experiment was lower than what literature proposed as optimal for maximal astaxanthin production, in retrospect a higher temperature could have been used. Nevertheless, heating is energy demanding so for this experiment and its purposes a lower temperature than recommended was chosen. It would however, be interesting to have done another experiment to compare the astaxanthin production with different temperatures.

When comparing the appearance of the cultures, the Kuhl- and the Enriched processed-cultures stand out as they look thicker, as Figure 15 and 16 shows. Even though the continuously mixing water keeps the cultures in movement, the thickened cultures may have suffered from light-limitation. The cultivation had an irradiance of 120 to 150 μmol photons $m^{-2}s^{-1}$, measured outside of the cylinders. Studies on *H. pluvialis* have recorded an increased cellular growth with a light intensity of 75 and 175 μmol photons $m^{-2}s^{-1}$ compared to 20 and 40 μmol photons $m^{-2}s^{-1}$ (Göksan et al., 2011). This is supported by studies indicating that an illumination of 75 to 177 μmol photons $m^{-2}s^{-1}$ is ideal for maximum cellular growth (Dominguez-Bocanegra, Legaretta, Jernimo & Campocosio, 2004). It is also shown that illumination above 500 μmol photons $m^{-2}s^{-1}$ is needed during the red phase to induce astaxanthin accumulation (Panis et al., 2016), while an increase in chl *a* was shown when changing illumination from 90 to 190 μmol photons $m^{-2}s^{-1}$ (Yong, Gong & Chen, 1997). This argues for a higher light intensity to be used for maximum pigment production, yet a sufficient intensity for cellular growth.

4.2 Content of nitrogen and phosphorus

The overall content of PO_4-P, NH_4-N and NO_3-N varied, both between the different cultures but also between the two methods used for PO_4-P-measurements. Significant difference between the two measurements was observed for the Kuhl-culture ($p < 0.05$). The measurements conducted at TBS, NTNU were estimated to be outside the limit value set to be 0.64 to 310 μg/L, which could be a source of error with inaccurate results.

Studies have shown that an optimal N:P ratio from weight measurements (mg L^{-1}) for maximum N and P uptake by microalgae is 3:1 (Su, Mennerich & Urban, 2011). Initial N:P ratio in this experiment was set to 0.97 in the Kuhl-cultures, 1.35 in the Enriched processed-cultures and 0.1 in the Processed water-cultures. All ratios decreased with time, see Appendix II. Based on

these ratios, the Enriched processed-cultures had the most sufficient initial amount of N and P. When comparing the range of variance between the Enriched processed and the Processed water, the highest variance was found in the Enriched processed cultures, which supports the study of Su et al. (2011) that the actual ratio has an influence on nutrient uptake. Based on these assumptions, it is possible to suggest that P in the Enriched processed cultures is utilized to a greater extent compared to the Processed water. This is supported by the cell density results, showing that the Enriched processed-cultures having a higher cell density compared to the other two, as shown in Table 3.

Gong and Chen (1997) state that the growth media is of major concern regarding cell productivity in *H. pluvialis*. As the cultures with Processed water were terminated first (day 8), an argument of nutrient limitation can be made. Higuara-Ciapara et al. (2006) showed in their study that vegetative cells of *Haematococcus* are very sensitive to deviations in environmental factors, such as nutrient content and light. It is therefore fair to predict a nutrient limitation in the Processed water-cultures early in the experiment, also supported by its colouring.

Results showed that the Kuhl-cultures had more N and P on day 1 compared to the two others. A study by Borowitzka et al. (1991) found that *H. pluvialis* had the best growth with a NO_3-N concentration of 0.5-1.0 g L^{-1} and a PO_4-P concentration of 0.1-1.0 g L^{-1}. Initial concentration in the Kuhl-cultures was 1.5 g L^{-1} NO_3-N and 1.6 g L^{-1} PO_4-P, which indicates a quite high initial nutrient concentration. For the Processed water-cultures, initial NO_3-N concentration of 0.014 g L^{-1} and 0.068 g L^{-1} PO_4-P was determined. According to the study of Borowitzka et al. (1991) and by comparing the concentration to the Kuhl-cultures, it is possible to predict a less sufficient start concentration for cellular growth in these cultures. This supports the argument that the Processed water-cultures had less optimal condition and therefore were transferred directly to the red phase, without entering the green vegetative phase, as discussed in Section 4.1A. However, in the same cultures a decrease of 79 % was shown in inorganic P while a 99 % decrease was shown in N, which indicates that the content of nutrients that were present actually was utilized. This implies that cultivation of *H. lacustris* in industrial processed water may reduce the amount of inorganic nutrients and could therefore be accepted as a contribution to water treatment.

