Improving the biomass of locally isolated green microalgae by nitrogen starvation and addition of glycerol

With a focus on the productivity of triglycerides for biodiesel

Yi Lu
Abstract

Microalgae are able to convert inorganic CO$_2$ or organic carbon into biomass highly efficiently. Biomass from microalgae is a promising feedstock for food additives, cosmetics, pharmaceuticals and biofuels. Biodiesel, deriving from triglycerides (TAGs) produced by microalgae, is a renewable and sustainable replacement for fossil fuels. In most circumstances, microalgal biomass consists mostly of proteins and carbohydrates whereas lipids contribute to the least part; however, the lipid content may increase when cells are exposed to the environmental stress. Nitrogen starvation (N-starvation) is a proven and effective strategy for enhancing TAG productivity in algal biomass. In this study, biomass productivity and lipid accumulation under N-starvation of six Nordic algal strains, including *Chlorella vulgaris* 13-1, *Coelastrum astroideum* RW10, *Desmodesmus sp.* RUC2, *Desmodesmus sp.* 2-6, *Coelastrella sp.* 3-4 and *Scenedesmus sp.* B2-2, which are adapted to low temperature and limited sunlight characteristic for northern climate, were investigated and compared with a culture collection strain *Scenedesmus obliquus* RISE (UTEX 417). *Desmodesmus sp.* RUC2 was the fastest N-consumer (5 days) and reached highest biomass concentration (3.718 g·L$^{-1}$) but only had 6.4% TAG content in the end. During N-starvation, biomass concentration of *C. vulgaris* 13-1 increased 1.9 folds (from 1.2 to 2.3 g·L$^{-1}$) but accumulated the most TAGs (from 1.4 to 11.4%); *Coelastrum astroideum* RW10 showed an increase in both TAGs (from 0.1 to 9.2%) and carbohydrates (from 22 to 69%). High lipid productivities of *Desmodesmus sp.* RUC2, *C. vulgaris* 13-1 and *Coelastrum astroideum* RW10 (55.9, 48.2 and 29.2 mg$\text{TAG}·\text{m}^{-2}·\text{d}^{-1}$, respectively) suggest that they are suitable for biodiesel production. Their TAG profiles showed that palmitic acid and oleic acid were the predominant fatty acids. Three strains, *Desmodesmus sp.* 2-6, *Coelastrella sp.* 3-4 and *Scenedesmus sp.* B2-2 did not show a significant TAG accumulation under N-stress. Glycerol is the by-product during biodiesel production, which can provide an ideal and cheap organic carbon source for some microalgal species. Microalgae have different growth abilities when cultivated autotrophically (with light and inorganic carbon), heterotrophically (without light and with organic carbon) and mixotrophically (with light and with organic carbon). In this study, *Desmodesmus sp.* 2-6 and *Coelastrella sp.* 3-4 were able to grow heterotrophically and mixotrophically with glycerol as a source of organic carbon, their growth rates during heterotrophic condition, however, were much lower than during autotrophic or mixotrophic conditions.
List of abbreviations

- DW: Dry weight
- FAs: Fatty acids
- FAMEs: Fatty acid methyl esters
- GC-FID: Gas Chromatography coupled with a Flame Ionized Detector
- MUFAs: Monounsaturated fatty acids
- OD: Optical density
- PUFAs: Polyunsaturated fatty acids
- Qy: Maximal quantum yield of Photosystem II
- SAFs: Saturated fatty acids
- SLP-FP: Short light path - flat panel
- SPE: Solid phase extraction
- TAGs: Triglycerides

Author contribution

I performed the whole procedures of nitrogen starvation experiments as well as experiments with glycerol addition, and the further biomass characterization, lipids extraction and quantification. Lorenza Ferro isolated and characterized the selected microalgae strains. Zivan Gojkovic constructed flat panel reactors and LED illumination panels and designed nitrogen starvation experiments. Intracellular nitrogen was determined by Andreas Hörnberg at Processum, Örnsköldsvik.
Table of contents

Abstract .......................................................................................................................... I
List of abbreviations ....................................................................................................... III
Author contribution ......................................................................................................... III
1. Introduction ................................................................................................................. 1
   Background .................................................................................................................. 1
   1.1 Cultivation conditions ......................................................................................... 1
   1.2 Chemical composition of algal biomass ............................................................... 2
      1.2.1 Proteins ........................................................................................................ 3
      1.2.2 Carbohydrates ............................................................................................ 3
      1.2.3 Lipids ......................................................................................................... 3
      1.2.4 Other components ...................................................................................... 4
   1.3 Biodiesel from microalgae ................................................................................... 4
   1.4 Strategies of enhancing TAG productivity ........................................................... 5
   1.5 Lipid analysis ....................................................................................................... 6
Aim of the diploma work ................................................................................................. 6

2. Popular scientific summary including social and ethical aspects .................................. 6
   2.1 Popular scientific summary .................................................................................. 6
   2.2 Social and ethical aspects .................................................................................... 7

3. Experimental Design .................................................................................................. 8
   3.1 Selected algae strains ......................................................................................... 8
   3.2 Inoculum and cultivation ..................................................................................... 8
      3.2.1 Medium ....................................................................................................... 8
      3.2.2 Cultivation conditions .................................................................................. 8
      3.2.3 Dry weight .................................................................................................. 10
      3.2.4 Optical density ........................................................................................... 11
      3.2.5 pH .............................................................................................................. 11
      3.2.6 Quantum yield ............................................................................................ 11
   3.3 Nitrogen determination ........................................................................................ 11
      3.3.1 Extracellular nitrogen .................................................................................. 11
      3.3.2 Intracellular nitrogen .................................................................................. 11
   3.4 Glycerol determination ........................................................................................ 11
   3.5 Carbohydrate determination .............................................................................. 12
   3.6 Protein determination ........................................................................................ 12
   3.7 Population density and cell volume ...................................................................... 12
   3.8 Lipid analysis ...................................................................................................... 12
      3.8.1 Extraction procedure ................................................................................... 12
      3.8.2 Identification and quantification .................................................................. 13

4. Results and Discussion ............................................................................................... 14
   4.1 Nitrogen starvation ............................................................................................... 14
      4.1.1 Nitrogen consumption and biomass growth .................................................. 14
      4.1.2 Fatty acid content and profile ....................................................................... 14
      4.1.3 TAGs productivity ....................................................................................... 18
   4.2 Glycerol .............................................................................................................. 23

5. Conclusions ................................................................................................................ 26

6. Outlook ....................................................................................................................... 26

Acknowledgement ......................................................................................................... 27
References ....................................................................................................................... 28
Appendix ........................................................................................................................ 33
1. Introduction

By 2050 the human population is forecast to expand from 7.5 to 9.2 billion. We will require 70% more food (United Nations, 2013), 50% more fuel (Taylor, 2010), and 50% more water (International Energy Agency, 2008). We also need to reduce CO$_2$ emissions by over 80% (EU-Commission, 2010). All of these will have to be achieved to ensure economic, social, political, climate, food, water and fuel security (Rodolfi et al., 2009). One approach to address this problem is to recycle CO$_2$ for fuel- or chemical-production using photosynthesis. Photosynthetic organisms use solar energy to incorporate atmospheric CO$_2$ into organic molecules. Therefore, photosynthesis mitigates climate change by counteracting increased levels of atmospheric carbon dioxide, the produced biomass also provides food and feed, and even can be used in technical processes to gain valuable products and biofuels. Replacing fossil oil with renewable energy resources is urgent. During the past decades, commercial biodiesel has been mainly produced from vegetable oils, animal fat or waste frying oil. However, producing these first generation biofuels requires a large amount of agricultural land and is competitive to human consumption. Microalgae and cyanobacteria are by far more productive photosynthetic organisms than higher plants, their average photosynthetic efficiency PE can reach up to 5% (Tredici, 2010), compared to territorial plants with only 1.8-2.2% (H. Chen et al., 2015). Biofuels from algae are considered to be a sustainable, renewable and environment friendly alternative energy source. Microalgae are easy to cultivate and can grow extremely fast with high energy conversion efficiency. In addition to the high growth rate, some microalgal strains have been demonstrated to produce more oil than conventional crops, particularly when exposed to different stress conditions (Chisti, 2007). Besides, microalgae can grow on non-arable land such as ponds and oceans, even on industrial and municipal wastewater (Mata et al., 2010). Algae perform well in wastewater reclamation and efficiently remove nitrogen, phosphorus, organic components, and even toxic elements (Vassilev & Vassileva, 2016). As a result, they are superior in comparison with other terrestrial feedstock for biofuels. Nevertheless, price of algal biofuels is still much higher than the conventional fossil fuels price (Richardson et al., 2014). All efforts in optimizing algal lipid production may lead in future to an algal fuel with competitive price comparable to fossil fuels, but several challenges have to be solved for a more ecological, renewable and considerable towards society and environment.

Background

1.1 Cultivation conditions

Most photosynthetic microalgae can be cultured in the presence, but also in the absence of light. Main cultivation conditions for microalgae are photoautotrophic, heterotrophic and mixotrophic.

