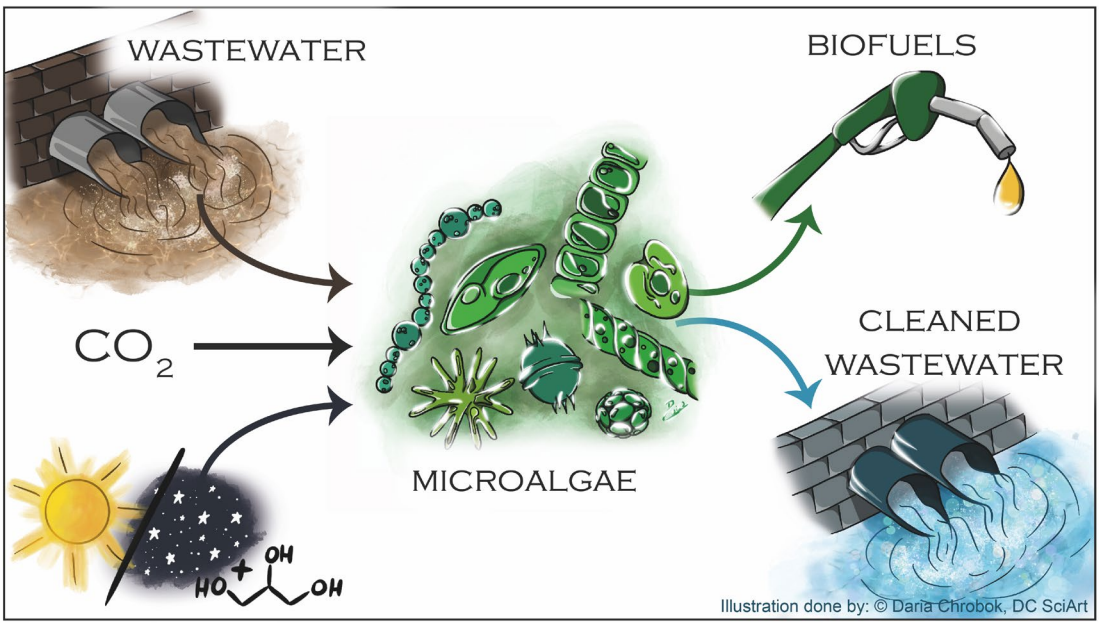

Autotrophic and heterotrophic culture of Nordic microalgae in wastewater for lipid production

Jean Claude Nzayisenga

UMEÅ UNIVERSITY

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Jean Claude Nzayisenga

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List of papers

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Paper 2. Nzayisenga, J. C.; Sellstedt, A. Metabolomics study of *Chlorella sp.* grown under heterotrophic condition (Manuscript)

Paper 3. Nzayisenga, J. C.; Farge, X.; Groll, S.; Sellstedt, A., Effects of light intensity on growth and lipid production in microalgae grown in wastewater. Biotechnology for Biofuels 2020, 13.

Paper 4. Nzayisenga, J. C.; Niemi, C.; Ferro, L.; Gorzsas, A.; Gentili, F. G.; Funk, C.; Sellstedt, A., Screening Suitability of Northern Hemisphere Algal Strains for Heterotrophic Cultivation and Fatty Acid Methyl Ester Production. Molecules 2020, 25, (9), 2107.

Abstract (English)

It is well established that society's main means for producing energy, the combustion of fossil fuels, is unsustainable and contributes to global warming. Microalgae have high potential for the production of biodiesel and energy source that can at least partially replace fossil fuels. In addition, microalgae are a valuable resource for cleaning up the wastewater that developed societies produce on a daily basis. The research presented in this thesis covers how various growth conditions affect the production of lipids – potential energy source – in Nordic microalgae species, and how these species can benefit municipal wastewater treatment.

The research presented in Paper 1 demonstrated that the combination of Fourier-Transform IR (FTIR) and Multivariate Curve Resolution-Alternating Least Squares (MCR-ALS) is a powerful tool for monitoring changes in the biochemical composition (lipids, carbohydrates and proteins) of microalgae grown under different conditions. Experiments showed that *Chlorella sp.* isolated from Umeå was able to grow under heterotrophic conditions using glycerol as a carbon source and, more importantly, demonstrated high lipid content. The substantial accumulation of lipids observed in *Chlorella sp.* corresponded to a decrease in carbohydrate content. Paper 2 covered the key metabolites associated with the observed high lipid content under heterotrophic conditions. The low carbohydrate content observed under these growth conditions may be linked to low levels of the metabolites involved in gluconeogenesis. Conversely, the increase in lipid content may be associated with an increase in fatty acid metabolites and/or certain amino acids. The research presented in Paper 3 showed that microalgae grown under high light intensity (300 µE m^{-2} s^{-1}) have higher lipid contents than microalgae grown under low light intensities (50 and 100 µE m^{-2} s^{-1}). The increase in lipid content under high light intensity conditions corresponded with a decrease in protein content. The research described in paper 4 demonstrated that among various Nordic strains, *Desmodesmus sp.* is the best candidate for biomass and lipid production under heterotrophic conditions with glycerol as the carbon source. Furthermore, the research covered in Papers 1, 3 and 4 demonstrated that the increase in lipid content under certain growth conditions corresponded to better biodiesel quality based on fatty acid composition. The experiments described in Papers 1,3 and 4 also showed that microalgae were able to remove most of nitrogen and phosphorus in wastewater, and thus, could be beneficial to municipal wastewater treatment plants.

In summary, we showed that coupling FTIR to MCR-ALS is useful for evaluating changes in the biochemical composition of microalgae. Nordic microalgae were able to produce high amounts of lipids, which showed a favorable fatty acid profile in terms of biodiesel quality, under certain growth conditions. Subsequent analyses provided insight into which metabolites were responsible for the observed changes in lipid accumulation. We also showed that Nordic microalgae can contribute to wastewater treatment.

Abstrakt (Svenska)

Den mest använda energikällan i samhället kommer från förbränning av fossila bränslen, vilken är ohållbar och tyvärr bidrar till den globala uppvärmningen. Därför behövs alternativ: Mikroalger utgör ett sådant alternativ därför att de har en stor potential när de gäller produktion av lipider som kan användas vid produktion av biodiesel, en energikälla som delvis kan ersätta fossila bränslen. Mikroalger har dessutom förmåga att rena avfallsvatten som kommer från våra kommuner och städer. Forskningen som presenteras i denna avhandling behandlar produktionen av lipider hos Nordiska arter av mikroalger odlade under olika tillväxtförhållanden, och hur de olika arterna kan användas för att behandla kommunalt avfallsvatten.

