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Optimized cold tolerant microalgae cultivation in photobioreactors

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Abstract

The aim of this research was to study selected cold tolerant algal species that can be grown at 6°C. *Chlamydomonas pulsatilla*, *Koliella sempervirens*, *Chloromonas platystigma*, *Chlamydomonas klinobasis* and *Macrochloris rubrioleum* have been cultivated to gather knowledge about their biochemical compositions, and describing their fatty acid and pigment content, which may be of the interest to aquaculture located in colder climates. The results indicate that all of these strains can be grown successfully under cold conditions with the recommended medium compositions of the culture bank. Their respective dry biomass yield after a 20 day cultivating period is between 2–4 g/l. The fatty acid data indicates that most of them produce PUFAs, such as C16:3 and 4, C18:2 and 3 (all strains), C18:4 is found in *C. platystigma*, *M. rubrioleum* and *K. sempervirens*, and the latter produces the very long PUFA C20:5. The oil content indicates that the highest average of lipids per dry biomass are found in *C. pulsatilla* (39%) and the lowest in *K. sempervirens* (28%) over a 20 day cultivation period. The major pigments produced by each strain was chlorophyll a (*C pulsatilla*: 31.5% of total pigments detected, *K. sempervirens*: 33%, *C. platystigma*: 23.7%, *C. klinobasis*: 29.9%) followed by lutein/zeaxanthin or other chlorophylls. The same was found in *M. rubrioleum* (41.5% of chlorophyll a of total pigments in 10 days and 35.2% in 20 day periods), however it produces secondary carotenoids that are not related to astaxanthin. The experiments with *Oocystis alpina* were carried out in the recommended environmental conditions (20°C). The experimental results show that they produce 2.7 g/l dry amount of biomass within a 10 day cultivation period, and the lipid measurement resulted the highest amount of oil content in the nitrogen deprived culture (58% lipid content per grams of dry cell mass), and a significant difference (p=0.043) between the individual treatments. The pigment composition revealed that *Oocystis alpina* contains chlorophyll a in the highest amount (57.6 % per dry cell mass) followed by violaxanthin (8.4%) and dinoxanthin (6.2%).

Table of Contents

Acknowledgment....i
Abstract....ii
1.0 Introduction....1
2.0 Materials and methods....4
2.1 Cultivation....4
2.1.1 *Nannochloropsis* sp. CCAP 211/78....4
2.1.2 Cold strains....4
2.1.3 *Oocystis alpina* UTEX 2541 (Reisige 1964)....5
2.2 Harvesting....6
2.3 Dry weight measurement....6
2.4 Optical density measurements....6
2.5 Fatty acid analysis preparation....6
2.5.1 Method:....7
2.5.2 Lipid weight preparation....8
2.5.3 GC preparation method....8
2.6 Pigment analysis preparation....8
2.6.1 Method....8
2.6.2 HPLC method....9
3.0 Results....11
3.1 *Nannochloropsis* sp....11
3.2 Cold strains....13
3.2.1. Growth....13
3.2.2. Dry weight measurements....13
3.2.3. Fatty acid content....14
3.2.4 Oil content....15
3.2.5. Pigment content....17
3.3 *Oocystis alpina*....20
3.3.1. Growth....20
3.3.2. Dry weight measurements....21
3.3.3. Total lipid content of *Oocystis alpina*....22
3.3.4. Pigment content....23
4.0 Discussion....24
4.1 *Nannochloropsis* sp. experiments....24
4.2 Cold strain experiments....24
4.2.1. Growth....24
4.2.2 Oil content....25
4.2.3 Fatty acid....26
4.2.4 Pigments....27
4.3 *Oocystis alpina* experiments....28
4.3.1 Growth....28
4.3.2 Lipid content....29
5.0 Conclusion....31
6.0 References....32
7.0 Appendices....35

1.0 Introduction

In this century of increasing concern over sustainable sources of food and renewable energy, there is heightened interest in the cultivation of microalgae for commercial applications (Draaisma *et al.* 2013, Li *et al.* 2010, Spolaore *et al.* 2006). Microalgae are rich in cellulose, starch, oils, antioxidants and pigments (Gouveia and Empis 2003). They do not require complex cultivation systems and can be relatively inexpensive and simple to produce at scale (Chisti 2007). Being photosynthetic organisms, algae convert solar energy to chemical energy by converting atmospheric CO_2 into biomass (Richmond and Hu 2013). Hence, they are potentially a source of renewable and carbon-neutral biofuels, as well as feed stock both for animal and for human consumption (Chisti 2007).

It has been demonstrated that algae has a high photon conversion efficiency (increased biomass yield per hectare), can be harvested all year around (short harvesting cycle: 1-10 days), can utilize sea- and waste water (Feng *et al.* 2011). Microalgae is reported to produce 15-300 times more oils than traditional land crops on a per area basis (Chisti 2007) and it results in less waste and pollution. (Mallick 2002).

Landmass used for algae production does not have to be arable, which leads to new economic opportunities for drought or salinity-affected regions (Wijffels *et al.* 2010). By use of marine and halophilic species, one does not require readily available freshwater, and cold tolerant strains can thrive in frigid temperature ranges (6-10°C) where other plant production would be problematic.

Only plants, algae and some fungal and bacterial species are capable of synthesizing carotenoids, and so it must be supplied in the feed of higher animals (Davis 1985). Microalgae are natural producers of these pigments, and the renowned pink coloration of salmonid flesh is gained by the fish consuming a diet rich in the pigment astaxanthin. They are also potent antioxidants, and astaxanthins antioxidant properties have been shown to be approximately 10 times greater than those of β-carotene, lutein, zeaxanthin or cantaxanthin (Miki 1991). In the wild this is accumulated through the trophic chain, while farm raised salmonids are usually fed with synthetic astaxanthin. The production of which is expensive, accounting for 50% of the total production cost of salmon farming, so finding culture-based production alternatives is a primary interest of aquaculture. Achieving the desired coloration with algal feed is important in an other commercial aspects, since it can be sold as “eco” food: these fish are labeled as organic food and have a high color intensity (Olesen *et al.* 2010). Cheaper and natural alternatives to produce other products (oils, vitamins and antioxidants) is in high demand for organic fish farming (Alfnes *et al.* 2006).

Microalgae use a wide variety of photosynthetic pigments that vary greatly in color. The literature distinguish between green chlorophylls, yellow, orange and red carotenoids, and red and blue-green biliproteins. They are capable of harvesting light effectively in a wavelength range of the visible spectrum 350-750 nm (Jeffrey and Wright 2006). They are found in the light-harvesting complexes of the plastids (chloroplast) where the solar energy is captured and transferred into the reaction centers to initiate the chemical reactions that are essential to photosynthesis. Photochemically the absorbance is related to the number of conjugated double bonds and the functional groups that are found in the chemical structure of the pigment (Richmond and Hu 2013).

The ecophysiological role of secondary carotenoids like astaxanthin, canthaxanthin and echinenone is not yet fully understood, but they are known to play a role in light stress responses such as shielding the photosystem (protein complexes that play a role in photosynthesis) against excessive irradiation (Cohen 1999). With chromatography it is possible to distinguish between these pigments, which can be used as a major criteria in field sampling to determine the taxonomy of different algal species. Algal pigments are also commonly used as an alternative of chemical dyes, and the demand for natural sources is increasing, as it has been found that carotenoid extracts have higher anti-oxidant properties than synthetic ones (Arad and Spharim 1998, Levin *et al.* 1997).

Lipid productivity is measured as the product of biomass productivity (grams per dry weight per litre per day) and lipid content (% dry weight) to measure the oil production on a basis of time and volume (Griffiths and Harrison 2009). Studies have shown that the quality and quantity of the lipid yield can increase with changes in light intensity and temperature along with varying nutrient media characteristics such as nitrogen, phosphate and trace metals composition, however the responses are highly species specific (Gouveia and Oliveira 2008) (Li Y *et al.* 2008). Cultivating algae in limited nutrient conditions is reported to increase the number of saturated (18:0), monosaturated (18:1), polysaturated (18:2) and unsaturated *n*-3 fatty acids (Harrison *et al.* 1990), and increase the relative fatty acid content (Reitan *et al.* 1994, Yeh and Chang 2011).

The optimum algal strain would possess the following qualities: grow fast, produce high amount of fatty acids (LC PUFAs [long chain polyunsaturated fatty acids] that are used for fish feed EPA [icosapentaenoic acid] and DHA [docosahexaenoic acid]) and high amounts of carotenoids. The quantity of fatty acids is the most important factor in species selection of algae for first-feeding fish larvae (Reitan *et al.* 1997).

Cold climate strains are an underrepresented algal subgroup in culture research, but due to their evolutionary adaptations they might possess qualities that would be interesting for aquacultural use (LC PUFAs, secondary carotenoids) (Spijkerman *et al.* 2012). On the far northern hemisphere the summer period is not particularly warm, yet the sunlight is available during day and night. This

could present new opportunities for algal production for temperate regions and during the cold seasons (Leya *et al.* 2009), since the main limiting factor for algal biomass production is light availability. Studying and modeling the kinetics of these factors is essential for the prediction of the final mass that can be harvested.

Objectives:

(1) The species used in this study were as follows: *Chlamydomonas pulsatilla* (Wollenweber 1926), *Koliella sempervirens* (Chodat Hindák 1963), *Chloromonas platystigma* (Pascher Korshikov ex H.Ehl 1970), *Chlamydomonas klinobasis* (Skuja 1956), *Macrochloris rubrioleum* (Kawasaki and Nakama, 2015). The cold strain algae that are used in these experiments were collected from Svalbard in Northern Norway, and relatively little is known about them. The main aim of this study was to expand the understanding of these strains, cataloging growth rates in cold temperatures, what amount of lipids they produce and what kind of PUFAs they contain, and to produce a pigment profile of these species in optimum medium conditions.

(2) *Oocystis alpina* UTEX no. 2541 (Reisige 1964) is reported to be a cold tolerant strain that was isolated from Toolik lake in Alaska (a tundra pool). When the project was planned, this strain was suggested to be the part of the study due to a supposed high content of fatty acids and their pigment composition have some interesting elements (Egeland pers. commun.). The aim with this strain was to test its growth rate at room temperature, identify the pigments it contains and determine the amount of lipids they produce in a variety of medium conditions.

2.0 Materials and methods

2.1 Cultivation

2.1.1 *Nannochloropsis* sp. CCAP 211/78

F2 medium (Guillard and Ryther 1962, Guillard 1975) was used with filtered and autoclaved seawater which was collected from the bay in Mørkved. A multi-cultivator (Multi-Cultivator MC 1000-OD, Photon Systems Instrument with HAILEA PWM cooling unit) was used. The bioreactor recorded light absorbance data at 680 and 720 nm with given time intervals. The environmental factors (light, temperature, aeration) were kept stable by the instrument. Air was supplied by a pump at 80 ml/min. Temperature was controlled at 20°C. The irradiance was the same in every tube (100 µE) except #5, see Table 1 for details. The instrument had eight 85 ml tubes to operate with for a 14 or 20 day period. The first tube was marked as control while the remaining seven had different medium treatments.

Table 1: Experimental treatments of *Nannochloropsis sp.*

#	Treatment	Description
1	Control group	F2 medium with no changes
2	Increased nitrates	F2 medium with double amount of $NaNO_3$
3	Increased phosphates	F2 medium with double amount of NaH_2PO_4
4	Increased aeration	F2 medium with double amount of aeration, 160 ml/min
5	Increased illumination	F2 medium with double amount of irradiance, 200 µE
6	Increased trace metals	F2 medium with double amount of trace metals
7	Decreased nitrates	F2 medium with half amount of $NaNO_3$
8	Decreased phosphates	F2 medium with half amount of NaH_2PO_4

2.1.2 Cold strains

The species that were used in this study were *Chlamydomonas pulsatilla* (Wollenweber 1926), *Koliella sempervirens* (Chodat Hindák 1963), *Chloromonas platystigma* (Pascher Korshikov ex H.Ehl 1970), *Chlamydomonas klinobasis* (Skuja 1956), *M. rubrioleum* (Meneghini Nomen cons. 1842). The strains were provided by The Culture Collection of Cryophilic Algae (CCCryo, Fraunholfer IZI-BB, Potsdam-Golm, Germany). The experiments included a triplicate of samples that were grown for 10 and 20 day periods, with recommended medium composition and light intensity, at 6°C. Individual treatments were not used. 250 ml flasks were prepared with 2N-BBM

medium (Nichols and Bold 1965, Nichols 1973) for the cultivation of each species. The algae was transferred from an incubation tube into a flask, and let grow in a modified refrigerator with a fluorescent tube (daylight spectrum) mounted on the door. The temperature was maintained at 2–5°C and no oxygen or CO_2 supply was added. The flasks were perturbed on a daily basis to maintain a suspension. After four to five weeks the cultures are ready for further cultivation.

Twelve 350 ml glass tubes were autoclaved and prepared in two racks with 310 ml medium added into each (see Appendix 1 for the structure). Twenty ml of algae from the starter flasks was added into a tube for a top (A) and bottom (B) rack. The placement of the species were randomized on both shelf A and B. The tubes were sealed and the racks were placed into an incubator with long daylight bulbs mounted on the back. The light intensity was 135 µE average. Carbon dioxide enriched air (1% CO_2) was supplied to each tube at a flow rate of 60 ml/min. The temperature was kept at 6°C. After the algae is mixed well within the medium, 20 ml sample was taken out with 50 ml syringes from each tube for dry weight and density measurement with a spectrophotometer, and for nutrient analysis.

2.1.3 *Oocystis alpina* UTEX 2541 (Reisige 1964)

The algae was transferred from an incubation tube into a 250 ml flask prepared with BBM medium (Nichols and Bold 1965, Nichols 1973) for the cultivation, and let grow in room temperature, exposed partially to light and no oxygen or CO_2 supply was added. After four to five weeks the cultures are ready for the experiment.

Six 350 ml glass tubes were autoclaved and prepared in two racks with 310 ml medium added into each. With the exception of the temperature, which was kept at 20°C, the preparations for the experiment was the same as with the cold strains described above. Aeration was set to 80 ml/min, and the light intensity averaged 135 µE. The treatments were as described in Table 2, with tubes placed randomly:

Table 2: Experimental treatments of Oocystis alpina.

#	Treatment	Description
1	Control group	BBM medium with no changes
2	Decreased nitrates	BBM medium with half amount of $NaNO_3$
3	Decreased phosphates	BBM medium with half amount of K_2HPO_4 and KH_2PO_4
4	Descreased trace metals	BBM medium with half amount of trace metals
5	Increased aeration	BBM medium with double amount of aeration, 160 ml/min

2.2 Harvesting

The cultures were harvested by centrifuging into a pellet, and the supernatant discarded. The algae was transferred into a microcentrifuge tube and stored in the freezer in –20°C.