In photoautotrophic cultivation, the algae use light (sunlight or artificial light) as their photosynthetically active photons source, and inorganic carbon (e.g., CO$_2$ or HCO$_3^-$) as the carbon source to convert to organic carbon and produce biomass. Photoautotrophic growth is the most common cultivation process and, when sunlight is used, also the cheapest one. However, light often becomes the limiting factor outdoors and therefore high production rates and high cell densities are hindered (C. Chen et al., 2011; Mata et al., 2010). Algae can be grown in closed photobioreactors or in an open pond system. In photobioreactors the more efficient use of light usually leads to higher cell densities, also less contamination by other microorganisms is expected. A cheaper system, which allows easier up-scaling, is the open pond; however, contaminations from bacteria and other algae species are common.
In heterotrophic cultivation, organic carbon, such as glucose, acetate, glycerol, fructose, sucrose, lactose, galactose and mannose provides both energy and carbon source. The algae are grown without light. Mixotrophic cultivation is a mix of different energy sources and carbon sources. The algae can be cultivated with both light and organic compounds, and intake both organic and inorganic carbon as carbon sources. CO₂, which is released during respiration, can be assimilated and reused by the algae during photosynthesis (Mata et al., 2010).

In heterotrophic and mixotrophic cultivation the algae can utilize not only common sugars (e.g., glucose), but also lignocellulosic sugars, or even carbon-rich waste water can provide the organic carbon (Lowrey et al., 2015), which has been shown to lead to a higher lipid production. It was reported that the marine algae *Chlorella sp.* and *Nannochloropsis sp.* produced notable more lipids in mixotrophic growth than in photoautotrophic or heterotrophic (Cheirsilp & Torpee, 2012). However, a major disadvantage of heterotrophic and mixotrophic cultivation is the relatively high cost of common sugar and need for closed bioreactors operated under strictly sterile conditions to avoid bacterial contamination. Glycerol, instead, is an ideal alternative as organic carbon source for microalgae. In biodiesel production from plant oil, every 10L of biodiesel production result in 1L of glycerol as by-product (Johnson & Taconi, 2007; Pragya et al., 2013). Cultivation of microalgae on industrial glycerol therefore is cost-effective and contributes greatly to a sustainable environment and economy. Many microalgal species have been reported to have both high biomass and high lipid productivity when grown in the presence of glycerol (Cabanelas et al., 2013; Cerón-Garcia et al., 2013; Y. Chen & Walker, 2011).

### 1.2 Chemical composition of algal biomass

<table>
<thead>
<tr>
<th>Alga</th>
<th>Composition</th>
<th>Conditions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proteins</td>
<td>Carbohydrates</td>
<td>Lipids</td>
</tr>
<tr>
<td><em>Chlorella vulgaris</em></td>
<td>41.51</td>
<td>20.99</td>
<td>15.67</td>
</tr>
<tr>
<td></td>
<td>6.00</td>
<td>51.00</td>
<td>43.00</td>
</tr>
<tr>
<td><em>Scenedesmus obliquus</em></td>
<td>30.38</td>
<td>13.41</td>
<td>4.66</td>
</tr>
<tr>
<td><em>Desmodesmus sp.</em></td>
<td>13-25</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Scenedesmus sp.</em></td>
<td>19</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>46</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td><em>Coelastrella sp.</em></td>
<td>25*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>16-37</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

WW: Waste water.
* Lipid content of *Coelastrella sp.* did not change when exposed to different concentrations of WW.
The organic composition of algal biomass is variable depending on the species or the growth conditions; nevertheless, as for all living cells, the general organic constituents of algae are: proteins > carbohydrates > lipids > nucleic acids. A general overview of the main organic constituents of various algal species is shown in Error! Reference source not found.. An organic composition of microalgal biomass is shown in Table 2.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mean</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteins</td>
<td>42.8</td>
<td>6.0</td>
<td>71.0</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>29.9</td>
<td>4.0</td>
<td>83.6</td>
</tr>
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<td>Lipids</td>
<td>23.4</td>
<td>0.9</td>
<td>77.0</td>
</tr>
<tr>
<td>Nucleic acids</td>
<td>3.9</td>
<td>1.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Sum</td>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(H. Chen et al., 2015; W. Chen et al., 2015; A. Demirbas, 2010; M. F. Demirbas, 2011; Huber et al., 2006; Noraini et al., 2014; Rabemanolontsoa & Saka, 2013; Raslavičius et al., 2014; Trivedi et al., 2015; Vassilev & Vassileva, 2016)

**1.2.1 Proteins**

Proteins are the main organic constituent in microalgal biomass; their content on nutritious proteins is comparable higher to plants (Becker, 2007). Algal biomass therefore can be used as protein source for human consumption and as animal feed (Becker, 2007; Safafar, 2017; Tokuçoğlu & Ünal, 2006). *Chlorella vulgaris*, a well-known green microalga, is marketed as a healthy food supplement for its high content of protein (Error! Reference source not found.) and other essential nutrients (e.g., chlorophylls, minerals and vitamins) (Konishi et al., 1996). However, the thick paste consistency, dark green colour and fishy odour of algae raw powder are the major obstacles for common use; the high cost of producing proteins from microalgae also limits the development for up-scaling (Becker, 2007). As a result, algal proteins are more recent used as animal feed with approximately 30% of the current world algal production (Becker, 2007).

**1.2.2 Carbohydrates**

The simple structure and high photosynthetic efficiency of microalgae contribute to the high content of carbohydrates in their biomass. Grown photoautotrophically algae capture CO$_2$ from the air in the photosynthetic process and convert it to glucose and further to a variety of polysaccharides such as starch and cellulose. Glucose and starch are stored in the plastids as energy reserves and the cellulose usually is used to build cell walls. Polysaccharides from algae can be fermented to bioethanol using appropriate microorganisms for anaerobic digestion. *Chlorococcum* and *Chlorella* are two common green microalgae genera used for bioethanol production (Trivedi et al., 2015).

**1.2.3 Lipids**

Lipid is a general term that refers to all hydrophobic compounds including triglycerides (TAGs), phospholipids, glycolipids, free fatty acids, sterols, fat-soluble vitamins etc. In algology, sterols, phospho- and glycolipids are classified as polar lipids, whereas TAGs are classified as neutral lipids (or non-polar lipids). The total lipid content in microalgae varies from 0.9-77% of dry weight (Table 2), depending on species and cultivation conditions. In normal conditions, algae synthesize mainly polar lipids since those are essential to build biological membranes and cell compartments (Harwood, 1998). Whereas some algae species are able to accumulate TAGs and store them in form
of oil globules within the cell, when they are exposed to stress conditions (Radakovits et al., 2010), because lipids generate more energy than carbohydrates upon oxidation, and therefore are the better reserve to rebuild after stress (Roessler, 1990).

Lipids can be classified into two main categories based on the length of their carbon chains; lipids with shorter-chain fatty acids (FAs) (C14-20) are mainly used as biofuels (Minhas et al., 2016a) and those with longer chains (C20 or longer) are used as food resources (Chew et al., 2017). FAs are further categorized as saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). Lipids originating from microalgae mainly contain SFAs and MUFAs such as C16:0 and C18:1, are suitable for biodiesel production. PUFAs are high valuable FAs for human health due to their ability of cardiac disorder prevention, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are commonly found in marine fishes, but are also produced by microalgae (Wang et al., 2015). Interestingly, fish cannot synthesize long-chain PUFAs themselves, but accumulate them by eating microalgae (Wang et al., 2015).

1.2.4 Other components

The minor components of microalgae are nucleic acids, pigments and inorganic elements (ash). Pigments from microalgae, mainly chlorophylls and carotenoids, also have high market value for different industries. Chlorophylls including chlorophylls a and b are primary pigments occurring in chloroplasts responsible for harvesting solar energy and converting it into algal biomass. Chlorophyll-rich microalgae, such as Chlorella vulgaris, can be used as colouring reagent in cosmetics or food (Gouveia et al., 2005).

Carotenoids are divided into primary and secondary carotenoids; primary ones are structural and functional components of the photosynthetic apparatus, while secondary carotenoids are produced after environmental stimuli. Lutein is a primary carotenoid as it maintains and protects the membrane, whereas astaxanthin is a secondary carotenoid. It is a highly valuable carotenoid produced mostly by microalgae Haematococcus pluvialis, which has been widely used in nutraceuticals, food and cosmetics due to its effective antioxidant and anti-inflammatory functions (Cerón et al., 2006).

Besides these organic compounds, algae biomass contains inorganic matter; algae biomass usually has a higher ash content (13.1-42.8%, mean 26.6%) compared to terrestrial biomass, since microalgae assimilate more nutrients in their rapid metabolism (Vassilev et al., 2013). Ash-forming elements are K, Na, Cl, Si, Ca, S, P, My, Fe, Al, Ti (Vassilev & Vassileva, 2016). Even sand and other contaminants contribute to high ash content, especially when algae are grown in open ponds. A high ash content of the biomass is not desired for biofuel production since the high content of inorganic matter decreases the heating value and therefore contributes a negative effect to the fuel quality (Vassilev et al., 2015).