Forskningen som presenteras i den första studien demonstrerar att man kan kombinera metoderna Fourier-Transform IR (FTIR) och Multivariate Curve Resolution-Alternating Least Squares (MCR-ALS) och få ett kraftfullt verktyg för att följa förändringar i den biokemiska sammansättningen (lipider, kolhydrater och proteiner) hos mikroalger odlade under olika förhållanden. Experimenten visade att *Chlorella sp.*, isolerad i Umeå kunde tillväxa under mörker (heterotrofa förhållanden) med glycerol som kolkälla och tillika, kanske ännu viktigare, hade högt lipidinnehåll. Den substantiella ackumuleringen av lipider som kunde observeras i denna art av *Chlorella* motsvarade en minskad mängd kolhydrater.

Nästa studie beskriver de nyckelmetaboliter som associeras med det observerade höga lipidinnehållet när algerna odlades under heterotrofa förhållanden. Det låga innehållet av kolhydrater som observerats under denna tillväxt kan länkas till låga nivåer av metaboliter involverade i glukoneogenesen och/eller vissa aminosyror.

Studie 3 visar att mikroalger som vuxit under hög ljusintensitet (300 µE m^{-2} s^{-1}) har högre lipidinnehåll jämfört mikroalger under låga ljusintensiteter (50 and 100 µE m^{-2} s^{-1}). Ökningen av lipider under hög ljusintensitet motsvarar minskning i proteininnehåll.
Slutligen visar studie 4 att bland olika Nordiska arter av mikroalger, så är *Desmodesmus sp.* den bäst lämpade kandidaten för biomassa och lipidproduktion under heterotrofa förhållanden med glycerol som kolkälla.

Sammanfattningsvis, har vi visat att Nordiska mikroalger har förmåga att producera stora mängder lipider under vissa tillväxtförhållanden, vilka dessutom visade sig ha en gynnsam fettsyra profil som är värdefullt för biodiesel kvaliteten. Ytterligare analyser klarlade vilka metaboliter som bidrog till den observerade förändringen i ackumulering av lipider. Nordiska mikroalger kan användas för behandling av avfallsvatten. Vi har också visat att det är mycket användbart att koppla metoderna FTIR till MCR-ALS för att utvärdera förändringar i biokemisk sammansättning hos mikroalger.

Abbreviations

DAG:	Diacylglycerol
FTIR:	Fourier-transform infra-red spectroscopy
GC:	Gas chromatography
LCMS:	Liquid chromatography mass spectrometry
MS:	Mass spectrometry
MCR-ALS:	Multivariate curve resolution-alternating least Squares
TAG:	Triacylglycerol
PCA:	Principal component analysis

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I. Introduction

Contemporary society has become dependent on the combustion of fossils fuels for energy production, a dynamic which is unsustainable and has impacted the global climate in a way that we have already began to witness **[1]**. Microalgae are considered a promising source of biofuels that can at least partially replace fossils fuels **[1]**. Furthermore, microalgae are also efficient at cleaning the wastewater that we produce every day **[2]**. The research presented in this thesis covers how various growth conditions affect the production of lipids – a precursor of biofuels – in Nordic microalgae species, and how these species can benefit municipal wastewater treatment.

1. Microalgae

1.1 What are algae ?

Algae are photosynthetic organisms that can be found all over the world, mostly in aquatic environments such as ponds, lakes, oceans and even wastewater **[3, 4]**. There are 30,000 to 1 million species of algae globally, and they produce nearly 70% of the oxygen in the atmosphere during photosynthesis **[5, 6]**. In contrast to plants, algae lack roots, stems or leaves but each cell is photosynthetic and can also absorb nutrients. They can survive in environments with high ranges of temperature, salinity, pH and light intensity **[7]**. They are eukaryotes that can be classified according to their size as microalgae or macroalgae. Microalgae are unicellular, with sizes of around 3 to 7 µm, while macroalgae (seaweeds) are multicellular and up to 70 m long **[8]**. Algae have different colors due to differences in photosynthetic pigments. Thus, they are often classified according to their pigment composition as green algae (Chlorophyceae), red algae (Rhodophyceae), brown algae (Phaeophyceae), diatoms (Bacillariophyce) and golden algae (Chrysophyceae) **[3]** (Fig. 1).

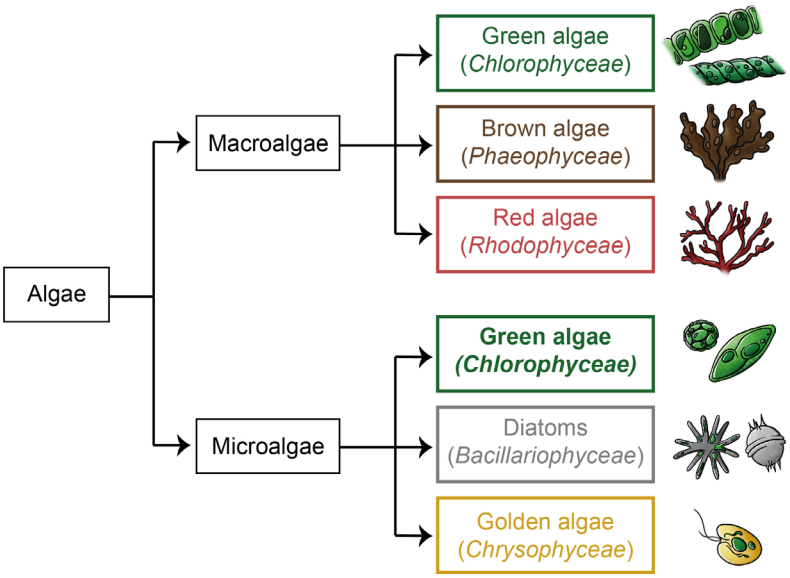

Figure 1: Classes of algae according to their photosynthetic pigments. Adapted from **[3]**.

Cyanobacteria, previously called blue green algae, are not only photosynthetic but also prokaryotic organisms. Therefore, many authors exclude cyanobacteria from algal groups [4]. Algae can produce various valuable compounds, such as health supplements, pharmaceuticals and cosmetics **[9]**. Algae have recently gained a lot of attention since clean, sustainable fuels that could replace fossil fuels can be generated from them. They can also be used in cheap, environmentally friendly wastewater treatment processes **[2]**. In this thesis, the term microalgae refers to green algae, and not other classes of microalgae (diatoms or golden algae), unless otherwise stated (Fig. 1).