4.3 Content of astaxanthin and chl *a*

For pigment analysis, a spectrophotometer and a HPLC were used. Both of the methods use separation techniques for determination of pigments from samples. For the content of chl *a*, both methods show highest amounts in the Kuhl-cultures. The large difference between the results from the two methods could be explained by the methods themselves. Studies have shown that extraction of astaxanthin from red-phased cultures of *Haematococcus* is challenging due to the thick walls of sporopollenin-material of the cells that act as a hinder for solvent extraction (Sarada et al., 2006). During the pre-treatment for HPLC analysis the samples were shaken but possibly not enough for the pigments to get released from the cells. This error was also the case with the spectrophotometric method, and could have led to inaccurate results. However, for the spectrophotometric method the HCl-concentration (4 M), temperature (70 °C) and treatment time (10min) were identical as described for maximal extractability, so the set-up of the analysis should not have had an impact on the results, according to Sarada et al. (2006).

The results from the two different analyse methods show large variations, as shown in Table 5. The HPLC-results suggested that the Kuhl-cultures contained more astaxanthin per cell than the Enriched processed-cultures, which are contrary to the spectrophotometrically results. The main difference between these cultures is the quantity of PO_4-P, NH_4-N and NO_3-N which may explain the differences. From nutrient analysis, it is shown that the Kuhl-cultures had higher concentrations of PO_4-P, NH_4-N and NO_3-N, which strengthens the idea that the amount of nutrient directly affects the content of chl *a*. Begum et al., (2016) states that a N-starvation in an algae culture may be visible as decreased amount of chlorophyll, since the chlorophyll molecule has four N-atoms in its structure. According to the high chl *a* result, this indicates that the Kuhl-cultures were suffering from a N-limitation, and not necessarily a P-limitation.

A study by Panis et al., (2016) shows an astaxanthin accumulation to be induced by nutrient depletion. Since the initial amount of nutrients in the Processed water-cultures is lower than the two other cultures, it is conceivable to indicate a higher amount of astaxanthin in these cultures, see Appendix II. However, results from the HPLC-method show that the Processed water-cultures had quite low astaxanthin content compared to the Kuhl-cultures. This could also be explained by methodical errors. In the HPLC-method, the samples were vacuum filtered before use in analysis, while harvested and centrifuged samples were used for the spectrophotometric

method. It is suggested for this experiment that the amount of dry mass differs between the two methods and that the amount collected for the HPLC-method was too low for a precise determination of pigments.

5 Concluding remarks

The cellular growth determined in this batch culture-experiment was as expected according to the growth phase diagram shown in Figure 1. The experiment has shown that the parameters Qy, Ft and turbidity can be used as proxy for cell density, with turbidity having the highest correlation. All cultures showed cellular growth with similar μ_{max} between the Kuhl- and the Processed water-cultures. μ_{max} was determined quite early in all three cultures indicating that the cultures relatively quickly got a less optimal environment for growth. The cell density and μ_{max} of the Processed water-cultures shows a possibility of using the industrial processed water for cultivation of *H. lacustris*. However, compared to the Kuhl-cultures it cannot be defined as suitable according to the first hypothesis, presented in Section 1.3. Based on these results, it is suggested that the cultures should be kept longer in the exponential phase in order to increase the biomass.

Large variation between the Processed water-cultures and the Kuhl-cultures suggests that higher amount of nutrients should be present in the industrial processed water in order to produce astaxanthin and chl *a*. The colouring of the cultures revealed that the Processed water-cultures never entered the green vegetative phase which is suggested needed to accumulate higher levels of pigments. Cultivation of *H. lacustris* for pigment production in industrial processed water without additional nutrients, as suggested in the second hypothesis, is therefore shown to be challenging.

This thesis has also shown that the amount of inorganic nutrients was reduced in the Processed water-cultures. This, along with the positive microalgae growth within the same cultures, indicate an opportunity of using *H. lacustris* for water treatment thus increasing its commercial interest.

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Appendix I

Illustration of the cell density (cells mL^{-1}) as a function of time ($days^{-1}$) in the three different cultures.

