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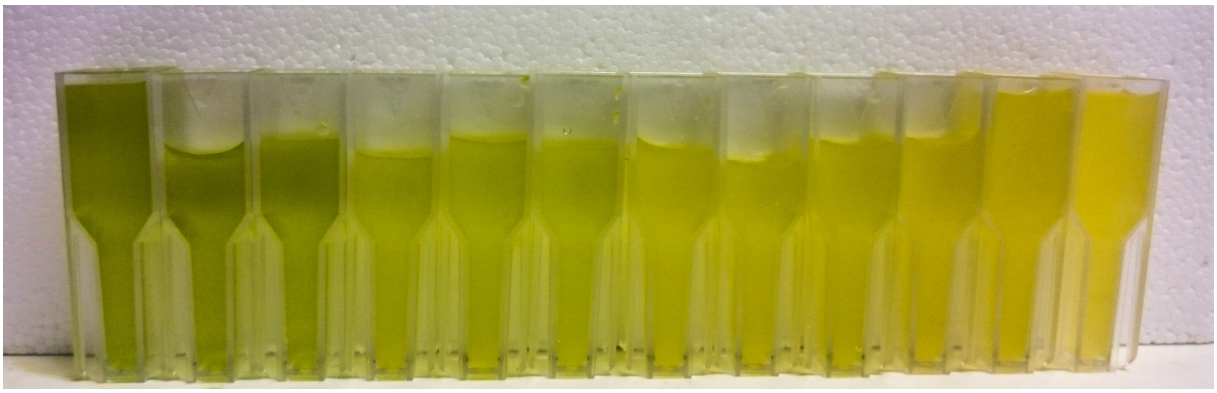

Mixotrophic growth of microalgae on crude glycerol

- Effects on productivity and biochemical composition

Master's thesis in biotechnology

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Abstract

The potential of microalgae to be grown for fuel purposes have long been known and have gained a lot of interest from the biofuel industry in recent years. This potential comes from being an environmentally friendly fuel alternative, utilizing the fast growing photosynthesizing algae as production organisms in a world where demand is constantly rising. However, fuels are not the only use for these organisms and other products of higher value could also be obtained. By looking wider and also investigating modes of growth not solely dependent on photosynthesis the useful applications and growth areas can be increased for example in regions with less sunshine. The aim of this study was to identify species of microalgae capable of utilizing glycerol as an added source of energy and carbon to increase productivity. Glycerol was chosen because it is a readily available byproduct from an increasing global biodiesel production. In this study we screened a small number of species connected to the genus *Scenedesmus* among green algae with regards to their growth rates, ability to utilize glycerol as a source of carbon and energy and their production of carotenoids. The species *Ankistrodesmus falcatus* was identified as having a fast exponential production (153.9 ± 48.6 $mgL^{-1}d^{-1}$) and it was further increased by addition of glycerol to the media (280.5 ± 10.8 $mgL^{-1}d^{-1}$). By further optimizing the growth conditions and increasing glycerol addition to this species an exponential production of 458.7 ± 14.4 $mgL^{-1}d^{-1}$ was measured. Productivity of both fatty acids and carotenoids was increased significantly verifying the potential of using glycerol addition to increase production, possibly in combination with lower light conditions.

Acknowledgments

I wish to thank my supervisors Joshua Mayers and Eva Albers for guiding me and giving me the freedom to learn from my mistakes. I would also like to thank the whole Industrial Biotechnology group for assisting and helping me when I felt lost in the lab.

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1 Introduction

1.1 Algae and Algal biotechnology

Algae are a diverse group of organisms ranging from small unicellular planktonic species up to macroscopic kelp. For this reason it is sensible to begin with a brief overview of what we mean by algae and where the algae treated in this report fit into the systematics of algae as a group. To make it even more complex there are many different types of algae and they are not necessarily closely related to each other. The main categories that people in general recognise is green (*Chlorophyta*), brown (*Phaeophyta*) and red (*Rhodophyta*) algae and among them the green are most closely related to plants that live on land (1). Algae have in common with the plants that they have chloroplasts that they use to conduct photosynthesis. These have been taken up as endosymbiotic cyanobacteria in the past and part of the systematics of algae are based on these uptake events (2).

The use of algae for biotechnical applications has been around for a long time and most of it has been focused on using them for production of biofuels. An early example of this is the U.S department of Energy´s aquatic species program that came out with a report on biodiesel production from algae, much due to the oil crisis in the 70´s (3). At that point the fuel production was deemed too expensive but in recent years the interest has gone up again. There have also been studies on how algae cultivation can result in several products simultaneously and by doing so increasing the overall economic feasibility (4-6).

The culturing of algae has been a subject of great scientific interest as the potential for highly productive organisms using photosynthesis generating fuels and more is attractive. Much research has been done in cross-disciplinary fields of biology and technology trying to understand the physiological requirements and optimizing growth reactors. One of the main issues for growing the microalgae in an effective manner is to provide them with adequate light in areas where the natural light might be limited during parts of the year. This in turn results in lower productivity and limits the economic feasibility (6).

The most attention has probably gone into optimizing conditions for photoautotrophic growth. This is the commonly known mode of metabolism for algae where inorganic carbon from carbon-dioxide and energy from light is utilized in photosynthesis. This is highly dependent on an effective light penetration and when higher culture densities are attained the problem of self-shading arises and thus reactor designs with effective mixing and short light path lengths become a necessity (7). This crucial dependence on light can also hamper production in northern latitudes and one way to solve this problem might be to provide the algae with growth supplements in the form of an additional carbon source suitable for a mixotrophic growth. Mixotrophy is when the organism use both photosynthesis (autotrophy) and additional carbon and energy sources (heterotrophy).

1.2 Utilization of Organic Carbon

If photoautotrophic and heterotrophic metabolisms are seen as the boundary modes of growth there are also intermediaries, namely mixotrophy and photoheterotrophy. Mixotrophy is when the algae makes use of both photoautotrophy and heterotrophic pathways to utilize both CO_2 and organic carbon sources, this may be time separated (*e.g*. day and night) or simultaneous. An extra level of complexity is seen in photoheterotrophic metabolism which is the mode where organic carbon is only utilized in the presence of light, this can easily be mistaken for mixotrophy.

Some algae may utilize heterotrophic pathways and grow on organic carbon sources without a light source. Several different carbon sources have been investigated and the most easily utilized is glucose that is readily used by many algae (8). The high price of glucose however make it prohibitively expensive to use in industrial scale. It can also be noted that glucose is used extensively in food production so there is a potential conflict. Other potential carbon sources include glycerol, acetate, xylose etc (Table 1). In this study the focus was on glycerol as a carbon and energy source which is known to be taken up and used in the metabolism of some algae (8). The actual pathways or the assimilation of glycerol by algae is not yet fully understood (8).

Table 1. A comparison of published specific growth rates of *Scenedesmus* species grown under mixotrophic conditions using various carbon sources.

Carbon Source	**Growth rates reported (d^{-1})**	**References**
Glucose	0.23-0.82	(9), (10)
Xylose	0.21	(11)
Fructose	0.63	(10)
Acetate	0.67	(10)
Glycerol	0.14-0.22	(9), (12)

1.3 Waste glycerol from biodiesel production.

As the development of a biobased economy is gaining more attention much of the focus is going into sustainable biofuels, for instance, biodiesel from residual fats and plant oils. During the process of refining the oils into a final biodiesel one of the main by-products is glycerol (approximately 10% w/w biodiesel) (13, 14). Whereas purified glycerol still has a reasonable market value, this crude glycerol is of a lesser quality and produced in such quantities that the value of such glycerol has decreased and the market has become saturated. The cost and energy requirement of refining abundant crude glycerol to a higher-quality product is now prohibitively expensive in relation to the market value, subsequently finding uses for it is becoming an issue (13). Production of other compounds of higher value from crude glycerol is now receiving considerable attention, including many biological routes (14, 15). By creating new products from what is now considered a waste product, the overall economic feasibility of the biodiesel industry will be improved substantially.

According to the European Biodiesel Board the total production of biodiesel in EU in 2013 was over 10 million tons but with a capacity of over 23 million tons (16). In a report from the Swedish ministry of energies the world production of biodiesel is over 26 million m^3 and rising (17). The European Commission has set a goal for year 2020 of 10% of all fuels in the transport sector to come from renewable sources so there is an incentive for governments to steer in the direction of biodiesel. EU also recognizes the issue of land use for fuel crops instead of food and it has been proposed that after 2020 only biofuels from sources not competing with food crops will be supported (18).

The crude glycerol that is made in the biodiesel production contains a number of impurities that make it less desirable for conventional uses but that does not necessarily pose a hindrance for biological conversion. The main impurities in crude glycerol is methanol and soap but depending on the oil source and production method various salts and pH levels may also be an issue. The contents vary depending on the biodiesel production process but typical values between 23- 37% by weight for methanol (19) and 0-31.4% soap by weight have been observed (20). A number of studies on various organisms have shown successful growth with crude glycerol as a carbon source (21-24)

1.4 Effects of mixotrophy and nutrient stress on biochemical composition

By altering the metabolic mode of the algae the biochemical composition of the biomass may also be altered. A number of studies have investigated the effect on oil content of microalgae under varying culture conditions, including mixotrophy. By providing the algae with an organic carbon and energy source it has been observed that apart from an increased growth there can also be an increased accumulation of oil in the cells (25-27). There has also been observed differences in pigment contents, both up and down-regulation, depending on the carbon source provided (27).

A tactic widely used for enhancing the oil content in microalga is to limit the nitrogen source in the media. It is generally seen that nitrogen promotes growth but not lipid accumulation so timing the nutrient starvation to after the major growth has taken place can be beneficial for production (8).

1.5 Algal biorefinery

After a big initial hype of algal biofuels as an alternative to fossil fuels it has become clear that to make this production economically viable an integrated approach to the production is needed (6). A number of recent articles treat the economics of algae cultivation and how integration into industry is needed to make it a reality (28, 29). To understand the concept of an algal biorefinery it is good to look at a definition of what a biorefinery is. One useful definition is: “A facility that integrates biomass conversion processes and equipment to produce fuels, power, materials and/or chemicals from biomass” (29). So it is about utilizing all the possible parts of the algae biomass for useful products. This might include some oils for diesel, some for food supplements, proteins for feed and pigments for pharmaceuticals among other possibilities. This is all on the end of what products come out of the algae but when one also considers the materials and processes that go into producing that biomass it truly becomes an integrated process. If CO_2, nutrients, carbon sources and water can be attained from other processes and thus make use of waste streams this would benefit both environment and overall profitability of the integrated system (30).

2 Aim

The aim of this study is to identify one or more species from the genus *Scenedesmus* capable of utilizing crude glycerol as a carbon and energy source for enhanced growth. Furthermore, the selected species will be characterized in terms of the biochemical composition and its response to growth on glycerol. This will be done from the perspective of identifying potential production organisms for an algal biorefinery. Especially the pigment and lipid composition will be investigated, as these are two groups of compounds identified as commercially interesting.

3 Methods

3.1 Common methods

The methods, species and media described in this section are relevant for all the experiments conducted.

3.1.1 Algae species used

Four algal species were used in this study and they are from or closely related to the genus *Scenedesmus* of green algae. The species were *Scenedesmus obliquus* strain CCAP 276/50, *Scenedesmus quadricauda* strain CCAP 276/21, *Ankistroddesmus falcatus* CCAP 202/15A, all from the Culture Collection of Algae and Protozoa. A natural isolate from the paper mill in Bäckhammar was also investigated, hereafter called *Scenedesmus sp* (backhammar) (31). All species are part of the local strain collection at the lab of Industrial Biotechnology, Chalmers University of Technology and has been subcultured at least every three weeks.

3.1.2 Growth media and preparation

The cultures were grown in a modified BBM media made up of stock solutions (Table 2)

Table 2. Stock solutions used to make up the BBM media.

Stock solution	Components	
Trace element solution	4.15 g	$Na_2EDTA \cdot 2\ H_2O$
	485 mg	$FeCl_3 \cdot 6\ H_2O$
	205 mg	$MnCl_2 \cdot 4\ H_2O$
	13.9 mg	$ZnCl_2$
	10 mg	$CoCl_2 \cdot 6\ H_2O$
	20 mg	$Na_2MoO_4 \cdot 2\ H_2O$
	1 Litre	Water
$NaNO_3$ Solution	250 g	$NaNO_3$
	1 Litre	Water
KH_2PO_2 Solution	14.8g	KH_2PO_2
	500 mL	Water
f2 vitamin 1	0.1 g	Thiamine·HCl
	100 mL	Water
f2 vitamin 2	0.01 g	Biotine
	0.01 g	Cyanocobalamin
	100 mL	Water
Solution 1	1.25 g	$CaCl_2 \cdot 2\ H_2O$
	3.75 g	$MgSO_4 \cdot 7\ H_2O$
	1.25 g	NaCl
	500 mL	Water

For the final media milliQ water was auotoclaved and filter sterilized (0.4 µm), the stock solutions added to a 1 L volumetric flask according to Table 3 and made up to one litre with the sterile water. In the case where an experiment required a different NO_3 concentration, more of this stock solution was added to the media.

Table 3. Preparation of BBM media.

Stock Solution	Amount
Solution 1	10 mL
$NaNO_3$ Solution	728 µL
KH_2PO_2 Solution	374 µL
Trace metal solution	1,2 mL
f/2 vitamin 1	1.2 mL
f/2 vitamin 2	100 µL
Water	To 1 litre

3.1.2.1 Crude glycerol

A sample of crude glycerol was kindly supplied by the Swedish company Eco-Bränsle (Karlshamn) accompanied with a data-sheet with average composition of the glycerol. This stated that the glycerol content was >75% w/w, methanol < 4% w/w, organic matter other than glycerol < 20% w/w. The actual glycerol content was measured in the same manner as for the cultures and was in reality closer to 45% w/w.

3.1.3 Analytical methods

3.1.3.1 Determination of biomass

For estimation of dry weight concentration, samples were filtered through preweighed glass fibre filters (47 mm, Whatman GF-C) that had been combusted at 550°C for 30 minutes to remove residual organic material and allowed to cool for at least 4 hours in a desiccator at room temperature. A known volume of sample was filtered onto each disk, washed twice with the volume of MQ water and then dried at 65°C for a minimum of 48 hours, cooled in a desiccator for 4 hours at room temperature before weighing again.

For estimation of culture biomass concentration, a standard curve was established for each species between the culture dry weight per litre (g L^{-1}) and culture optical density (OD). OD was measured at the wavelengths 450 nm, 650 nm, 680 nm and 750 nm with a Thermo Scientific Genesys 10 UV scanning spectrophotometer. These wavelengths relate to the following components in the algae, 450 nm for carotenoids, 650 nm for chlorophyll *a*, 680 nm for chlorophyll *b* and 750 nm for the biomass concentration. To get relevant samples, cultures were grown under both autotrophic and mixotrophic conditions, and under nutrient starvation, and were sampled regularly throughout their growth curves. The OD and the dry weight was measured in triplicates.

3.1.3.2 Pigments Quantification

At set time points in experiments 1-3, biomass samples were taken for subsequent quantification of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and total carotenoid (Car) content. A known volume of culture (0.5 – 2 mL depending on biomass concentration) was centrifuged in 2 mL Eppendorf tubes for 12 minutes at 13,500 x*g* (Eppendorf Centrifuge 5424). The majority of the supernatant was removed and then samples were centrifuged a further 5 minutes and the remaining supernatant discarded. Thereafter the samples were stored in a -20°C freezer until further analysis. Quantification was performed using the extinction coefficient equations for different solvents presented by Wellburn (32) (Table 4).