1.2 Factors influencing growth of microalgae

Microalgal growth is affected by diverse biotic and abiotic environmental factors. Temperature, light and carbon source are examples of abiotic factors, whereas initial algal cell density, algal pathogens and zooplankton are examples of biotic factors **[10, 11]**. Some of the most important microalgal growth factors are discussed below.

1.2.1 Carbon source

The carbon source is the most crucial factor for cultivation of microalgae. Like plants, microalgae can grow **autotrophically** using inorganic carbon, especially CO_2, to produce chemical energy through photosynthesis. Certain microalgae, such as *Chlorella pyrenoidosa* and *Scenedesmus obliquus* can grow efficiently in high CO_2 concentrations (5-20%) **[12]**. Therefore, microalgae are considered good candidates for taking up CO_2 from industrial flue gases and thus reduce CO_2 emissions into the atmosphere that are strongly contributing to global warming. In addition to autotrophy, some microalgae can grow **heterotrophically**, using organic carbon compounds as sources of energy, in the absence of light **[13]**. Glucose is the mostly widely used source of organic carbon by heterotrophic microalgae and assimilated through hexose/H^+ symport systems **[14, 15]**. Other organic carbon sources that are known to be utilized by some microalgae include acetate, xylose and glycerol **[15, 16]**. Different species can use different organic carbon sources and, for example, the ability to assimilate glucose is not necessarily accompanied by ability to assimilate xylose or glycerol **[16]**. Heterotrophic microalgae can play important roles in commercial scale microalgal production in boreal regions where there are long winters and low solar fluxes. In addition, evolution of the ability to grow heterotrophically may have been an adaptive requirement for microalgae to survive in boreal regions. Thus, screening for the ability to grow in heterotrophic conditions could be important for large-scale microalgal production. Microalgae that can assimilate and use organic carbon and at the same time CO_2 photosynthetically are called **mixotrophic [13]**.

1. 2. 2 Light

Both the quality and quantity of light are extremely important factors for growth of microalgae as they are photosynthetic organisms. Different light sources have different spectra, and thus different effects on growth of microalgae, as the photosynthetically active wavelengths depend on their pigments **[3]**. Light intensity also strongly influences their growth, and three important ranges for microalgal growth can be defined: light limitation, saturation and inhibition **[17]**(Fig. 2). In the light limitation range, the microalgal growth rate increases as light intensity increases until a saturation threshold is reached. Beyond this threshold the photon absorption rate exceeds energy

conversion and associated production rates of key metabolites: the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) and adenosine triphosphate (ATP) **[18]**. Above the light saturation threshold, light inhibits growth of microalgae and their photosynthetic apparatus is irreversibly damaged **[11]**. The optimum light intensity for maximum biomass growth varies among microalgal species **[11]**, so it is interesting to investigate the optimum light intensity of isolated microalgae.

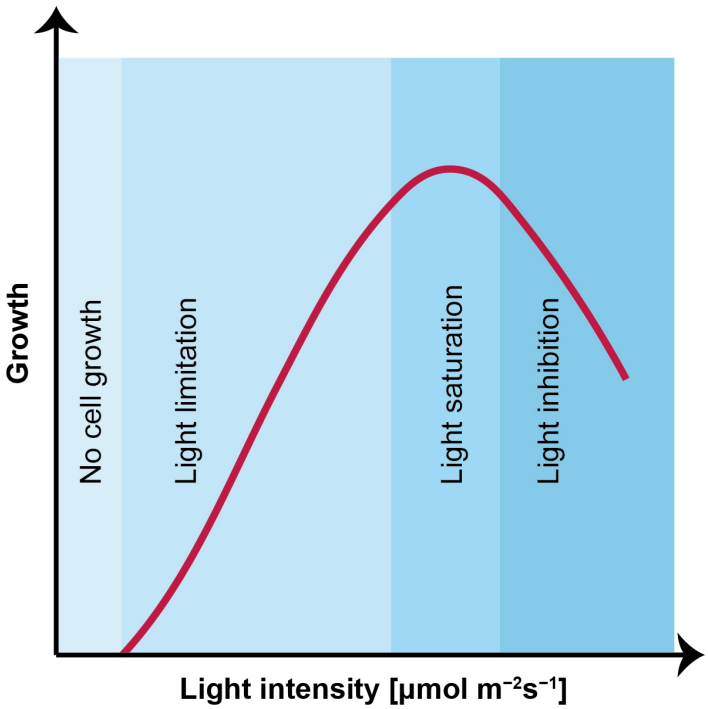

Figure 2: Effect of light intensity on the specific growth rate of microalgae under phototrophic cultivation. Adapted from **[17]**.

1.2.3 Nutrients

Like plants, microalgae need various micro and macronutrients to grow. However, nitrogen (N) and phosphorus (P) are the two major crucial macronutrients for their growth **[19]**. Nitrogen is needed to synthesize important biochemical compounds, including DNA, RNA, amino acids and pigments such as chlorophyll **[19, 20]**. It occurs in several inorganic forms that can be assimilated by microalgae, including nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+) and nitric oxide (NO). Assimilated nitrogen is processed by the glutamine synthetase system, which catalyzes the formation of

the amino acid glutamine from ammonium and glutamate. NO_3^- must be converted to NO_2^-, and NO_2^- to NH_4^+ before it can be used by this system **[20]**. Thus, NH_4^+ is the most preferred nitrogen source since the least energy is required for its uptake and assimilation. NO can also be used to grow microalgae. This can be obtained from flue gases with high contents of nitrogen oxides (NOx), which are hazardous pollutants, and thus reduce emissions of NOx into the atmosphere **[20]**.

Phosphorus is an important element for major components of organisms, such as DNA, RNA, membrane phospholipids and ATP. Phosphorus is present in environments in various natural forms, including orthophosphate, polyphosphate, pyrophosphate, and metaphosphate as well as the organic forms **[20]**. Phosphorus is actively absorbed by cells in orthophosphate form ($H_2PO_4^-$ or HPO_4^{2-}) and certain enzymes are involved in transforming different phosphorus forms into orthophosphate. However, small amounts of various form of phosphorus can also be absorbed passively **[20]**. Microalgae can assimilate more phosphorus than they need and store it as 'polyphosphate granules **[21]**. Phosphorus stored in polyphosphate granules can be used when phosphorus is scarce in the environment, and the ability of microalgae to accumulate high phosphorus contents in granules gives them attractive potential for removing excessive phosphorus in wastewater **[21]** (which can have damaging environmental effects, as outlined in section 4.1).