In the case of *Nannochloropsis* sp., after centrifuging the remaining sample was re-suspended in isotonic ammonium formate (30 g/l) to remove the salt, and centrifuged once more. The washing was repeated and the sediments were transferred into microvials and stored in the freezer in –20°C.

2.3 Dry weight measurement

The first batch was harvested after 10 days (group A), and the dry weight measurement repeated. After 20 days group B is harvested with the same method. 47 mm glass fibre filters (VWR Norway) with nominal pore size of 1 µm were weighed and used in each experiment. Eighteen ml algae (with culture medium) from the cultivating vials were drawn and filtered through with a help of a vacuum pump. A plate was prepared from aluminium foil and were marked with the ID's of each strain accordingly (A1–6, B1–6) and dried in an oven on 90°C for two days. After drying the weight of each plate was measured.

2.4 Optical density measurements

Every day 2 ml sample was drawn from the cultures to measure the optical density. A spectrophotometer (HACH DR 3900) was used to determine and record the biomass growth. The samples were transferred in 2 ml Eppendorf tubes: 1 ml of the sample was transferred into a plastic cuvette and used to measure the optical density at 540 and 680 nm. The data was registered and noted every second day during each experiment. After the optical density reached 1.0, the samples were diluted accordingly with distilled water. The samples were marked with the current date and correct strain ID.

2.5 Fatty acid analysis preparation

The method of the extraction procedure was described by Breuer *et al.* (2012). After the harvest of each strain, 2 ml Eppendorf tubes were filled with algal suspension and frozen at –80°C in preparation for freeze drying. A freeze dryer (SP Industries, INC. VirTis BenchTop “K” Series Freeze Dryer) was used to dry the samples, the sample tubes were opened on the top while the instrument ran for three days to complete the process. 8–12 mg of sample was weighed on a precision scale, using VWR Universal Fit Screw Caps with O-Rings as containers (VWR European)

and were transferred into 2 ml micro tubes (Freestanding micro tube with cap and graduations 2.0 ml, VWR European). The tubes were put for a minute into a centrifuge to settle the algae on the bottom. Approximately 0.2–0.3 ml, 0.1 and 1.0 mm glass beads (SI Cell Disruption Media) were added into the samples, the bigger diameter ones were used on the *M. rubrioleum* and *Oocystis alpina* samples due to difficulties in the extraction with those species.

2.5.1 Method:

1: One ml of a mixture of chloroform to methanol (4:5) was added into each sample, and the cap is secured on the microtubes.

2: A shaker (MagNA Lyser) was used at 6000 beats per min^{-1}, two times for 30 seconds. The samples were placed on ice to cool for a minute.

3: The samples were transferred into a centrifuge to settle the sample and the beads at 10.0 rpm on 6°C for 60 seconds.

4: The liquid phase was transferred into glass vials (Kimax culture tubes with screw caps 16×100mm) that were assigned into each sample without transferring any of the solid material or beads.

5: Step 1-4 was repeated 4 times in total, until all of the algae is transferred. At the final step, cell components might be transferred along.

6: Two and a half ml buffer solution (Tris buffer, Breuer *et al.* 2012) were added on the top to extract the methanol from the chloroform phase.

7: Each sample was secured with their caps and mixed with a vortex three times for 30 seconds to separate the chloroform phase to the bottom.

8: Samples were kept in the freezer until usage with the buffer on for lipid analysis.

The samples stood in –20°C in the freezer for 2–5 days to complete the extraction. They were vortexed again, then centrifuged at 2500 RPM for 1 minute. The chloroform phase was transferred into a new vial with a glass Pasteur pipette, taking care to avoid adding debris. One ml chloroform was added to the original sample which was followed by centrifuging and transferring of the chloroform phase. The method was repeated 4 times in total until all the samples were transferred, and the chloroform phase lost all of its color.

2.5.2 Lipid weight preparation

The vials were placed into an evaporator (Reacti-Therm I #TS-18822 Heating Module) and all the chloroform was evaporated with a steam of gasous nitrogen. After the vials were dry, 0.5 ml chloroform was added and was centrifuged at 3900 rpm for 5 minutes. HPLC microvials (Agilent Vial insert, 250 µl pulled pointed glass) were weighed and have been assigned to each sample. The microvials were placed into common HPLC vials (Agilent Technologies, 2 ml vials with cap) to keep vertical. Three times 90 µl (270 µl total) sample was transferred into the microvials and the solvent was evaporated with gasous nitrogen. The dried microvials were removed from the common vials and weighed on a balance.

2.5.3 GC preparation method

Fatty acid extraction and derivatization was performed as described by Breuer *et al.* (2013) using sample preparation method 2 (see in detail at 2.5.1), followed by the procedure of the fatty acid extraction step 25-35. Fatty acid methyl esters (FAMEs) were separated and quantitated using a gas chromatograph fitted with a flame ionization detector (Scion GC-FID, Bruker, USA). The column used for the analysis was an Agilent DB-23 column. Linear calibration was performed using a Supelco 37 component FAME standard and additional (C16:2, C16:3, C18:4) FAME standards (Larodan, Sweden).

2.6 Pigment analysis preparation

2.6.1 Method

The procedure was identical to that described at 2.5.1, with the following additional steps. For the pigment preparation the fume hood was kept as dark as possible, and the samples were kept in the darkness whenever possible to avoid pigment degradation. After step 7 (2.5.1), the buffer solution was removed from the top of the samples in the same day the samples were prepared. The sample was transferred into a pear shaped vial and chloroform phase was evaporated with a rotary-evaporator. Before the vial was taken off, it was flushed with N_2, and 0.5 ml propan-2-one was added to dissolve the remaining pigment sample. The samples were filtered through 0.2 µm filters (GHP Acrodisc® 13 mm Syringe Filter with 0.2 µm GHP Membrane, PN 4554T) to remove any particulate debris. The samples were pipetted into a plungerless 2 ml BD Plastipak Syringe, and the filter was placed on the mouth of the syringe. A brown HPLC vial (Agilent Technologies, 2 ml Vials with cap) was placed under the filter, and the plunger was placed back to the syringe. With care the

sample was pressed trough the filter into the HPLC vial, and flushed with nitrogen before sealing with a cap.

2.6.2 HPLC method

The method was a modified version of the UN Method – The Fifth SeaWIFS HPLC Analysis Round-Robin Experiment (SeaHARRE-5)(Egeland 1995) with a modification to the solvent composition, adding at least 1% hexane during the whole analysis, which has been reported to improve the separation (Lundales *et al.* 2014). An Agilent 1200 HPLC instrument (including a vacuum degasser, thermostated autosampler with enlarged injection loop, quarternary pump, thermostated column compartment and diode array detector) was used to analyze the samples. The eluents were A: 1.0 M ammonium acetate, B: methanol, C: propan-2-one, D: hexane. Two identical C_{18} columns coupled after each other and were used to separate the pigments (ACE 5 C_{18} part no. ACE-121-2564, 4.6×250 mm each with 5 µm packing) with a separate guard column (ACE), and were kept at 25°C with a flow rate 0.5 ml/min during the analysis. The samples were placed into the autosampler at 4°C with the illumination turned off. The injection volume was set to 5 µl, and the run time was 130 minutes per sample. The detection wavelengths were 390 (DAD1 A), 420 (DAD2 B), 450 (DAD3 C) and 480 nm (DAD4 D). Table 3 summarizes the solvent composition and timing of the eluents.

Table 3: Solvent table for the method used for the pigment analysis. The eluents were A: 1.0 M ammonium acetate, B: methanol, C: propan-2-one, D: hexane.

Time	A [%]	B [%]	C [%]	D [%]
0	19.9	80	0	0.1
60	0	70	29.9	0.1
100	0	30	50	20
110	0	0	40	60
120	0	99.9	0	0.1
130	19.9	80	0	0.1

Calibration of quantification was done before the sample analysis. The pigments were determined by their retention time and spectrum compared with their respective external standards. Dry pigments were dissolved in known amounts of solvents using a measuring pipette, and 5th fold dilutions were recorded by a common spectrophotometer. All five dilutions were tested in the HPLC

instrument, and the calibration line was made by the program ChemStation. The standards used for the calibration were from the institute's collection.

3.0 Results

3.1 *Nannochloropsis* sp.

The main goal was to model growth over a 14 day period with different medium composition treatments, and determine whether there is a significant difference in outcome.

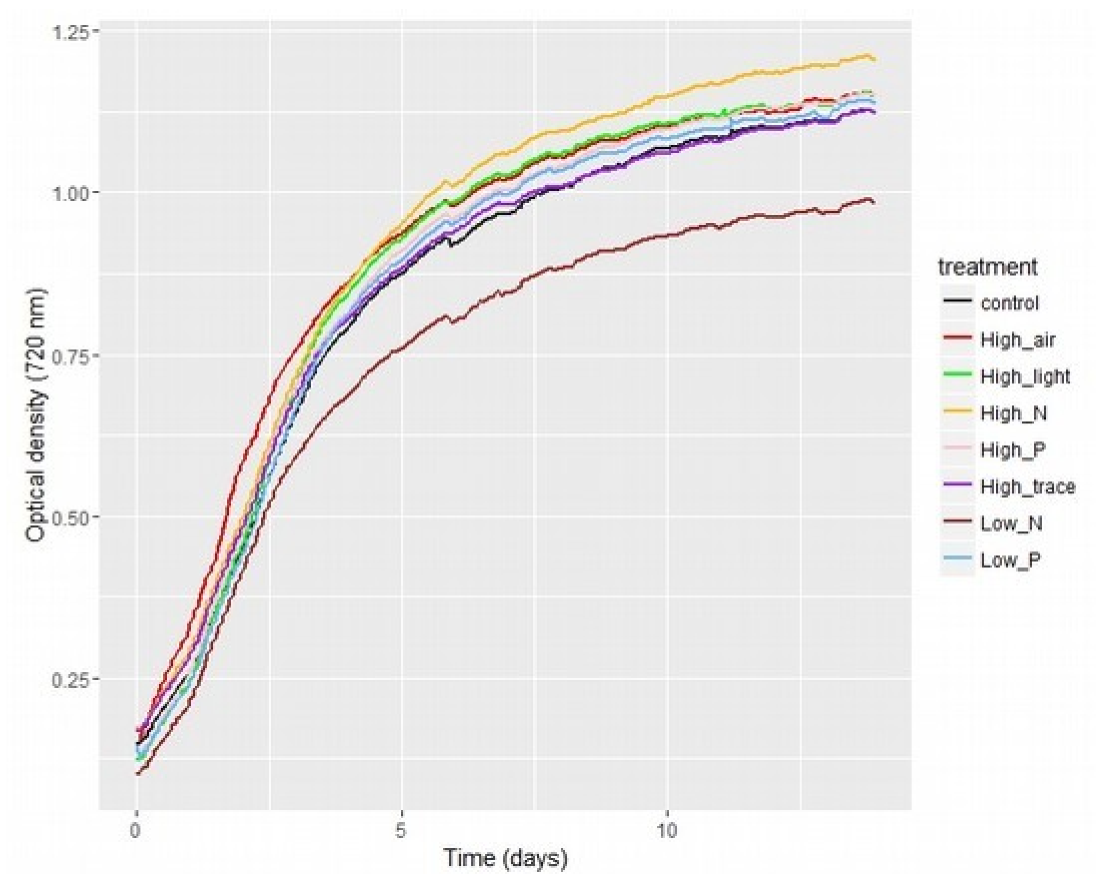

Fig 1: Averaged growth rates of the three *Nannochloropsis* sp. experiments.

As illustrated in Figure 1, the recorded optical density data indicates that the highest growth and biomass amounts was achieved with the high nitrate sample, while the algae that was treated with low nitrate resulted in a slower growth and less biomass at peak. The other samples had similar outcomes to the control group.

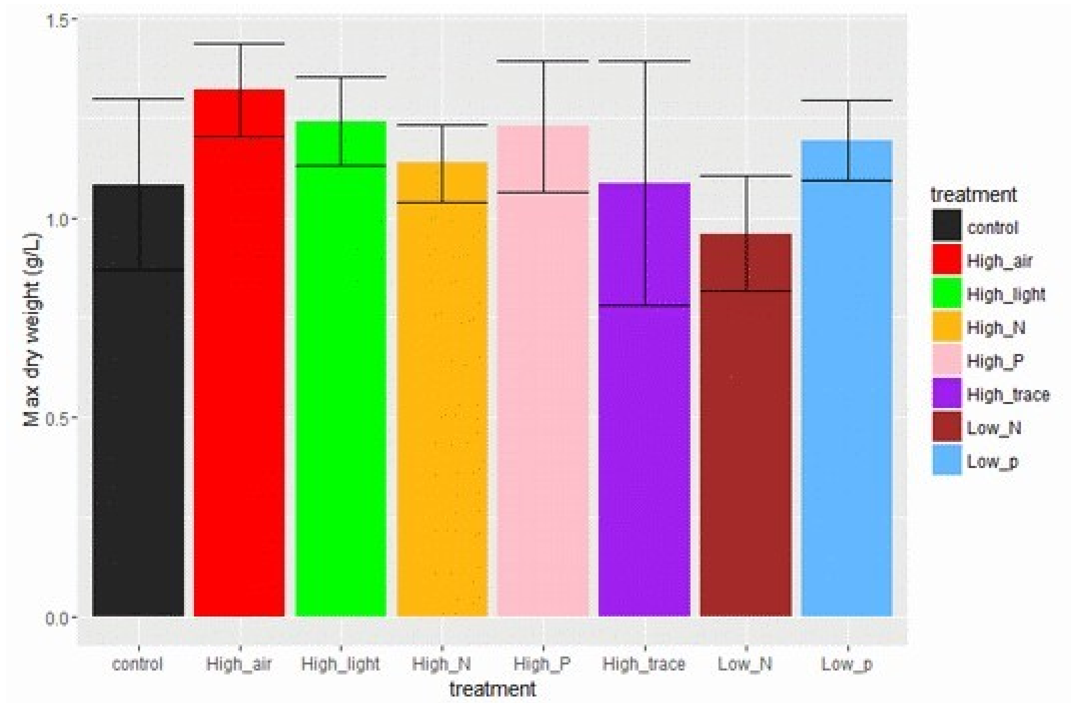

Figure 2: Averaged dry weight measurements (g/l) of *Nannochloropsis* sp. Bars show maximum dry weight as averages of three samples. Error bars indicate the standard deviation.

Figure 2 shows the averaged dry weight measurements of the three runs. The figure displays that the increased aeration treatment has produced the most dry biomass amounts, while the low nitrate treatment resulted in the least amount of dry biomass. The error bars in the figure indicate the standard deviation. Bars show maximum dry weight as averages of three samples. To verify, a one-way ANOVA test was performed on the dry weight data. Table 4 summarizes the results.