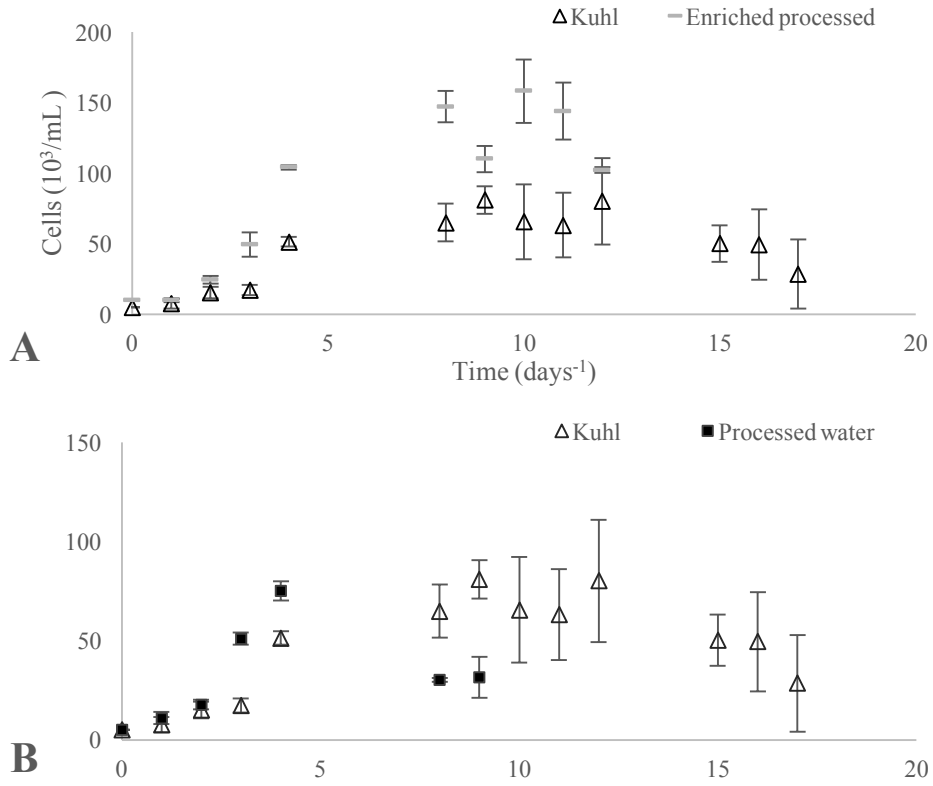

Figure 19 Number of cells from day 0 until day 18. Kuhl-cultures shown in both A and B. ***A****: Number of cells (cells mL^{-1}) over time ($days^{-1}$).* $n_{Kuhl} = 2$ *and* $n_{Enriched\ processed} = 3$. ***B****: Number of cells (cells mL^{-1}) over time (days).* $n_{Kuhl} = 2$ *and* $n_{Processed\ water} = 3$.

Appendix II

Results of nutrient analysis of NH_4-N, NO_3-N and PO_4-P (µg/cell) from the three different cultures.

Table 6 Amount of NH_4-N, NO_3-N and PO_4-P (µg/cell) determined in the three different cultures. Results are shown as mean±SD, $n_{Kuhl} = 2$, $n_{Enriched\ processed} = 3$ and $n_{Processed\ water} = 3$.

	Kuhl	**Enriched processed**			**Processed water**	
Day	*1*	*1*	*7*	*10*	*1*	*7*
NH_4-N [2]		0.011	$5.5x10^{-6} \pm 6.0x10^{-7}$	$8.8x10^{-6} \pm 3.4x10^{-6}$	0.011	$1.5x10^{-5} \pm 6.7x10^{-7}$
NO_3-N [1]	0.31	0.039			0.028	
NO_3-N [2]		0.112	$5.7x10^{-4} \pm 9.3x10^{-4}$	$3.4x10^{-4} \pm 5.3x10^{-4}$	0.0014	$< 5.8x10^{-6}$
PO_4-P [1]	0.32	0.04			0.014	
PO_4-P [2]		0.083	0.0032 ± 0.02	0.05 ± 0.03	0.42	0.09 ± 0.02
PO_4-P [3]		0.0028	$1.5x10^{-4} \pm 4.1x10^{-6}$	$1.6x10^{-4} \pm 3.9x10^{-6}$	0.0018	$9.4x10^{-4} \pm 4{,}1x10^{-5}$
N:P ratio*	0.97	1.35	0.21	0.21	0.10	$1.8x10^{-4}$

*N:P-ratio calculated from NO_3-N [2] and PO_4-P [2], except ratio calculated in Kuhl where NO_3-N [1] and PO_4-P [1] were used. [1]: Results from industrial processed water and Kuhl-media. [2]: Results from Eurofins-report from day 1, 7 and 10. No results from Processed water-culture on day 10. [3]: Results from PO_4-P measurements by Kjersti Andresen at TBS, NTNU.