1.2.4 Temperature.

The optimum temperature for microalgal growth is species- or strain-dependent, and mostly varies between 15 and 40 ^oC **[19]**. However, they can grow at up to 15 ^oC less than their optimum temperature. For example, authors (date) found that *Chlorella vulgaris* grew maximally at 15 ^oC, but could survive and grow at 4^oC **[11]**. Moreover, **[22]** recently showed that species isolated from Nordic regions can grow at very low temperatures (5^oC) and produce as much biomass as when grown at high temperature (25^oC). At low temperature, the fluidity of cell membranes declines, so there are reductions in both membrane transport and overall microalgal growth rates. In adaptive responses, the ratio of unsaturated to saturated fatty acids in membrane generally rises as temperature declines since unsaturated fatty acids can increase cell membranes' fluidity **[19, 23]**.

1.2.5 pH

Another major factor influencing microalgal growth is the pH of the medium or extracellular environment, as diverse cellular processes (including enzyme activities and uptake of both CO_2 and nutrients) are affected by the medium or extracellular pH **[11]**. Most microalgae grow optimally in the pH 7-9 range, although some can grow at very low or very high pH**[24]**. Lower than optimal (acidic) pH interferes with nutrient absorption and diverse cellular functions while effects of higher than optimal (alkaline) pH include reductions in CO_2 affinity and impairment of cell cycling **[11]** .

As most microalgae live in water, the CO_2 they absorb during photosynthesis is dissolved in water and may be in three forms: H_2CO_3, HCO_3^- or CO_3^{2-}. The pH determines which carbon form dominates, and the carbon forms influence the pH. HCO_3^- is the major form at pH around 8. When HCO_3^- is used as a CO_2 source, OH^- is released into the medium, thereby raising the pH (Equation 1)[**25]**. At high pH, CO_3^{2-} becomes the major form, but OH^- is still released if CO_3^{2-} is used as a CO_2 source, so the pH of cultures will become even higher (Equation 2). It is recommended to use a buffer in large-scale cultivation of microalgae to avoid excessive pH fluctuations**[25]**. Another problem associated with high pH is formation of ammonia, which is very toxic even at very low concentrations, from ammonium present in the environment.

Equation 1: $HCO_3^- \rightarrow CO_2 + OH^-$ **[25]**

Equation 2: $CO_3^{2-} + H_2O \rightarrow CO_2 + 2OH^-$ **[25]**

2. Lipid production in microalgae

2.1 What are lipids ?

Lipids are crucial components of organisms, but there is no universally accepted definition. They can be simply defined as fats and oils, or chemically as molecules that are insoluble in water but soluble in organic solvents such as chloroform, ethers and alcohols **[26]**. According to the latter definition, diverse compounds, such as sterols, carotenoids, triacylglycerols (TAGs) and phospholipids, are all lipids, despite major differences in their chemical structures and biosynthesis **[26]**. However, various substances that are generally regarded as lipids are almost as soluble in water as in organic solvents, so more biologically significant definitions have also been proposed, notably "Lipids are fatty acids and their derivatives, and substances related biosynthetically or functionally to these compounds **[27].**". Fatty acids are major components of numerous types of TAGs, phospholipids and other diacylglycerols (DAGs), which are the main constituents of biological fats, oils and membranes (see section 3.3). In these substances, the fatty acids are covalently linked to a glycerol 'backbone' (Fig. 3).

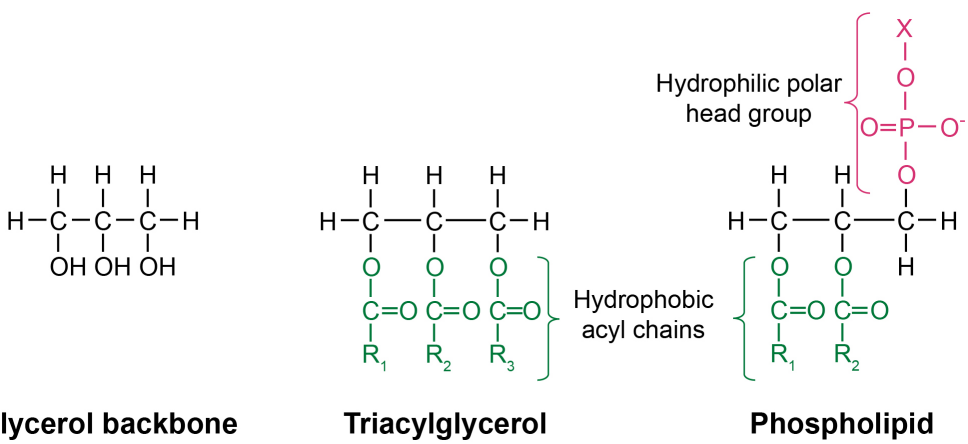

Figure 3 : The 'glycerol backbone' and two main groups of fatty acyl lipids: TAGs (neutral) and phospholipids (polar). Adapted from **[26]**.

There are numerous fatty acids, with different chemical structures and associated variations in both chemical properties and physiological roles. Hereafter, the term 'lipid'

refers to TAGs and DAGs, unless otherwise stated. Fatty acids and lipids derived from them are used commercially in the food industry, for example as cooking oils and butter, and in the chemical industry, for example, as detergents and lubricants **[26]**.

2.2 Structure of fatty acids

A fatty acid is a carboxylic acid with a long chain of carbon and hydrogen atoms (Fig. 4), which provides its 'fatty' (hydrophobic) properties. Formic acid (HCOOH) and carboxylic acids with short chains (less than around six carbons) lack this hydrophobicity and thus are not classified as fatty acids **[26]**. Fatty acids differ in numbers of carbons in their hydrocarbon chain, and both numbers and positions of double bonds in the chain. Fatty acids structures determine the properties of lipids **[28]**. For example, lipids composed of fatty acids with no double bonds (which are called saturated, because they are 'saturated' with hydrogen) are solid at room temperature and mostly known as fats or waxes. Lipids composed of fatty acids with double bonds (unsaturated) are liquid at room temperature and mostly known as oils **[28]**.

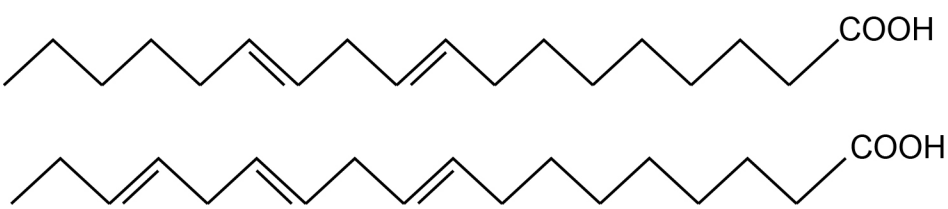

Figure 4 : Examples of fatty acids: Linoleic acid (18:2) and linolenic acid (18:3). The first number in the numerical notation (here 18) refers to the number of carbon atoms in the chain, the second number refers to the number of double bonds.