Table 4. Equations used to calculate the pigment concentrations.

Spectrophotometer	Carotenoids (µg x mL^{-1})	Chlorophyll *a* (µg x mL^{-1})	Chlorophyll *b* (µg x mL^{-1})
High detail Spectrophotometer (<1 nm resolution)	$C_{car} = (1000A_{480,0} - 1.29C_A - 53.78C_B)/220$	$C_{Chla} = 12.47A_{665,1} - 3.62A_{649,1}$	$C_{Chlb} = 25.06A_{649,1} - 6.5A_{665,1}$
Low detail Spectrophotometer (≈ 1 nm resolution)	$C_{car} = (1000A_{480,0} - 2.14C_A - 70.16C_B)/220$	$C_{Chla} = 12.19A_{665} - 3.45A_{649}$	$C_{Chlb} = 21.99A_{649} - 5.32A_{665}$

Before analysis of experimental samples, an evaluation of extraction solvent and pre-treatments was conducted to determine which method gave the highest pigment yield. Samples of *S. obliquus* was used for this trial taken at the same time and the same amounts (1 µg). The evaluation was an iterative process that aimed at finding an optimal combination of solvent and treatment for the algae studied. The solvents evaluated was Methanol, 80% Acetone, Diethyl ether (DEE) and Dimethyl sulfoxide (DMSO) and the pre-treatments were shaking in a heated incubator, bead-beating (Qiagen TissueLyser II) and sonication (Elmasonic P). In addition, the effect of increased temperature on extraction yield was evaluated for some of these methods.

A comparison of the highest extraction concentrations from each solvent was made (Figure 1). Then a comparison between different treatments using the same solvent was conducted to determine the method to be used (Figure 2).

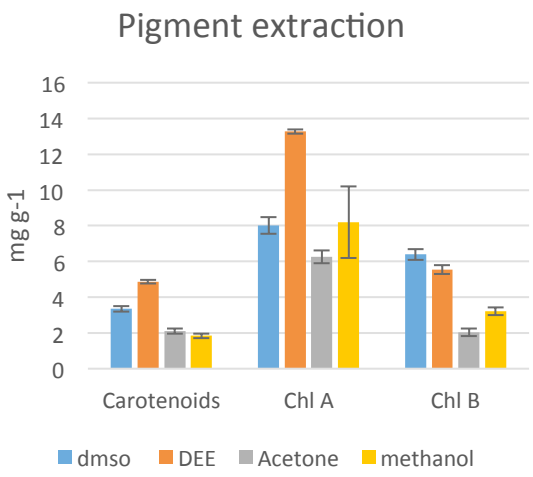

Figure 1. Pigment concentrations per dry weight using different solvents. Error bars =±1SD, measured in duplicates.

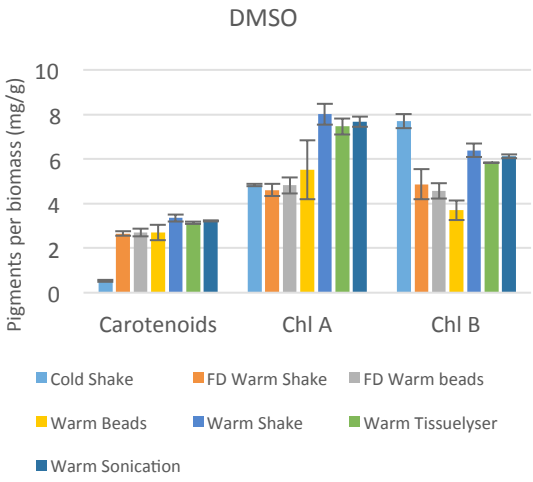

Figure 2. Different approaches to extracting the pigments with DMSO as the solvent. Error bars =±1SD, measured in duplicates.

The reason that DMSO was chosen over DEE was a combination of ease of handling, that DEE required mechanical disruptions of the cells in a tissue-lyser and that the ratio of Chl a to Chl b seemed quite different to what was observed with other methods. In addition, this altered ratio has an effect on the calculation of the carotenoid content was together with ones previously mentioned arguments against DEE. Using DMSO as the solvent made for easier and safer handling combined with consistent results.

Based on these results the best method for pigment extraction was the incubation of samples in DMSO at 60 °C in an Eppendorf Thermomixer comfort shaker (700 rpm) for 30 min followed by centrifugation for 5 minutes to recover the supernatant.

The absorbance of pigment extracts for the initial trial were measured using a Beckman Coulter DU800 spectrophotometer at the appropriate wavelengths (Table 5) and thereafter, experimental samples were measured using a Thermo Scientific Genesys 10UV Scanning spectrophotometer

Table 5. The wavelengths used for analysis of pigments in the two spectrophotometers used.

Spectrophotometer	Wavelenghts (nm)
Beckman Coulter DU800	480.0, 649.1, 665.1. 750.0
Thermo Scientific Genesys 10UV	480, 649, 665, 750

3.1.4 Nutrient concentration

Two main nutrients, glycerol and nitrogen, were analysed throughout the experiments to provide a more detailed picture of the metabolism of the studied algae.

3.1.4.1 Glycerol

Glycerol was the additive investigated in this study as an organic source of carbon and energy and thus the uptake rates of this nutrient was very important to monitor. Culture samples were filtered through a 0.2 µm cellulose filter to remove cells prior to analysis. Filtered samples were stored in HPLC vials in a -20°C freezer until analysis. For each culture two samples were taken.

The samples were then measured using a Dionex Ultimate 3000 HPLC equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) and a Phenomenex Security Guard AJO-4490 guard column. Column temperature was set to 60°C and 5 mM H_2SO_4 was used as the eluent at a flow rate of 0.6 mL min^{-1}. A refractive index detector (Shodex) was used for the detection and quantification of glycerol. A standard curve of glycerol was prepared in the range 6.25-1000 mg mL^{-1}.

3.1.4.2 Nitrate

The nitrate samples for nitrate analysis were filtered in the same manner as for glycerol analysis and stored in Eppendorf tubes in a -20°C freezer until analysis.

The analysis was conducted through an enzymatic assay adapted from Ringuett et al. (33) and was carried out in duplicate. To get in the range of the enzymatic assay (1 - 50 µM) the samples were diluted according to estimates of the nitrate concentration based on the initial values and the growth. A summary of the solutions used in this assay is described in Table 6.

To test the stated range of the method (0 - 50µM) a sample of 60 µM was added as an extra standard to see if the linearity held at this concentration. It was clear that this was not the case and the stated range was verified (Figure 3).

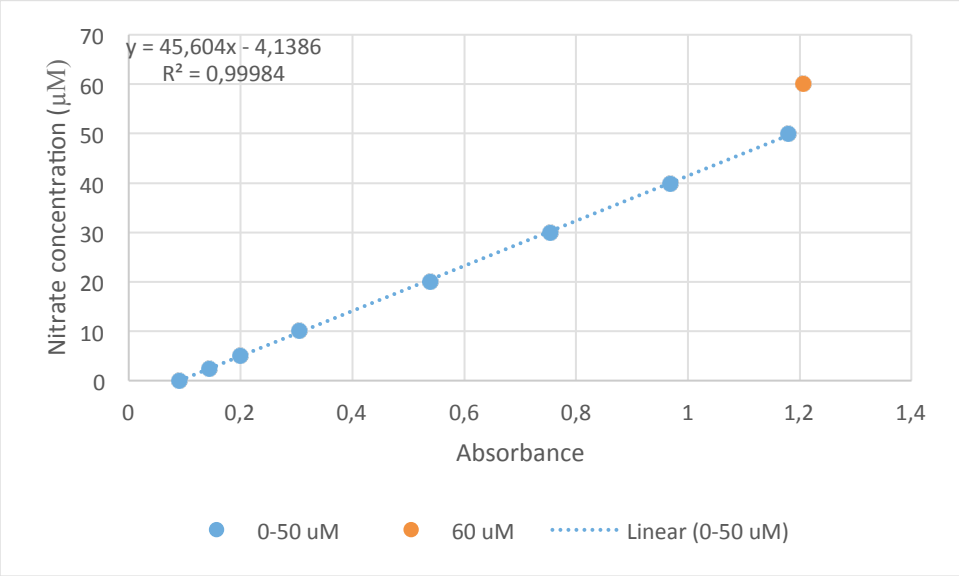

Figure 3. Standard curve for nitrate measurements with 60 µM added as a separate series.

Table 6. Solution used in the nitrate quantification assay.

Solution	Content
Buffer	27.6 mM potassium dihydrogen phosphate 25 mM potassium hydroxide 25 mM EDTA
NADH mix	2.4 mg NADH 1.5 mL MilliQ water
Nitrate Reductase mix	1 mL Nitrate reductase (The nitrate elimination company inc) 0.5 mL NADH mix 9 mL Buffer
Reagent 1	58 mM sulfanilamide in 3,6M HCl
Reagent 2	3.86 mM N-(1-naphtyl)ethylenediamine dihydrochloride (NED)
Standard solution	600 µM sodium nitrate

The assay was carried out in microplates. To each well 100 µL of sample was added and then 50 µL of the enzyme solution. The plates were put on a shaker at 900 rpm for 60 minutes at room temperature to react. When reaction was complete 50 µL of reagents 1 and 2 was added and the plate put on the shaker for an additional 10 minutes at 600 rpm. This resulted in a pink color that could be quantified by absorbance at 540 nm in a BMG Labtech FLUOstar Omega plate reader. On each plate a set of standards between 0 - 60 µM sodium nitrate was added to create a unique standard curve for each plate.

3.1.5 Fatty acid composition analysis

To quantify the fatty acid profile a larger sample was taken from the cultures (approximately 50 mL) and centrifuged in a Sigma 4K15 centrifuge at 5525 xg for 15 minutes and the supernatant removed. The pellet was then washed twice with Milli Q water, frozen at -80°C and then freeze-dried for a minimum of 48 h at -60°C using a Heto drywinner freezedrier with an Edwards RV8 pump. The dry pellet was ground to a fine powder and freeze-dried once more for 24 h. The fatty acids were then extracted, transesterified and analysed according to a protocol adapted from (34) as follows. Approximately 5 mg of dried biomass was weighed out and recorded into 11 mL glass tubes fitted with PTFE-lined caps. To each tube 0.5 mL of a chloroform/methanol mix (2:1) was added and the tubes were sonicated on ice for 60 min at 37.5 Hz. After sonication, 0.25 mL of an internal standard was added (equalling 200 µg of C17 in methanol) as well as 0.75 mL of a transesterification mix. This mix was made up of HCl/Methanol (5 % v/v) with 0.01 % (w/v) butylated hydroxytoluene. The tubes were thoroughly vortexed for 15 seconds. The samples were heat treated in an 85°C oven for 90 min and shaken halfway through. After given time to cool down 2 mL of hexane was added and the tubes mixed by vortexing and 20 minutes of gentle shaking on a rotating board. To separate the two phases the tubes were centrifuged for five minutes at 2000 xg at 10°C. Using a Pasteur pipette the upper hexane layer was transferred to clean tubes. 2 mL of fresh hexane was added to the biomass tubes and the shaking procedure was repeated and followed by centrifuging and collection of the hexane layer. The two hexane collections from each sample was combined and stored in a -80°C freezer until further analysis. When thawed, the samples were evaporated using a GeneVac miVac Quattro concentrator centrifugal evaporator at 30°C and then re-suspended in 1 mL GC grade hexane. The re-suspended samples were transferred to GC- vials and diluted ten times. The analysis was carried out according to Khoomrung et al. 2012 (35) in a Focus GC ISQ single quadrupole GC-MS (Thermo Fisher Scientific) and with a Zebron (ZB-WAX) GC column (Phenomenex).

3.2 Experimental Design of algae cultivation trials

Three types of experiments was planned and executed in this study, differing in scale and aim. In the first experiment, the aim was to assess the differences in growth between the species and under autotrophic vs mixotrophic growth to find a good candidate to move forward with. In the bottle experiments, the scale was increased and gave the opportunity to have more detailed sampling and analysis of the biochemical composition. In the bottles, more conditions were investigated. To be able to quickly assess growth under addition of inhibitors a plate screening method was used.

3.2.1 Screening trials in small flasks

The initial screening of all 4 species was carried out in 100 mL Erlenmeyer flasks equipped with air in- and outlet as well as a sampling tube. Each species was grown autotrophically and mixotrophically with an addition of 250 mg L^{-1} glycerol (equating to 100 mg carbon L^{-1}), each treatment was carried out in triplicate.

The flasks were placed on a modified rotary shaker running at 160 rpm. The board had been equipped with an up-ward facing light array providing light from underneath the flasks to provide an even distribution without interference from condensation or shading from tubing over the bottles. The light array consisted of cold white LEDs strips with an output at 5600 K. The LED strips used was organized in a 1x2 cm grid. The light output was approximately 100 µM photons m^{-2} s^{-1}. The light:dark cycle was set to 18:6 h. Samples were taken at the end of the light period each day for OD measurements and every 2 days for pigment quantification. The cultures was provided with filtered

(0.2 µm) and humidified ambient air bubbled through the flasks at a rate of 0.2 L L^{-1} min^{-1}. A total of twelve flasks were grown simultaneously and they were surrounded by blank flasks containing water to provide an even light distribution for the flasks at the edges compared to those in the centre.

3.2.2 Bottle trials

In further experiments on *A. falcatus*, a setup permitting larger sampling volumes to be taken was implemented. This system consisted of three parallel magnetic stirrers with five position that were set between perpendicular light racks so all bottles were illuminated evenly from two sides. Each light rack consisted of 2 (4 in total) 8W T8 cool white LED tubes (OSRAM) and cultures were grown under a 18:6 h light:dark regime at 150 µM photons s^{-1} m^{-2}. On each stirrer row, four 500 mL bottles were placed in the middle and blank bottles with water were placed on the ends to maintain even light distribution. Each bottle was filled with 400 mL of culture and stirred at 350 rpm. Each bottle was equipped with a gas inlet permitting filtered (0.2 µm) and humidified gas to be bubbled through the cultures at a rate of 0.2 L L^{-1} min^{-1}. During the glycerol concentration trials this ambient air was provided, whereas in the following experiments 2.5 % carbon dioxide was added.

Sampling was carried out at the end of each light period with 4 mL taken out with a syringe through the air inlet that was temporarily blocked. At day 4 and day 8 a larger volume was taken for fatty acid composition analysis (see section 3.1.5)

3.2.3 Plate trials

To provide a fast and simple way of screening different conditions and the effect of different inhibitors on algal growth, a microplate system was used. In 24 well plates, conditions were tested in replicates of 6 so each plate could hold 4 conditions. Each well was loaded with 2.4 mL of culture and the plate was placed on the same bottom lit shaker system that was used in the flask screening trials. To allow gas transfer and prevent build-up of condensation a semipermeable membrane (DiversifiedBiotech Breathe-EASIER) was placed over the wells. The cultures were grown under a 18:6 h light:dark regime and sampled at the end of the light period each day. 100 µL of each well were transferred to a 96 well plate and absorbance measured using a BMG Labtech FLUOstar Omega plate reader at 450 nm, 650 nm, 680 nm and 750 nm. The inhibitors evaluated and concentrations are presented in Table 7. The potassium was added in the form of the salt KOH and the values shown are recalculated for the actual potassium content.