Table 4: One-way ANOVA test carried out on dry weight measurements of Nannochloropsis. The test returns a *p* value of >0.05 and the H_0 is accepted based on the data analysis.

	df	**Sum Sq**	**Mean Sq**	**F value**	***p***
treatment	7	0.270	0.0385	1.33	0.301
residuals	16	0.465	0.029		

The null hypothesis assumes no relationship between the treatment variables and measured biomass outcomes. Because the *p* value is higher than the critical value 0.05 the null hypothesis is accepted. The different medium compositions did not effect significantly the final biomass yield of the cultures.

3.2 Cold strains

3.2.1. Growth

Three sets of 10 and 20 day growth cycles were studied with each cold strains. Figure 3 illustrates the recorded optical density measurements.

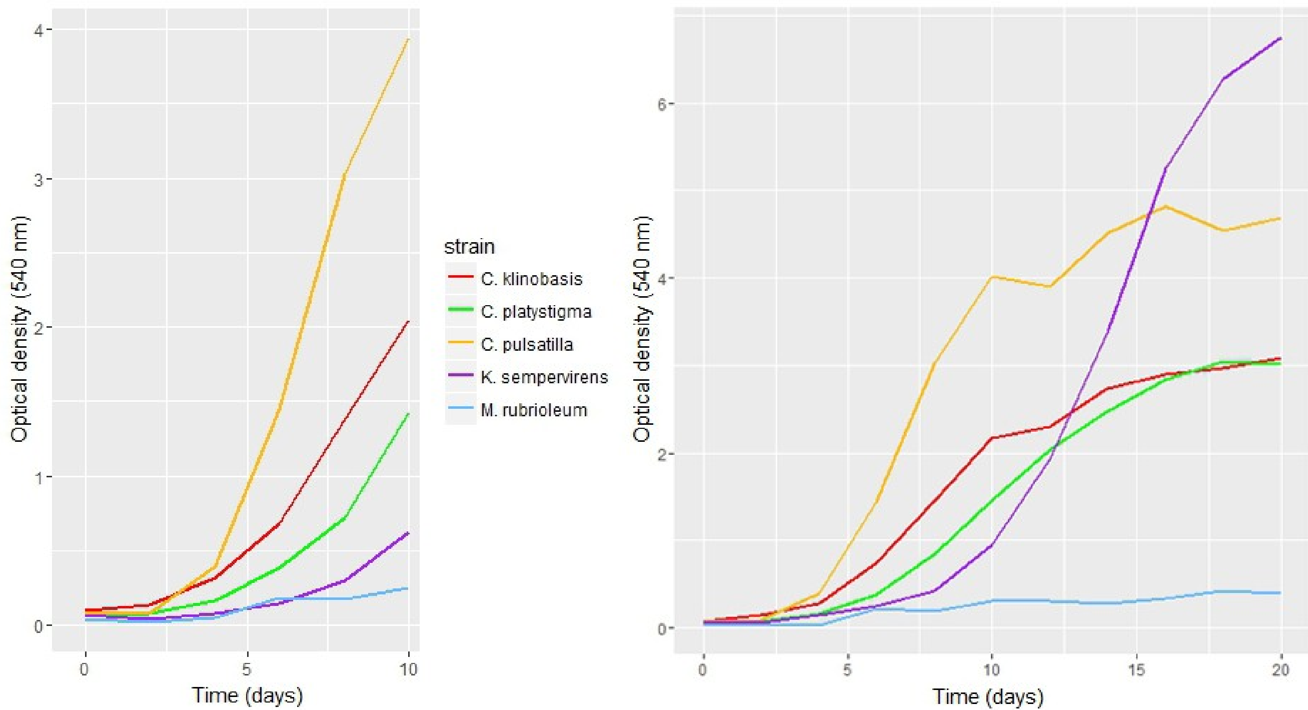

Figure 3: Averaged growth curves of the cold strain experiments. The first set shows the 10 day cultures while the second set to the right shows the 20 day cultures. The data in each panel is the mean optical density (540 nm) of three replicate growth curves.

3.2.2. Dry weight measurements

Figure 4 illustrates the mean dry weights (g/l) measured after the 20 day cultivation period. The data indicates that *C. pulsatilla* increased the most amount of biomass over the given period while *M. rubrioleum* produced the least amounts.

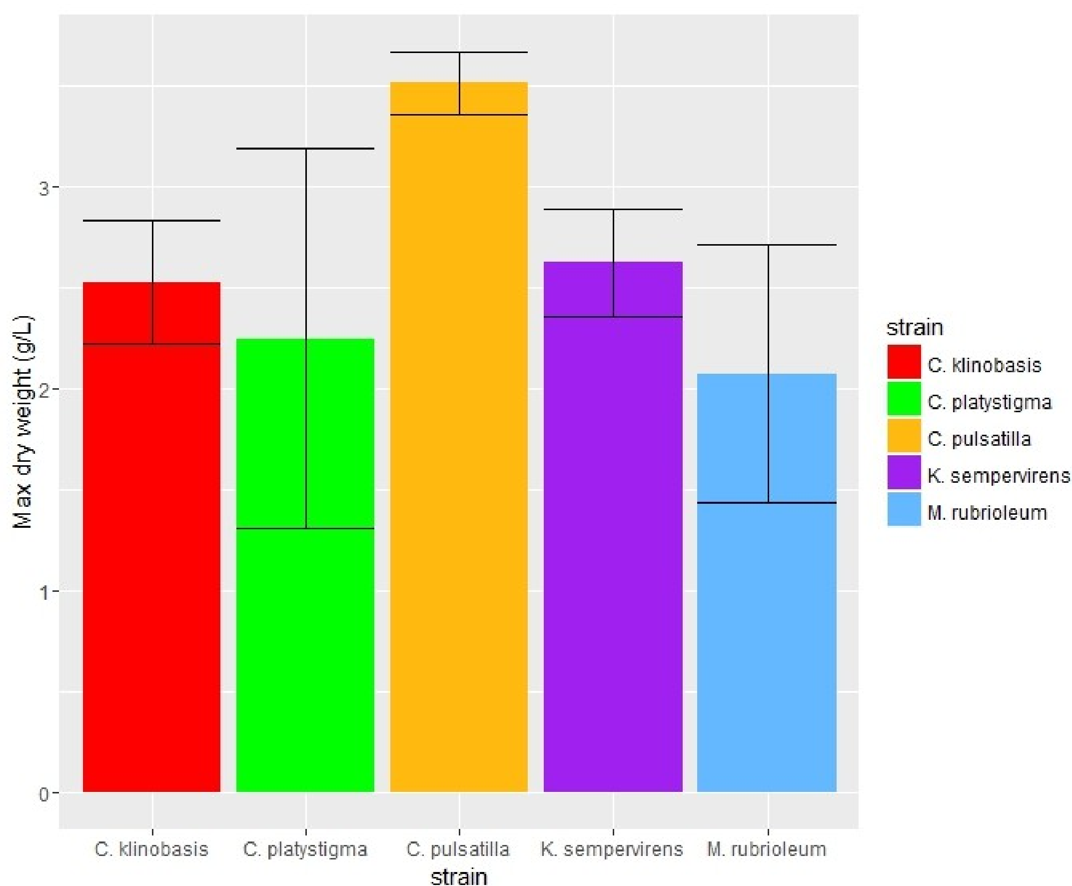

Figure 4: Averaged dry weight measurements (g/l) of the cold strains that grew for a 20 day period. Bars show maximum dry weight as averages of three samples. Error bars indicate the standard deviation.

A one-way ANOVA was carried out and the results are shown in Table 5. The *p* value is above the critical value 0.05, and hence there is no statistically significant difference between the amount of biomass growth of the cold tolerant strains.

Table 5: One-way ANOVA test carried out on dry weight measurements of cold strain algae. The test returns a p value of >0,05 that indicates no statistically significant variance of growth rate means.

	df	Sum Sq	Mean Sq	F value	*p*
strain	4	3.723	0.93	3.13	0.065
residuals	10	2.97	0.30		

3.2.3. Fatty acid content

Table 6 summarizes the results of the gas chromatography. The results are averaged from the triplicate 10 and 20 day data recorded. The header 'Total' shows the mean total amount of fatty acids

found within each strain (mg/g), and the percentage (%) shows the mean total percentage of fatty acid found in each treatment.

Table 6: Summary of the averaged fatty acid content of the different cold strains. The amounts in the bracket indicate standard error (n=3 replicate cultures). 'Total' shows the averaged total amount found within each strain (mg/g), and the percentage (%) shows the averaged total percentage of fatty acid found in the strain. Fatty acids marked with 'b' are isomers.

Strain	Day	C14:0	C16:0	C16:1	C16:2	C16:3	C16:3[b]	C16:4	C18:0
C. klinobasis	10	2 (2)	10 (2)	7 (2)	10 (4)	8 (4)	29 (7)	16 (3)	15 (0)
C. klinobasis	20	8 (2)	19 (1)	10 (0)	20 (1)	11 (3)	55 (9)	30 (4)	16 (1)
C. platystigma	10	-	4 (1)	8 (3)	-	14 (1)	-	11 (2)	16 (0)
C. platystigma	20	-	11 (4)	9 (0)	17 (1)	25 (7)	4 (4)	41 (10)	16 (0)
C. pulsatilla	10	1(0)	10 (3)	4 (0)	8 (0)	4 (4)	27 (2)	22 (4)	16 (1)
C. pulsatilla	20	6 (1)	25 (2)	4 (0)	8 (0)	6 (0)	50 (4)	45 (1)	16 (1)
M. rubrioleum	10	-	7 (2)	6 (3)	11 (5)	14 (1)	-	8 (1)	16 (1)
M. rubrioleum	20	-	18 (3)	5 (0)	16 (1)	16 (1)	6 (0)	9 (1)	16 (1)
K. sempervirens	10	-	5 (0)	-	9 (0)	13 (4)	-	3 (1)	18 (0)
K. sempervirens	20	-	11 (0)	4 (0)	17 (1)	15 (1)	-	1 (0)	16 (1)

Strain	Day	C18:1	C18:1[b]	C18:2	C18:3	C18:4	C20:5	Total	%
C. klinobasis	10	21 (2)	10 (4)	16 (3)	15 (1)	-	-	161 (25)	16 (1)
C. klinobasis	20	34 (3)	14 (4)	33 (6)	22 (1)	-	-	271 (26)	27 (3)
C. platystigma	10	20 (1)	18 (1)	7 (2)	23 (2)	7 (2)	-	129 (8)	13 (1)
C. platystigma	20	33 (5)	25 (3)	13 (2)	66 (21)	4 (0)	-	265 (50)	26 (5)
C. pulsatilla	10	23 (1)	8 (0)	21 (3)	22 (3)	-	-	167 (16)	17 (2)
C. pulsatilla	20	33 (2)	17 (1)	43 (2)	39 (2)	-	-	292 (11)	29 (1)
M. rubrioleum	10	26 (7)	22 (3)	18 (6)	21 (2)	10 (1)	-	157 (30)	16 (3)
M. rubrioleum	20	74 (13)	34 (5)	47 (10)	23 (2)	11 (1)	-	275 (38)	27 (4)
K. sempervirens	10	26 (4)	-	8 (3)	17 (1)	11 (0)	12 (1)	123 (8)	12 (1)
K. sempervirens	20	67 (7)	-	14 (1)	20 (2)	7 (2)	10 (1)	182 (5)	18 (0)

3.2.4 Oil content

Triplicate samples from ten and twenty day cultures were prepared for oil content analysis, and their averaged percentage results can be found in Figure 5. Error bars indicate the standard deviation of the mean.

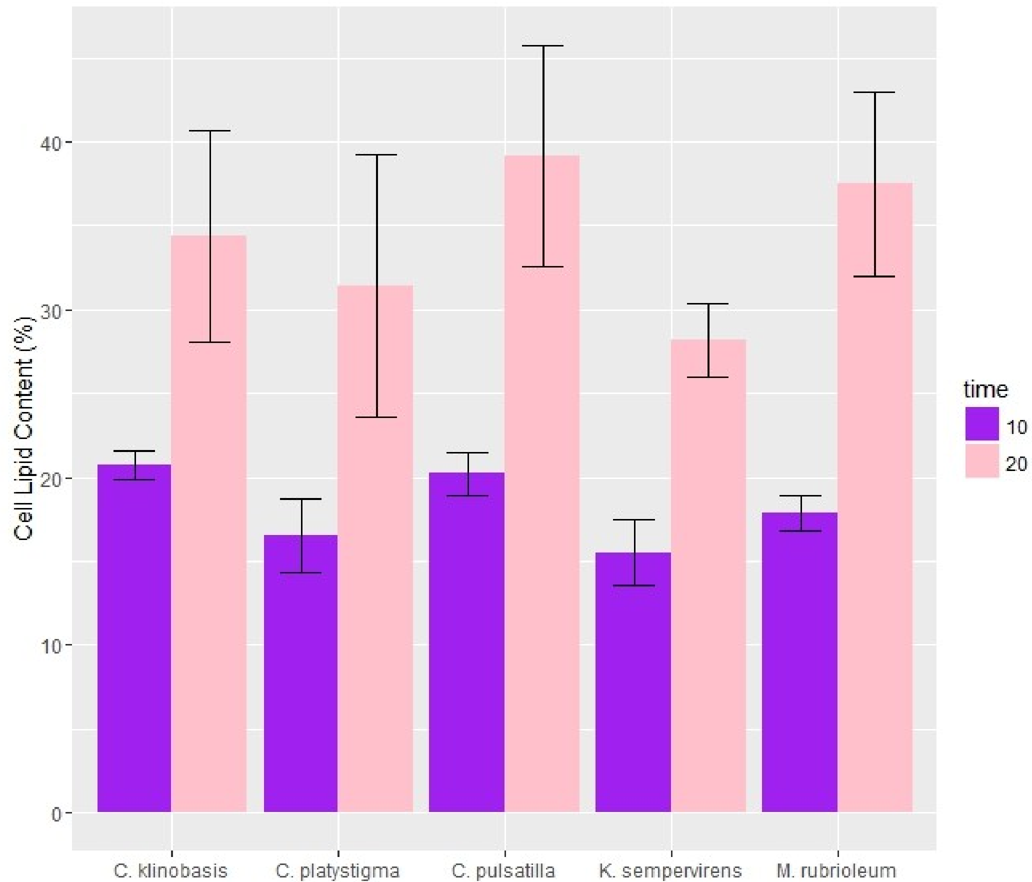

Figure 5: 10 and 20 day oil percentage yields by each cold strains. Colored bars and error bars indicate the mean and the standard deviation of the total cell lipid content (% dry weight).