2.3 Major forms of microalgal lipids and their functions

Lipids can be classified as polar or neutral. Fatty acids in free form are toxic, and their content is minimized by their combination with another molecule, usually glycerol in cells. The carboxyl (-COOH) group of a fatty acid can react with any of the three hydroxyl (-OH) groups of glycerol. Most carboxyl groups are charged at cellular pH, but this reaction results in loss of fatty acids' charge, and glycerol is not charged **[26, 29, 30]**.

Thus, TAGs (in which fatty acyl groups are linked to all three hydroxyl groups of glycerol) are neutral. In a DAG, two of the three hydroxyl groups of glycerol are attached to fatty acids, but the third hydroxyl is attached to a charged molecule (Fig. 3). Two of the main polar lipids of microalgae are phospholipids and glycolipids, in which the charged molecule is a phosphate and carbohydrate, respectively. Phospholipids and glycolipids are key structural and functional components of cell membranes, while TAGs are usually present in organelle-like structures called lipid droplets or lipid bodies, each of which usually contains various types of TAGs **[31, 32]**. The physiological roles of TAGs are not well characterized, but they are involved in energy and carbon storage, possibly because fat has around twice as high energy content per unit mass than protein or starch **[32]**. Due to their high energy content and lack of charged groups, TAGs are potentially strong clean, sustainable alternatives to fossil fuels **[30, 32]**.

2.4 Fatty acids and TAG synthesis in microalgae.

Like plants, microalgae synthesize fatty acids in chloroplasts. Unlike plants, in which fatty acids can be synthesized and stored in special cells, tissues or organs, the whole fatty acid synthesis process occurs within single cells in microalgae **[30, 33]**. Acetyl-CoA produced from glycolysis is the starting point of fatty acid synthesis. The initial step of dedicated fatty acid synthesis is transformation of acetyl-CoA into malonyl-CoA (Fig. 5). This is also regarded as the most limiting step for synthesis of fatty acids as acetyl-CoA is also used in energy production via the TCA cycle **[34]**. Malonyl-CoA then reacts with acyl carrier protein (ACP), yielding malonyl ACP (Fig. 4). A multisubunit enzyme called fatty acid synthase then starts the elongation of fatty acids, using malonyl coA and acetyl CoA as substrates (Fig. 5). The reaction is repeated several times, adding malonyl CoA (and hence two carbon atoms) each time to a growing fatty acid chain. The reaction may be stopped at various points to release a completed fatty acid, but usually a saturated fatty acid called palmitic acid (16:0) is released. This 16:0 is then used as a precursor to synthesize longer, unsaturated or polyunsaturated fatty acids such as 18:0, 18:1 or 18:3. Produced free fatty acids can later be used for *de novo* TAG synthesis **[33]**.

De novo TAG synthesis starts with a transfer of free fatty in the form of acyl CoA from chloroplast to cytoplasm, where they get linked to glycerol to make TAG (Fig.5). To make one TAG molecule, two acyl CoA provide acyl groups to positions 1 and 2 of glycerol 3 phosphate, in a reaction sequence that results in phosphatidic acid (PA)

formation (Fig. 5). PA is later dephosphorylated to diacylglycerol (DAG). Finally, another acyl group provided by acyl CoA is added to the DAG, resulting in a TAG (Fig. 5). In addition to *de novo* synthesis, TAG can be produced via acyl CoA-independent pathways. In that case, phospholipids from plasma or chloroplast membrane may be recycled and reused to make TAGs (Fig. 5)**[33]**.

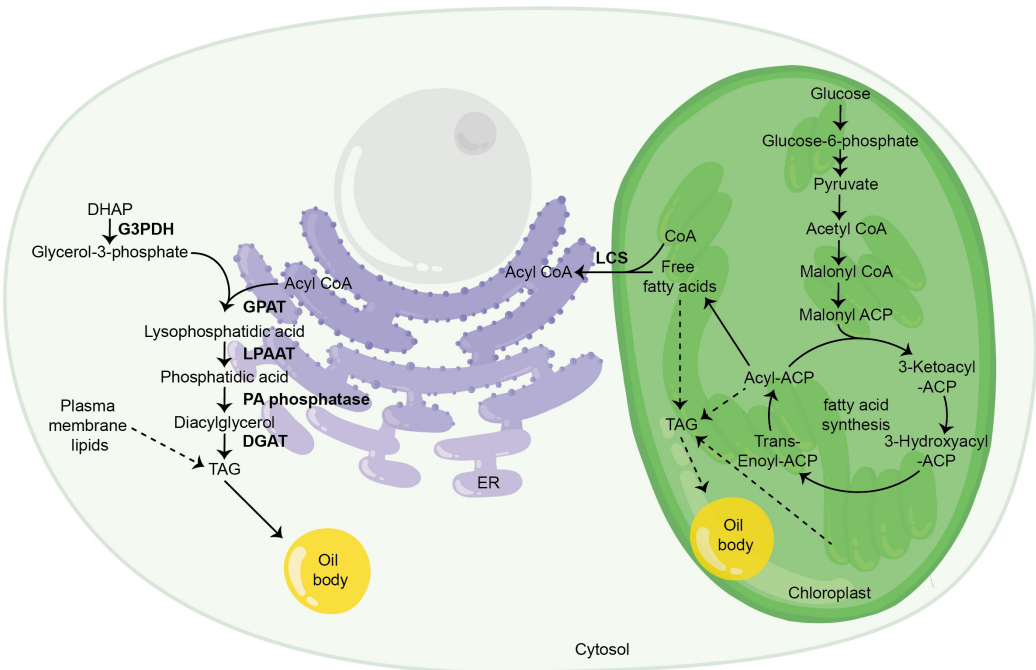

Figure 5 : Fatty acids and TAG synthesis in microalgae. Adapted from **[33]**

2.5 Measurement of lipid contents

The traditional method to measure lipid contents is based on extraction and purification of lipids followed by their gravimetric determination, i.e. weighing them using an analytical balance. To separate lipids from other cell components, organic solvents are used, most frequently a mixture of chloroform and methanol **[35, 36]**. However, gravimetric methodology has several disadvantages. Most importantly, for measuring fatty acyl lipids, there are obvious risks of overestimating their contents through inclusion of other lipids, such as pigments or steroids. This problem can be solved by using gas chromatography (GC) to detect and quantify solely fatty acids **[37]**. For this, fatty acids in extracted lipids are first methylated via a process called transesterification,

which results in fatty acid methyl esters (FAMEs). Transesterification neutralizes the polarity of fatty acids' carboxyl groups, which otherwise hinders GC analysis **[38]**. Another more important benefit of using GC is that fatty acids are individually measured, so it provides a 'profile' showing percentages of each specific fatty acid in samples **[37]**.