1.3 Biodiesel from microalgae

Biofuels are fuels produced via biological processes and include biodiesel, bioethanol and biogas.

Biodiesel is produced from fatty acid alkyl esters, mainly fatty acid methyl esters (FAMEs), deprived from vegetable oil or animal fat through transesterification catalysed by acids or alkalis. The physical and chemical properties of FAMEs are similar to traditional diesel fuel, sometimes FAMEs perform even better. High quality biodiesel should have low viscosity for operability, high cetane number (CN) for higher combustion efficiency, few long-chain saturated FAMEs for a lower melting point and
a low concentration of polyunsaturated FAMEs to avoid oxidation (Selvarajan et al., 2015). As a result, microalgal biomass used for biodiesel production should not only contain a high amount of lipids, but the lipids also have to be composed of the proper fatty acids with a considerable amount of C16-C18.

1.4 Strategies of enhancing TAG productivity

Several strategies of enhancing the TAG productivity in microalgae were studied and compared (Error! Reference source not found.) (Benvenuti et al., 2015; Breuer et al., 2012; Ho et al., 2012; Mujtaba et al., 2012; Rézanka et al., 2011; Sun et al., 2014). The lipid composition of microalgae does not change significantly during normal growth conditions, however, when exposed to environmental stress, algae prevalently accumulate large quantities of TAGs and (or) starch, while the amount of proteins is diminished.

Nitrogen starvation

Nutrient starvation (N-starvation) is one of the most common strategies to induce high TAG productivity. The nutrients, which affect biomass composition, are nitrogen, phosphate and sulphate. Among all the nutrients needed for algae growth, nitrogen is of the most importance, as it accounts for 1-10% of total dry weight (DW) (Wijffels et al., 2010). Intracellular nitrogen mainly exists in the form of protein and makes a range from 59.3-96.8% of total nitrogen. In addition, inorganic nitrogen (nitrate, nitrite and ammonium) are the most important non-protein nitrogen molecules (0.4-40.4%), followed by nucleic acids (0.3-12.2%) and chlorophylls (0.1-1.8%) (Lourenço et al., 2004).

Nitrogen is important for efficient photosynthesis as this essential element is part of chlorophyll and of proteins. Microalgae can use nitrogen from liquid culture in form of nitrate, nitrite, ammonium and urea. When cultures are exposed to nitrogen deprivation, photosynthesis proceeds at a lower rate. Proteins and chlorophyll are degraded as nitrogen source to maintain cell growth (Su et al., 2011). Instead of assimilating proteins, organic carbon is used to accumulate lipids (Rodolfi et al., 2009). The lipid content of Chlorella vulgaris was found to increase from 16% to 43%, whereas its protein content decreased from 41% to 6% during nitrogen deprivation (L. Xu et al., 2011). It is worth noticing that lipid accumulation is often associated with a decrease of biomass growth rate. Thus, lipid content and biomass productivity are means to characterize lipid productivity. N-starvation not only affects the composition of algal biomass, but also causes a morphological change of some algae. Scenedesmus sp. commonly exists unicellular, but forms four or eight-celled coenobia during N-starvation. An increase in cell size was also observed with a decrease of the cell width (Pancha et al., 2014). In addition, some algal cells stop division and accumulate lipids within the cell under stress conditions (Dean et al., 2010; Sigee et al., 2007).

Depending on the application of the algal biomass, there are three different strategies to grow microalgae: 1) cultivate the cells in a nutrient-sufficient medium in order to obtain high biomass concentration with relative low lipid content (one-phase strategy); 2) cultivate the algae in a nutrient-limiting medium with low biomass productivity, but high lipid content (one-phase strategy); 3) cultivate the cells first in nutrient-rich medium to receive high biomass, when the nutrients are consumed a nutrient deprivation phase follows with the aim of high lipid production (two-phase strategy), known as progressive starvation strategy.

Light intensity is another factor influencing the growth of microalgae and the composition of FAs in the biomass. A light intensity of 100-200 µmol photons m$^{-2}$ s$^{-1}$ is considered to be optimal for algal growth. High light stress increases not only the lipid
productivity of microalgae, but changes also the composition of FAs. Some algae species accumulate SFAs and MUFAs under high light intensity instead of PUFAs (Khotimchenko & Yakovleva, 2005). While high light intensity boosts the lipid production, at the same time the algae suffer from photoinhibition, causing damages to the macromolecules within the cell (Tredici, 2010). Therefore microalgae are often cultured in a two-phase strategy, first at low light for biomass production and then shifted to high light (Minhas et al., 2016b).

Other stresses influencing lipid production are high temperature, pH, and high salinity (Mata et al., 2010).

1.5 Lipid analysis

To date, lipid profiles of algal biomass have been defined in numerous studies (Benvenuti et al., 2015; Breuer et al., 2013; Choi et al., 1987; Rodolfi et al., 2009; Van Vooren et al., 2012; Wang et al., 2015). FAs in microalgae usually are analysed after transmethylating them to FAMEs to change the volatility of the lipids and to improve their separation by gas chromatography (Liu, 1994). As mentioned above, the yield of lipids depends on many factors, such as algal species, cultivation conditions, environmental factors, but also the extraction method. Different pretreatments of the biomass and extraction methods lead to significantly different yield and FA profile of obtained lipid mixture. Comparing different pretreatment methods such as boiling the biomass in isopropanol, freezing, oven-drying or freeze-drying showed that freeze-dried microalgal biomass resulted in lowest yields. The FAMEs content in biomass of Coelastrella sp. contained 36.6% of total lipids before, but only 15% of total FAMEs after purification steps (Lage & Gentili, 2018). A one-step total lipid extraction method (Folch et al., 1957) modified by Axelsson & Gentili (2014) showed to be a simple and fast method to receive a crude lipid content (Lage & Gentili, 2018). However, this method does not allow information about the composition of the lipids and their FA profiles. Also, this method was reported to over-estimate the lipid content (Pruvost et al., 2009). The authors further compared several extraction methods in combination with or without purification steps, such as thin-layer chromatography (TLC), solid-phase extraction (SPE) and direct transesterification (Lage & Gentili, 2018); TLC and SPE gave the best and reproducible results but one should keep in mind that TLC leads to oxidation of PUFAs and this method is labour intensive.

Aim of the diploma work

This study is within the project lead by the consortium “MicroBioRefine”, which investigates the potential of locally isolated microalgal and cyanobacterial strains to produce biomass during wastewater reclamation in Northern Sweden. The aim of my study was to enhance the lipid production of six selected Swedish green microalgal strains in lab scale by exposing them to different stress conditions and different cultivation conditions. The best performing microalgal strain(s) will be potential candidate(s) for biodiesel production coupled with wastewater reclamation.

2. Popular scientific summary including social and ethical aspects

2.1 Popular scientific summary

Microalgae are unicellular photosynthetic microorganisms living in freshwater and marine systems, that can either live solitary or in chains or groups. Due to their high photosynthetic efficiency, their fast growth, their resistance to environmental factors and
the quality of their biomass they are widely used in biotechnological applications providing food additives, cosmetics, pharmaceuticals and biofuels. Depending on the species algal biomass contains different amounts of proteins, carbohydrates and fats. Biomass composition can be changed to obtain more of the desired product by exposing the cells to different environmental conditions. My work has been performed within the “MicroBioRefine” consortium, a collaboration of ten research groups of three universities from northern Sweden and industrial partners to investigate the potential of Nordic wild microalgal strains in wastewater reclamation and biomass production. Within this consortium a culture collection of microalgae was established with local strains, which can grow at low temperatures and with limited light availability. While these local strains are well-adapted and are able to clean wastewater even in the cold and dark Nordic climate, their biomass is quite poor in lipids, which are used for biodiesel production. The aim of my work was to test, if I could improve their lipid production by exposing selected algal strains to stress. Analysis of the lipids then showed if they are suitable for commercial biodiesel production. The stressors I applied were nitrogen starvation and heterotrophic/mixotrophic growth.

Nitrogen (N) is an essential element for plants to build proteins and chlorophylls. If nitrogen-compounds are removed from the growth medium, algae are “starved”. They still perform photosynthesis, but as N is missing, they are not able to build proteins. Therefore carbohydrates or lipids are synthesized instead of proteins to provide energy reserve to the algae once the starvation stress is removed so that they can get recover afterwards. Heterotrophic growth is another cultivation strategy. It is induced by letting the algae use organic carbon instead of CO\(_2\), in the absence of light. Not all algae are able to grow heterotrophically; sometimes they also need some light. This mixture of photosynthetic (autotrophic) growth and heterotrophic growth is called mixotrophic. I added glycerol, which is considered as a useless industrial by-product, as a carbon and energy source for microalgae during heterotrophic/mixotrophic growth.

Three out of the six tested local algal strains showed high oil productivity during nitrogen starvation, and could be tested in larger scale for commercialization in the future. Growth in the presence of glycerol worked for two algae strains, which produced more biomass than the control.