Appendix III

Kuhl medium – "1x Kuhl"

The growth media used in this experiment were modified from Kuhl and Lorentzen (1964).

	Mixing volume (L)	g/L	hydration
	Solution A		
Potassium Nitrate	KNO_3	101,1	
Calcium Chloride	$CaCl_2$	1,47	
	Solution B		
Magnesium Sulfate Hepta Hydrate	$MgSO_4*7H_2O$	24,66	
	Solution C		
MonoSodium Phosphate Anhydrous	NaH_2PO_4	54	anhydrous
DiSodium Phosphate Dihydrous	$Na_2H_2PO_4*2H_2O$	8,9	dihydrous
	Solution D		
Boric Acid	H_3BO_3	0,061	
Manganese Sulfate MonoHydrous	$MnSO_4*H_2O$	0,169	
Zinc Sulfate MonoHydrous	$ZnSO_4*H_2O$	0,287	
Copper Sulfate PentaHydrous	$CuSO_4*5H_2O$	0,0025	
Ammonium molybdate tetrahydrate	$(NH_4)_6MO_7O_{24}*4H_2O$	0,0124	
	Solution E		
Iron Sulfate MonoHydrate	$FeSO_4*H_2O$	6,95	
Ethylene Diaminetetra Acetic Acid	EDTA	9,3	

Solutions A, B, C, D and E are all stock solutions.

To mix the medium that can be used for cultivation, do the following:

- Mix 10 mL of solution A, B and C and 1 mL of D and E into a final volume of 1 L water. Add first plenty of water to avoid chemical reactions.

Appendix IV

Analysis report performed by Eurofins of the industrial processed water used in this experiment.

eurofins

SWEDAC ACKREDITERING 1125 ISO/IEC 17025

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AR-17-SL-194162-01

EUSELI2-00462057
Kundnummer: SL8461182

Analysrapport

Provnummer:	177-2017-09121432	Ankomsttemp °C	12,9
Provbeskrivning:		Provtagningsdatum	2017-08-28
Matris:	Avloppsvatten		
Provet ankom:	2017-09-12		
Utskriftsdatum:	2017-10-16		
Provmärkning:	NO-SeWa-Non_1-1		

Analys	Resultat	Enhet	Mätо.	Metod/ref	
Klorid	27	mg/l	10%	SS-EN ISO 10304-1:2009	b)
TOC	60	mg/l	10%	SS EN 1484:1997	b)
Ammonium-nitrogen (NH4-N)	4.3	mg/l	15%	SS-EN 11732:2005	b)
Fosfor P	0.68	mg/l	10%	SS-EN ISO 15681-2:2005	b)
Kväve N	5.8	mg/l	10%	ISO 29441:2010	b)
Natrium Na (uppslutet)	210	mg/l	20%	SS-EN ISO 15587-2 utg 1 / SS-EN ISO 11885:2009 ut	b)
Kalium K (uppslutet)	23	mg/l	20%	SS-EN ISO 15587-2 utg 1 / SS-EN ISO 11885:2009 ut	b)
Kalcium Ca (uppslutet)	100	mg/l	15%	SS-EN ISO 15587-2 utg 1 / SS-EN ISO 11885:2009 ut	b)
Järn Fe (uppslutet)	0.66	mg/l	20%	SS-EN ISO 15587-2 utg 1 / SS-EN ISO 11885:2009 ut	b)
Magnesium Mg (uppslutet)	5.3	mg/l	20%	SS-EN ISO 15587-2 utg 1 / SS-EN ISO 11885:2009 ut	b)
Mangan Mn (uppslutet)	1.2	mg/l	20%	SS-EN ISO 15587-2 utg 1 / SS-EN ISO 11885:2009 ut	b)
Aluminium Al (uppslutet)	0.040	mg/l	15%	SS-EN ISO 15587-2 utg 1/SS-EN ISO 17294-2 utg1 mo	b)
Antimon, Sb (uppslutet)	< 0.0010	mg/l	25%	SS-EN ISO 15587-2 utg 1/SS-EN ISO 17294-2 utg1 mo	b)
Arsenik As (uppslutet)	< 0.00050	mg/l	35%	SS-EN ISO 15587-2 utg 1/SS-EN ISO 17294-2 utg1 mo	b)
Barium Ba (uppslutet)	0.19	mg/l	20%	SS-EN ISO 15587-2 utg 1 / SS-EN ISO 11885:2009 ut	b)*
Bly Pb (uppslutet)	< 0.00050	mg/l	35%	SS-EN ISO 15587-2 utg 1/SS-EN ISO 17294-2 utg1 mo	b)
Bor, B (uppsluten)	0.16	mg/l	20%	SS-EN ISO 15587-2 utg 1 / SS-EN ISO 11885:2009 ut	b)
Kadmium Cd (uppslutet)	< 0.00010	mg/l	35%	SS-EN ISO 15587-2 utg 1/SS-EN ISO 17294-2 utg1	b)