There are also other techniques to measure contents of lipids that do not require their extraction. These include fluorescent methods, which are also faster and require smaller numbers of cells to determine lipid contents than gravimetric methods. Nile red and BODIPY are two fluorescent dyes that are commonly used to determine lipid contents, since they can stain neutral lipids in cells. Fluorescence microscopy and spectroscopy are then used to analyze images and quantify lipid contents **[39, 40]**. The main drawback of fluorescence methods is that the thickness and rigidity of some microalgae species' cell walls impair the dyes' penetration into the cells **[39]**. FTIR is another method to measure lipid contents that does not require lipid extraction. In standard spectroscopy, samples are exposed to a beam of monochromatic (single-wavelength) light, and possibly beams of light with one or more other wavelengths after changing settings. In contrast, in FTIR, samples are exposed to a polychromatic light beam, each molecule in the sample has specific absorption peaks, and the sum totals of these peaks form an infrared absorption spectrum (which is measured)**[41]**. The areas or heights of peaks in infrared spectra correspond to concentrations of specific molecules in the samples. Carbohydrates are associated with three bands between 900 and 1100 cm^{-1} due to stretching vibrations of *v*C–O–C bonds in polysaccharides. Proteins are associated with two bands, at 1650 and 1540 cm^{-1} due to stretching of *v*C=O and N–H bonds of amides (amide I and II, respectively). A band at 1740 cm^{-1} arising from C=O stretching is assigned to lipids (Fig. 6)**[42]**. The protein amide I peak has been used as an internal reference to estimate lipid and carbohydrate contents in analyses of FTIR spectra, because the protein content is not supposed to vary substantially. However, in some cases it may vary significantly between samples, so standard FTIR data analysis is unreliable **[43, 44]**. In recent efforts to resolve the problem, MCR-ALS has been used to analyze FTIR spectra of animal and plant samples **[45]**. This resolves numbers of spectral components assigned to specific chemical classes that differ between samples, and relative contributions of each component to every sample's FTIR spectrum **[45]** (Fig. 6). The combination of FTIR and MCR ALS can also be reliably used in analyses of algae. In addition to its rapidity, a major advantage

of FTIR over other methods is the ability to measure three major biochemical components of microalgae (lipids, carbohydrate and protein) simultaneously.

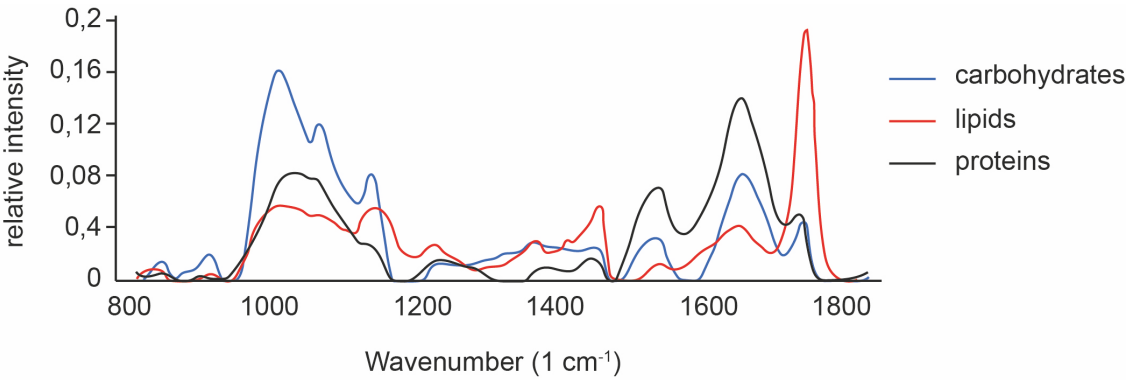

Figure 6. Results of FTIR data analysis using MCR-ALS, showing three resolved components: Components (**blue**), (**red**) and (**black**) respectively assigned to carbohydrates, lipids and proteins **[46]**.

2.6 Microalgal lipids as a source of biofuel

Demand for energy will probably continue to increase, and fossil fuels will eventually be depleted. In addition, combustion of the fossil fuels that we currently use results in emission of high amounts of CO_2 into the atmosphere, thereby contributing to global warming **[1]**. Therefore, alternative fuels are needed that are renewable, environmentally friendly and abundant. Biodiesel, produced from fatty acids in TAGs obtained from suitable organisms, has been considered a good alternative (Fig. 7) **[47]**. Combustion of biodiesel releases amounts of CO_2 into the atmosphere, which are very similar to the amounts absorbed by the plants or algae during the photosynthesis that generated the fuel **[48]**. However, before fatty acids in TAGs and DAGs can be used they must be transesterified, because biological fats and oils are too viscous for use in combustion engines, at least without expensive additional fuel treatment systems **[49]**. To generate biodiesel, fatty acids in the source material are transferred to an alcohol, generally methanol, because it is cheap **[50]**. This results in a kind of biodiesel consisting of fatty acid methyl esters (FAMEs) (Fig. 7). To date, biodiesel has been mostly produced from plants, for example soybean in USA and rapeseed in Sweden **[51]**. This raises the question of food vs fuel because the price of food may increase significantly if a large portion of food crop is used for biodiesel production. Even if the biodiesel can be produced from non- food crop, the agricultural land for cultivation will still be

needed and therefore still the food vs fuel problem **[1]**. Biodiesel can also be produced from microalgae with several advantages compared to plants. Microalgae have much higher growth rates than plants, as they can double their biomass in less than 5 hours during exponential growth phases **[1]**. They do not need agricultural land to grow, thus avoiding competition with agricultural crops and the food vs fuel dilemma. In addition, the biomass of some algal species contains high amount of TAGs, the most suitable type of lipids for biodiesel production **[9]**.