Table 7. Inhibitors and concentrations tested in plate trials.

Test	Treatment 1	Treatment 2	Treatment 3	Treatment 4
Methanol – Low	0,01%	0,05%	0,1%	
Methanol – High	0,1%	0,25%	0,5%	1%
Potassium	50 mg L^{-1}	75 mg L^{-1}	100 mg L^{-1}	125 mg L^{-1}
Crude Glycerol	~250mg L^{-1}	~500mg L^{-1}	~750mg L^{-1}	

3.3 Statistical analysis

The statistical analysis was done with one-way ANOVA in the program IBM SPSS 20 utilizing Tukey´s post hoc test and homogenous subgroupings.

4 Results

4.1 Biomass measurements

To facilitate continuous measurements of biomass in the cultures, a standard curve was created for each species correlating dry weight with optical density measured in a spectrophotometer. The relationships in ***Table 8*** were subsequently used for all experiments.

Table 8. Relationship between dry weight and optical density measured at 750 nm.

Species	Equation	R^2-value
S. obliquus	DW = 0.8859xOD_{750nm}	R^2=0.9945
S. quadracauda	DW = 0.7763xOD_{750nm}	R^2=0.9617
S. sp. backhammar	DW = 0.9249xOD_{750nm}	R^2=0.9810
A.falcatus	DW = 0.8867xOD_{750nm}	R^2=0.9250

4.2 Species Screening Trial

4.2.1 Growth dynamics, biomass production and glycerol uptake

An initial screening of the four species was carried out to identify which had the highest growth rates, biomass production as a response to glycerol addition in the media. The highest exponential growth rate was shown by *S. sp* backhammar (1.01 d^{-1}) and all four species showed an increase in growth rate when grown under mixotrophic conditions (

Figure ***4*** A). The final biomass concentrations of *A. falcatus* were among the highest in both autotrophic and mixotrophic treatments (Figure 4, Figure 5) but this strain also showed a noteworthy increase in the exponential biomass productivity (280 mg L^{-1} d^{-1}) under mixotrophic conditions compared to all other species and treatments(Figure 4 B; $P < 0.05$).

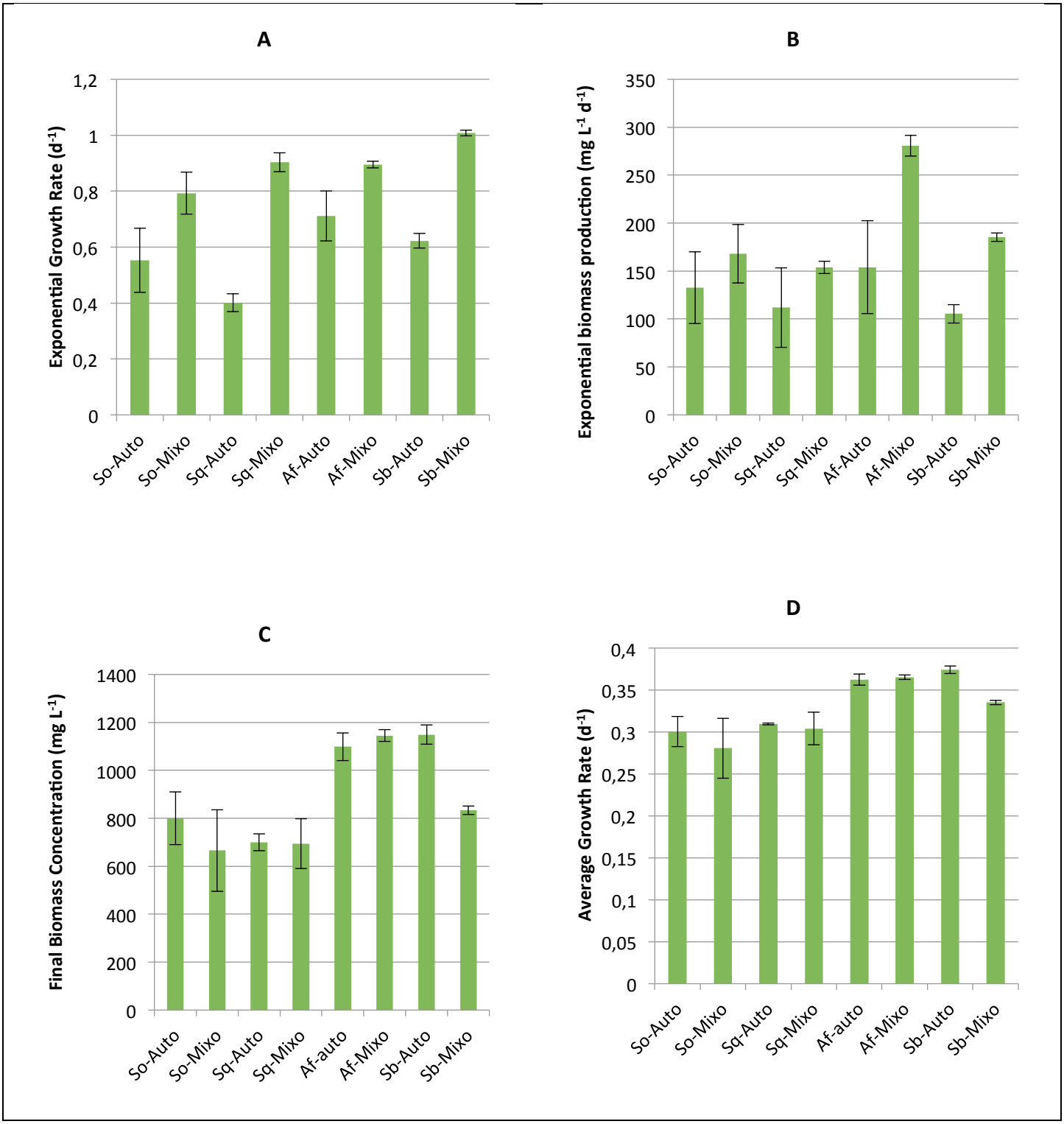

Figure 4. Growth data for the initial screening experiment. In A the exponential maximal specific growth rate (day-^{1}) is displayed. B shows the biomass production (mg L^{-1} d^{-1}) in the exponential growth phase. The final biomass concentrations is shown in C. D shows the average growth over the full 8 days. Error bars represent ±1SD. So – *S. obliquus*, Sq – *S. quadracauda*, Af – *A. falcatus*, Sb – *S. sp* (backhammar).

The data from the flask screening is also presented numerically with subgroupings shown (Table 9). Here it was seen that there was a clear difference between the growth rates between autotrophic and mixotrophic cultures for all species examined. There was also a difference in the average glycerol uptake rate between all the species.

Table 9. *Growth data from the screening experiment presented numerically ±1SD. Groupings within each variable (column) is depicted by letters in superscript. The experiment was run for 8 days and treatments were done in triplicate (n = 3).* So – *S. obliquus,* Sq – *S. quadracauda*, Af – *A. falcatus*, Sb – *S. sp (*backhammar*).*

Culture	*Exponential Growth Rate (d^{-1})*	*Maximum biomass production (mg L^{-1} d^{-1})*	*Final Biomass Concentration (mg L^{-1})*	*Average Growth Rate (d^{-1})*	*Average biomass production (mg L^{-1} d^{-1})*	*Average glycerol uptake rate (g g^{-1} d^{-1})*
So-Auto	0.553 ± 0.115^{ab}	132.6 ± 37.4^{a}	800.6 ± 110.3^{a}	0.301 ± 0.018^{ac}	91.1 ± 13.8^{a}	n/a
So-Mixo	0.793 ± 0.075^{cd}	168.0 ± 30.5^{a}	665.4 ± 169.9^{a}	0.281 ± 0.036^{a}	74.6 ± 21.4^{a}	0.105 ± 0.021^{a}
Sq-Auto	0.401 ± 0.032^{b}	111.8 ± 41.4^{a}	699.9 ± 35.1^{a}	0.310 ± 0.001^{ac}	80.1 ± 4.1^{a}	n/a
Sq-Mixo	0.903 ± 0.034^{de}	153.8 ± 6.4^{a}	694.3 ± 103.7^{a}	0.304 ± 0.019^{ac}	79.2 ± 12.9^{a}	0.174 ± 0.020^{b}
Af-auto	0.711 ± 0.089^{ac}	153.9 ± 48.6^{a}	1098.7 ± 58.0^{b}	0.362 ± 0.007^{b}	129.8 ±7.2^{b}	n/a
Af-Mixo	0.895 ± 0.012^{de}	280.5 ± 10.8^{b}	1144.9 ± 24.6^{b}	0.365 ± 0.003^{b}	135.4 ± 3.1^{b}	0.066 ± 0.003^{c}
Sb-Auto	0.622 ±0.026^{ac}	105.4 ± 9.7^{a}	1149.0 ± 40.0^{b}	0.374 ± 0.004^{b}	136.4 ± 5.0^{b}	n/a
Sb-Mixo	1.007 ± 0.010^{e}	185.4 ± 4.4^{a}	833.0 ± 17.7^{a}	0.335 ± 0.003^{bc}	97.0 ± 2.2^{a}	0.303 ± 0.007^{d}

4.2.2 Glycerol uptake

The glycerol concentration was measured daily for the first three days of the screening experiments. As can be seen (Figure 5) the initial concentrations were quite varied, which may be due in part to a slight delay in the measurement of the glycerol from inoculation. But some trends are still visible from the data. Both *S.* backhammar and *S. quadricauda* assimilated glycerol rapidly (0,303 d^{-1} and 0,174 d^{-1}, respectively) from the start and run out after two days whereas *S. obliquus* showed very gradual uptake over the whole growth period (0,105 d^{-1}) (Figure 5 and Table 9). *A. falcatus* showed a

lag phase before beginning a more gradual glycerol uptake than the other species (average 0,066 d^{-1}) over the same period (days 0-3). When comparing the biomass generated (**Table 9**) after three days between the autotrophic controls and the mixotrophic cultures divided with the glycerol taken up during the same time period this gives a yield of biomass on glycerol. The lowest figures were seen for *S. sp* (backhammar) (0,44 g_{DW} g_{Gly}^{-1}) and *S. obliquus* (0,84 g_{DW} g_{Gly}^{-1}) while higher figures were seen for *S. quadricauda* (1,51 g_{DW} g_{Gly}^{-1}) and *A. falcatus* (1,72 g_{DW} g_{Gly}^{-1})

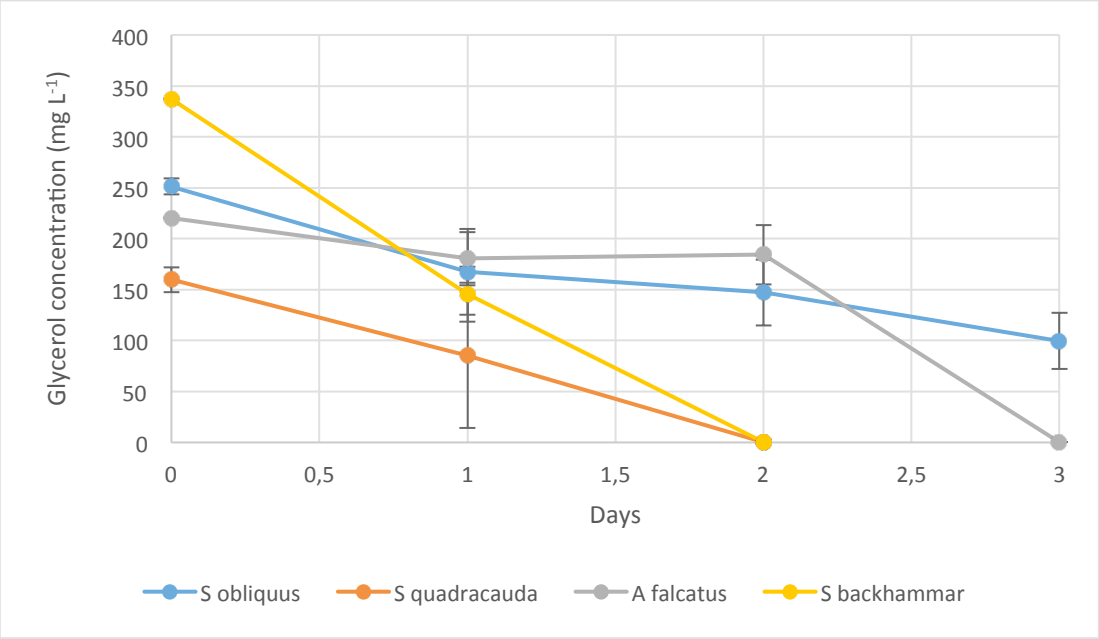

Figure 5. Screening experiment with different levels of glycerol concentrations measured daily for the first three days of the experiments and carried out in duplicate. Error bars = ±1SD

4.2.3 Pigment Composition

The pigment contents from the screening experiment are presented in two ways, as a concentration per volume of culture (Figure 6 A+B) and on amount per dry weight basis (Figure 6 C+D)). Large fluctuations can be observed in the data for the screening experiments. But the quick response times for increase of the carotenoids in the beginning of the growth phase could be distinguished in *S. quadricauda.* *A. falcatus* had the highest concentration of carotenoids both per volume and biomass in the mixotrophic cultures at both day 4 and 8 while day 6 is characterized by high standard deviations.