### 2.2 Social and ethical aspects

Fossil fuels are non-renewable energy resources; at the current consumption rate, coal and oil reserves worldwide are estimated to last around 110 and 50 years, respectively (World Coal Association, 2016). Meanwhile, CO\(_2\) emissions from conventional fossil fuel combustions exacerbate global warming. We therefore need renewable and sustainable fuels in order to fulfil the increasing needs of energy. Photosynthetic organisms use solar energy to incorporate atmospheric CO\(_2\) into organic molecules. Therefore, photosynthesis not only prevents climate change, the produced biomass also provides food and feed, and even can be used in technical processes to gain biofuels. Algae and cyanobacteria are without doubt the most productive photosynthetic organisms on Earth. Their fast growth allows harvesting after short cultivation times, while higher plants require growth periods of few months to several years. Furthermore, the aquatic growth of microorganisms in tanks or ponds requires marginal land space and allows a continuous, automated system with very high flow-through capacities. And best of all, microbial cultures are able to grow in waste streams. The algae will receive their energy from organic carbon sources found in the sewage and/or from sun light. Therefore wastewater can be cleaned and the biofuels can be extracted from the energy-rich biomass. For biodiesel production in Sweden it is of a great interest to identify algae adapted to the Nordic climate, which are still able to produce biomass with high oil content.
3. Experimental Design

3.1 Selected algae strains

Six microalgal strains selected for N-starvation experiments were isolated from different water sources in Sweden (Table 3). While the strains used in this study are wild Swedish strains, one algal strain originating from the culture collection was included additionally. This strain, *Scenedesmus obliquus* RISE (UTEX 417), had been grown in an open pond in middle Sweden for three years and was used as control. *Desmodesmus sp.* 2-6 and *Coelastrella sp.* 3-4 were selected to investigate their growth on glycerol.

<table>
<thead>
<tr>
<th>Strain IDs</th>
<th>Species</th>
<th>Origins</th>
<th>Latitudes-longitudes</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-6</td>
<td><em>Desmodesmus sp.</em></td>
<td>Skåne - Lake Ringsjön (FW)</td>
<td>55.86-13.55</td>
</tr>
<tr>
<td>3-4</td>
<td><em>Coelastrella sp.</em></td>
<td>Umeå (MWW)</td>
<td>63.80-20.28</td>
</tr>
<tr>
<td>13-1</td>
<td><em>Chlorella vulgaris</em></td>
<td>Umeå (MWW)</td>
<td>63.80-20.28</td>
</tr>
<tr>
<td>B2-2</td>
<td><em>Scenedesmus sp.</em></td>
<td>Dåva (MWW)</td>
<td>63.87-20.40</td>
</tr>
<tr>
<td>RW10</td>
<td><em>Coelastrum astroideum</em></td>
<td>Bäckhammar (FW)</td>
<td>59.16-14.18</td>
</tr>
<tr>
<td>RUC 2</td>
<td><em>Desmodesmus sp.</em></td>
<td>Umeå - River Campus (FW)</td>
<td>63.82-20.30</td>
</tr>
<tr>
<td>RISE (UTEX 417)</td>
<td><em>Scenedesmus obliquus</em></td>
<td>UTEX culture collection</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Selected microalgal strains, their places of isolation with latitudes and longitudes (according to Ferro et al., 2018)

Water source: FW=fresh water; MWW=municipal wastewater

3.2 Inoculum and cultivation

3.2.1 Medium

The algal cultures were inoculated in liquid Bold’s Basal Medium (BBM), modified from Culture Collection of Algae and Protozoa (2015), for photoautotrophic cultivation, and for heterotrophic and mixotrophic cultivation with glycerol addition. pH was adjusted to 6.7. The composition was as follows (in g·L⁻¹ unless otherwise indicated): NaNO₂, 0.250; MgSO₄·7H₂O, 0.075; NaCl, 0.025; K₂HPO₄, 0.075; KH₂PO₄, 0.175; CaCl₂·2H₂O, 0.025; trace element solution, 1 mL. Trace element solution consists (mg·L⁻¹ unless otherwise indicated): ZnSO₄·7H₂O, 0.082; MnCl₂·4H₂O, 0.144; MoO₃, 0.071; CuSO₄·5H₂O, 0.157; Co(NO₃)₂·6H₂O, 0.049; H₃BO₃, 1.142; Na₂MoO₄·2H₂O, 0.018; Na₂EDTA, 5.000; KOH, 3.100; FeSO₄·7H₂O, 0.498; H₂SO₄, 0.001 mL·L⁻¹.

NaNO₂ was increased to 1.500 g·L⁻¹ for part of the glycerol addition experiments. Glycerol was added in two concentrations, 25 mM and 2% (equivalent to approximately 217 mM). In this study, cultivation with 0.250 g·L⁻¹ (5 mM) NaNO₂ and 25 mM glycerol is called “nutrient limit condition” and with 1.500 g·L⁻¹ (30 mM) NaNO₂ and 2% glycerol is called “nutrient sufficient condition”.

3.2.2 Cultivation conditions

All microalgae were pre-cultured in flasks with BBM in an orbital shaker at 25 °C under continuous irradiance of 100 µmol photons m⁻² s⁻¹. Prior to use all flasks had been sterilized to prevent any contaminations.
To expose the microalgae to N starvation, cells were cultivated in a short light path - flat panel (SLP - FP) (Width × Height × Depth = 30 × 30 × 1.5cm) with a working volume of 1.2 L (Error! Reference source not found.). Each experiment was performed two times with two technical replicates. Each flat panel was illuminated from one side by white LED panel with adjustable light intensity of 45 to 650 µmol photons/m²/s with 12:12h light and dark cycles for the entire growth period (12-20 days). Light intensity was increased daily to provide sufficient irradiance. The culture suspensions were mixed by bubbling filtered and pre-humidified air (with a flow rate 1L/min) through a silicon tube with small holes placed horizontally in the bottom of the culture chamber. Extra CO₂ (3% v/v of CO₂/air) was mixed with the air as the only carbon source.

**Figure 1.** SLP-FPs for N-starvation experiments

SLP-FP photobioreactor used in this study is characterized by optimal light availability for the culture that is able to achieve very high biomass densities and has higher biomass productivities than tubular reactors or open ponds (Cuaresma et al., 2009). In the current study, this type of vessel was used in order to achieve highest biomass quantity per culture volume and maximize effect of light exposure on TAG accumulation.

Cultures were inoculated from pre-cultures at the exponential growth phase to achieve biomass concentrations between 0.05-0.10 g·L⁻¹ at the start of the experiment. The amount of inoculum was calculated based on calibration curve of OD₇₅₀ and its relation to DW. Samples were taken daily to measure Optical density (OD), Quantum yield (Qₚ), DW, pH and extracellular nitrogen concentration. Samples were taken every second day for biomass characterization, starting when the extracellular nitrogen in the medium was removed. The samples were centrifuged and the supernatant was discarded, the pellets were washed with demineralized water and centrifuged again to eliminate any impurities. Subsequently the pellets were lyophilized for 72 hrs and stored in −18 °C until further analysis.
To investigate the growth on glycerol, algae cultures were inoculated in 200 mL flasks with a working volume of 50 mL for 14 days, two biological replicates per algal strain. Temperature was maintained at 25 °C and irradiance intensity was 100 µmol photons m⁻² s⁻¹ constantly. Biomass concentrations were measured at day 7 and day 14. As described in Figure 2, one flask containing medium with 5 mM NO₂⁻ and 25 mM glycerol and one with 30 mM NO₂ and 2% glycerol were wrapped with aluminium foil to provide heterotrophic condition for 7 days (day 0-7), and then exposed to light to switch to mixotrophic growth for another 7 days (day 8-14). Control cultures without additional glycerol but with the same inoculum were exposed to light constantly during the entire period. One flask containing growth medium with 30mM NO₂ in the absence of glycerol and another one without NO₂ and glycerol were grown autotrophically and used as controls.

![Figure 2](image)

**Figure 2.** Heterotrophic and mixotrophic cultivations in the presence of different glycerol concentrations. Two biological replicates of *Desmodesmus* sp. 2-6 and *Coelastrella* sp. 3-4 were grown in an orbital shaker with constant light; some of them were wrapped with aluminum foil to isolate from light for heterotrophic growth

### 3.2.3 Dry weight

DW concentration, expressed as [g·L⁻¹], was measured by filtration using Ø47mm Whatman glass fibre filters with a pore size of 1 µm (Gojkovic et al., 2014). The culture broth was first filtered through a pre-weighed filter and washed by demineralized water; filters were dried at 70°C for 16 hours or more and then cooled down in a desiccator for 2 hrs. The filter weight was measured on an analytical balance with a precision of 0.00001g. DW was determined in triplicates and calculated according to the following formula:

$$\text{DW (g·L}^{-1}) = \frac{(w_2 - w_1)}{x} \times 1000$$

Where $w_1$, $w_2$ (g) are the weights of the dry filter before and after filtration, $X$ (ml) is the volume of culture broth.
3.2.4 **Optical density**

OD of the samples was measured daily spectroscopically at 530, 680, and 750 nm (Varian Cary 50 Bio) and was used as an indicator of the cultures daily performance.