A two-way ANOVA was performed, and the results indicate that there is a statistically significant difference between the oil percentage yield between the strains (p<0.001) and a statistically significant difference between the oil yields of the 10 day and 20 day samples (p<0.001). Table 7 summarizes the ANOVA results.

Table 7: Two-way anova test results carried out on 10 and 20 day averaged oil weight percentages of cold strains.

	df	**Sum Sq**	**Mean Sq**	**F value**	***p***
strain	4	478.9	119.7	6.2	<0.001
time	1	3825.2	385.2	199.4	<0.001
strain:time	4	118.0	29.5	1.5	0.205
residuals	50	959.0	19.2		

3.2.5. Pigment content

Within each of the following pigment content summary tables, amounts are given in nanograms of pigments detected within the sample that is prepared from the amount of dry cell mass measured in miligrams. The percentage ratios are computed from the amount of each pigment (ng) found in the total amount of pigments detected (ng). The pigment marked as unknown stands for the carotenoids that have astaxanthin-like peaks (see description in detail at 3.2.4.2.). The measured values that the instrument recorded (three individual values in ng) are added together to be able to describe the percentage amount within the sample.

3.2.5.1. Typical Green algal strains

The result chromatographs can be seen as follows: *Chlamydomonas pulsatilla (*Appendix 3), *Koliella sempervirens (*Appendix 4), *Chloromonas platystigma* (Appendix 5), *Chlamydomonas klinobasis* (Appendix 6). Table 8 summarizes the amount of pigments and their respective percentage ratio that were found in each typical green strain. The values are calculated from 20 day old samples.

The major pigments found was chlorophyll a in each strain (*C pulsatilla*: 31.5% of total pigments detected, *K. sempervirens*: 33%, *C. platystigma*: 23.7%, *C. klinobasis*: 29.9%) followed by lutein/zeaxanthin or other chlorophylls. 7.3% of violaxanthin (of total pigments detected) was found in *K. sempervirens,* which is a relatively high amount of orange pigment compared to the other cold strains.

Table 8: Summary of the pigment composition of the typical green algal strains. The list is based on retention time. Amounts are measured by pigment weight (ng) against amount of dry cell mass (mg). 'Nd' indicates lack of that certain pigment within the species, 'd' indicates that a certain peak was found but no calibration was done for it, while 'na' stands for not available.

Pigments	*C. pulsatilla*		*K. sempervirens*		*C. platystigma*		*C. klinobasis*	
	Amount (ng/mg)	%	Amount (ng/mg)	%	amount (ng/mg)	%	Amount (ng/mg)	%
Chlorophyllide b	d	na	d	na	nd	na	d	na
Chlorophyllide a	$1.7*10^2$	5.2	$1.0*10^2$	9.7	8.1*10	1.4	$4.5*10^2$	8.8
Pheophorbide a	2.4*10	0.7	2.8*10	2.7	nd	na	7.2*10	1.4
Cis-Neoxanthin	7.2*10	2.2	8.7	0.8	8.7*10	1.6	5.8*10	1.1
Violaxanthin	1.1*10	0.3	7.7*10	7.3	$2.7*10^2$	0.5	5.7*10	1.1
Luteoxanthin	nd	na	d	na	d	na	d	na
Antheraxanthin	d	na	4.2*10	3.9	1.2*10	0.2	9.4*10	1.8
Lutein, Zeaxanthin	$7.2*10^2$	21.7	$3.4*10^2$	32.3	$8.7*10^2$	15.4	$1.1*10^3$	20.9
Cantaxanthin	8.3	0.3	nd	na	nd	na	nd	na
Chlorophyll b	$6.8*10^2$	20.4	5.4*10	5.1	$9.0*10^2$	16	$8.8*10^2$	17.1
Chlorophyll a	$1.1*10^3$	31.5	$3.5*10^2$	33	$1.3*10^3$	23.7	$1.5*10^3$	29.9
Echinenone	nd	na	nd	na	nd	na	3.2	0.1
ψ,ψ-carotene	d	na	nd	na	nd	na	nd	na
Pheophytin a	nd	na	8.2	0.8	$7.3*10^2$	12.9	2.0*10	0.4
β,ψ-carotene	d	na	nd	na	d	na	d	na
β,ε-carotene	4.0*10	1.2	nd	na	1.5*10	0.3	d	na
β,β-carotene	5.8*10	1.8	1.1*10	1	$3.1*10^2$	5.4	$2.7*10^2$	5.2
Total amount detected (ng)	$5.9*10^2$		$2.3*10^2$		$1.1*10^3$		$6.4*10^2$	
Amount dry cell mass (mg)	17.9		21.9		19.2		12.4	

3.2.5.2. *M. rubrioleum*

The result chromatographs of *M. rubrioleum* can be found in Appendix 7 (10 day sample), Appendix 8 (20 day sample) and Appendix 9 (unknown carotenoids). Appendix 10 has the results of the hydrolysis done on 20 day samples. Table 9 summarizes the amount of pigments and their respective percentage ratio that were found in *M. rubrioleum.* The hue has turned from olive green to bright red after 12-14 days, and the suspicion was that the algae might contain astaxanthin or a diester due to the intense coloration.

Table 9: Summary of the pigment composition of *M. rubrioleum* and the results of the hydrolysis that have been performed on 20 day samples. The list is based on retention time. Amounts are measured by pigment weight (ng) against amount of dry cell mass (mg). 'Nd' indicates lack of that certain pigment within the species, 'd' indicates that a certain peak was found but no calibration was done for it, while 'na' stands for not available.

Pigments	***M. rubrioleum* 10 day**		***M. rubrioleum* 20 day**		***Hydrolysis***	
	Amount (ng/mg)	%	amount (ng/mg)	%	Amount (ng/mg)	%
Chlorophyllide b	d	na	nd	na	nd	na
Chlorophyllide a	$1.0*10^2$	0.5	2.1*10	0.3	nd	na
Pheophorbide a	3.3*10	0.2	7.4	0.1	nd	na
Cis-Neoxanthin	$3.6*10^2$	1.7	9.9*10	1.2	nd	na
Violaxanthin	$5.1*10^2$	2.5	1.5*10	1.9	nd	na
Luteoxanthin	d	na	d	na	nd	na
Antheraxanthin	6.5*10	0.3	8.7	0.1	d	na
Lutein, Zeaxanthin	$1.8*10^3$	8.7	$5.8*10^2$	7.2	$1.2*10^2$	12.9
Cantaxanthin	$2.9*10^2$	1.4	$1.7*10^2$	2.1	2.1*10	2.2
Chlorophyll b	$3.2*10^3$	15.5	$9.6*10^2$	11.9	nd	na
Chlorophyll a	$8.5*10^3$	41.5	$2.8*10^3$	35.2	nd	na
Echinenone	$1.0*10^3$	5	$6.2*10^2$	7.7	87	9.5
ψ,ψ-carotene	nd	na	nd	na	nd	na
Pheophytin a	$1.2*10^2$	0.6	3.8*10	0.5	nd	na
β,ψ-carotene	d	na	d	na	nd	na
β,ε-carotene	d	na	1.5*10	0.2	nd	na
β,β-carotene	$1.1*10^2$	5.6	$6.0*10^3$	7.5	7.9*10	8.6
Unknown	$1.9*10^3$	9.6	$1.5*10^3$	18.4	nd	na
Total amount detected (ng)	$2.4*10^3$		$1.2*10^3$		$3.3*10^2$	
Amount dry mass (mg)	1.2*10		1.5*10		3.7*10	

After testing the 20 day samples with HPLC, multiple astaxanthin-like peaks were found in the results, but with different retention times compared with astaxanthin (see 4.2.4 for more details). To clarify that these are not related to astaxanthin, an anaerob hydrolysis was performed, and no trace of astaxanthin was found, and the hydrolysis removed the unknown pigments completely.

3.3 *Oocystis alpina*

3.3.1. Growth

The first three batches were grown in the same way as *Nannochloropsis* sp. with the same medium composition adjustments (see 2.1.1) but applied to the BBM medium. Due to issues with settling that could not be avoided, the experiment used the equipment that was made for the cold strains (see 2.1.2 and Appendix 2), and some of the experimental treatments had to excluded.

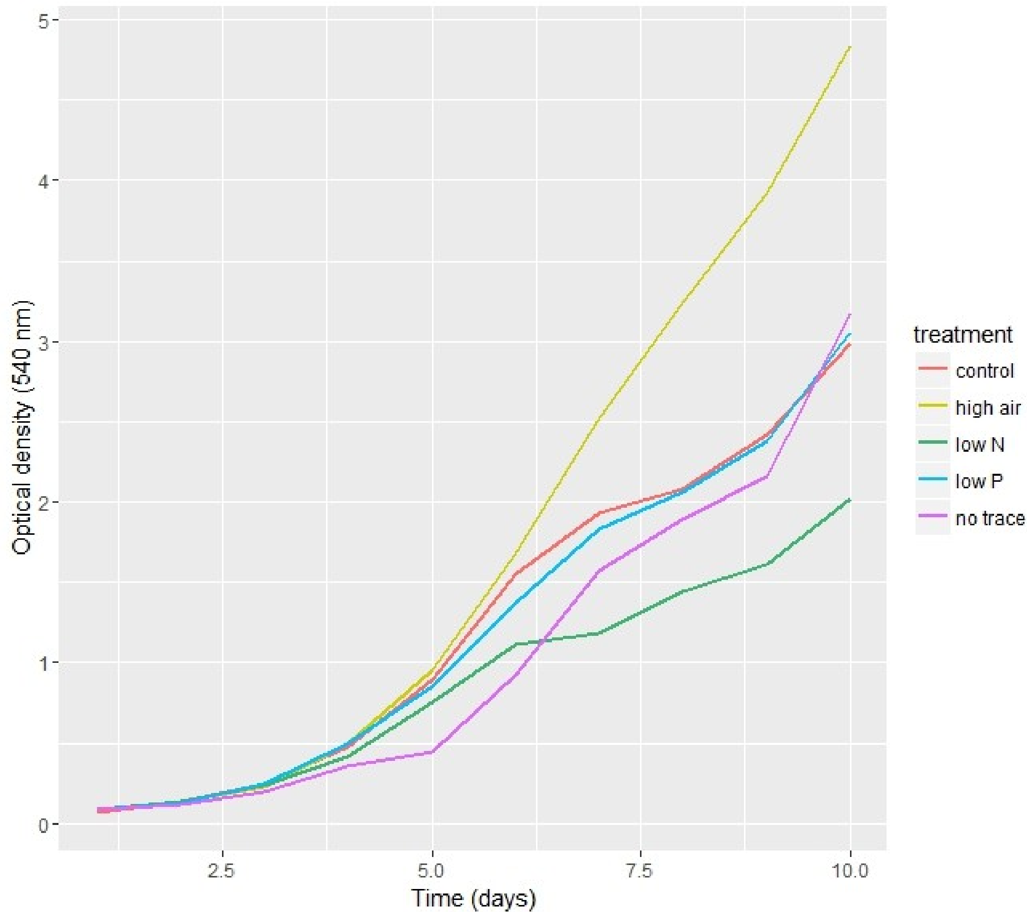

Figure 6: Averaged growth rate of the three *Oocystis alpina* experiments.

The averaged optical density data of the three individual runs are shown in Figure 6. The results indicate that the culture with the increased aeration produced the highest amount of biomass, while the lower nitrates produced the least amount.

3.3.2. Dry weight measurements

The dry weight measurements have attended similar results to the optical density. Figure 7 summarizes the results of the three individual runs, showing the maximum averaged dry weight values measurements (g/l).

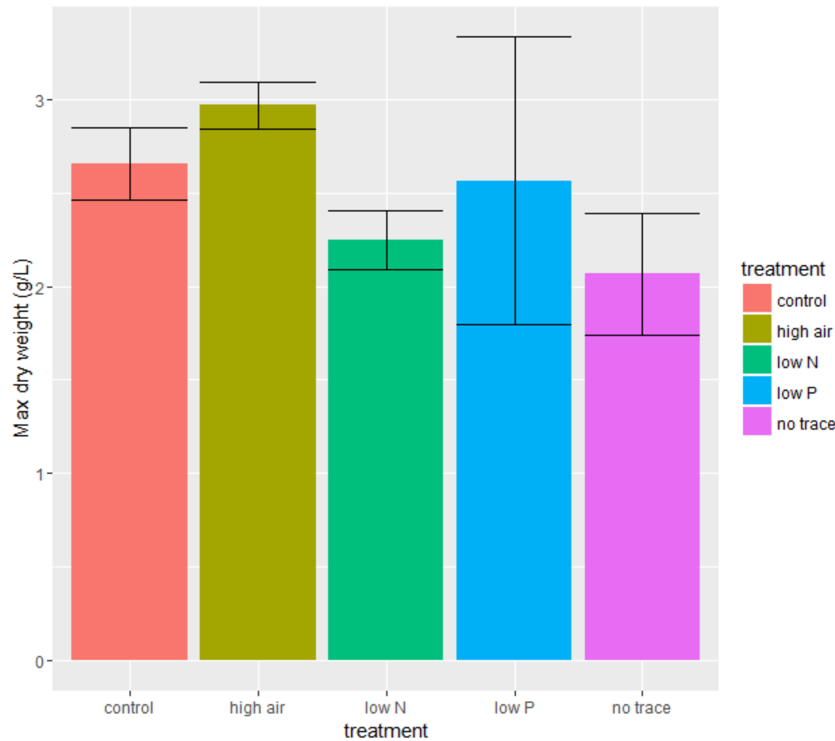

Figure 7: Averaged dry weight measurements of the three experimental runs of *Oocystis alpina*. Error bars indicate the standard deviation.

The null hypothesis states that there is no significant difference between the different treatments.

A one-way ANOVA test was performed on the dry weight measurements, and the results can be seen in Table 10. As the *p* value (0.044) is below the critical value 0.05, there is significant difference between the treatments used on *Oocystis alpina* and the H_0 is rejected.

Table 10: One-way ANOVA test carried out on dry weight measurements of *Oocystis alpina*. The test returns a *p*-value of <0.05, indicating statistically significant variance of dry weight means.

	df	**Sum Sq**	**Mean Sq**	**F value**	***p***
treatment	4	1.99	0.50	3.2	0.044
residuals	15	2.34	0.16		

3.3.3. Total lipid content of *Oocystis alpina*

The averaged percentage oil content results can be found in Figure 8. A one-way ANOVA was carried out, and the results indicate that there is no statistically significant difference between the oil percentage yield within the individual treatments ($p = 0.061$). Table 11 summarizes the results.