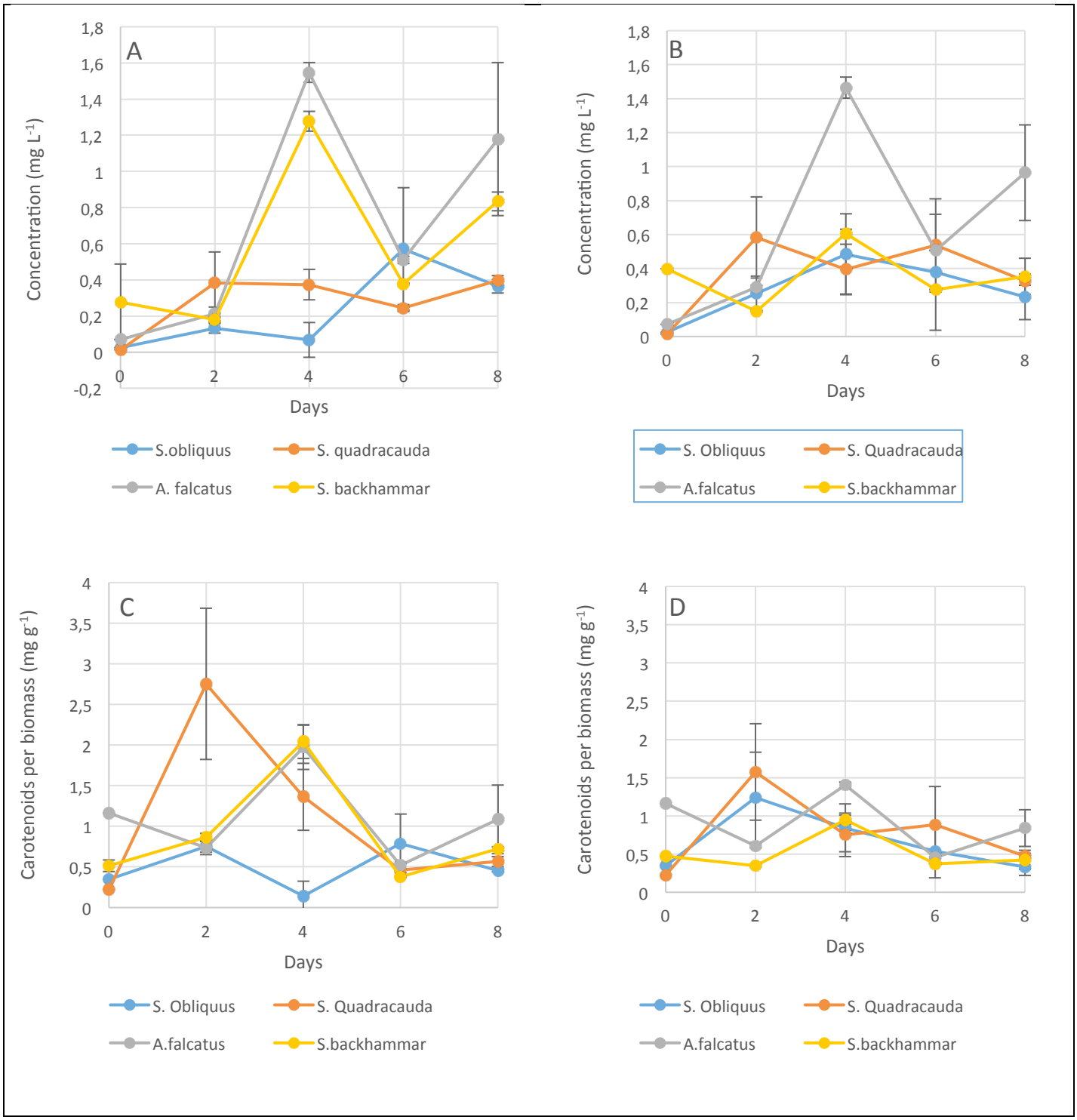

Figure 6. Carotenoid contents for auto- (A+C) and mixotrophic (B+D) cultures of the four species and shown both per volume of culture (A+B) and per dry weight (C+D). Error bars = ±1SD.

4.3 Glycerol concentration trials

For the subsequent experiments, the species *A. falcatus* was chosen based on the results from the screening experiments. In a scaled-up experiment, the response in growth and biochemical composition was investigated in relation to three concentrations of glycerol.

4.3.1 Growth dynamics and biomass production

In the glycerol concentration trials, a clear positive response in growth was seen with increasing concentration of glycerol (Figure 7). Both the growth rate and the final biomass increased in the mixotrophic cultures (Table 10; one-way ANOVA, df_8, F = 7.83 and 313.42, $P < 0.01$). The 300 mg C L^{-1} supplementation resulted in the highest growth rate of 1.06 d^{-1} in ($P < 0.01$) and the highest biomass concentration 1032 mg L^{-1} after 4 days ($P < 0.01$). Biomass concentration did not appear to vary from day 4 onwards in the two highest glycerol supplied treatments, but increased linearly from 504 mg L^{-1} to 643 mg L^{-1} between day 4 and 8 in the culture with lowest glycerol addition. The autotrophic condition demonstrated a linear growth rate throughout the experiment, reaching a final biomass concentration of 340 mg L^{-1}. The autotrophic culture showed a lower exponential growth rate in this setup compared to the screening but growth in the culture with 100 mg C L^{-1} was similar (Table 9).

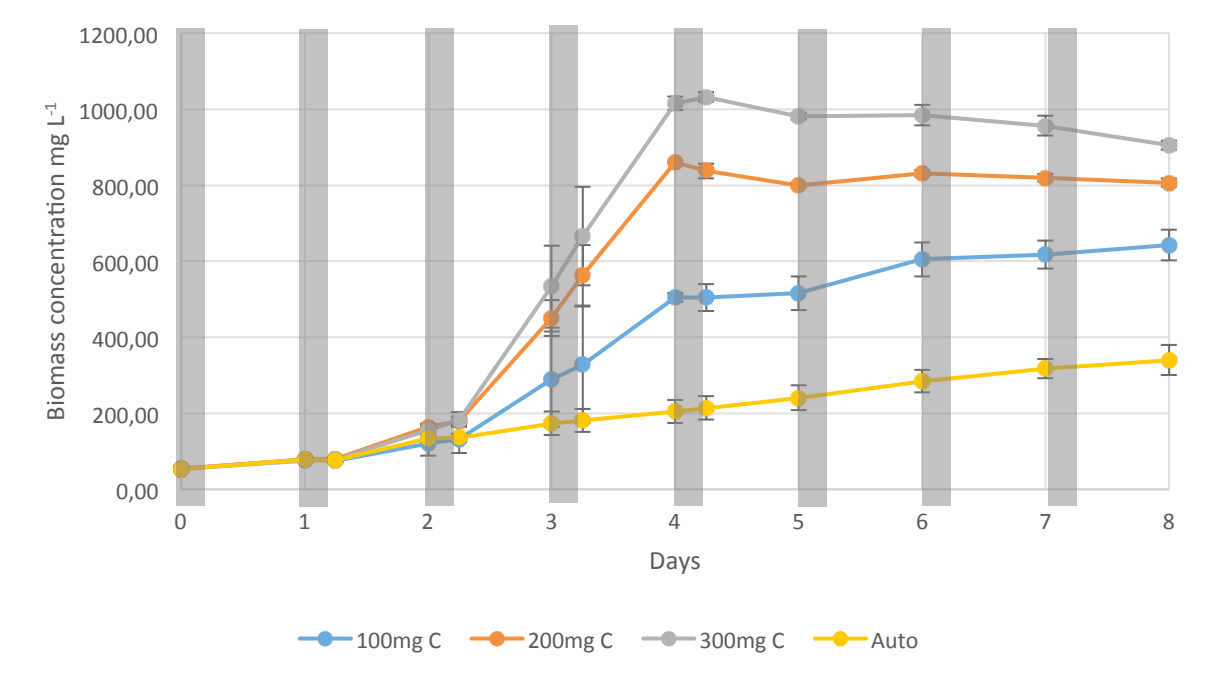

Figure 7. Growth response to increased glycerol concentrations over eight days. The dark regions show the dark hours of the day. Data are means of triplicate experiments (n =3), with samples analysed in duplicate, error bars = *±1SD*.

Table 10. Growth data from the glycerol concentration experiment presented numerically ±1SD. Groupings within each variable (column) is depicted by letters in superscript.

	Exponential Growth Rate (d^{-1})	Maximum biomass productivity (mg L^{-1} d^{-1})	Maximum Biomass Concentration (mg L^{-1})	Average Growth Rate (d^{-1})	Average biomass production (mg L^{-1} d^{-1})	Average glycerol uptake rate (d^{-1})	Average Nitrogen uptake (d^{-1})
Auto	0.599 ± 0.192[a]	58.0 ± 24.6[a]	340.1 ± 40.0[a]	0.233 ± 0.012[a]	35.97 ± 4.9[a]	n/a	0.00956 ± 0.00084[a]
100mg C	0.905 ± 0.068[ab]	218.1 ± 15.5[b]	643.0 ± 40.6[b]	0.310 ± 0.007[b]	73.65 ± 5.0[b]	0.847 ± 0.251[a]	0.01456 ± 0.00106[b]
200mg C	0.967 ± 0.086[b]	271.4 ± 12.2[b]	859.8 ± 2.3[c]	0.692 ± 0.008[c]	201.44 ± 0.8[c]	0.817 ± 0.126[a]	0.00804 ± 0.00017[ac]
300mg C	1.060 ± 0.110[b]	333.6 ± 31.4[c]	1032.4 ± 12.7[d]	0.695 ± 0.007[c]	230.24 ± 3.2[d]	0.642 ± 0.002[a]	0.00712 ± 0.00028[c]

4.3.2 Nutrient uptake

4.3.2.1Glycerol uptake

The uptake of glycerol in the glycerol trial is presented in Figure 8, where a clear lag phase was seen until day 2 before the glycerol was utilized by *A. falcatus* within the next 2 – 2.5 days (by day 4), which was the same time when a clear difference in growth was visible (Figure 7). In addition, it appeared that glycerol uptake initially only took place during dark phases of day 2 to 3, before uptake took place during the light phase (Figure 8). The two lower supplied treatments depleted extracellular glycerol by the end of the light phase on day 4, but the highest supplied treatment did not reach full depletion until the end of the corresponding dark phase. The uptake rates did not differ significantly between the treatments (Table 10). When comparing the carbon incorporated in the biomass against that added from the glycerol we could see that for all three treatments there is no large difference (Table 11). This was based on a 50 % carbon content in the biomass (36) and the initial concentration of glycerol in each treatment.

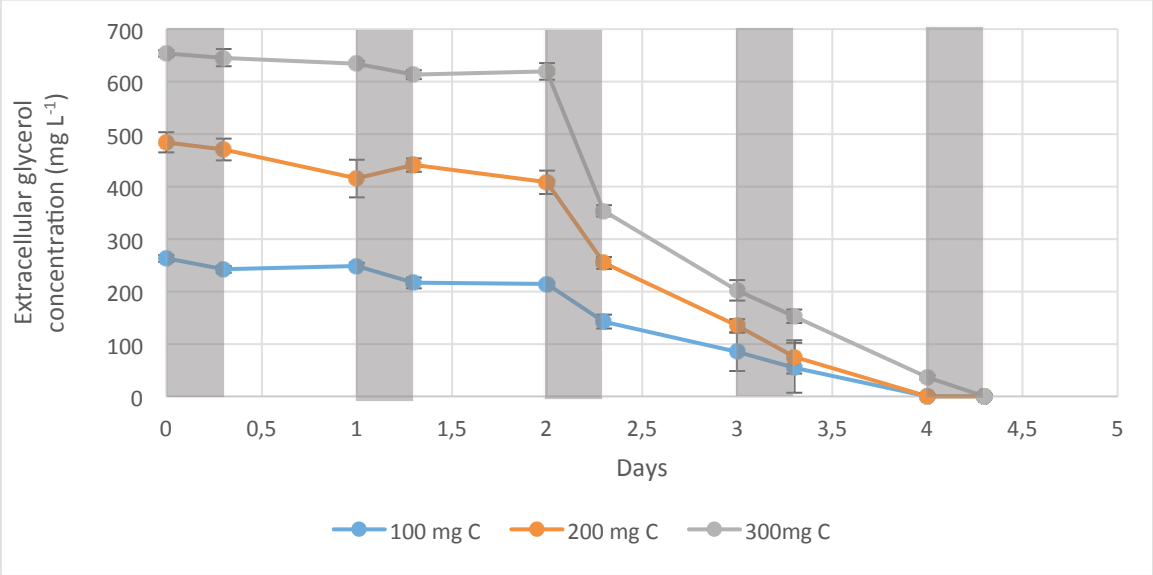

Figure 8. Media glycerol uptake during 8-day batch cultures of *A. falcatus* grown with different concentrations of glycerol. Samples were taken at the start and end of the light phase, with the dark phase indicated in grey. Data are means of triplicate experiments (n =3), with samples analysed in duplicate, error bars = ±1SD.

Table 11. A comparison of the carbon incorporated in the biomass against the carbon from glycerol added to the cultures. Based on the maximum biomass concentrations.

	Carbon increase against auto (mg L^{-1})	**Carbon from Glycerol (mg L^{-1})**	**Ratio**
100 mg C	152	103	1,48
200mg C	260	190	1,37
300mg C	346	256	1,35

4.3.2.2Nitrogen uptake

The uptake of nitrate from the media (starting concentration = 30 mg N L^{-1}) was also measured throughout the experiment. The nitrogen uptake for the two cultures with highest glycerol concentrations were nearly identical and may suggest that nitrogen limitation has started to affect them (Figure 9). From **Error! Reference source not found.** one might draw the conclusion that the nitrogen uptake rate would be higher in the cultures with glycerol but when the biomass production is taken into account it becomes clear that is not actually true (Table 10). The mixotrophic cultures have run out or nearly run out of nitrogen by day 4, while the autotrophic cultures still have nitrogen left by day 8. This is the same time that the glycerol was depleted and growth came to a halt.

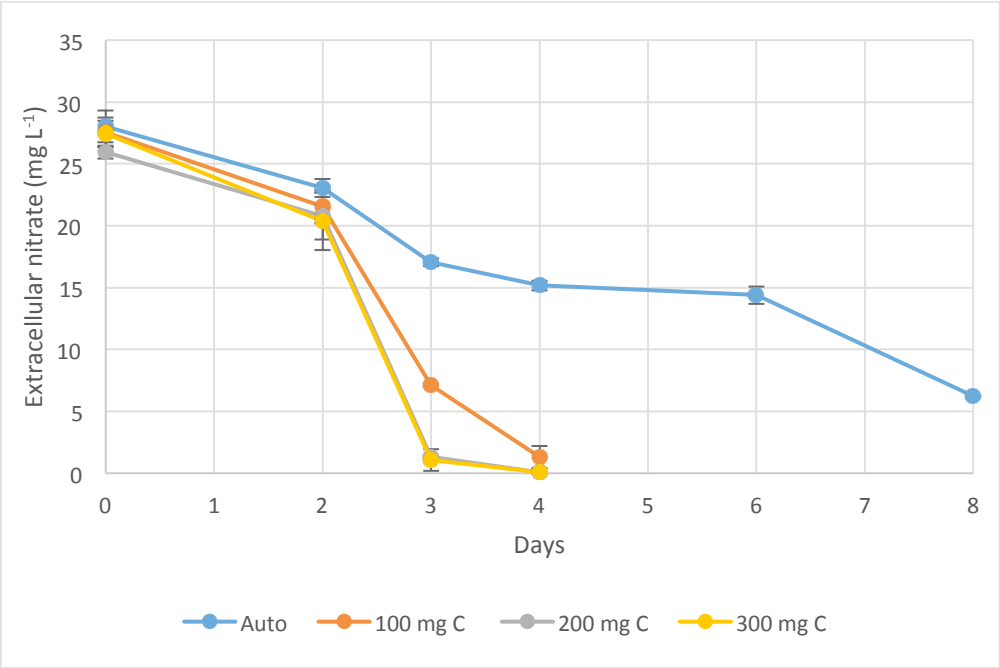

Figure 9. Media nitrate uptake during 8-day batch cultures of *A. falcatus* grown with different initial concentrations of glycerol and an autotrophic control. Starting nitrate concentration of the media was 30 mg NO_3 L^{-1}. Data are means of triplicate experiments (n =3), with samples analysed in duplicate, error bars = ±1SD.

4.3.3 Biochemical composition

4.3.3.1Pigment Composition

The concentration of total carotenoids in the cells varied slightly during the growth but seemed to be stabilized somewhat after the first four days. In the autotrophic cultures, the content nearly triples to a concentration of 4.05 mg g^{-1}, whereas mixotrophic treatments resulted in between 30 to 50 % lower concentrations (1.9 – 2.7 mg g^{-1}), with the lowest glycerol supplementation having significantly higher pigment content than the highest glycerol supplementation ($P < 0.01$). The chlorophylls were clearly lost from the mixotrophic cultures and more so as the glycerol concentration was higher ($P < 0.05$). The large standard deviations seen here at day 2 led to the switch of spectrophotometer and after that we saw more stable data.