3.2.5 **pH**

The culture pH was maintained in the range of 7.2 ± 0.5 during the experiment by maintaining the balance between simultaneous CO₂ addition (which acidifies medium and decreases pH) and gradual N assimilation by alga (a net uptake of protons which increases pH) (Scherholz & Curtis, 2013; Wang & Curtis, 2016). The pH was daily measured and CO₂ addition was increased manually, if necessary, to correct sudden pH increase provoked by eventual fast culture growth.

3.2.6 **Quantum yield**

Photosystem II (PSII) maximal quantum yield (Q_Y) was determined by measuring the chlorophyll fluorescence in a portable pulse-amplitude-modulated fluorimeter AquaPen (CAP-C100, Czech Republic; emission peak: 620 nm, saturating light pulse: 2100 µmol photons m⁻² s⁻¹). Cultures were diluted in demineralized water to an OD of 0.4 in a cuvette with a light path of 10 mm and adapted to dark conditions for 15 min prior to the measurement.

Q_Y is a measure of the PSII efficiency. A healthy algae culture usually has a Q_Y value around 0.6-0.7 (Benvenuti et al., 2015).

3.3 **Nitrogen determination**

3.3.1 **Extracellular nitrogen**

Fresh algae samples were centrifuged and the supernatant was diluted for extracellular nitrogen determination, based on the method described by García-Robledo et al. (2014). In brief, 1 mL of the sample and 50 µL of Griess-reagent (equal portions of sulphanilamide reagent and NED reagent) were transferred into 1.5 ml Eppendorf tubes to determine NO₂⁻ by Greiss reaction and subsequently 100 µL of Vanadium chloride (VCl₃) reagent was added to reduce any possible NO₃⁻. The samples were gently mixed and incubated at 60 °C for 25 min and cooled down to room temperature. The absorbance was measured at 540 nm.

3.3.2 **Intracellular nitrogen**

Intracellular nitrogen (% DW) in algal biomass was measured for alga *Chlorella vulgaris* 13-1 and *Coelastrella sp.* 3-4 during the cultivation period starting with a population density of 2.0 × 10⁶/mL and 0.6 × 10⁶/mL, respectively. 20 mg freeze-dried algal biomass was weighted in 1.5 mL Eppendorf and combusted by CHN628 Series Elemental Determinator, Leco. NOₓ was then detected by a thermal conductivity cell.

3.4 **Glycerol determination**

Glycerol concentration in the supernatant was determined by a method described by Bondioli & Della Bella (2005). The culture was first centrifuged for 3 min at 13,000 rpm; 0.5 mL of the diluted supernatant was transferred into a test tube, 1.5 ml working solvent, 1.2 mL 10 mM potassium periodate and 1.2 mL 0.2 M acetylacetone were added afterwards. The mixture was water bathed at 70 °C for 1 min and the absorbance was measured at 410 nm.
3.5 Carbohydrate determination

Total carbohydrate content in the algal biomass was measured by the widely used phenol-sulfuric acid colorimetric method with a hydrolysis pre-treatment (DuBois et al., 1956). Polysaccharides were first hydrolysed by a strong acid into monosaccharide, and then underwent two reactions, of which the monosaccharides first were dehydrated into furfural and hydroxymethyl furfural. In the second reaction the furan derivatives form aromatic complexes with phenol and yield a yellow colour change.

2 mg of freeze dried algal biomass were dissolved in HCl and incubated at 90°C for 3 hrs. The same amount of NaOH was added for neutralization. 5% phenol was added to 0.05 mL of pre-treated sample with 0.45 mL ddH2O. 2.5mL concentrated sulfuric acid was added rapidly to the liquid surface. After incubating for 30 min in a water bath at 35°C, the absorbance was measured spectroscopically at 483 nm. Glucose was used for calibration.

3.6 Protein determination

The method to determine the total protein amount was adapted from (Lowry et al., 1951) with a protein precipitation from Slocombe et al. (2013). 1.5 - 3.0 mg biomass was re-suspended in 200 µL 24% trichloroacetic acid, the homogenates were incubated on a heating block for 15 min at 95°C. Protein quantification was performed using a Lowry assay kit (BIO-RAD). 25 µL of sample were mixed with 125 µL RC Reagent I and 125 µL RC reagent II. The mixture was centrifuged at 15,000xg for 5 min and the supernatant was discarded. Freshly made Reagent A and Reagent B were added to the pellet and incubated for 15 min at room temperature. The absorbance of the samples was read at 750 nm.

3.7 Population density and cell volume

Population density and total volume of the cultures was measured using Beckman Coulter Multisizer 3 cell counter, according to the manufacturer instructions (aperture size 70 µm, analytical volume 100 µl, sample dilution 200x or 500x). Population density was calculated using dedicated Multisizer software. The volume of a single cell was therefore calculated by dividing total cell volumes and population density:

\[ V_{cell} (\mu m^3) = V_{total} (\mu m^3) / Num_{total\ cell} \]

3.8 Lipid analysis

3.8.1 Extraction procedure

TAGs and polar lipids were extracted from the algal biomass and their fatty acid profiles were quantified as described by Breuer et al. (2013). The whole procedure was performed in three steps.

1) Cell disruption and solvent based lipid extraction

A determined amount of lyophilized microalgal powder was dissolved in 4:5 (v/v) chloroform:methanol with glass beats and treated in the bead beater until the cells were disrupted. dd H2O with 50 mM Tris and 1 M NaCl was added to the mixture for phase separation. The mixture was vortexed and centrifuged and subsequently the bottom chloroform phase was taken and evaporated under nitrogen gas stream with a spider.

2) Solid phase extraction
In this step, TAG and polar lipids were separated. The extracted lipids were re-suspended in 7:1 (v/v) hexane: diethylether and separated by a pre-equilibrated silica column (Sep-pak Vac Silica cartridge 6cc/1000mg). TAGs were eluted by 5 column volumes of 7:1 (v/v) hexane: diethylether and polar lipids were eluted by 5 column volumes of 2:2:1 (v:v:v) methanol : acetone : hexane. All solvents were evaporated under nitrogen.

3) Transesterification of FAs to FAMEs

Methanol with H$_2$SO$_4$ was added in all samples and incubated for 3 hrs at 70°C for transesterification. Pentadecanoic acid (C15:0) was added in the methanol/H$_2$SO$_4$ mixture as an Internal Standard (IS). After the transesterification process, hexane and H$_2$O were added and the mixtures were vortexed and centrifuged. The upper layers (hexane) were taken and injected into a Thermo Scientific™ TRACE™ 1310 Gas Chromatography coupled with a Flame Ionized Detector (GC-FID) (Hägersten, Sweden), 1 µL sample was injected into a 30m FAMEWAX column (Restek Corporation, Bellefonte, Pennsylvania, USA). The temperature program used was the following: the initial temperature was 195°C and then increased with a rate of 1.8°C/min to 240°C, where it was held for 2.8 min. Nitrogen was used as the carrier gas with a constant flowrate of 1.5 mL/min. Run time for each sample was 29 min.

3.8.2 Identification and quantification

FAME used as calibration standard for GC were methyl laurate (C12:0), methyl myristate (C14:0), methyl palmitate (C16:0), methyl palmitoleate (C16:1), methyl stearate (C18:0), methyl oleate (C18:1), methyl linoleate (C18:2), methyl linolenate (C18:3), methyl arachidate (C20:0), methyl eicosapentaenoate (C20:5) and methyl behenate (C22:0) from (Larodan, Sweden). The standards were diluted 1, 2, 5, 10, 20, 50 and 100 times in hexane for GC calibration as well as for quantification of the samples based on Relative Response Factors (RRFs) (Rome & McIntyre, 2012). Each FAME has a response factor, which can be calculated from the ratio between peak area and concentration, the RRF of each FAME is the ratio of response factor and the response factor of the IS as described in the equations below:

\[
\text{Response factor} A = \frac{\text{Peak area } A}{\text{Concentration } A}
\]

\[
\text{Relative response factor (RRF)} = \frac{\text{Response factor } A}{\text{Response factor IS}}
\]

Thus, with a known concentration and peak area of IS (C15:0), it is possible to calculate the concentration of each analyte with its peak area RRF.

\[
\text{Concentration } A = \frac{\text{peak area } A}{\text{peak area IS}} \times \frac{1}{\text{RRF } A} \times \text{concentration IS}
\]

It is beneficial to use RRFs when multiple samples are analysed and one of the samples is the IS. The retention times (RTs) and RRFs of each standard are shown in the table below:

<table>
<thead>
<tr>
<th>Common names (of FA)</th>
<th>Names</th>
<th>Retention time</th>
<th>RRF</th>
</tr>
</thead>
<tbody>
<tr>
<td>C12:0</td>
<td>Methyl Laurate</td>
<td>2.25</td>
<td>0.98</td>
</tr>
<tr>
<td>C14:0</td>
<td>Methyl Myristate</td>
<td>2.93</td>
<td>0.97</td>
</tr>
<tr>
<td>C15:0</td>
<td>Methyl Pentadecanoate (IS)</td>
<td>3.46</td>
<td>1.00</td>
</tr>
<tr>
<td>C16:0</td>
<td>Methyl Palmitate</td>
<td>4.15</td>
<td>1.18</td>
</tr>
<tr>
<td>C16:1</td>
<td>Methyl Palmitoleate</td>
<td>4.40</td>
<td>1.02</td>
</tr>
<tr>
<td>C18:0</td>
<td>Methyl Stearate</td>
<td>6.27</td>
<td>1.12</td>
</tr>
<tr>
<td>C18:1</td>
<td>Methyl Oleate</td>
<td>6.54</td>
<td>0.98</td>
</tr>
<tr>
<td>C18:2</td>
<td>Methyl Linoleate</td>
<td>8.23</td>
<td>0.92</td>
</tr>
<tr>
<td>C18:3</td>
<td>Methyl Linolenate</td>
<td>8.29</td>
<td>1.24</td>
</tr>
<tr>
<td>C20:0</td>
<td>Methyl Arachidate</td>
<td>9.54</td>
<td>0.97</td>
</tr>
</tbody>
</table>
Table 4.