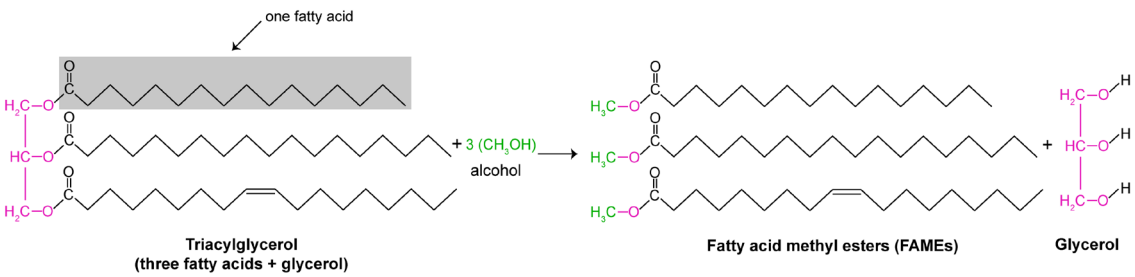

Figure 7 : Biodiesel production using methanol as an alcohol substrate. Adapted from **[52]**.

Different microalgae species have different types of fatty acids, and the quality of biodiesel is determined by the length and saturation of the constituent fatty acid carbon chains (Fig. 4) **[53]**. The tendency of biodiesel to be degraded by oxidation is also affected by its FAME (or, more generally, Fatty Acid Alcohol Ester) composition, and storage conditions. Biodiesel with high contents of di- or higher unsaturated hydrocarbon chains (e.g. 18:2, 18:3 and 20:4) is most vulnerable to oxidation, because the presence of double bonds make biodiesel susceptible to oxygen attack. The 'cold flow properties' (fluidity at low temperatures) of biodiesel are also important **[54]**. As the temperature declines, the fluidity of biodiesel decreases and crystals may form in it that can clog filters. This tendency is correlated to its contents of saturated FAMEs (e.g. 16:0 and 18:0) **[54]**. Double bonds in fatty acids cause kinks in their chains, while fatty acids with saturated hydrocarbon chains are straight. Therefore, saturated fatty acids can pack together easily, which reduces fluidity **[55]**.

3. Wastewater treatment

3.1 What is wastewater ?

Wastewater can simply be defined as any ''used water'' from different human activities. Wastewater have different contaminants depending on the origin of wastewater **[56]**. For example, industrial wastewater is rich in heavy metals (e.g.: cadmium and zinc), chemical toxins (e.g.: biocides and surfactants) and poor in nitrogen and phosphorus content **[57, 58]**. In contrast, municipal wastewater which is wastewater from our homes such as wastewater from baths, dishwasher, toilets, are rich in nitrogen and phosphorus **[59]**. Wastewater has to be treated to remove contaminants before reuse or discharge into aquatic environments, such as lakes, rivers or the sea. Releasing untreated wastewater contaminated with heavy metals or other toxins into aquatic environments can cause massive damage to ecosystems directly, while release of water with high nitrogen and phosphorus contents leads to excessive growth of algae and plants ('eutrophication')**[60]**. When these algae and plants die, they are decomposed by bacteria, which consume nearly all the available oxygen in the process. This results in 'hypoxic' conditions, i.e. too low oxygen concentrations for fishes and various other aquatic organisms to survive **[61]**.

3.2 Wastewater treatment

Wastewater treatment generally involves three phases: primary, secondary and tertiary **[62-64]**. Primary treatment refers to the mechanical or physical removal of solid objects by passing wastewater through screens, a comminutor and grit chamber (Fig. 8). Screens prevent the passage of most debris, the comminutor grinds any residual debris, then the ground material sediments in the grit chamber and a tank called a primary clarifier. The sedimented solid is called primary sludge **[64]**. Secondary treatment refers to the decomposition of soluble organic compounds by microbes in the wastewater (Fig. 8). Air is supplied in secondary treatment to facilitate metabolic processes **[63]**. One of the most widely used types of secondary treatment involves use of an aerated tank for microbial activities followed by another tank (a secondary clarifier) for sedimentation of solid products (mainly bacteria) called 'activated sludge'**[64]**. Some of the activated sludge is re-used in the aerated tank with new wastewater while the rest is disposed of in an appropriate fashion (Fig. 8). The wastewater obtained can be

disinfected and either released into a recipient surface water body or subjected (if necessary) to tertiary treatment **[64]**.

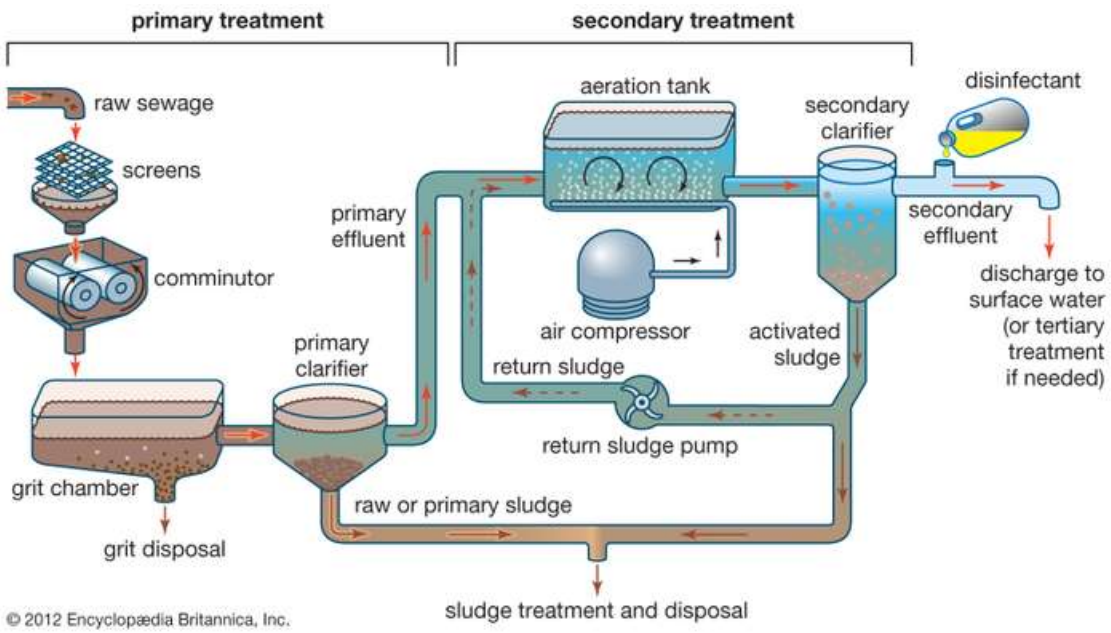

Figure 8 : Schematic diagram of primary and secondary wastewater treatment procedures **[64]**.