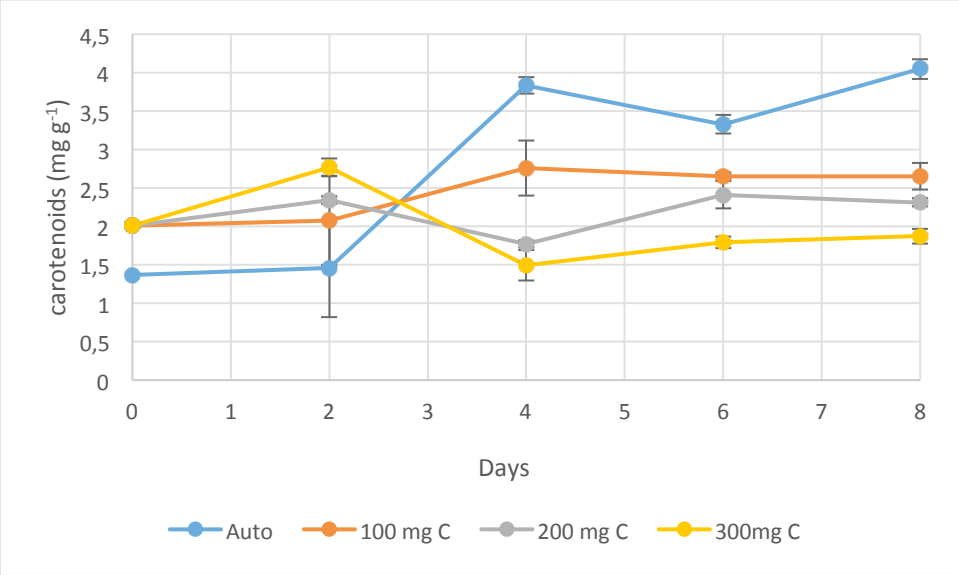

Figure 10. Carotenoid content in glycerol supplementation and autotrophic (Auto) cultures run over eight days in triplicate (n = 3). Measurements were done in duplicate and error bars = ±1SD.

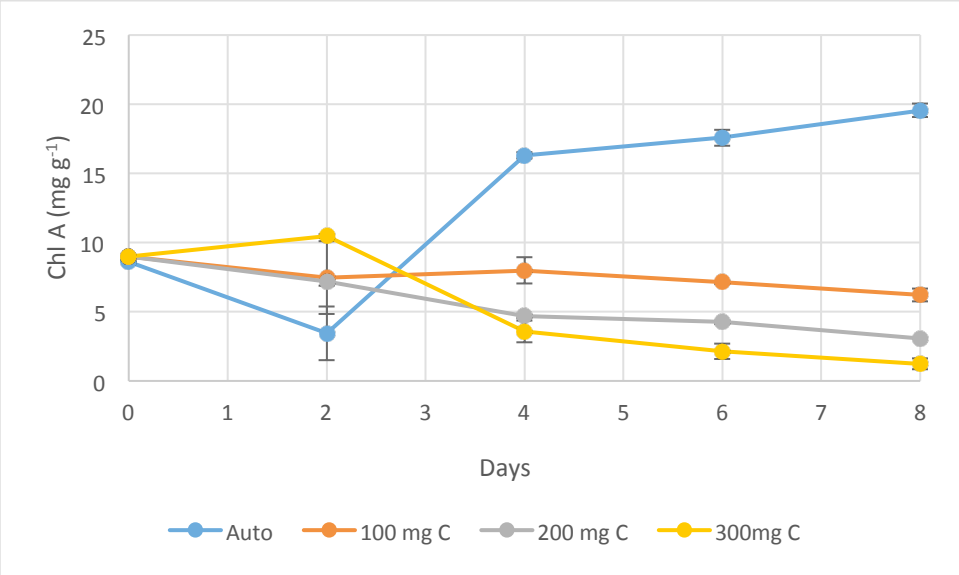

Figure 11. Chlorophyll a content in glycerol supplementation and autotrophic (Auto) cultures run over eight days in triplicate (n = 3). Measurements done in duplicate and error bars =±1SD.

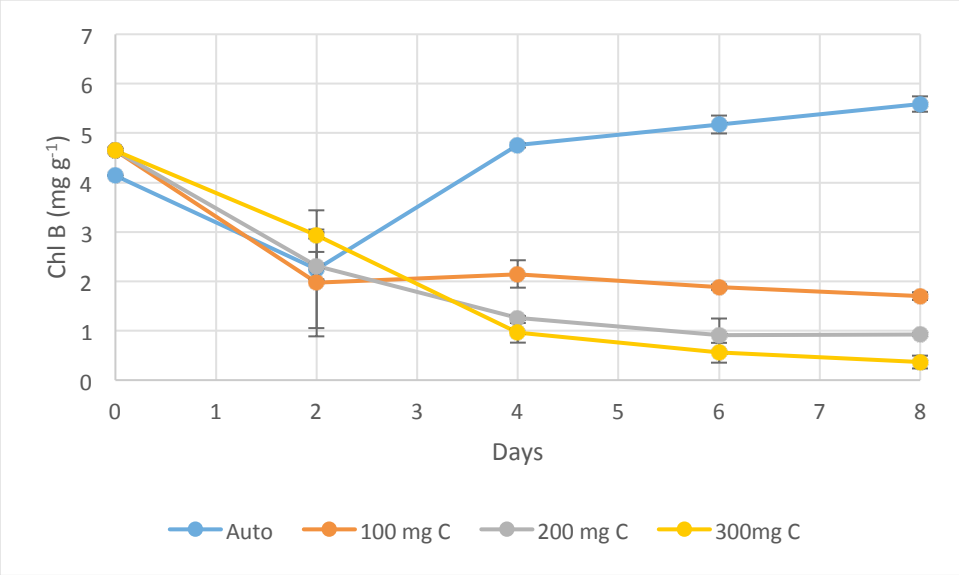

Figure 12. Chlorophyll b content in glycerol supplementation and autotrophic (Auto) cultures run over eight days in triplicate (n = 3). Measurements done in duplicate and error bars = ±1SD.

4.3.3.2Fatty acid composition

The fatty acid composition was analysed on the final biomass on day eight and an increase in total lipid content was observed in relation to the added glycerol (Figure 13, one-way ANOVA, df_8, F = 12,507, P < 0,05). A wide range of unsaturated fatty acids were observed (Figure 14)

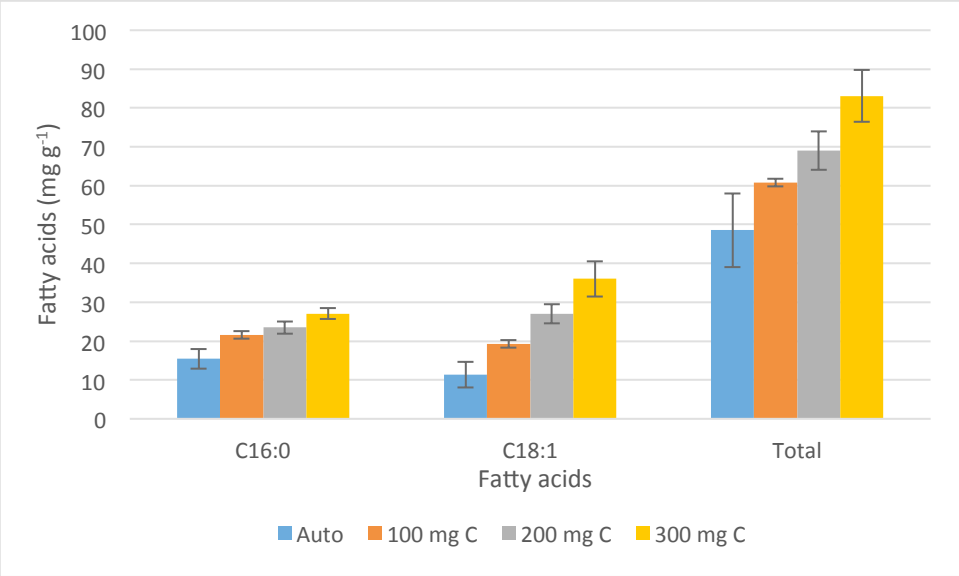

Figure 13. Major constituents and total fatty acids (mg g^{-1}) at day eight of glycerol supplementation trials. Displayed with error bars = ±1SD, n=3

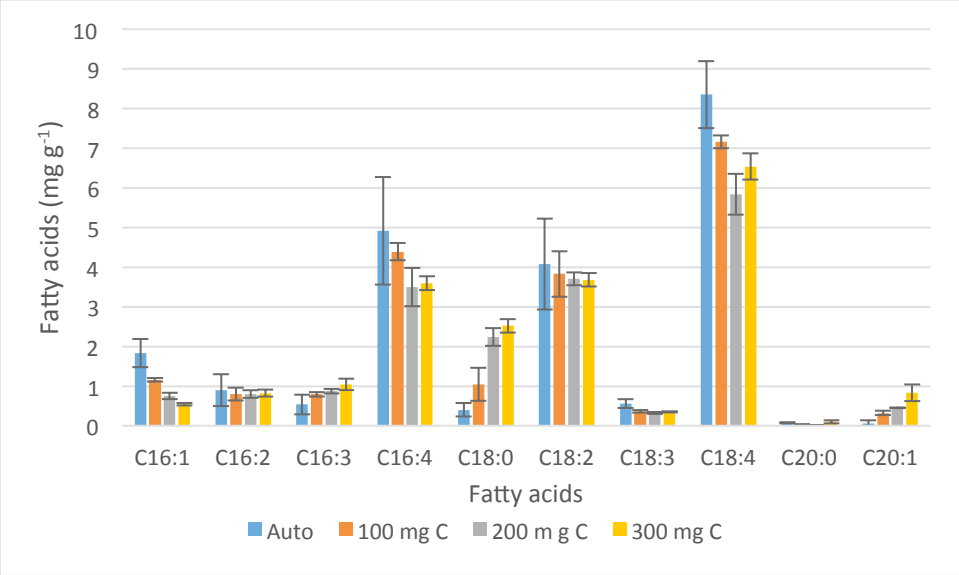

Figure 14. Minor constituents of fatty acids at day eight of glycerol supplementation trials. Displayed with error bars = ±1SD, n=3

4.4 Improving biomass production

4.4.1 Growth data

Carbon dioxide (2.5 %) was added by the gas inflow to the cultures to examine the effects of supplementation of an inorganic and organic carbon source on growth and biomass productivity. Mixotrophic treatments were supplied with 750 mg glycerol L^{-1} as this concentration elicited the highest growth rate and biomass production of those tested. The growth rates increased and the maximum specific growth rate achieved was in the mixotrophic culture with CO_2 addition and high N (1.25 d^{-1}) (Figure 15). Between the mixotrophic with low nitrate, with and without CO_2 addition (Table 10 and Table 12) there seem to be little difference. The next comparison would be with same glycerol concentration and higher nitrate, once again no real difference was observed. With an addition of CO_2 and higher nitrate in combination the increase in growth rate becomes more pronounced.

There were also high final biomass concentration in these cultures (Figure 16). The highest reached was in the autotrophic, high N and CO_2 culture (2.32g L^{1}). However, the mixotrophic culture under the same conditions were very close (2.25 g L^{-1}) and there was no statistical difference between them ($P < 0.05$).

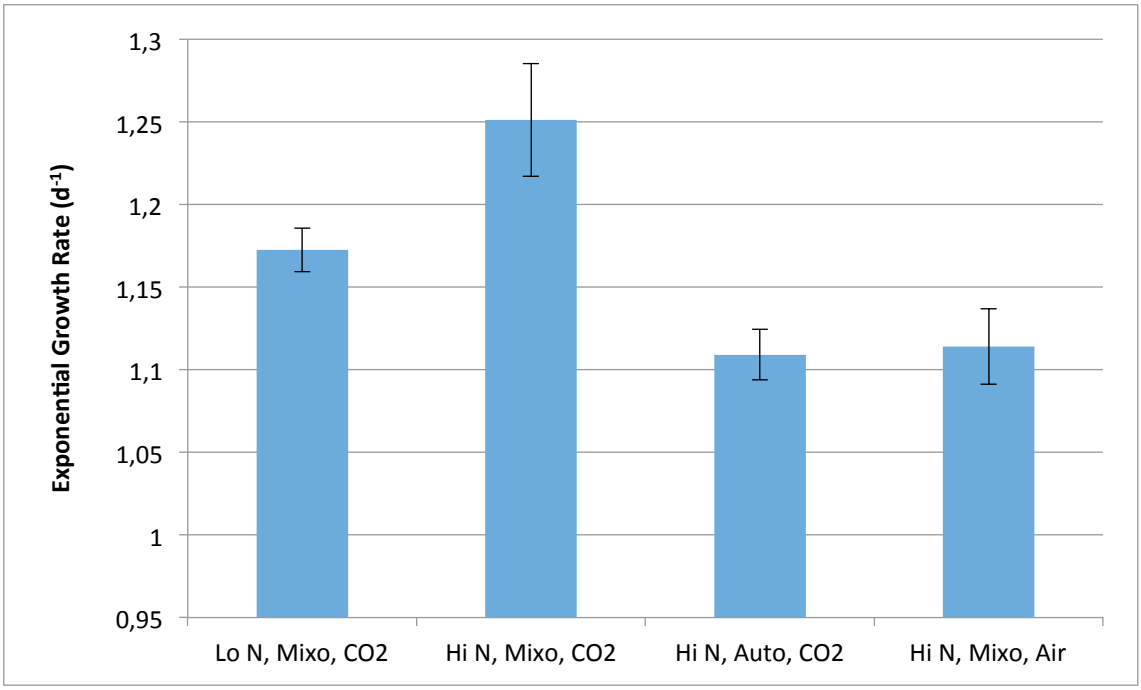

Figure 15. Exponential growth rates for varied nitrogen and CO_2 conditions, n = 3, error bars = ±1SD

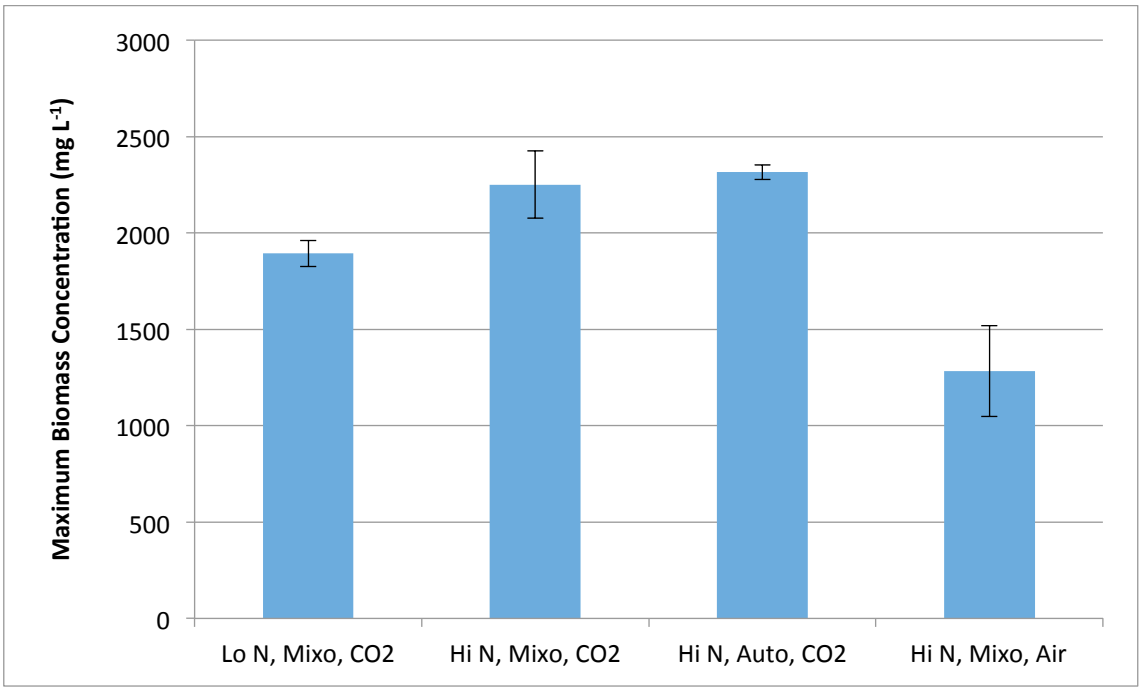

Figure 16. Maximum biomass concentrations reached in varying nitrogen and CO_2 conditions, n = 3, error bars = ±1SD

Table 12. Growth data from the improved production experiment presented numerically ±1SD. Groupings within each variable (column) is depicted by letters in superscript.