<table>
<thead>
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<th>Retention time</th>
<th>RRF</th>
</tr>
</thead>
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<td>C12:0</td>
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<td>4.40</td>
<td>1.02</td>
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<td>C18:0</td>
<td>Methyl Stearate</td>
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<td>0.98</td>
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<td>8.29</td>
<td>1.24</td>
</tr>
<tr>
<td>C20:0</td>
<td>Methyl Arachidate</td>
<td>9.54</td>
<td>0.97</td>
</tr>
<tr>
<td>C20:5</td>
<td>Methyl Eicosapentaenoate</td>
<td>13.56</td>
<td>1.34</td>
</tr>
<tr>
<td>C22:0</td>
<td>Methyl Behenate</td>
<td>14.07</td>
<td>1.06</td>
</tr>
</tbody>
</table>

4. Results and Discussion

4.1 Nitrogen starvation

4.1.1 Nitrogen consumption and biomass growth

To investigate if N-starvation will induce lipid production in our Nordic algal strains, *Chlorella vulgaris* 13-1, *Coelastrum astroideum* RW10, *Desmodesmus sp.* RUC2, *Desmodesmus sp.* 2-6, *Coelastrella sp.* 3-4, *Scenedesmus sp.* B2-2 were compared to the culture collection strain *Scenedesmus obliquus* RISE. Cultures were inoculated from flasks during their exponential phase to SLP-FPs as describe in Chapter 3.2. The biomass concentration based on DW and daily extracellular nitrogen concentrations in the medium are presented in Figure 3. Starvation day was counted from the day when NO$_3^-$ concentration in the liquid medium was detected to be zero. All tested algae strains had consumed the nitrogen present in the growth medium after five to eleven days and entered the starvation phase. *Desmodesmus sp.* RUC2 was the fastest N-consuming alga which entered starvation phase at the fifth day, whereas *Scenedesmus sp.* B2-2 was the slowest N consumer (11 days).

The growth curves showed the typical lag phase in the beginning of the experiment, followed by an exponential growth phase, in which the biomass concentration increased dramatically, until a stationary phase was reached. For some algae strains (*Desmodesmus sp.* RUC2, *Coelastrella sp.* 3-4 and *Scenedesmus sp.* B2-2) even a reduction of their biomass concentration was observed at the end of the experiment. *Desmodesmus sp.* RUC2 reached a highest biomass concentration of 3.718 ± 0.05 g·L$^{-1}$, followed by *C. vulgaris* 13-1 (2.479 ± 0.02 g·L$^{-1}$) and *Coelastrella sp.* 3-4 (2.240 ± 0.06 g·L$^{-1}$). The biomass concentrations of the algae strains, such as *Coelastrella astroideum* RW10, *Desmodesmus sp.* 2-6, *S. obliquus* RISE and *Scenedesmus sp.* B2-2, were relatively low with around 1.932 ± 0.02 g·L$^{-1}$ or less.

4.1.2 Fatty acid content and profile

When grown at standard conditions, protein makes the predominant organic compound in most green microalgae (Vassilev & Vassileva, 2016). Nitrogen deprivation induces a reduction of protein and induction of carbohydrates and lipids (Harrison et al., 1990). In Error! Reference source not found., the content of the organic compounds in our algal strains is shown, day 0 being the first day of starvation, which means day 5-11 after culture start (see Figure 4), depending on the species. Not all algal strains were found to have abundant protein content at onset of starvation (Error! Reference source
not found.), *C. vulgaris* 13-1, *Desmodesmus sp.* 2-6, *Coelastrella sp.* 3-4 contained more carbohydrates than protein at the first day of starvation. *C. vulgaris* 13-1 for example, contained 48% of carbohydrates on day 0 compared to only 23% of protein. This result may be reflected by the fact that starvation already occurred earlier since only extracellular N content was measured, which might not reflect the actual intracellular N-stress.

From the second day of N deprivation, the carbohydrate content surpassed protein amount in all algal strains except for *S. obliquus* RISE. *Coelastrum astroideum* RW10 reached the highest carbohydrate content at the end of the experiment with 69 ± 9%, which had increased throughout N-starvation. Carbohydrate content of *C. vulgaris* 13-1 increased to 53 ± 2% at Day 2, but decreased continuously after reaching 37 ± 1% at day 8. The other strains, however, had relatively low content of carbohydrates ranging from 24-35% (Day 2) to 27-32% (End Day), which did not change significantly.
Figure 3. Biomass concentrations and amount of extracellular N of selected algal strains during the time course of cultivation. Each experiment was performed in two biological replicates. While the duplicate showed the same trend, only one representative experiment is shown here, because different light intensities given by LED panels resulted in differences of captured CO₂ and different amounts of biomass achieved, even when cultivated in parallel.
Lipids were observed to contribute the least to the microalgal biomass; however, they are the most important constituents for biodiesel production. The highest total lipid content was found in *C. vulgaris* 13-1 with 13% at the end of the experiment. In addition, *Coelastrum astroideum* RW10, *Desmodesmus* sp. RUC2 and *S. obliquus* RISE also had noticeable total lipid content (11%, 7% and 4%, respectively). *Desmodesmus* sp. 2-6, *Coelastrella* sp. 3-4 and *Scenedesmus* sp. B2-2 also increased their lipid content, but only with 2%.

One should keep in mind that the displayed results might be underestimated due to:

1) The loss during each extraction and analysis procedure, especially when separating TAGs and polar lipids, where some lipids might be difficult to elute from the silica column.

2) The difficulty of breaking the hard algal cell walls (Tredici, 2010; Van Vooren et al., 2012). Only 42-63% of the biomass of *Desmodesmus* sp. 2-6, *Coelastrella* sp. 3-4 and *Scenedesmus* sp. B2-2 was obtained, when the amount of proteins, carbohydrates and lipids are summarized. Therefore also large standard deviations were obtained from the data received from these strains at day 0 of N-starvation: the cell walls were comparatively intact at the beginning of N-starvation, and degraded afterwards, which resulted in an easier cell-break. We can conclude that algae species with hard cell walls are not suitable for biodiesel production due to the extra cost of cell-breaking.

3) A pink phase appeared after centrifugation of the biomass, in particular the strain *S. obliquus* RISE (Figure 4), which is suspected to be the leakage of lipids. A high speed of centrifugation may lead to a loss of 40% of lipids in microalgae with thinner cell walls (Y. Xu et al., 2015). Same phenomenon was also observed by Safafar (2017) using *Chlorella sorokiniana* and *Nannochloropsis salina*. The pink phase from *N. salina* biomass contained 83% lipids of DW, whereas the green phase has only 9%.

4) Some extra peaks were observed in the chromatogram, which could not be assigned to any of the standards used and non-optimal peak separation, in some cases

![Figure 4](image.png)

*Figure 4.* Pink phase separated from the green phase in the biomass of the strain *S. obliquus* RISE biomass (left) after centrifugation, compared to one without phase separation (right)

Figure 6 shows the ratio of polar and non-polar lipids (TAGs) in the algal biomass. All algal strains accumulated TAGs during N-starvation, but with different accumulation abilities. TAG content was increasing during N-starvation for all algae except for *Desmodesmus* sp. 2-6 (Day 3), *Coelastrella* sp. 3-4 (Day 6) and *Scenedesmus* sp. B2-2 (Day 4 and 6). *C. vulgaris* 13-1 was the best performing alga with the highest TAG content at the end of the experiment (11.41 ± 1.7 % of DW). Good TAG producers
were also *Coelastrum astroideum* RW10 (7.93 ± 1.97%) and *Desmodesmus sp.* RUC2 (6.43 ± 1.13%), which surpassed the TAG content of the control strain, *S. obliquus* RISE (3.35 ± 1.01%). In contrast, *Scenedesmus sp.* B2-2 reached 2.23 ± 1.33% TAGs content at the fourth day of starvation, followed by a dramatic decrease to 0.60 ± 0.22%. Likely the algae metabolized the TAGs to receive energy for biomass production. However, also technical mistakes in the measuring procedure cannot be excluded.