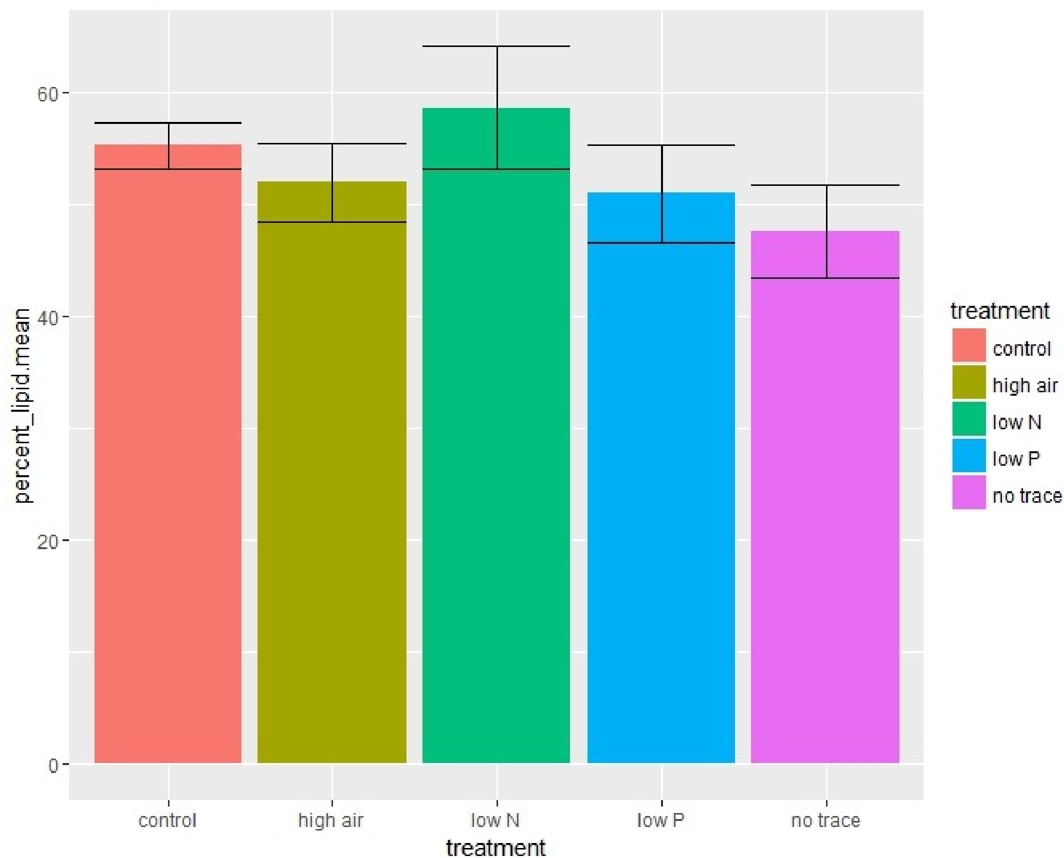

Figure 8: Averaged oil content percentages found in the experimental *Oocystis alpina* samples. Error bars indicate highest and lowest measured values.

Table 11: One-way ANOVA test carried out on averaged oil content percentages of *Oocystis alpina*. The test returns a *p*-value of >0.05, indicating that there is no statistically significant variance between the oil percentage yield of the individual treatments.

	df	Sum Sq	Mean Sq	F value	*p*
treatment	4	212.9	53.2	3.21	0.062
residuals	10	166.0	16.6		

3.3.4. Pigment content

The result chromatographs of *Oocystis alpina* UTEX 2541 can be found in Appendix 13. Table 12 summarizes the amount of pigments and their respective percentage ratio that were found in this strain. The values are calculated from 10 day old samples.

Table 12: Summary of the pigment composition of *Oocystis alpina* UTEX 2541. The list is based on retention time. Amounts are measured by pigment weight (ng) against amount of dry cell mass (mg). 'Nd' indicates lack of that certain pigment within the species, while 'na' stands for not available.

Pigments	amount (ng/mg)	%
Chlorophyllide b	nd	na
Chlorophyllide a	1.9*10	0.3
Dinoxanthin	$3.6*10^2$	6.2
Pheophorbide a	nd	na
Cis-Neoxanthin	nd	na
Violaxanthin	$4.8*10^2$	8.4
Luteoxanthin	$2.0*10^2$	3.4
Antheraxanthin	4.5*10	0.8
Lutein	$2.3*10^2$	3.9
Zeaxanthin	4.2*10	0.7
Cantaxanthin	6.2	0.1
Chlorophyll b	nd	na
Chlorophyll a	$3.3*10^2$	57.6
Echinenone	2.1	0
ψ,ψ-carotene	nd	na
Pheophytin a	2.0*10	0.3
β,ψ-carotene	nd	na
β,ε-carotene	nd	na
β,β-carotene	$2.6*10^2$	4.4
Total amount detected (ng)	$8.5*10^2$	
Amount dry mass (mg)	1.5*10	

With the same method, this was the only strain where lutein and zeaxanthin separated from one another. Oocystis aplina has been reported to turn color (Culture bank 2016), however a flask was kept for 4 weeks with aeration, then two more weeks without aeration. Furthermore then the culture was stored along with the other strains, yet no color change was observed and no further chromatography was performed.

4.0 Discussion

4.1 *Nannochloropsis* sp. experiments

Nannochloropsis sp. is an abundant and extensively studied family of marine microalgae that is widely used in research due to its high growth rate and lipid content (Converti *et al.* 2009, Rodolfi *et al.* 2009). In this study it was used as the model organism for testing growth and different medium compositions and extraction methods. To test if there was a difference in the dry mass yield due to differences of medium compositions, the average results of the three separate runs were examined further. A one-way ANOVA test was performed on the collected dry mass data, and the results indicate that the p value (0.301) is higher than the critical value 0.05, hence the null hypothesis is accepted. The individual medium compositions during the 14 day period of growth did not effect significantly the final biomass yield of the cultures.

4.2 Cold strain experiments

Chlamydomonas pulsatilla, *Koliella sempervirens*, *Chloromonas platystigma*, *Chlamydomonas klinobasis and Macrochloris rubrioleum* were chosen for this study to obtain more information about these species. The strains were isolated in Svalbard (Northern Norway), and the findings may encourage further study opportunities of the aquaculture with temperate climates. Two sets of experiments were carried out with the strains. The first set included a 10 day long observation of the growth performance at 6°C with an optimal light (135 μE) and recommended medium composition (2N-BBM). The second set of experiments were carried out the same way, but the cultures were allowed to grow for 20 days instead to see if there is a difference in lipid and pigment accumulation due to nutrient depletion, as it has been reported with other cold tolerant strains (Leya *et al.* 2009, Spijkerman *et al.* 2012).

4.2.1. Growth

To be able to grow these strains in a relatively cold environment, a specially built bioreactor was constructed. Containing 310 ml culture vessels, a wooden rack, air pressure stabilizers and connection to the pump, and a fridge was modified with light bulbs mounted on the back (see in detail: 2.1.2 and Appendix 1).

The cultures grew well at low temperature, and the recorded optical density data indicates that *C. pulsatilla* biomass increased the most during the cultivation period, while *M. rubrioleum* has shown the weakest growth pattern (shown in Figure 3). The 20 day observations show that the

biomass of *K. sempervirens* increased the most while *M. rubrioleum* seemingly performed poorly. Even though the optical density measurements implied that *M. rubrioleum* is not growing well, the culture vials were observed to contain a lot of biomass. This strain has begun to clump together into clusters of cells in suspension, and the suspicion is that this resulted in aberrant optical density measurements. Visually the appearance was that the algae had stronger biomass growth, but unfortunately this could not be verified via spectrophotometry.

The averaged dry weight measurements of the 20 day old samples indicate that *C. pulsatilla* has the highest amount of dry biomass (4 g/l), while *M. rubrioleum* has the least (2 g/l) among the five cold strains (see Figure 4). The study was unable to find a significant difference between the amount of biomass yield between the strains, however in the statistical analysis by one-way ANOVA returned a *p* value very close to the critical value of 0.05. It is possible that a larger sample size might yield clarifying data, further experiments are required to test this.

Few directly comparable studies are available in the literature examining growth dynamics of cold strain algae. In their study of psychrophilic algal species harvested from snow fields, Leya *et al.* (2009) noted growth rates given in units of weight change over time, but takes care to emphasize that their experimental setup was not optimized for this purpose.

4.2.2 Oil content

Biochemical composition changes usually occur under nutrient starvation or other species-specific stress factor (Chisti 2007, Rodolfi *et al.* 2009). Nutrient-replete lipid content of green algae is on average 23% dry weight, which sees a significant rise to 41% under nitrogen deprivation. Under nitrogen-deficient conditions algal cells will synthesize products such as fatty acids and carotenoids rather than nitrogen-dependent molecules like proteins. Studies have shown that there is great variety between species pertaining to lipid production under nutrient deficient conditions, and the two often trade off to a certain degree (Griffiths and Harrison 2009). In these experiments the results show similar percentage ratios during the 10 and 20 day periods to previous studies (Leya *et al.* 2009, Piepho *et al.* 2012, Roessler 1990, Spijkerman *et al.* 2012), in the latter the cultures most likely absorbed all the available nutrients (see Figure 5 for details).

In the 10 day cultivation period, the strains produced the following amounts of averaged lipid percentages by dry cell biomass weight. In decreasing order: *C. klinobasis* (21%), *C. pulsatilla* (20%), *M. rubrioleum* (18%), *C. platystigma* (17%) and *K. sempervirens* (15%). In the 20 day samples increased lipid accumulation has been observed, most likely due to nutrient depletion. The strains produced the following amount of lipid percentage (averaged, by dry weight) in a decreasing

order: *C. pulsatilla* (39%), *M. rubrioleum* (37%), *C. klinobasis* (35%), *C. platystigma* (32%) and *K. sempervirens* (28%).

A two-way ANOVA test was performed to compare oil yield of the different species and the results indicate that there is a statistically significant difference, both between species and the 10 and 20 day groups. The oil yield results could possibly be enhanced further with additional stress factors, such as longer growth periods, extremes of light, aeration, medium composition (phosphorous, nitrogen, iron), salinity, etc. (Piepho *et al.* 2012, Spijkerman *et al.* 2012). Determining species specific stress factors that induce lipid accumulation is highly recommended.

4.2.3 Fatty acid

The fatty acid data indicates that all of the cold strains produce PUFAs, ranging from C14:0 to C20:5. *C. klinobasis* and *C. pulsatilla* show taxonomic similarities both in fatty acid composition and the total amounts in both 10 and 20 day periods. These two strains are the only ones where C:14:0 was detected, the rest of the studied species have been rich in C16 and C18 saturated and non-saturated fatty acids. The major fatty acids in *C. klinobasis* and *C. pulsatilla* was C16:3, in *K. sempervirens* was C18:1, and *C. platystigma* along with *M. rubrioleum* had C18:3 most common.

Three strains was found with C18:4, but only *K. sempervirens* has produced EPAs (C20:5) both in 10 and 20 day samples. The latter algae produces mainly C18 and C20 fatty acids, however the overall percentage yield is found to be the lowest both in 10 (12%) and 20 (18%) days. Because the growth rate was recorded to be the highest over a 20 day period, and the dry mass being moderate (see Figure 4.) this might be an interest for aquaculture for feeding, especially if the biochemical composition could be enhanced with an additional stress factor for increased lipid production (Reitan *et al.* 1997). Further research on this matter is recommended.

The relative composition of fatty acids differs between algal species and also strongly depends on environmental factors such as temperature, nutrient availability, and solar radiation (Teoh *et al.* 2004 Roessler 1990). The majority of research on lipid characteristics of algae are done on temperate algal species. Rhodolfi *et al.* (2009) have presented findings indicating that 16-18 carbon long fatty acids accumulate the most in a variety of fresh- and saltwater algal species cultured under stressed conditions. What is known about cold-strain algae is that generally they will have a higher ratio of polyunsaturated to saturated fatty acids (C18:1 found to be the most dominant), as these are advantageous to maintain cell membrane fluidity, flexibility, and functionality under cold conditions. One recent study by Spijckerman *et al.* (2012) compared fatty acid composition of snow algae with cultured cryophilic species grown under nitrogen replete and

nitrogen deficient conditions. The results of this paper's study broadly falls in line with the findings of these authors.

4.2.4 Pigments

The HPLC chromatography results were calculated from 20 day old samples. The strains has been examined to determine the ratio of dry cell mass amount containing a certain pigment (nanograms of pigment per grams of dry cell mass) and their respective percentage amount based on the total amount of pigment detected (see Table 8 in detail). The major components are found to be chlorophlyll a in every examined species, followed by lutein/zeaxanthin or other chlorophylls, and no secondary carotenoids are found in the four typical green strains. *M. rubrioleum* has shown similar pigment composition pattern within the 10 and 20 day samples. It contains chlorophyll a in the highest amounts, however it produced secondary carotenoids by the end of the 20 day experiments. Various environmental factors such as nitrogen depletion, exposure to high light, oxidative stress, pH, high salinity or a drastic change in temperature are known to induce secondary carotenoid synthesis (Fábregas *et al.* 2001, Ip and Chen 2005), and since the light intensity has not been changed during these experiments, the algae most likely have been under the effect of nutrient depletion. Visually it had turned from the original dark green color to dark red, and the suspicion was that it has accumulated astaxanthin (or its esthers, isomers, etc). These peaks were found to be very dominant on the result chromatogram (see Appendix 8, peaks marked under 'unknown'), and the spectras of these pigments were strongly resembling to astaxanthin (see Appendix 9 for spectras). Calibrations have been made for both astaxanthin and its diester, but the retention times of the standards were not matching with any of the unknown carotenoids. To exclude any possibility that these pigments might relate to astaxanthin, an anaerob hydrolysis was performed, and no trace of astaxanthin was found (see Appendix 10). The unknown pigments are probably a type of carotenoid esters with one or two keto group attached. Their combined weight were the second highest in the 20 day samples (18.4 ng/g dry cell mass), and since the β,β-carotene and the echinenone are the ones that are most likely responsible for the red coloration were only approximately 15% of the total pigment detected, further experiments are recommended to identify the unknown carotenoids. It is described by Masojídek *et al.* (2000) that lutein and chlorophyll a and b decrease under nitrogen defficiency, salinity and high irradiance stress in a *Chlorococcum* sp., resulting secondary carotenoid synthesis and change in color, and these results are comparable to the findings of the *M. rubrioleum* experiments.

Before the real analysis, a sample of each strain was tested with the HPLC, but the results were a bit weak. It was decided to keep the samples in the freezer for 3 days after the preparation (see 2.5.1) instead of doing the same method that is described in 2.7. The water-based buffer was left on the top and the samples were not flushed with nitrogen neither, and the resulting chromatograms have produced more isomers and peak overlaps. Unfortunately, repeat measurements following proper procedure could not be completed due to lack of time. The testing had to go on with the remaining 15 samples, which were all 20 day samples due to randomized batch preparation. Hence the pigment results lack the 10 day dataset. Since only *M. rubrioleum* produced secondary carotenoids, it might not be relevant as such, but a confirmation would be recommended.