	Exponential Growth Rate (d^{-1})	Exponential biomass production (mg L^{-1} d^{-1})	Maximum Biomass Concentration (mg L^{-1})	Average Growth Rate (d^{-1})	Average biomass production (mg L^{-1} d^{-1})	Average Glycerol uptake rate (d^{-1})	average nitrogen uptake
Lo N, Mixo, CO_2	1.173 ± 0.013^{a}	386.0 ± 2.7^{a}	1894.1 ± 68.0^{a}	0.417 ± 0.003^{a}	228.4 ± 8.4^{a}	0.371 ± 0.028^{a}	0.00651 ± 0.00014^{a}
Hi N, Mixo, CO_2	1.251 ± 0.034^{b}	458.7 ± 14.4^{b}	2251.1 ± 175.7^{ab}	0.436 ± 0.012^{a}	272.8 ± 22.1^{ab}	0.419 ± 0.025^{a}	0.01088 ± 0.00159^{bc}
Hi N, Auto, CO_2	1.109 ± 0.015^{c}	331.1 ± 9.6^{c}	2315.5 ± 37.9^{b}	0.435 ± 0.003^{a}	280.5 ±4.8^{b}	n/a	0.01283 ± 0.00105^{cd}
Hi N, Mixo, Air	1.114 ± 0.023^{ac}	439.1 ± 26.4^{b}	1284.3 ± 236.3^{c}	0.367 ± 0.025^{b}	152.1 ± 29.6^{c}	0.551 ± 0.034^{b}	0.01437 ± 0.00142^{d}

4.4.2 Nutrient uptake

4.4.2.1Glycerol uptake

Although the variations in the analysis of the glycerol in this experiment were quite high, some interesting trends can still be seen (Figure 17). In this experiment like the others, there seemed to be a lag phase before the glycerol was taken up and there was a higher uptake rate when air was supplied instead of CO_2 (*Table 12*). The cultures with high nitrogen addition ran out of glycerol by day 3, whereas the cultures with lower nitrogen hadn't run out by day 6 when the last glycerol measurement was taken.

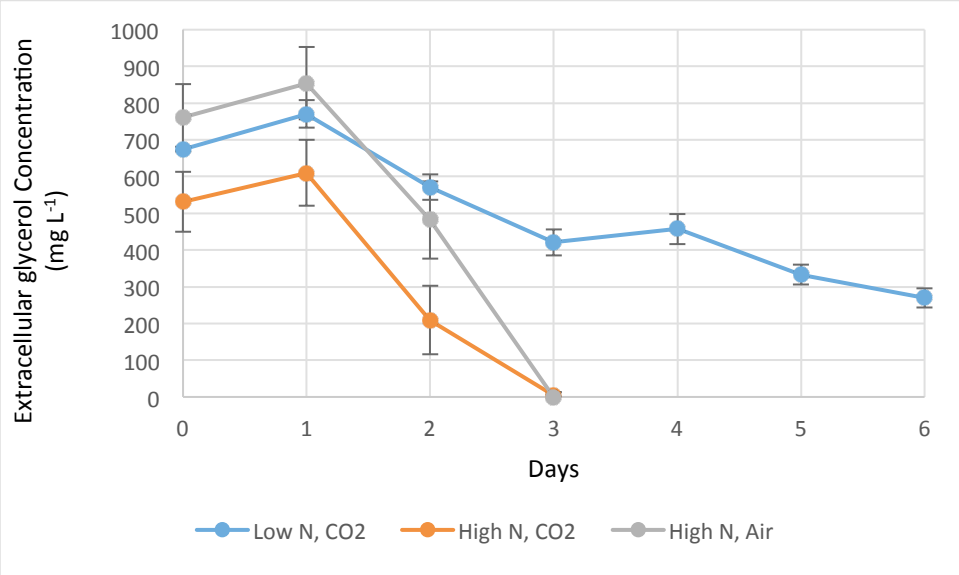

Figure 17. Glycerol uptake in CO_2 and nitrogen experiment run over eight days in triplicates (n = 3). Measurements carried out in duplicate and error bars = ±1SD.

4.4.2.2Nitrogen uptake

The nitrate uptake profiles with the different combinations of CO_2 vs air and mixotrophy vs autotrophy suggest that the mixotrophic cultures with CO_2 addition quickly become nitrogen-limited (by day 3) even at higher concentrations (Figure 18) and there is room for further optimization of the algal growth. The autotrophic cultures and the mixotrophic cultures without CO_2 run out of nitrogen by day 4.

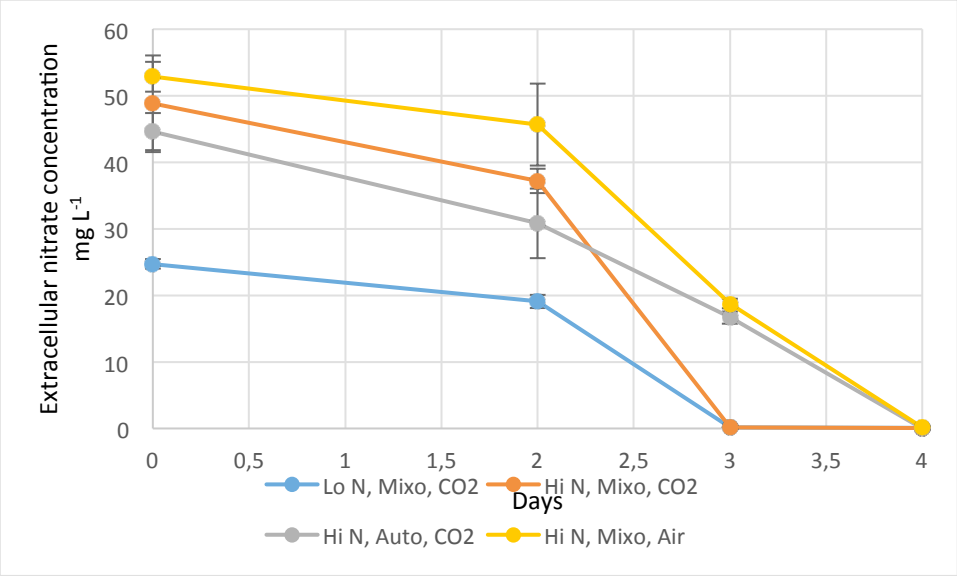

Figure 18. Nitrogen uptake in CO_2 and nitrogen experiment run over eight days in triplicates (n = 3). Measurements carried out in duplicate and error bars = ±1SD

4.4.3 Biochemical analysis

4.4.3.1Pigment Composition

The total carotenoid content of the cultures seemed to go down in every treatment (Figure 19) but less so in the autotrophic cultures and there is a difference in the final concentrations ($P < 0,001$). There was also a clear difference when it comes to the chlorophylls (Figure 20 and Figure 21) ($P < 0,001$). The treatment that did the most interesting thing in respect of pigment content was the high nitrogen, mixotrophic culture with air supplied. It initially followed the same downward trend as the other cultures with all pigments decreasing. Then from day 4 the chlorophylls didn't decrease anymore and the carotenoids actually went up slightly between day 4 and day 8 ($P < 0,05$). One explanation could be that the glycerol had run out (day 3, Figure 17) but the nitrogen lasted a bit longer (day 4, *Figure 18*) so they can still spend nitrogen on forming pigments, when the growth on glycerol is no longer an option.

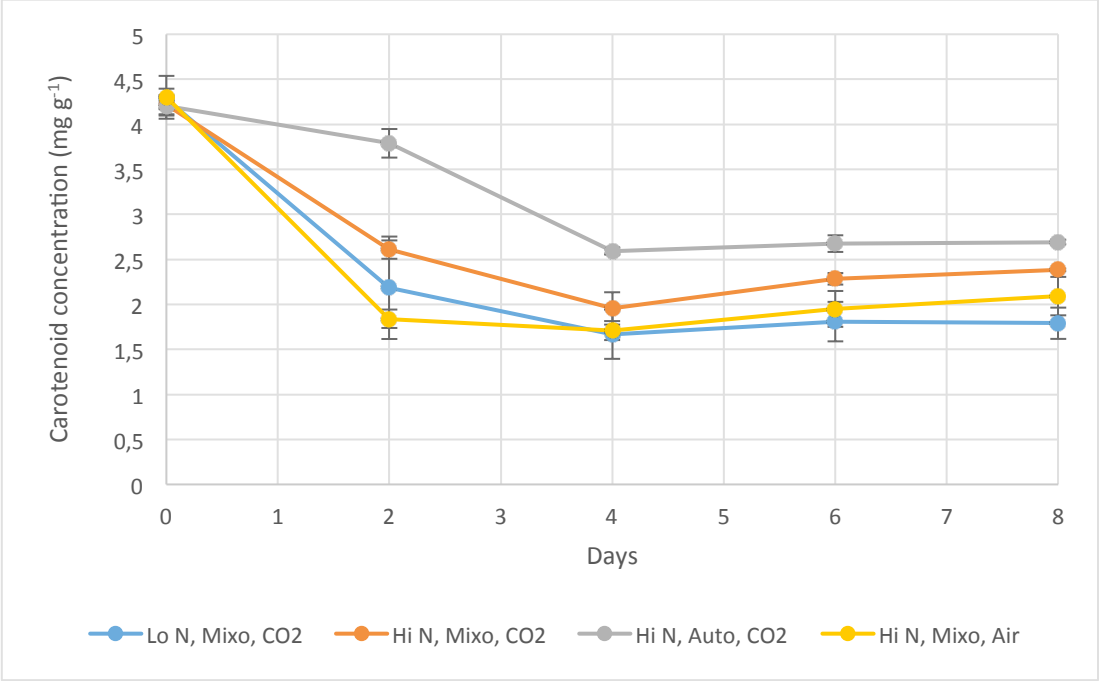

Figure 19. Carotenoid content in CO_2 and nitrogen experiment run over eight days in triplicates (n = 3). Measurements carried out in duplicate and error bars = ±1SD

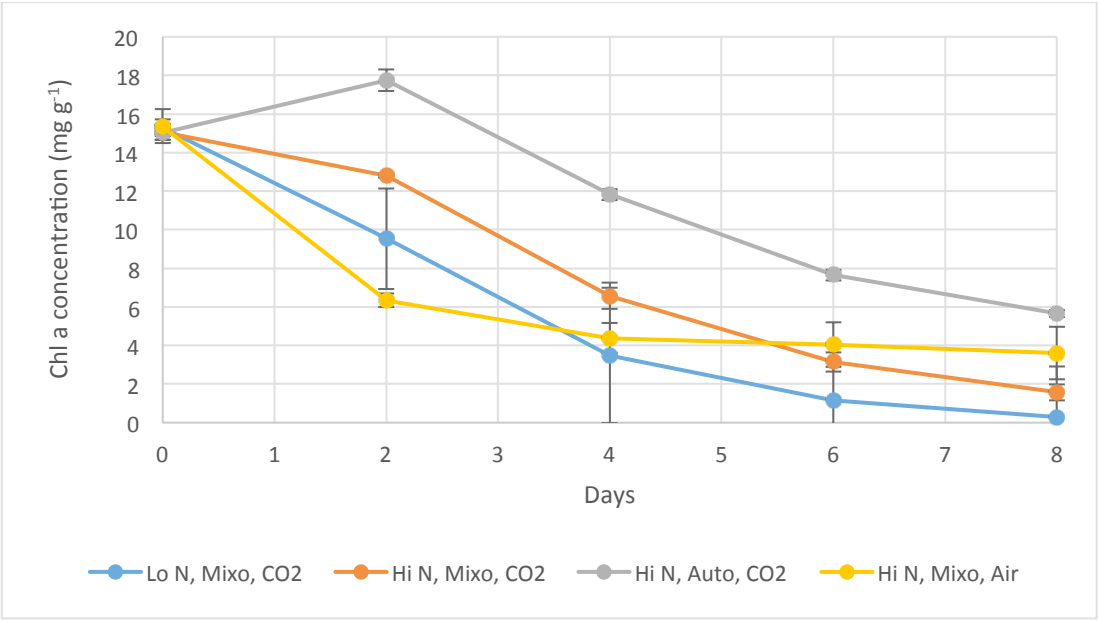

Figure 20. Chlorophyll *a* content in CO_2 and nitrogen experiment run over eight days in triplicates (n = 3). Measurements carried out in duplicate and error bars = ±1SD

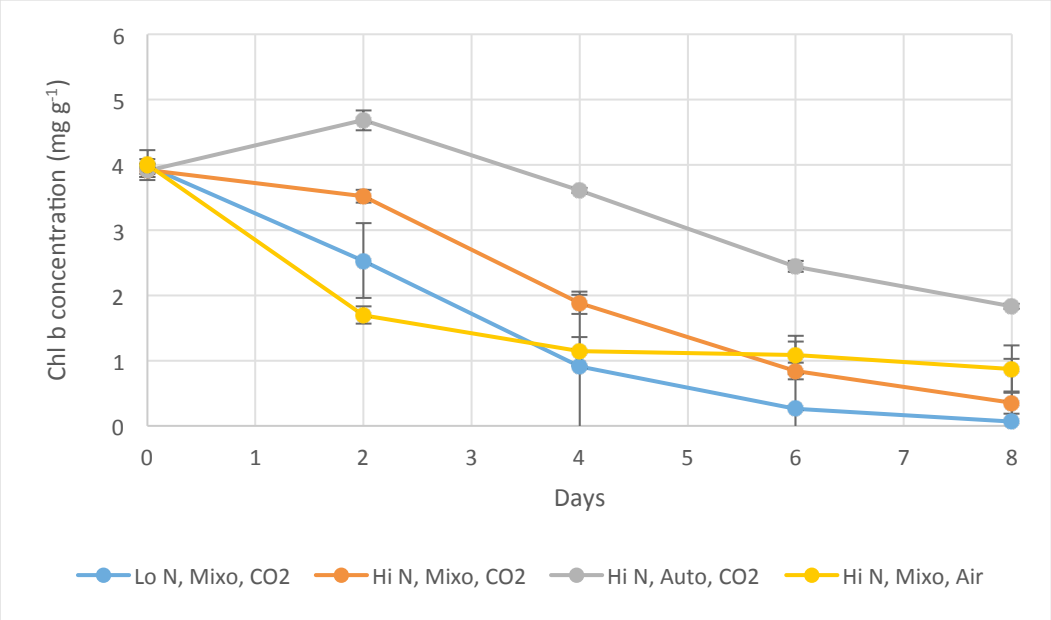

Figure 21. Chlorophyll *b* content in CO_2 and nitrogen experiment run over eight days in triplicates (n = 3). Measurements carried out in duplicate and error bars = ±1SD

4.4.3.2Fatty acid composition

During the trials with CO_2 and nitrogen levels, the changes in fatty acids (Figure 22) were not as pronounced as in the glycerol concentration trial (Figure 13). There are trends that warrant further experiments to gain more data for statistical analysis. When comparing the total fatty acids in 300 mg C L^{-1} with high (55,9±1,7 mg g^{-1}) and low nitrogen (79,3±3,1 mg g^{-1} , see section 4.3.3.2) a difference can be observed confirming the accumulation of lipids in response to nitrogen starvation (t-test, $P < 0,05$).