Only TAGs can be processed in the biodiesel industry (Bondioli et al., 2012), polar lipids therefore should not be accounted in the total FA profile regarding biodiesel production. It therefore is interesting to note that the percentages of polar lipids decreased during N starvation in most algal species. Structural and functional membrane-associated-lipids may have been degraded during N starvation. Similar results were also observed by Suen et al. (1987) with the macroalgae *Nannochloropsis sp.*

It is worth noticing that the TAG content of the strains *C. vulgaris* 13-1 and *S. obliquus* RISE increased even when the culture was in stationary phase from starvation at Day 4-8 (Cultivation day 12-16) and at Day 4-6 (Cultivation day 9-11), respectively (Figure 3), indicating that these algae still converted light energy into lipids, even when they were not duplicating any longer. A similar conclusion was obtained by Suen et al. (1987), who observed with alga *Nannochloropsis sp.* which was able to accumulate lipids and gain biomass from newly fixed carbon, and they continued to synthesize lipids on the expense of other cellular components in the stationary phase.

Figure 7 shows the daily fatty acid profiles of the algal strains exposed to N starvation. The most predominant FAs in selected strains were C16:0 and C18:1 FAs, which goes in accordance with FA profiles of the majority of other green algae strains (Biondi et al., 2012; Converti et al., 2009; Su et al., 2011). While the FA composition did not change significantly during the starvation period, C18:1 presented the most in FA profile in all algal strains, except for *Scenedesmus sp.* B2-2 (Day 0), followed by C16:0. A small quantity of one PUFA, C18:3, was observed in most algal strains. *C. vulgaris* 13-1 showed to contain relatively more C18:3 (approximately one third) in TAGs compared to the other strains, which did not vary during starvation. High amount of C16-C18 and low concentration of PUFAs suggest a suitable FA composition for biodiesel production.

### 4.1.3 TAGs productivity

Overall, biomass concentration of selected algal strains increased 1.9 - 3.8 folds during N starvation, TAG content increased gradually during N starvation in all algal strains with exceptions for *Desmodesmus sp.* 2-6, *Coelastrella sp.* 3-4 and *Scenedesmus sp.* B2-2 (Table 5). At the last day, *C. vulgaris* 13-1 accumulated the most TAGs with 30.9 mg, followed by *Desmodesmus sp.* RUC2 (26.9 mg) and *Coelastrum astroideum* RW10 (17.5 mg), which indicates those three algal strains have the best abilities for capturing and fixing CO2 under N-starvation. As discussed above, biomass concentration and lipid content should be considered spontaneously for lipid productivity. As a result, although *C. vulgaris* 13-1 accumulated the most TAGs with 11.41%, it is not the most lipid-productive alga strain. *Desmodesmus sp.* RUC2, instead, achieved highest average areal daily productivity (55.9 gTAG/m² day⁻¹) and therefore, is the best candidate for biodiesel production among all tested algal strains. *Coelastrum astroideum* RW10 is also a promising alga for its ability of accumulating both lipids and carbohydrates (Error! Reference source not found.), which can be processed as a potential feedstock for both biodiesel and bioethanol. On the other hand, *Scenedesmus sp.* B2-2 consumed nitrogen in the medium extremely slow (11 days) and performed neither a significant biomass growth nor high lipid content, which makes this strain
unsuitable for lipid production in large scale outdoors systems. This is the first study, to our knowledge, that investigates lipid profile of *Coelastrum astroideum* and *Coelastrella sp.* and their behaviours under nitrogen stress. Both of these species are recently isolated and characterized by large cell size, fast growth and high resistance to cold stress that can be used in future for wastewater reclamation and bulk chemicals production (Ferro et al., 2018). Besides, high TAG productivity as well as carbohydrates accumulation ability makes *Coelastrum astroideum* RW10 an interesting candidate for further investigation.

*Figure 5.* Carbohydrates, proteins and lipids composition of the algal strains; Total lipids are calculated by summing up TAGs and polar lipids. Day 0 is the first day of nitrogen starvation (day 5-11 after cultivation as seen in Figure 3).
Figure 6. Compositions of FA content of the microalgal strains. TAGs are shown in blue and polar lipids in pink.
Figure 7. FA profile of TAGs from all seven algae strains during the time course of N starvation (% TAG)
Table 5. Summary of the presented results; numbers of days refer to the starvation days

<table>
<thead>
<tr>
<th>Strain IDs</th>
<th>Names</th>
<th>Starvation (Cultivation) duration</th>
<th>Cx</th>
<th>Cx increase during starvation</th>
<th>fTAG</th>
<th>Pcum</th>
<th>PTAG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>day</td>
<td>gL⁻¹</td>
<td>Fold</td>
<td>% TAG of DW</td>
<td>mgTAG</td>
<td>gTAG/m²·day⁻¹</td>
</tr>
<tr>
<td>13-1</td>
<td>Chlorella vulgaris</td>
<td>Day 0</td>
<td>1.2 ± 0.1</td>
<td>3.9 ± 0.2</td>
<td>1.4 ± 0.2</td>
<td>7.2 ± 1.3</td>
<td>11.4 ± 1.7</td>
</tr>
<tr>
<td>RW10</td>
<td>Coelastrum astroleuim</td>
<td>Day 0</td>
<td>0.4 ± 0.0</td>
<td>2.8 ± 0.0</td>
<td>0.1 ± 0.1</td>
<td>4.2 ± 0.1</td>
<td>9.2 ± 0.4</td>
</tr>
<tr>
<td>RISE</td>
<td>Stonedelus obliquus</td>
<td>Day 0</td>
<td>0.6 ± 0.1</td>
<td>2.8 ± 0.0</td>
<td>0.3 ± 0.1</td>
<td>2.4 ± 0.4</td>
<td>3.4 ± 1.0</td>
</tr>
<tr>
<td>RUC2</td>
<td>Desmodesmus sp.</td>
<td>Day 0</td>
<td>1.0 ± 0.1</td>
<td>3.6 ± 0.1</td>
<td>1.5 ± 1.2</td>
<td>5.2 ± 0.6</td>
<td>6.4 ± 1.1</td>
</tr>
<tr>
<td>2-6</td>
<td>Desmodesmus sp.</td>
<td>Day 0</td>
<td>1.1 ± 0.3</td>
<td>1.6 ± 0.1</td>
<td>1.2 ± 0.5</td>
<td>2.4 ± 0.4</td>
<td>2.0 ± 0.5</td>
</tr>
<tr>
<td>3-4</td>
<td>C. vulgaris</td>
<td>Day 0</td>
<td>1.2 ± 0.1</td>
<td>1.9 ± 0.1</td>
<td>0.7 ± 0.3</td>
<td>3.0 ± 0.7</td>
<td>2.8 ± 1.1</td>
</tr>
<tr>
<td>B2-2</td>
<td>Scenedesmus sp.</td>
<td>Day 0</td>
<td>0.7 ± 0.1</td>
<td>2.5 ± 0.1</td>
<td>0.3 ± 0.2</td>
<td>2.2 ± 1.3</td>
<td>0.6 ± 0.2</td>
</tr>
</tbody>
</table>

* Day 2 for Desmodesmus sp. 2-6
** Day 8 for C. vulgaris 13-1 and Coelastrum astroleuim RW10, Day 3 for Desmodesmus sp. 2-6

Cx: Biomass concentration (DW) [g L⁻¹]
fTAG: TAG content of biomass (gTAG/g DW)
Cumulative TAG Productivity: \( P_{\text{cum}} = C_x \times f_{\text{TAG}} \times V_R \) [gTAG]; \( V_R \), reactor volume = 1.2 L
Average Areal Daily Productivity: \( P_{\text{TAG}} = (P_{\text{cum}} - P_{\text{start}})/A_{\text{illumin}} \times \Delta t \) [gTAG/m²·day⁻¹]
Aillumin: Surface of reactor receiving light, 0.25 m × 0.3 m = 0.075 m²
\( \Delta t \): Duration of starvation in days
Effect of N starvation on population density in the FP and a single cell size of algal *C. vulgaris* 13-1 and *Coelastrella* sp. 3-4 is shown in Figure 8. Each alga was inoculated with a population density of $2 \times 10^6$/mL and $0.6 \times 10^6$/mL, respectively, based on calibration curves between OD$_{750}$ and cell number. NO$_2^-$ concentrations were measured daily until they reached zero (at day 6 for both algal cultures). However, algal cells were still dividing at the expense of intracellular nitrogen (% of DW) until a minimum (day 6 to 8). For the alga *Coelastrella* sp. 3-4, no significance trend on cell volume was found before day 8. However, a single cell size increased continually from $206.3 \pm 5.0$ µm$^3$ (day 7) to $281.0 \pm 4.2$ µm$^3$ (day 12) when the stationary phase reached with stable (or even decreasing) population density. During that period microalgae tended to store lipids bodies in the cytosol, matching with the microalgae *Chlamydomonas reinhardtii* and *Scenedesmus subspicatus* (Dean et al., 2010).