4.3 *Oocystis alpina* experiments

Choosing the right species with relevant properties for specific conditions and products is essential for successful mass-production culture (Pulz and Gross 2004). To enhance the biomass production of microalgae, there are factors that should be considered, such as temperature, aeration, light, water quality (pH, salinity), nutrient availability, and growth parameters (density, cell fragility, settlement, etc) (Rosello *et al.* 2007). The experiments with *Oocystis alpina* were carried out in the recommended environmental conditions (see 2.1.3 for details) but the medium compositions were different from each other. Some of the chosen stress factors should enhance the biochemical composition of the strain, pressuring the cultures to produce more lipids in a short period of time.

4.3.1 Growth

The first three cultures were grown in the multi-cultivator that was used during the *Nannochloropsis sp.* experiment, and it was clear that the algae requires more space and gas flow than what the instrument can support. In the 85 ml vessels the algae settled on the bottom of the vessel during the third day, and modifications were made to increase the turbulence and the direction: flow rate was increased to the maximum of 90 ml/min. Rubber tubes were added to the end of glass tubes to increase the length and create more turbulence. Tubes were shaken daily to re-suspend the biomass, yet the density measurements by the instrument were unreliable despite these efforts (see Appendix 2). Due to these issues the experimental cultures were grown by using the 310 ml tubes and the rack that was made originally for the cold strains.

Switching to the other instrument did not completely eliminate the settling, and on day 3 of the cultivation still vessels had to be perturbed once daily. With such treatment the settling was however minimized and, the cultures grew satisfactorily with the experimental treatments (see 2.1.3 for details on the medium composition). The optical density measurements indicate that the highest amount of biomass was produced under increased aeration treatment, and the lowest was produced by the low nitrate medium cultures (see Figure 6 for details). The averaged dry weight of three individual experiments was measured after a 10 day cultivation period, and it was found that the increased aeration treatment produced the most biomass within this time frame (3 g/l dry mass) while the medium lacking trace metals produced the least amount of algae (2.1 g/l), see Figure 7 for details. A one-way ANOVA was performed on the averaged dry weight (p=0.044), and it was found that there is statistically significant difference between the dry biomass yield of the individual treatments.

The results are in line with previously reported growth rates in multiple studies (Converti *et al.* 2009, Harrison *et al.* 1990, Li *et al.* 2008, Reitan *et al.* 1994). It is shown that the medium containing no trace metals are the strongest limiting factor in the case of this particular strain, followed by the low nitrates and low phosphates, while the high aeration enhanced the biomass growth, supplying more CO_2 to the cells (with increased turbulence). As nitrogen starvation induces lipid accumulation, the expectation was to find the highest lipid yield within those cultures.

4.3.2 Lipid content

Oocystis alpina was tested to determine the averaged lipid content of the three experimental runs. The values have been calculated to percentage yield by dry cell mass, and the results describe very high amount of lipids in a short growth period. The expectation was that the cultures with the low nitrates will produce more lipids per dry biomass, as it was shown in previous studies with other strains (Breuer *et al.* 2012, Feng *et al.* 2011, Griffiths and Harrison 2009). The culture which was grown in a limited nitrogen medium has produced the highest lipid content (58%) while the least amount of oil was found in the cultures with no trace metals added (47%). These results put *Oocystis alpina* into the high lipid producing strains, according to a previous study done by Griffiths and Harrison (2009).

To verify if there is a significant difference between the variables, a one-way ANOVA was performed (p=0.060), and the results indicate that the medium compositions have not affected the lipid yield significantly. Because the p value is very close to the critical value, it is recommended to increase the amount of experimental runs to test if more data would make a difference between the

variables. The other option would be to increase the incubation time, because longer time given for the cultures may enhance their lipid accumulation further, or give a secondary stress factor, however it might result in net biomass loss (Converti *et al.* 2009). Since *Oocystis alpina* has produced really high amount of lipids in a short period of time, further study on the fatty acid composition is encouraged.

4.3.3 Pigment content

The HPLC chromatography results were calculated from 10 day old samples. The pigment composition revealed that *Oocystis alpina* contains chlorophyll a in the highest amount (57.6 % per dry cell mass) followed by violaxanthin (8.4%) and dinoxanthin (6.2%). Being a green algae, no chlorophyll b was found, and the amounts of β,β-carotene were low ($2.6*10^2$ ng/g dry cell mass, 4.4% of total pigments detected), and no chlorophyll b was detected. This strain was reported to produce secondary carotenoids (Culture bank 2016), however in the 10 day periods the cultures did not changed in color. A single culture in a flask was prepared and let grow for 21 days with optimal conditions (evaporated medium was replaced with the recommended BBM), and it was left for an other 30 days with no more nutrients added, only the evaporated medium was re-filled with distilled water. The culture did not change in color, and due to lack of time the pigments were not prepared for another HPLC analysis. In the 10 day samples 2.1% of echinenone was found, and it might increase with a longer incubation time, just like in *M. rubrioleum.*

5.0 Conclusion

The cold strains grew well in low temperature, producing between 2-3.5 grams of biomass per litre medium over the course of 20 days. The oil accumulation was consistent with other studies using similar warm-water algae (Griffiths and Harrison 2009). These strains produce a lot of polyunsaturated fatty acids (C16:2 and longer), and *K. sempervirens* produces some EPA. The pigment composition was found to be noteworthy in *M. rubrioleum* which have turned red over a 20 day cultivation period and the β,β-carotene and cantaxanthin might be interest of aquacultural use. In overall more experiments are recommended, especially with *K. sempervirens* to see if the fatty acid composition and the amounts can be enhanced, and with *M. rubrioleum* the unknown carotenoids are encouraged to be identified.

Oocystis alpina grew very well in a 10 day period, producing on average 2-3 grams of biomass per litre. The oil production is exceptionally high, especially under nitrogen depletion, and this strain is highly recommended to study further and determine the fatty acid composition. The pigments are mainly containing chlorophyll a, however the violaxanthin and the β,β-carotene might be an interest for aquaculture.

6.0 References

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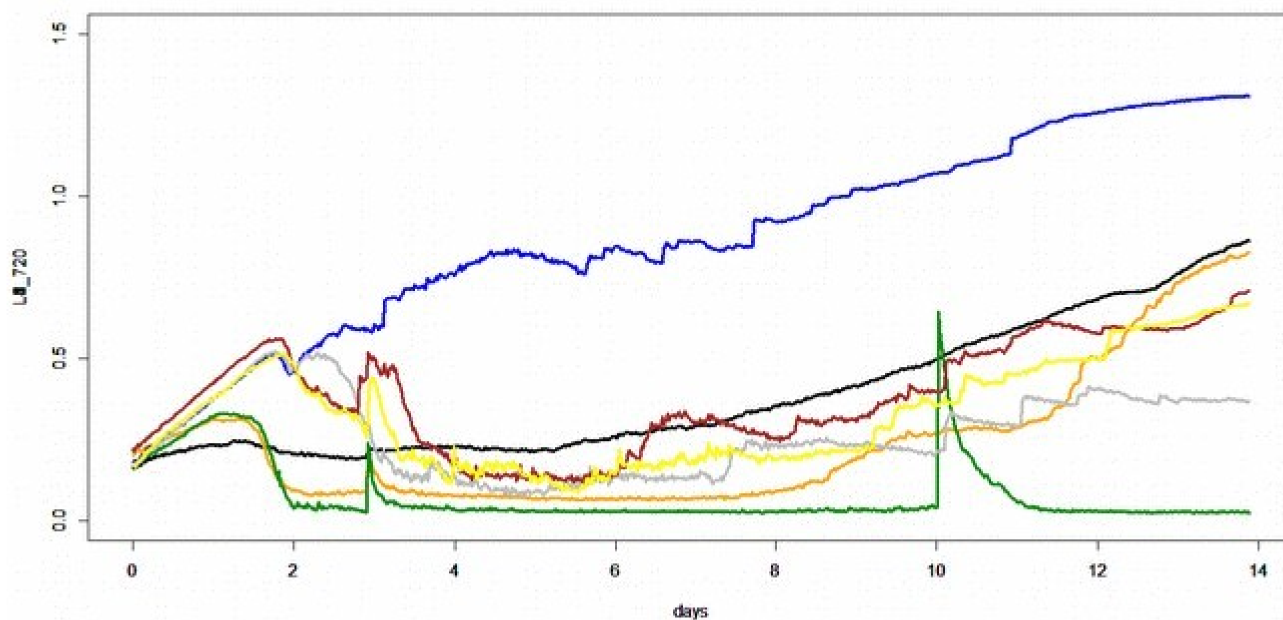
7.0 Appendices

Appendix 1: Structure of the cold strain multi-cultivator device.

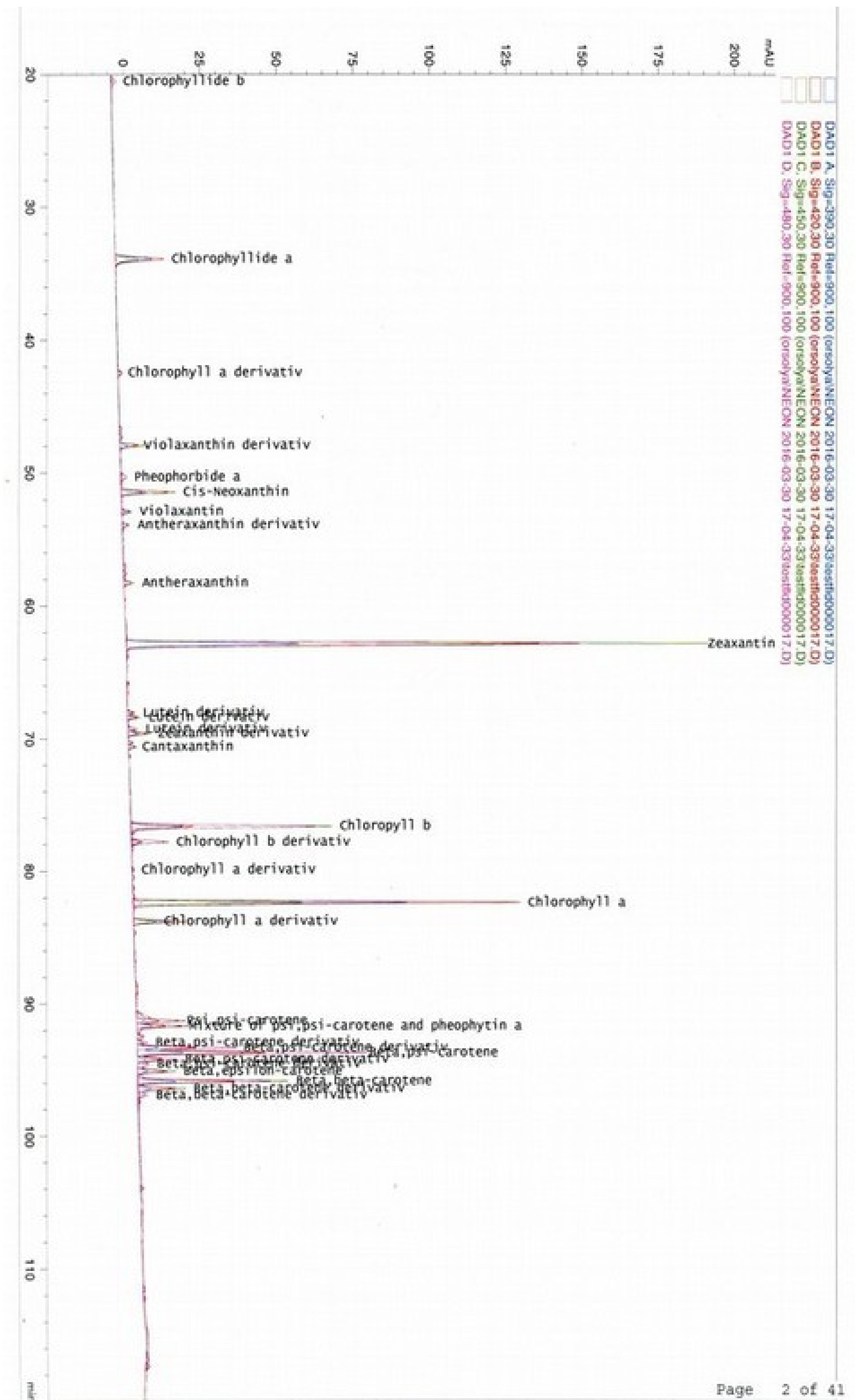
The refrigerator has been modified to be separated to two levels. The installation has been built up from wooden panels screwed together to support the 310 ml vials (8 in total) standing in a vertical position. Six of them were used in the experiment, the two outermost in left and right side were only place holders to avoid false data collection due to inefficient light absorption. The vials are sealed in the top, a glass tube entering to the bottom supplied the aeration. The gas pressure is controlled by a regulator for each vial individually. Samples were drawn from a secondary glass tube added to reach to the middle of the culture.