Förklaringar

AR-003v46

Laboratoriet/laboratorierna är ackrediterade av respektive lands ackrediteringsorgan. Ej ackrediterade analyser är markerade med *

Mätosäkerheten, om inget annat anges, redovisas som utvidgad mätosäkerhet med täckningsfaktor 2. Undantag relaterat till analyser utförda utanför Sverige kan förekomma. Ytterligare upplysningar samt mätosäkerhet och detektionsnivåer för mikrobiologiska analyser lämnas på begäran.

Denna rapport får endast återges i sin helhet, om inte utförande laboratorium i förväg skriftligen godkänt annat. Resultaten relaterar endast till det insända provet.

d

				mo	
Kobolt Co (uppslutet)	0.0036	mg/l	20%	SS-EN ISO 15587-2: utg 1/SS-EN ISO 17294-2 utg1 mo	b)
Koppar Cu (uppslutet)	0.0060	mg/l	15%	SS-EN ISO 15587-2: utg 1/SS-EN ISO 17294-2 utg1 mo	b)
Krom Cr (uppslutet)	0.0022	mg/l	25%	SS-EN ISO 15587-2: utg 1/SS-EN ISO 17294-2 utg1 mo	b)
Molybden, Mo (uppslutet)	0.0051	mg/l	40%	SS-EN ISO 15587-2: utg 1/SS-EN ISO 17294-2 utg1 mo	b)
Nickel Ni (uppslutet)	0.0010	mg/l	25%	SS-EN ISO 15587-2: utg 1/SS-EN ISO 17294-2 utg1 mo	b)
Selen, Se (uppslutet)	< 0.0030	mg/l	35%	SS-EN ISO 15587-2: utg 1/SS-EN ISO 17294-2 utg1 mo	b)
Silver Ag (uppslutet)	< 0.00050	mg/l	20%	SS-EN ISO 15587-2: utg 1/SS-EN ISO 17294-2 utg1 mo	b)
Svavel, S (uppslutet)	120	mg/l	20%	SS-EN ISO 15587-2: utg 1 / SS-EN ISO 11885:2009 ut	b)
Titan, Ti (uppslutet)	< 50	µg/l	20%	SS-EN ISO 15587-2: utg 1 / SS-EN ISO 11885:2009 ut	b)
Vanadin V (uppslutet)	0.0018	mg/l	30%	SS-EN ISO 15587-2: utg 1/SS-EN ISO 17294-2 utg1 mo	b)
Wolfram, W (syrauppslutet)	0.0025	mg/l	20%	SS-EN ISO 15587-2: utg 1/SS-EN ISO 17294-2 utg1 mo	b)*
Zink Zn (uppslutet)	0.019	mg/l	15%	SS-EN ISO 15587-2: utg 1/SS-EN ISO 17294-2 utg1 mo	b)
Kväve Kjeldahl	7.2	mg/l	10%	SS-EN 25663:1994	a)
Kemisk kommentar Pga misstag på lab har analys av Hg uppslutet ej kunnat utföras på detta prov.					

Utförande laboratorium/underleverantör:

a) Eurofins Food & Feed Testing Sweden (Lidköping), SWEDEN
b) Eurofins Environment Testing Sweden AB, SWEDEN

Paola Nilson, Rapportansvarig

Denna rapport är elektroniskt signerad.

e