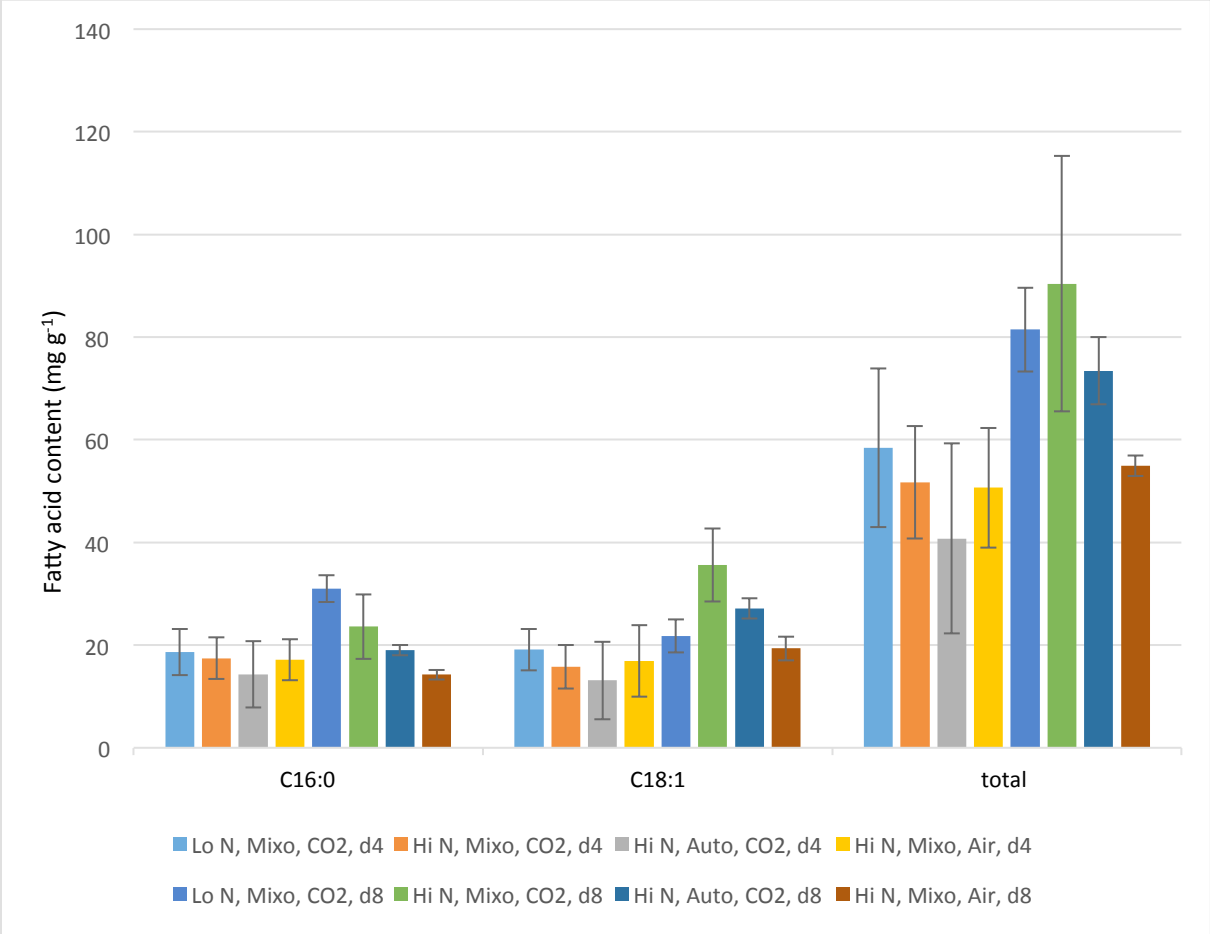

Figure 22. Major constituents of and total fatty acids in carbon dioxide and nitrogen trials. Error bars = ±1SD, n=3

4.5 Inhibitors and Crude Glycerol

A summary of the results from growth experiments with suspected inhibitors and crude glycerol is presented in Table 13 and discussed in the following sections.

Table 13. Summary of the growth results from plate experiments. Shown with ±1SD and with eventual subgrouping for each variable (column) marked with letters in superscript (P<0,05) . Potassium and Crude Glycerol share the same control.

	Methanol			Potassium			Crude Glycerol	
	Exponential Growth (d^{-1})	Final Absorbance		Exponential Growth (d^{-1})	Final Absorbance		Exponential Growth (d^{-1})	Final Absorbance
0.10%	1.15 ± 0.08^{a}	1.06 ± 0.09^{a}	50 mg L^{-1}	1.29 ± 0.20	0.51 ± 0.10	250 mg L^{-1}	1.32 ± 0.27	0.52 ± 0.12^{a}
0.25%	1.20 ± 0.17^{ab}	1.08 ± 0.14^{ab}	75 mg L^{-1}	1.30 ± 0.16	0.53 ± 0.08	500 mg L^{-1}	1.12 ± 0.11	0.35 ± 0.01^{b}
0.50%	1.54 ± 0.09^{c}	1.23 ± 0.10^{b}	100 mg L^{-1}	1.23 ± 0.10	0.54 ± 0.03	750 mg L^{-1}	1.21 ± 0.18	0.29 ±0.01^{b}
1.00%	1.36 ± 0.05^{b}	1.24 ± 0.09^{b}	125 mg L^{-1}	1.29 ± 0.09	0.59 ± 0.05	Control	1.44 ± 0.20	0.58 ± 0.04^{a}

4.5.1 Methanol

When *A. falcatus* was grown with added methanol to the media no adverse effects were visible as is demonstrated in

Figure 23 were increasing concentrations of methanol were added to cultures. Instead it was found that there can be a positive effect of a certain methanol addition (P < 0.05) .

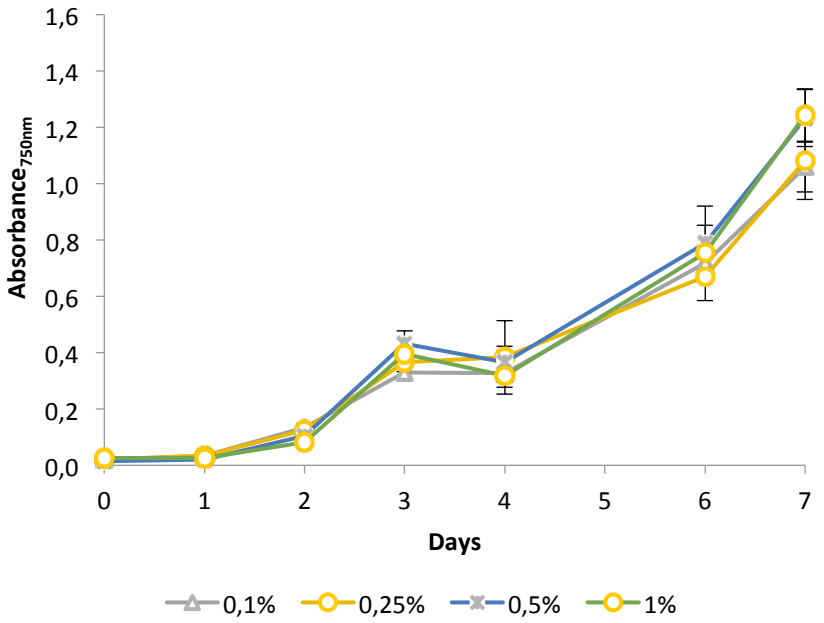

Figure 23. Growth profile with addition of methanol run over seven days (n = 6). Error bars = ±1SD

4.5.2 Potassium

The effects of potassium added to the media is seen in (Figure 24) and no visible or statistical differences were clear from this experiment in growth rate or final absorbance in relations to the control treatment.

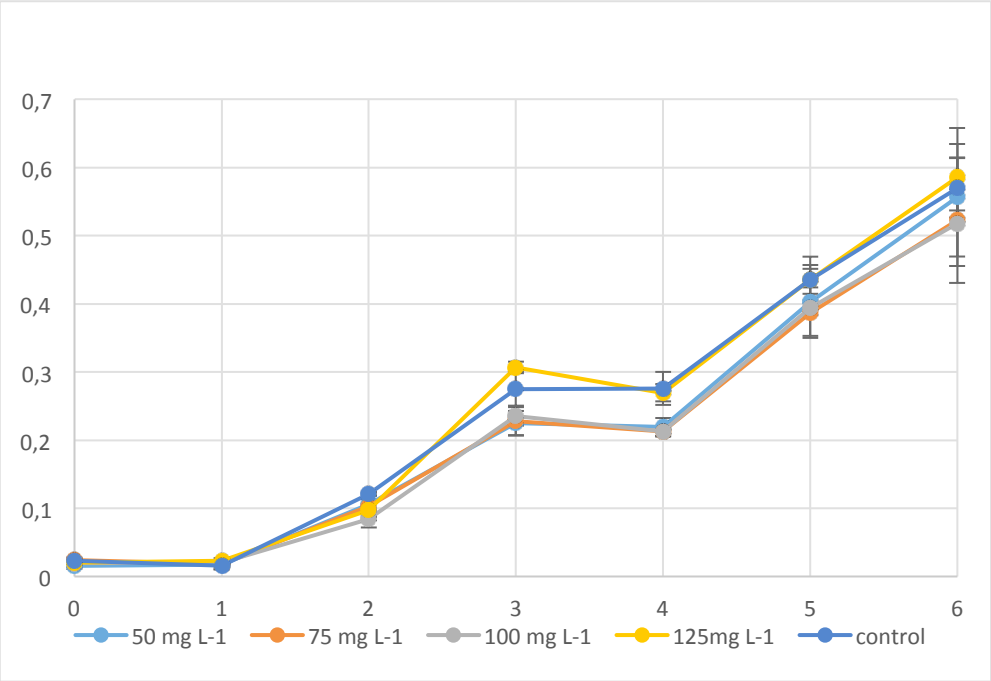

Figure 24. Growth with increased potassium levels run for six days (n = 6). Error bars = ±1SD.

4.5.3 Crude Glycerol experiment

When cultures were supplied with crude glycerol as a carbon and energy source with a technical glycerol addition as control (300 mg L-1). Cultures exposed to the lowest concentration at 250 mg L-1 was growing in a nearly identical way as the control. However, with increasing additions of the crude glycerol there seemed to be an inhibition of growth (Figure 25). It is worth noting that the cultures with crude glycerol showed a tendency to form aggregates that made correct readings of the absorbance harder to obtain.

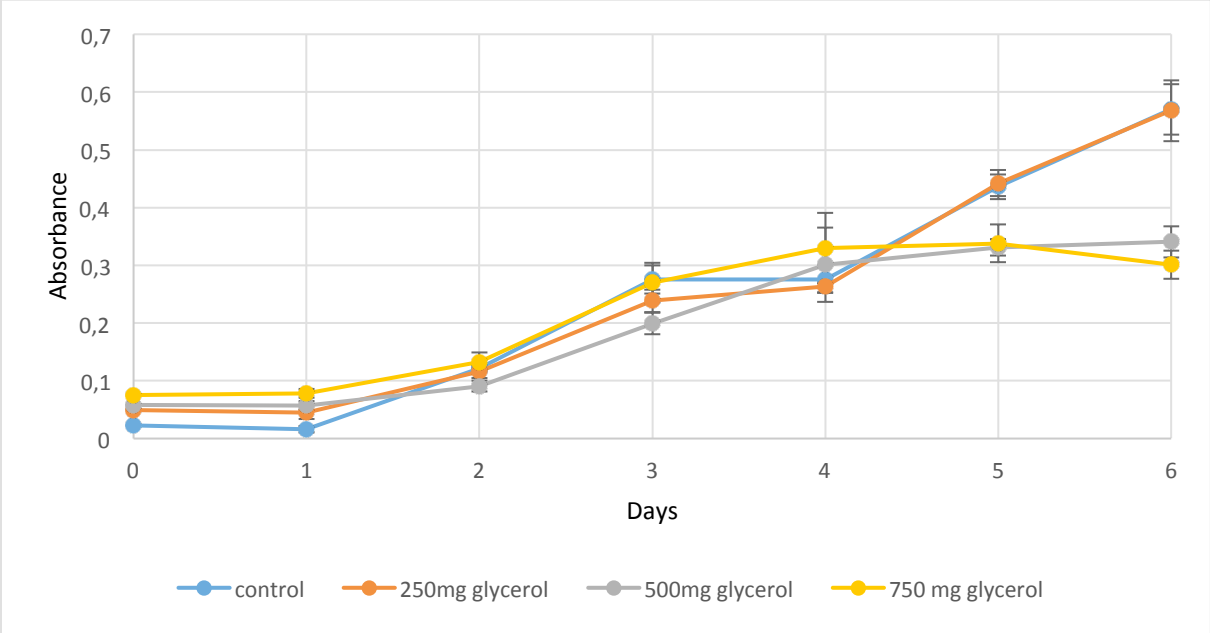

Figure 25. Growth on crude glycerol as a carbon and energy source run for six days (n = 6). Error bars= ±1SD.

4.6 Growth test with the photosynthetic inhibitor DCMU

To investigate the mode of metabolism in the species studied, an experiment with the photosystem inhibitor DCMU was conducted. As can be seen all the autotrophic cultures showed a similar flat line when DCMU is added, which is exactly what is expected. In the heterotrophic cultures, all species showed some growth and a bit more so in *S.* backhammar and *S. quadricauda,* which is consistent with the uptake rates shown in section 4.2.2. In the mixotrophic cultures, the differences found suggest different metabolic modes. For *A. falcatus* and *S. obliquus* (Figure 26, A2 and C2) the cultures with DCMU added showed no growth whereas both *S.* backhammar and *S. quadricauda* did (Figure 26, B2 and D2). *S. quadricauda* in mixotrophic culture without DCMU showed a clear difference in the growth curve compared to the autotrophic culture (Figure 26, D1 and D2).