Appendix 2: Problems with Oocystis grown in the multi-cultivator (MC 1000-OD, Photon Systems Instrument)

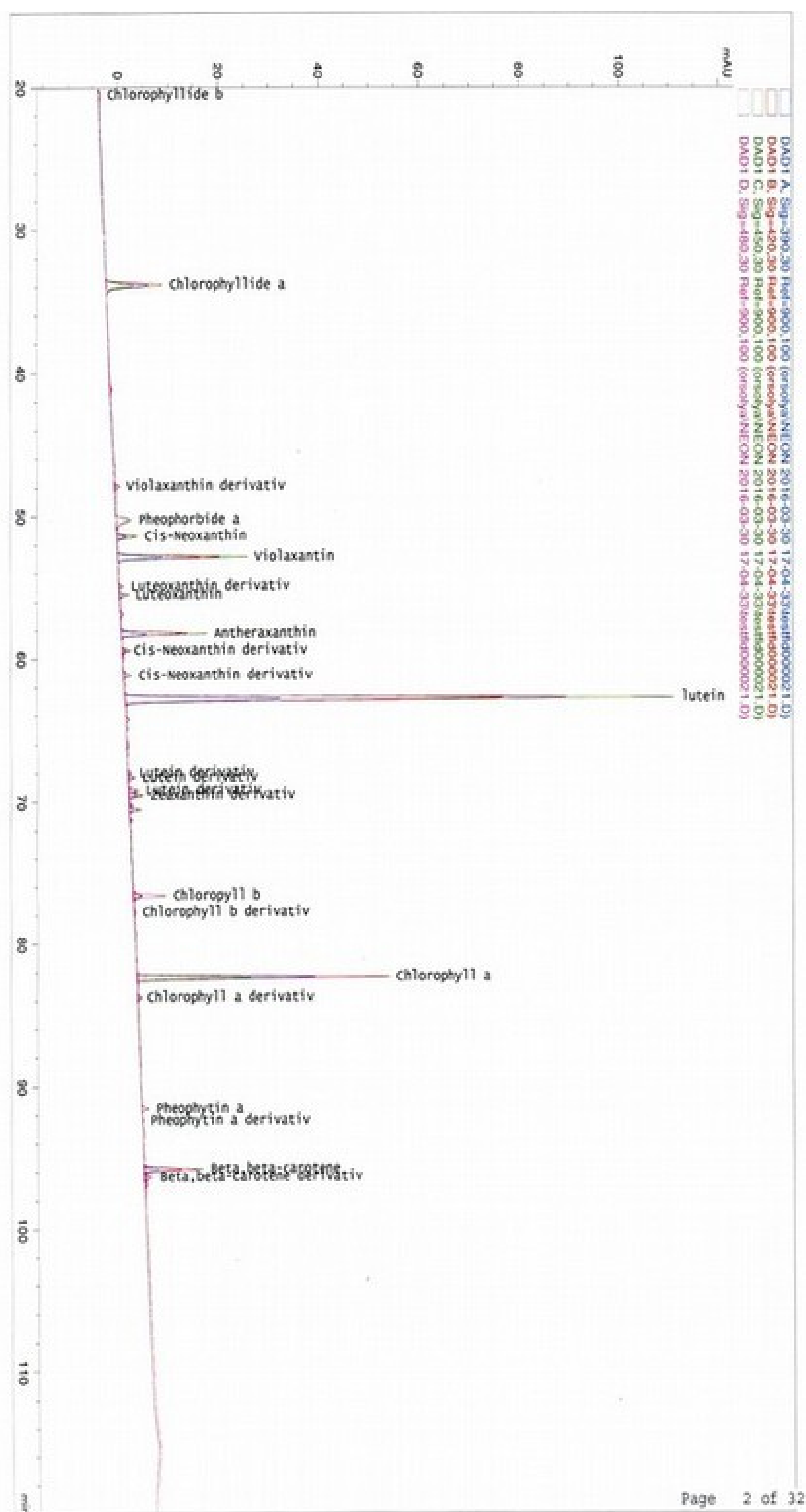
On the attached photograph it can be seen that the algae has settled in the bottom of the tubes. Due to this error the instrument could not record the optical density properly, which resulted in an inaccurate growth curve in all cultures that were cultivated in this instrument. See an example below. Biomass density is plotted against time.



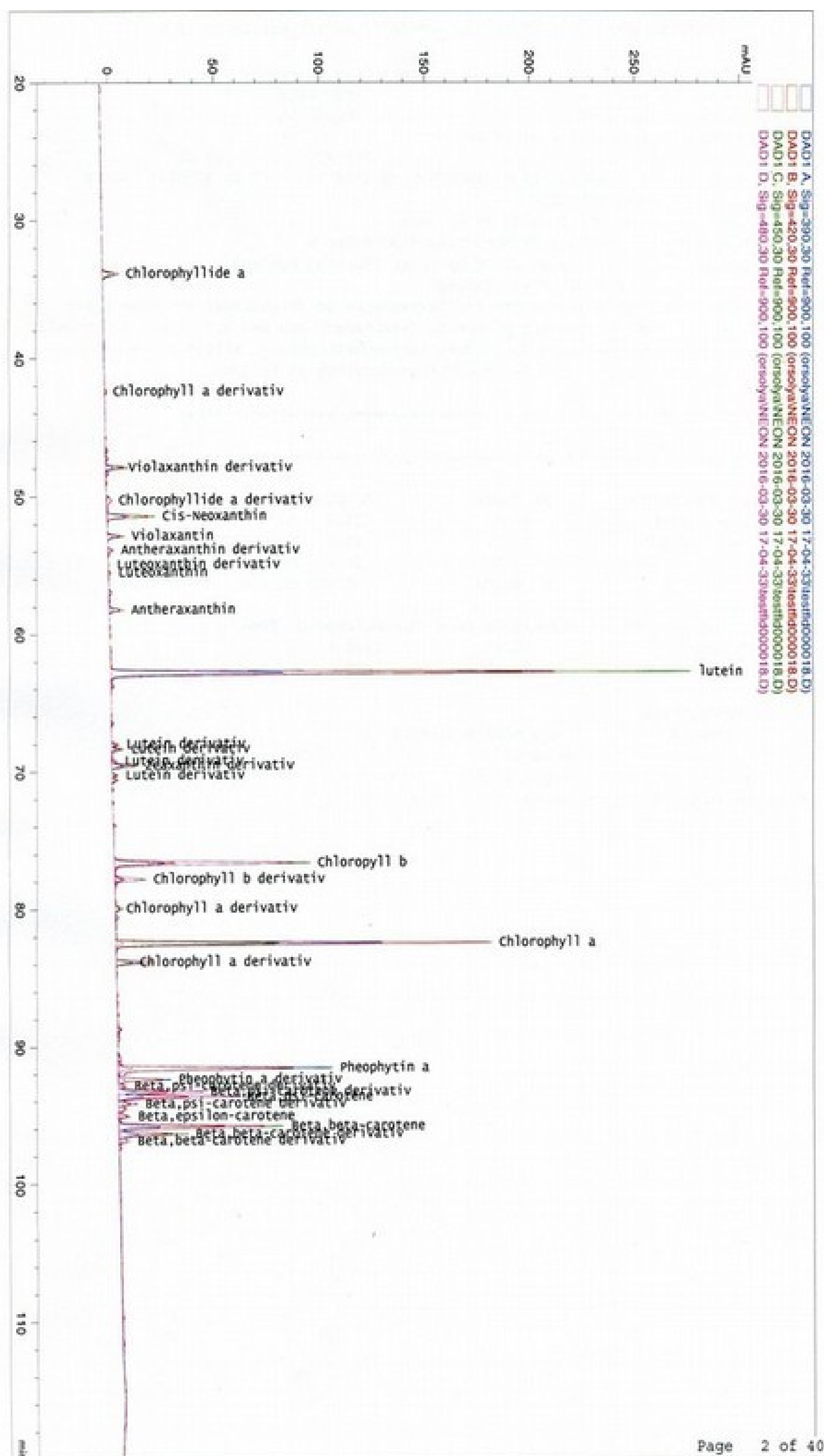
Appendix 3: Chromatograph of *Chlamydomonas pulsatilla* (20 day sample)



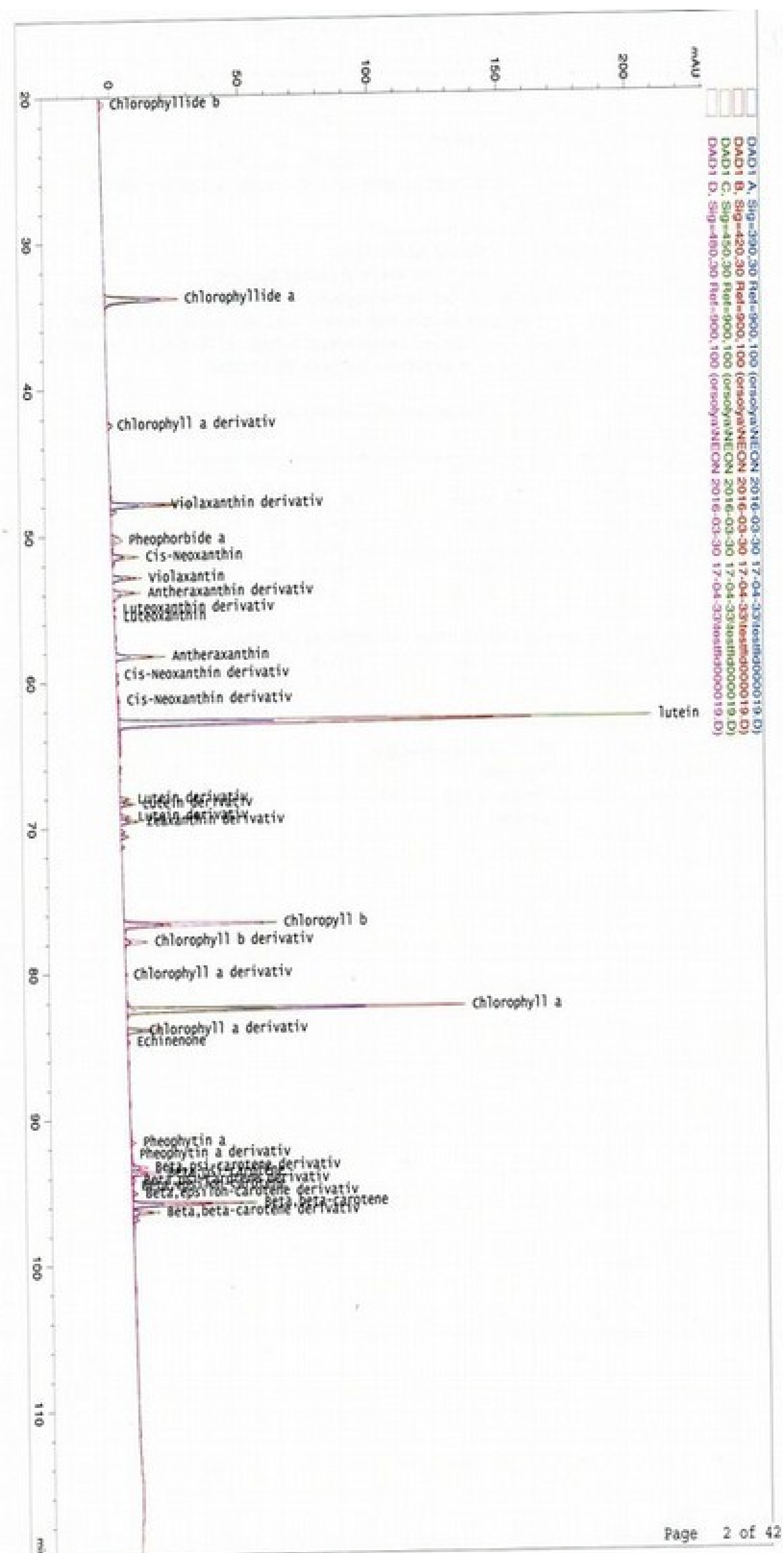
Appendix 4: Chromatograph of *Koliella sempervirens* (20 day sample)



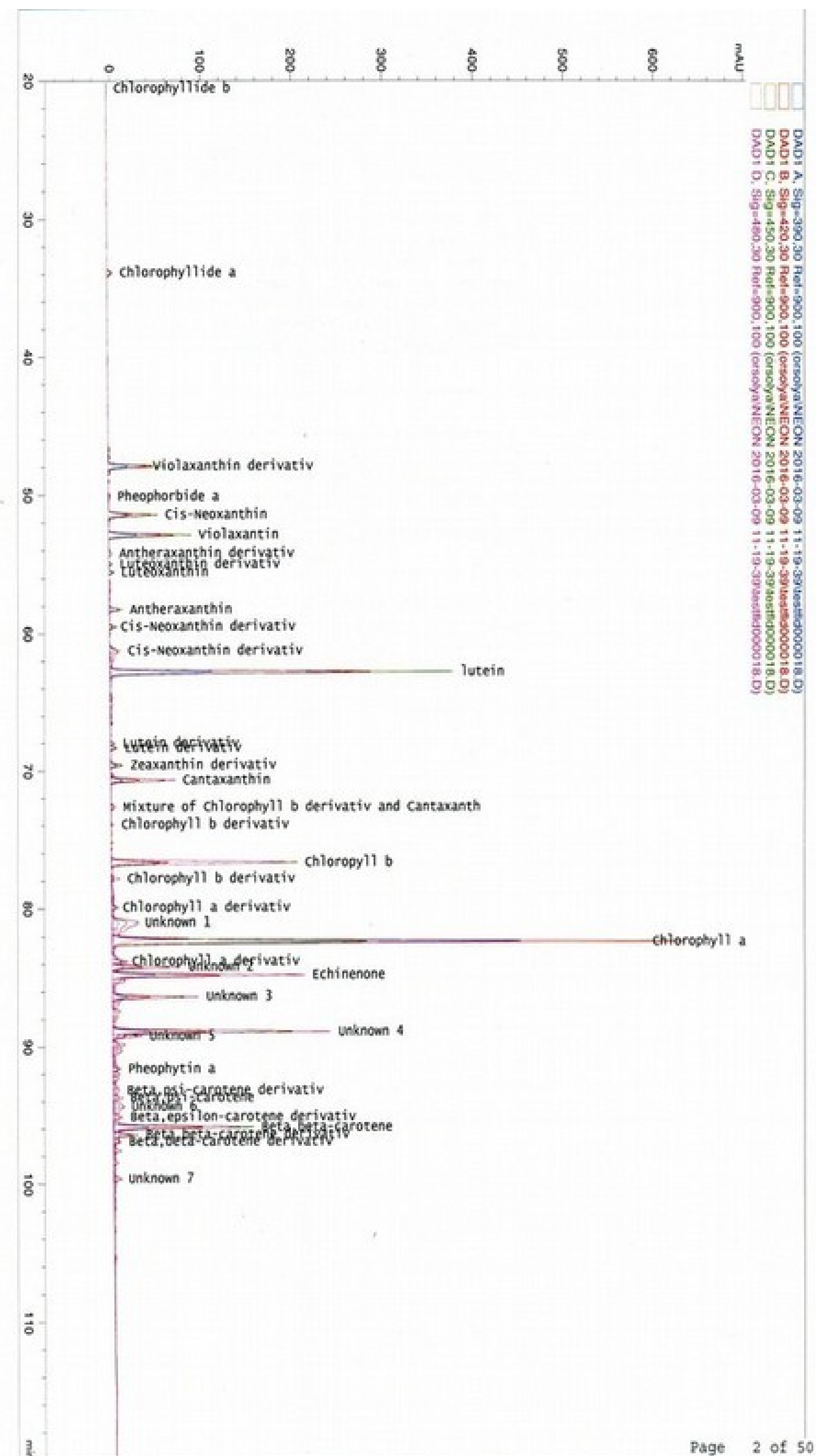
Appendix 5: Chromatograph of *Chloromonas platystigma* (20 day sample)