**Figure 8.** Changes of population densities and single cell sizes of algal strains *C. vulgaris* 13-1 and *Coelastrella* sp. 3-4 during the time course of cultivation. N$_{ext}$: extracellular nitrogen NO$_2^-$ (mM) in liquid medium, N$_{int}$: intracellular nitrogen (% of DW).

### 4.2 Glycerol

To test other cultivation conditions for selected microalgae, they were pre-tested on Petri-dish with BBM and glycerol and incubated in dark for several days. Two algal strains, *Desmodesmus* sp. 2-6 and *Coelastrella* sp. 3-4 were able to grow heterotrophically with glycerol as the carbon source. They were exposed to different concentrations of NO$_2^-$, glycerol, and cultivated in the presence or absence of light, as described in chapter 3.2.2. The biomass concentration at day 7 and day 14 for each culture condition is presented in Figure 9. Both *Desmodesmus* sp. 2-6 and *Coelastrella* sp. 3-4 did not show a significant growth in the presence of 2% glycerol and the absence
of N, independent of the presence or absence of light. Grown mixotrophically, both algal strains reached higher biomass concentrations (Desmodesmus sp. 2-6 with 2.302 g·L⁻¹ in nutrient sufficient conditions, 2.240 g·L⁻¹ in nutrient limit; Coelastrella sp. 3-4 with 2.420 g·L⁻¹ in nutrient sufficient conditions, 2.135 g·L⁻¹ in nutrient limit) compared to autotrophic conditions (Desmodesmus sp. 2-6 and Coelastrella sp. 3-4 with 1.825 g·L⁻¹ and 1.685 g·L⁻¹, respectively). Meanwhile, as shown in Figure 10, when both algae were grown mixotrophically for 14 days, biomass of algae cultivated in nutrient sufficient condition (2.302 g·L⁻¹ and 2.420 g·L⁻¹ for Desmodesmus sp.2-6 and Coelastrella sp.3-4, respectively) were always higher than cultivated in nutrient limit condition (2.240 g·L⁻¹ and 2.135 g·L⁻¹ for Desmodesmus sp.2-6 and Coelastrella sp.3-4, respectively) even if both nitrogen and glycerol were not fully consumed in all cases (data not shown). Interestingly, when growing algae in heterotrophic condition for 7 days, they showed a better growth in the next 7 days compared to those in mixotrophic condition constantly within the same period (red dash lines in Figure 9). These algae seem to adapt better. However, the reason behind this phenomenon has not yet been fully understood.

![Figure 9](image_url) Biomass of Desmodesmus sp. 2-6 and Coelastrella sp.3-4 at day 7 and day 14 grown at different concentrations of nutrients mixotrophically or heterotrophically; Mix: illuminated with light during the whole period; Het + Mix: grown in dark for 7 days and switched to mixotrophic growth for another 7 days; nutrient sufficient stands for 30mM NaNO₃ + 2% glycerol; nutrient limit stands for 5mM NaNO₃ + 25mM glycerol; each cultivate condition was performed with two biological replicates, data with standard deviations are shown in Appex 1.
The abnormal growth was observed in *Coelastrella* sp. 3-4 at nutrient sufficient conditions, the biomass surpassed other conditions at day 7 with 0.153 g·L⁻¹, but only reached a much lower biomass concentration (0.780 g·L⁻¹) at day 14, most likely is due to bacterial contaminations (Appex 2). This alga was not completely axenic, the bacteria competed with algae especially in the absence of light, where is unfavourable for algae. High concentration of nutrients provided an ideal environment for bacteria when algae could not perform photosynthesis.

![Graph showing comparison of mixotrophic growth and heterotrophic + mixotrophic growth of alga Desmodesmus sp. 2-6 and Coelastrella sp. 3-4](image)

**Figure 10.** Comparison of mixotrophic growth and heterotrophic + mixotrophic growth of alga *Desmodesmus* sp. 2-6 and *Coelastrella* sp. 3-4
5. Conclusions

In this study, I investigated the potential of microalgae to produce lipids under nitrogen deprivation. Six Nordic species were compared to the culture collection strain Scenedesmus obliquus RISE (UTEX 417). I was able to show that the microalgal strains Chlorella vulgaris 13-1, Coelastrum astroideum RW10, S. obliquus RISE and Desmodesmus sp. RUC2 could be stressed by nitrogen starvation and accumulated a noticeable higher content of TAGs. Coelastrella sp. 3-4, Desmodesmus sp. 2-6 and Scenedesmus sp. B2-2 showed only slightly increased TAG content shortly after starvation. High TAG productivity and biomass concentration are important factors for a strain suitable for biodiesel production. Two Norvic algal strains C. vulgaris 13-1 and Desmodesmus sp. RUC2 are suggested to be selected for their potential value in biodiesel production because of their high TAG productivity (53.7 mgTAG/m²·d⁻¹ and 55.9 mgTAG/m²·d⁻¹, respectively). In addition, Coelastrum astroideum RW10, which has a moderate TAG productivity (29.2 mgTAG/m²·d⁻¹), but produced biomass with high TAG content (9.2% of DW), has the ability to accumulate both lipids and carbohydrates during nitrogen starvation.

Only two of the selected microalgae, Desmodesmus sp. 2-6 and Coelastrella sp. 3-4, were able to grow heterotrophically with glycerol as carbon source. Growth of both algal strains under heterotrophic conditions was much lower than under mixotrophic conditions.

6. Outlook

Six algal strains were selected for their nitrogen and phosphorus removal abilities in wastewater previously, this study further showed their biomass growths and lipid contents when N was deprived. A combination of municipal wastewater reclamation by microalgae and producing biodiesel from accumulated lipids is a feasible and promising strategy to maximize the commercial value of microalgae. The best TAG producers Chlorella vulgaris 13-1, Coelastrum astroideum RW10 and Desmodesmus sp. RUC2 are promising candidates for biodiesel production and therefore are suggested for large scale cultivations. Lipids in algal biomass from heterotrophic and mixotrophic conditions should be evaluated to find the best growth strategy for highest TAG productivity in comparison with photoautotrophic growth. Furthermore, the currently-used pretreatment and analytical methods are non-specific for microalgae; therefore, a method should be validated and standardized for an efficient extraction.
Acknowledgement

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Last but not least, I would like to thank my family; they always stand by me no matter what decision I made, also thank for their financial support.
References


## Appendix

### Appendix 1

**Apex 1.** Cultivation conditions and algal biomass of *Desmodesmus sp.* 2-6 and *Coelastrella sp.* 3-4 at day 0, day 7 and day 14; values without standard deviation were calculated based on calibration curve between OD$_{750}$ and DW.

<table>
<thead>
<tr>
<th>Alga</th>
<th>Nutrient</th>
<th></th>
<th>Cultivation Condition</th>
<th>Biomass (g·L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nitrogen</td>
<td>Glycerol</td>
<td></td>
<td>Day 0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>2%</td>
<td>Mixotrophic</td>
<td>0.052</td>
</tr>
<tr>
<td></td>
<td>5mM</td>
<td>25mM</td>
<td>Mixotrophic</td>
<td>0.049</td>
</tr>
<tr>
<td></td>
<td>5mM</td>
<td>25mM</td>
<td>Heterotrophic+Mixotrophic</td>
<td>0.049</td>
</tr>
<tr>
<td></td>
<td>30mM</td>
<td>2%</td>
<td>Mixotrophic</td>
<td>0.049</td>
</tr>
<tr>
<td></td>
<td>30mM</td>
<td>2%</td>
<td>Heterotrophic+Mixotrophic</td>
<td>0.049</td>
</tr>
<tr>
<td></td>
<td>30mM</td>
<td>0</td>
<td>Autotrophic</td>
<td>0.051</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>2%</td>
<td>Mixotrophic</td>
<td>0.034</td>
</tr>
<tr>
<td>2-6</td>
<td>5mM</td>
<td>25mM</td>
<td>Heterotrophic+Mixotrophic</td>
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</tr>
<tr>
<td></td>
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<td>25mM</td>
<td>Mixotrophic</td>
<td>0.026</td>
</tr>
<tr>
<td></td>
<td>30mM</td>
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</tr>
<tr>
<td></td>
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<td>Mixotrophic</td>
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</tr>
<tr>
<td></td>
<td>30mM</td>
<td>0</td>
<td>Autotrophic</td>
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</tr>
</tbody>
</table>
Appendix 2

Appex 2. Culture flasks pictures. In each picture, from left to right are: N+G+L 1, N+G+L 2, N+G+nL 1, N+G+nL 2, N+L, N+nL; N, NO2- added; G, glycerol added; L, light for 14 days; nL, no light for 7 days and switched to light afterwards; a-1 to f-1: Desmodesmus sp. 2-6; a-2 to f-2: Coelastrella sp. 3-4; a&d, Day 0; b&e, day 7; c&f, day 14; a-c, “nutrient limit”; d-f, “nutrient sufficient”