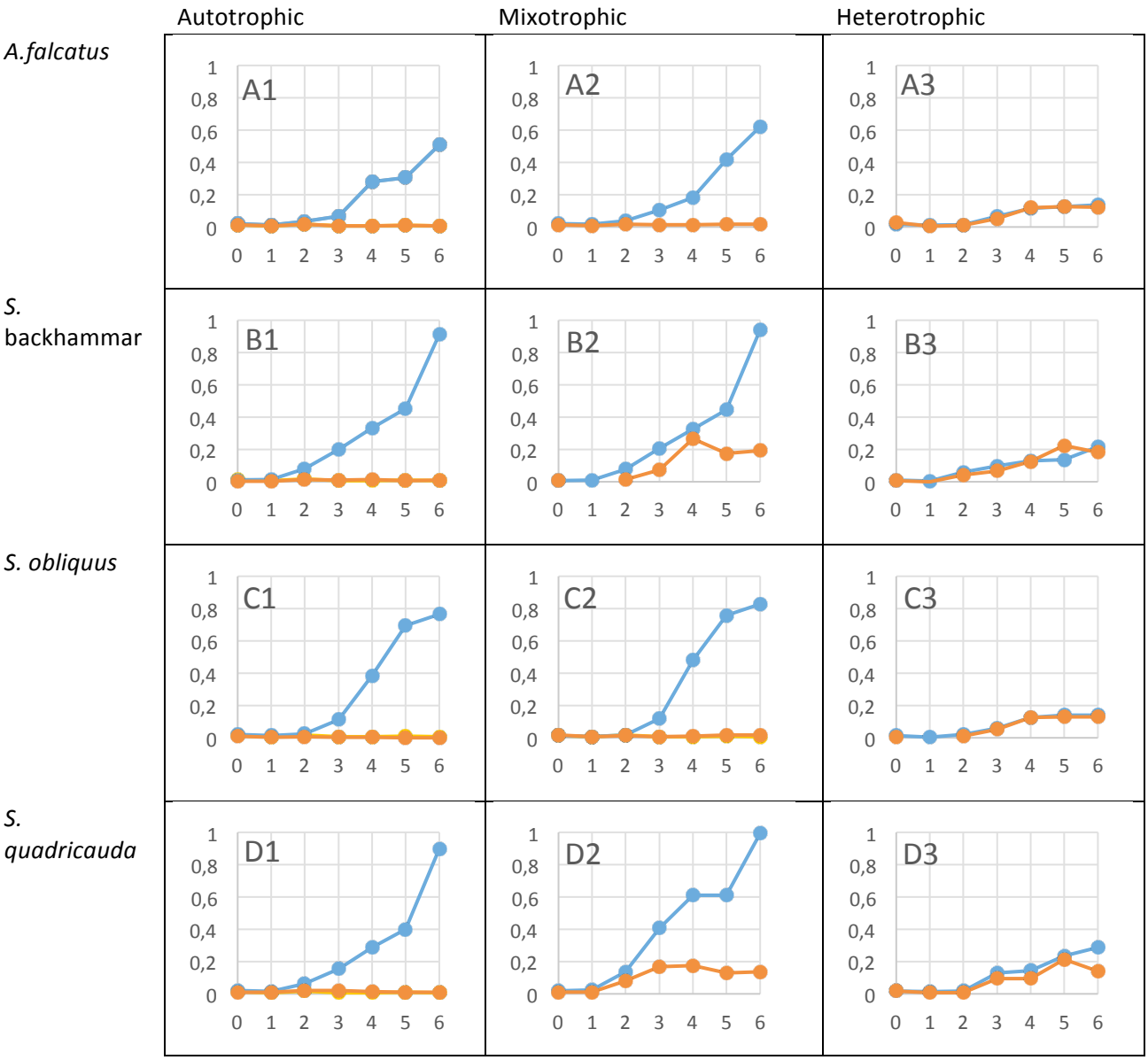

Figure 26. Metabolic modes of the four investigated species. Each mode is here shown with (orange) and without (blue) DCMU that blocks the photosynthesis. Each treatment was carried out in groups of six (n = 6). Horizontal axis = days, vertical axis = absobance$_{750nm}$.

5 Discussion

The main aim of this study was to investigate the growth of green microalgae with glycerol as a carbon and energy source. This was done by an initial screening of four species of freshwater microalgae. Then one species was selected for further experiments with varying conditions to better understand and improve the biomass production.

5.1 Effects of Glycerol on growth mode

In the screening experiments (section 4.2) and in the DCMU test (section 4.6), it became clear that the different species showed different responses to the addition of glycerol in the media, but that it generally has a favourable effect on exponential growth rate in comparison to autotrophic growth alone. Under dark conditions, and with glycerol available (heterotrophic), a small but noticeable increase in biomass occurred in all species (Table 9). This suggest that they can utilize the glycerol as a substrate. When grown under mixotrophic conditions with DCMU added to inhibit photosynthesis, only *S. backhammar* and *S. quadricauda* demonstrated growth on glycerol. To get a clear picture of what metabolic mode is utilized in each case is complicated and the basic DCMU studies might not be enough to be sure. One must also consider that the metabolism might well change over time in the cultures as the environment changes.

When considering the results of the photosynthetic inhibition, screening and glycerol concentration experiments together, it can be observed that *A. falcatus* at least initially favours photosynthetic growth and only takes up glycerol during dark hours (Figure 8) or when light becomes limiting, as such, the cells start taking up glycerol more rapidly and growth continues. This behaviour would suggest that the utilisation of glycerol is independent from the photosynthesis and is temporally separated with photosynthetic growth during the day and heterotrophic metabolism of the glycerol during the night. However, when light becomes limiting, cells shift to utilising glycerol during the light phase, possibly simultaneously with photosynthesis. Both of these scenarios would be classified as mixotrophy. But seeing that the uptake starts when the density of the cultures is still relatively low, light limitation seem improbable and the lag phase might instead be an acclimatization period. However, continuous measurements of eventual light limitation in the cultures as they grew denser was not conducted in this study so this would need further experiments. When considering that there is a decrease in overall pigment content in the cultures growing on glycerol compared to autotrophic conditions (Figure 10-Figure 12), this down-regulation of chlorophyll synthesis and related pigments would suggest that the cells shift from photosynthetic growth to heterotrophic utilisation of glycerol as they virtually lack chlorophylls once the uptake of glycerol commences. We have not seen any previous studies investigating the processes of this mixotrophy but encourage further studies.

5.2 Biomass production

As was already discussed a positive effect on growth rate and biomass production upon glycerol addition was observed and increased with increasing glycerol concentration (Table 10). If one consider 50 % (36) as a relative carbon content per biomass one can look at the effectiveness of biofixation of the glycerol added (Table 11) and see that the ratio is above 1. This indicates that the effect of mixotrophic growth is not simply the autotrophic + heterotrophic growth. Carbon dioxide is the only other carbon source supplied so this is where the extra incorporated carbon must come from. In future experiments it would be interesting to investigate this further by detailed

measurements of both glycerol and CO_2 uptake simultaneously to get a fuller picture of the carbon fixed and if there is a synergistic effect here.

5.3 Biochemical composition

That the addition of glycerol has an effect on the metabolism of the alga *A. falcatus* is clear from the study done and we have also showed that this altered metabolism changes the biochemical composition in terms of pigment composition and fatty acids content and profile. The biochemical composition of microalgae can vary significantly and is dependent on many factors during cultivation such as growth conditions (temperature, light pH), media constituents and nutrient stress (37, 38). Subsequently, depending on the products one wish to focus on, an optimization process must take place that may lead to growth conditions or production strategies that are specific for that particular product. Here we are beginning this work by describing the effects of glycerol concentration, CO_2 addition and to some extent nitrogen starvation. It is easy to visually get a sense that something has changed when looking at *A. falcatus* samples grown under different conditions or for different times as they tend to shift from green to a more orange/yellow tint. We have shown that this effect is due to a loss of chlorophylls (Figure 10-Figure 12 and Figure 19-Figure 21) when a shift to mixotrophic growth occurs.

If one considers that the loss of pigments leads to a lowered capacity for photosynthesis this means that in a batch culture where the glycerol run out the cells are left with few options for growth with no pigments to carry out photosynthesis and no glycerol to use for heterotrophic growth. The cells are seen to accumulate more lipids in correlation with the added glycerol (Figure 13) and we also saw that the lower nitrogen level resulted in higher lipid content, consistent with other studies (39). Seeing that our study only stretched for eight days there might not have been enough time for lipids to accumulate properly, which would explain why we did not see as high levels as other studies that grew the algae for a longer time period (39). To fully appreciate the changes in biochemical composition, we suggest that additional experiments are conducted with longer timespans monitoring both fatty acid and pigment composition.

5.4 Inhibitors and growth on crude glycerol

We have seen that main impurities in the crude glycerol such as methanol and high potassium levels does not have an adverse effect on the growth when considered individually. Methanol even showed a small but significant positive effect on final culture density, which is favourable as it can be found at levels around 20 % in crude glycerol depending on the source (20, 23). One of the other main impurities that still needs to be investigated is the soap fraction that is formed in the transesterification process of making biodiesel. The studied levels of inhibitors has been based on typical composition of the crude glycerol obtained from a Swedish company producing biodiesel. This means that if future growth optimization leads to higher glycerol concentrations in the media it is important to also study the effects of the increased levels of inhibitors that would follow. The optimal level of crude glycerol used might be a compromise between supplying glycerol and keeping the level of inhibitors below concentrations that may negatively affect growth. This also suggests that strategies employing a fed-batch mode of growth using crude glycerol (where it is continuously added upon depletion), must consider the potential build-up of inhibitory compounds during

cultivation, which may eventually deleteriously effect growth even if the concentrations of inhibitors in the feed initially are within an acceptable range.

If some of these compounds prove to be inhibitory (possibly the fatty acid salts/soap) it does not need to be a great hindrance to crude glycerol as a substrate, as there are well established ways to remove certain fractions (especially soap) from the glycerol, for instance by acidification (40). Other options include precipitation with calcium, which was found to improve the ability of fungal species *Rhodopseudomonas palustris* to ferment glycerol for hydrogen production (41). There will be an added cost associated with any pre-treatment that is done on the glycerol so the less is needed the better.

5.5 Implications of a biorefinery on crude glycerol

The alga *A. falcatus* has previously been identified as a potential species for biofuel production (42) and under the conditions studied here we have seen growth rates and biomass productivities that as far as we are aware, are significantly greater than those currently in the literature (42, 43). This suggests that there is much we can do to further optimise algal production for both maximum biomass yield and product composition. However, even with high biomass productivities and lipid contents, a single product approach has little chance of profitability (4, 29). By considering the whole biomass as the raw product and then fractionating it into various components a higher overall value is feasible. For a long time the sole interest in algae has been oil for biodiesel, but once we start analysing the biomass we see that there is much more of value there. The biomass can be divided up in lipids both for diesel production (bulk), feed and for food in terms of polyunsaturated fatty acids (high value). The pigments are highly sought after as antioxidants and for example the carotenoid lutein is today primarily produced from Marigolds in Asia, which leads to a seasonal production (44). The production also heavily rely on cheap labour for picking the flowers and processing. A bad year due to drought will quickly affect the market price. If an alternative method with a microalgae-based biorefinery can produce lutein of a comparable quality this market can be accessed with production closer to the buyers. This market was 2010 estimated to be around $233 million dollars and a predicted annual growth of 3.6 % (44) .

Each situation of constructing a viable setup for a biorefinery is unique and will be affected by the local industries that can be connected to it. In this case, we have investigated the use of crude glycerol as a source of carbon and energy and have seen that it can significantly improve the biomass productivity, as well as change the biochemical composition of the cells. If one consider other requirements for the cells, other nutrients must be added to the media and one option is to use waste water to provide such nutrients (45). This not only provides an environmentally positive effect but it can also add value as waste water treatment can be a costly process and industries are under regulations on what they are allowed to release. The glycerol itself is, as we have discussed, one of the main side products of biodiesel production and can be procured for a low price. Another additive that greatly improves the growth is CO_2, which is also an unwanted product from many industries. This could be added in the form of flue gas (46). When using either waste water or flue gas one must also consider other compounds therein and the effects they may have on the algae and potential impurities that need to be treated downstream. We would suggest an approach were these options are evaluated from the point of view of their price, the environmental benefits, and how the product quality is affected. Ideally, most of the substrates for the algal cultivations can be obtained from

various industrial waste streams, so one must also consider the streams coming out from the cultures. An effective way of separation of various fractions from the biomass is critical and it requires further research (6). Other than the products mentioned above, a fraction of carbohydrates will be left from the biomass and this could potentially be utilised as a substrate for fermentation by yeast (29, 47). By combining the growth of algae with a fermentation process one could also use the CO_2 that is a waste product from the fermentation to improve the growth of the algae. Algae instead of carbon dioxide produce gaseous oxygen, which is a potentially valuable by-product. By linking the growth of algae with other aquaculture, such as fish-farming, the water for fish larvae can be oxygen-enriched and the fish can be fed a portion of the algae produced (protein fraction might be used for feed). To sum up, a thought through approach to all the streams of compounds going in and out of the algae cultivation can not only improve the production, but also minimize waste and give higher profits.

5.6 Alternate pigment production strategies

When producing many different products it can be hard to know how the optimization process would look and as we have seen in the previous section many aspects must be taken into consideration. However, there is also many opportunities. The overall productivity of a desired compound or group of compounds is highly affected by the growth rate and biomass productivity. How this can be raised by a mixotrophic approach using crude glycerol has been shown here. To improve the yield of a specific product, further optimization of the growth conditions must be conducted. In this case, we will discuss from the point of producing carotenoids as they are high-value products and to improving the productivity of them even by small amounts can have a greater effect on the overall profit. The other compounds such as lipids, carbohydrates and protein will still be produced simultaneously. As we have seen, the mixotrophic growth makes the growth rate to increase but the pigmentation goes down, primarily in the chlorophylls. The carotenoid content however seem to stay relatively stable (Figure 19-Figure 21) and this leads to an interesting opportunity. By alternating the autotrophic and mixotrophic growth periods in the cultures it is not unreasonable to expect that the pigments will be kept and even increased as the cell density increases and light becomes limiting. Promising results with this alternating approach has been shown for the productivity of carotenoids in other species (48). As the carotenoids have a protective role in the cells, where they direct and dissipate excess light as well as act as antioxidants (49), a high irradiance level and oxidative stress might induce the production of these compounds. Once again, this is a trade-off between inducing the production of these compounds through stress and not stressing the cells so much that they do not grow. We propose conducting experiments in a fed-batch reactor where the glycerol addition can aid growth at certain times and quickly produce high cell densities, but that the algae, at least initially, are grown autotrophically under conditions that induce high pigmentation. An alternative approach might be to grow the inocula in dense autotrophic cultures, which will promote pigmentation due to light limitation and that the production cultures subsequently are grown mixotrophically with some source of mild oxidative stress that gives an incentive to retain their antioxidative carotenoids.

6 Conclusions

We have successfully identified the species *A. falcatus* as a green microalgae capable to grow on glycerol as a source of organic carbon and energy. This significantly improves the growth rate and raise the potential of growing this species even under light-limited conditions such as high latitudes. The growth rates observed are among the highest observed and reported in the literature for this species, as far as we are aware. The metabolic mode is proposed to be mainly mixotrophic under these conditions, but with temporal changes between mainly photoautotrophic during light hours to mainly heterotrophic during the dark phase. Furthermore, we have seen that the growth on glycerol enhanced media improves the lipid content but potentially has a small negative effect on carotenoid content, which can be compensated for with the increase in growth rate giving an increased net productivity. To fully appreciate the potential of this species as a production organism in a biorefinery approach further studies in optimizing growth conditions must be conducted but the initial results are intriguing and warrants that such studies are undertaken.

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