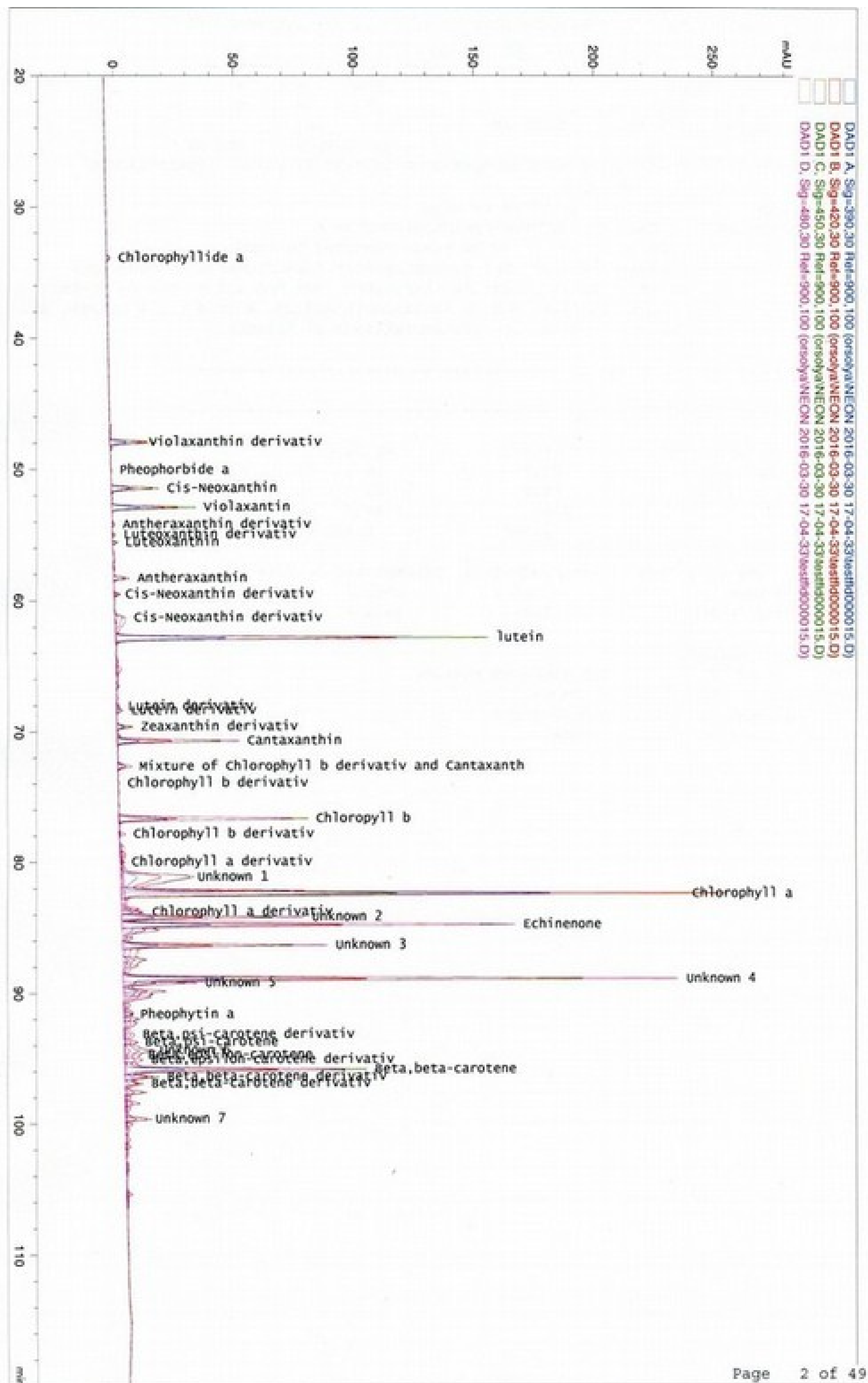
Appendix 6: Chromatograph of *Chlamydomonas klinobasis* (20 day sample)



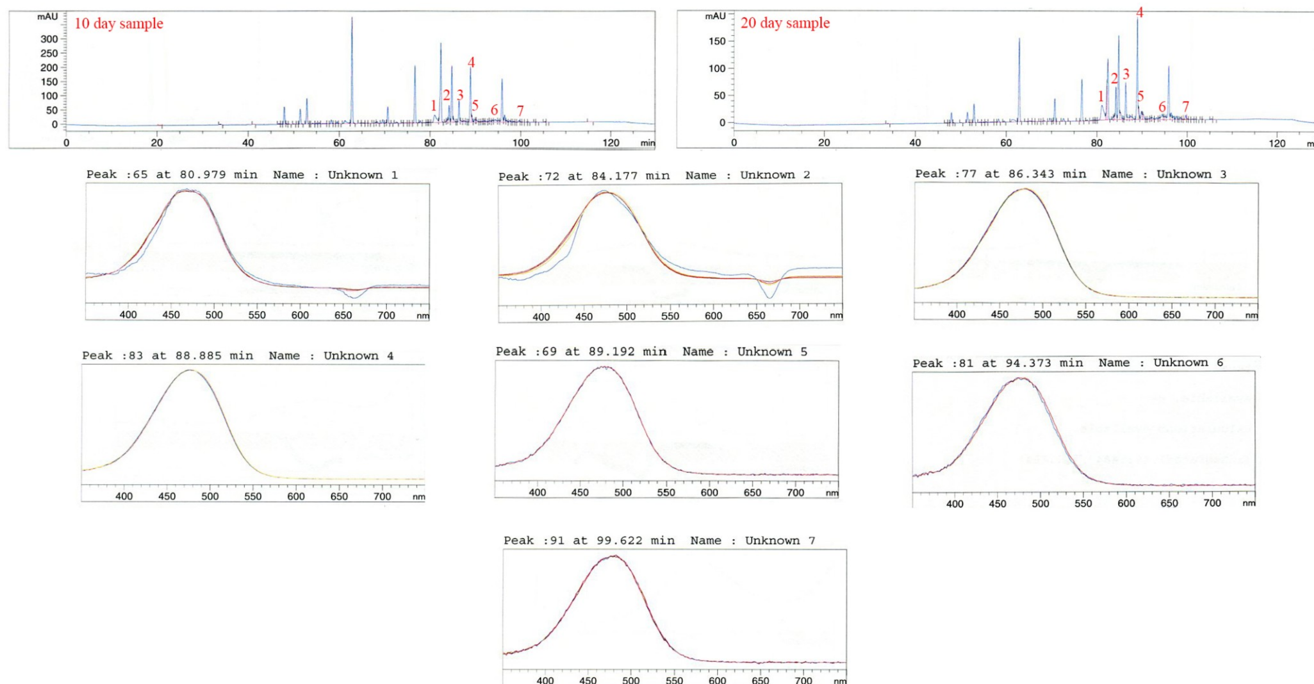
Appendix 7: Chromatograph of *M. rubrioleum* (10 day sample)



Appendix 8: Chromatograph of *M. rubrioleum* (20 day sample)

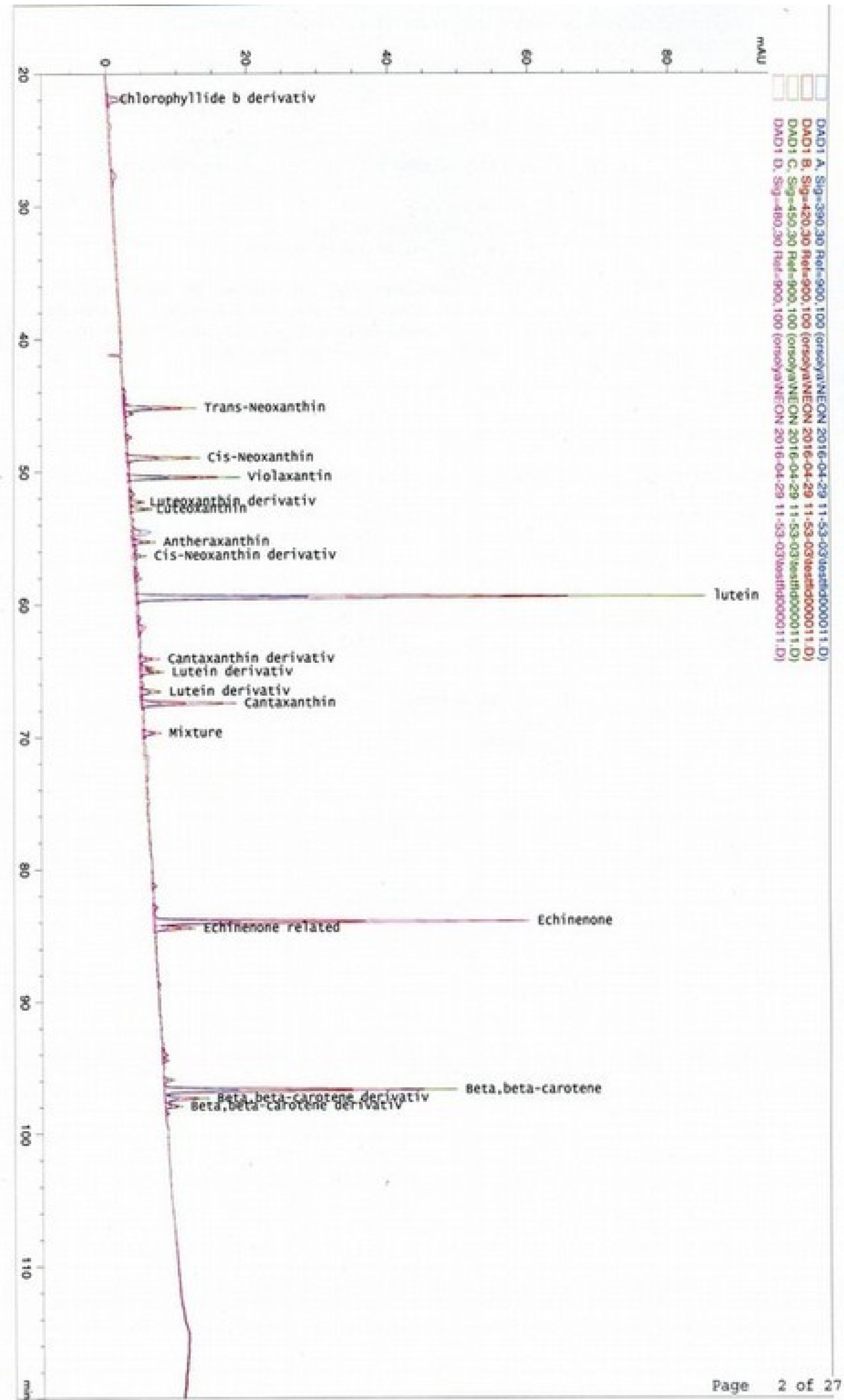


Appendix 9: Spectras of the unknown pigments found in *M. rubrioleum* (10 and 20 day samples)

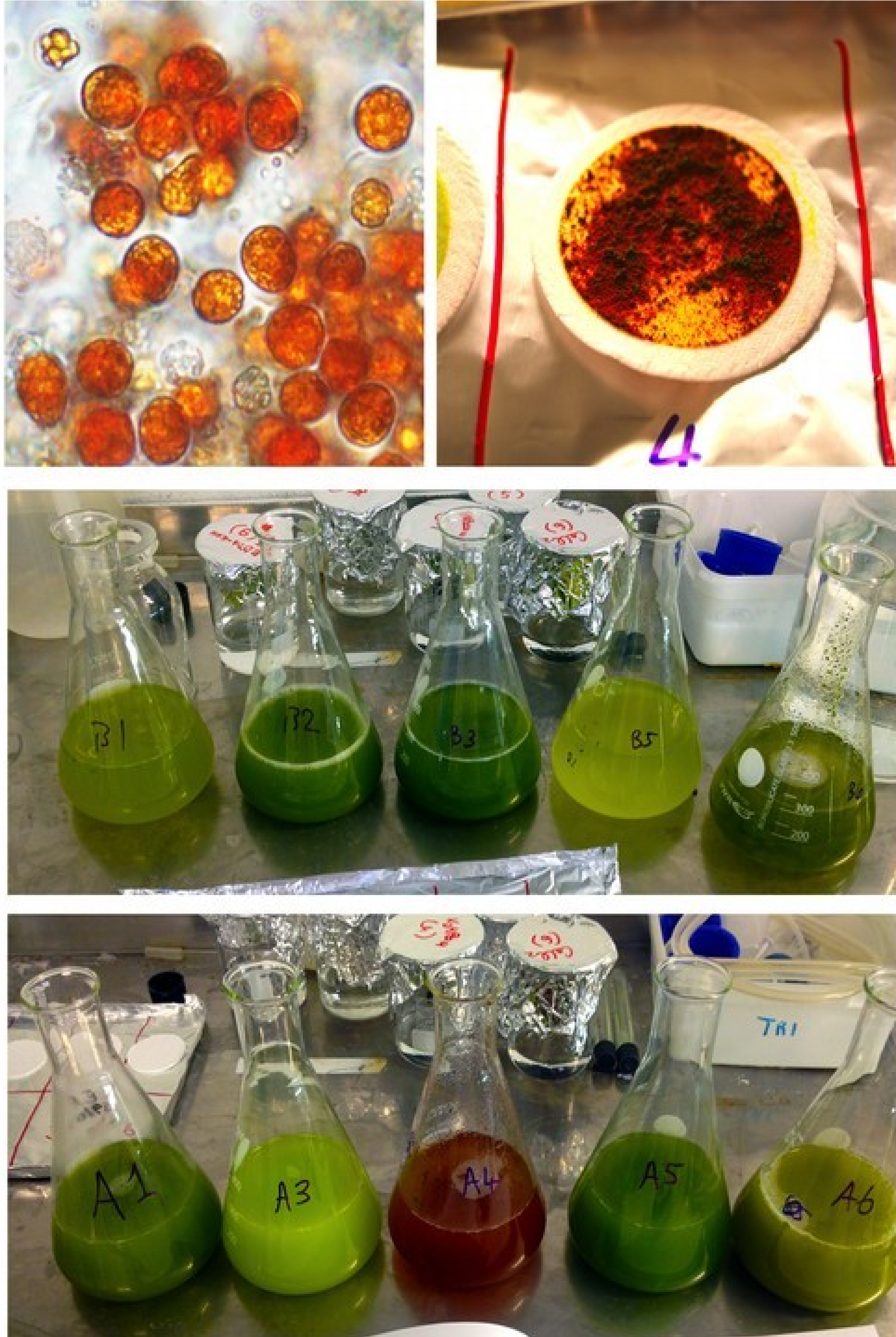


Simplified 10 and 20 day chromatograms are shown in the top row, marked with the respective spectras. 'Unknown 1' is placed as '1' on the timescale and so on with the others. The spectras are very similar both in shape and wavelength, however the timing was completely off.

Appendix 10: Chromatograph of hydrolysis result performed on 20 day old *M. rubrioleum* sample



Appendix 12: Photos taken during the cold strain experiments



The photos on the top are made of 20 day old samples of *M. rubrioleum,* the first one with a microscope, the second one after filtration. The middle image with the culture flasks are made of 10 day old samples, while the bottom one are 20 day old. The color change in culture B6 and A4 (*M. Rubrioleum*) is very profound, while the other cultures mainly just increased in biomass density.

Appendix 13: Chromatograph of *Oocystis alpina* (10 day sample)