Tertiary treatment is the final cleaning phase before wastewater is discharged to the environment. The purpose of this phase of wastewater treatment is to remove any contaminants that were not removed during secondary treatment. For example, inorganic compounds such as nitrogen and phosphorus are removed during this phase **[62, 65]**. Nitrogen can be removed using both chemical or physical methods, e.g., ion exchange and air stripping of ammonia, respectively, neither of which are particularly cost-effective. The air stripping of ammonia is based on adding lime to wastewater in order to volatilize ammonia, after which the volatile compounds are transferred from the liquid to an air stream, while ion exchange removes ammonium by using an ion exchange resin (e.g. clinoptilolite) that selectively captures ammonium. Nitrification-denitrification is a biological method that is commonly used to remove nitrogen from wastewater. In this method, specific bacteria first convert ammonia into nitrate, after which another set of bacteria transform nitrate into nitrogen gas, which is released into the air. However, nitrification-denitrification can be rather expensive due to the costs

associated with constant aeration and the maintenance of large settling tanks. Phosphate can be chemically removed from wastewater by relying on precipitation with compounds such as aluminum or ferric ions, but this method is also expensive. A biological method for phosphorus removal also exists, as certain bacteria are able to accumulate phosphate and store excess amounts as polyphosphate. For instance, microalgae are an example of phosphate-accumulating microorganisms **[21, 66]**.

3.3. The relevance of microalgae to municipal wastewater treatment

The global population is currently experiencing a long-term trend of urbanization, which will increase daily water consumption, and hence, municipal wastewater production, in urban areas. Municipal wastewater with high levels of nitrogen and phosphorus is the optimal environment for microalgae growth **[59, 60]**. However, wastewater cannot be considered as an artificial growth medium because of the constant changes in wastewater composition. For example, ammonia concentrations may periodically exceed the levels that support microalgae growth **[67]**. Moreover, nitrogen and phosphorus concentrations in wastewater can vary depending on the origin of the wastewater as well as the time of year. Another example of the complexity of wastewater is that wastewater may contain predatory zooplankton that prey on microalgae, and/or bacteria that consume organic compounds and compete with microalgae **[58, 68]**. Despite the complexity of wastewater, different microalgae have been shown to thrive in wastewater and remove efficiently the total nitrogen and phosphorus **[2, 4]**. Importantly, after wastewater treatment, microalgal biomass can be used to generate biodiesel and bioethanol via the chemical transformation of microalgal lipids and carbohydrates, respectively **[59, 69]**. This use of wastewater for growing microalgae could decrease the cost of producing biodiesel to a point that such projects are economically feasible **[59]**. Microalgal uptake of nutrients from wastewater varies between microalgae species and, in some cases, strains of the same species. As such, certain microalgae are more efficient than others at removing nutrients from wastewater. For example, the microalgae that already inhabit wastewater are usually highly effective at removing target compounds, most likely due to the fact that they have adapted to the environment, i.e., these algae survive alongside other naturally occurring organisms **[58]**. In addition, microalgae found near the site of the wastewater plant can also be effective bioremediation agents since they are adapted to the local environment. This factor has recently been noted to be

important for large-scale microalgae cultivation **[58]**. When focusing on biodiesel production, researchers should try to identify a local strain that is able to remove nutrients from wastewater as well as accumulate significant amounts of lipids.

4. Metabolomics

4.1 What is metabolomics?

Metabolomics can be defined as a tool for identifying and quantifying metabolites (e.g., amino acids, peptides, carbohydrates, lipids) in living organisms **[70, 71]**. While other "omics" approaches (genomics, transcriptomics, proteomics) are concerned with what may happen in an organism, metabolomics shows the current state of an organism (Fig. 9). For example, it is difficult to connect mRNA or protein levels to metabolism because of various biochemical processes, such as RNA splicing and post translation **[71, 72]**. Metabolites are most often quantified using mass spectrometry (MS) since this methodology can detect diverse metabolites at very low concentrations **[73]**. Various separation techniques may precede MS, with liquid chromatography MS (LCMS) and gas chromatography MS (GCMS) the most commonly applied systems **[72]**. The selection of separation technique depends on the metabolite of interest. For example, GCMS is employed when the sample contains volatile compounds that should be derivatized (e.g., via methylation), while LC-MS can be applied to samples that do not require derivatization and already exist in the liquid phase **[72]**. Environmental changes can significantly influence organisms over various levels; for example, a change in temperature may alter transcription, which stimulates metabolic changes, and subsequently, impacts both phenotype and physiology **[74]**. In microalgae-based biodiesel production systems, the environmental changes that impact lipid production and accumulation are of paramount importance, as lipids are the basis for biodiesel production. Various previous studies have failed to increase lipid production by targeting the expression of key enzymes, which suggests that our knowledge of lipid metabolism in microalgae is lacking **[75]**. Therefore, it is important to use metabolomics as a tool to follow, and begin to understand, lipid production in microalgae.

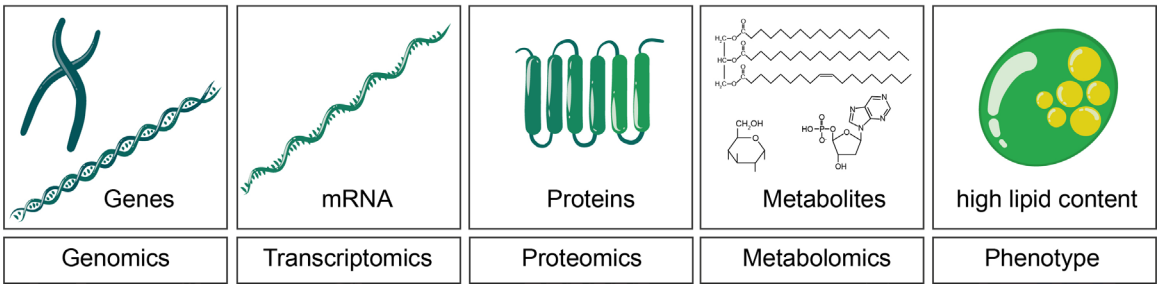

Figure 9: An illustration of how different "omics" approaches can be used to study how cells and organisms function. Adapted from **[76]**.

4.2 Metabolomic studies of algae under auto- and heterotrophic conditions

As described above, certain microalgae are able grow in a light-deprived environment by relying on an external carbon source. Importantly, some of these heterotrophic microalgae show higher accumulation of lipids, especially fatty acids, under heterotrophic conditions. The mechanism which explains this change in lipid metabolism under heterotrophic conditions is not well understood, but different pathways have been suggested (Fig. 10). The first theory focuses on glucose - the most common source of organic carbon - which is taken up through the hexose/h+ symporter system **[14]**. During glycolysis, glucose is broken down over several steps to yield pyruvate. Acetyl-CoA, a precursor of lipid synthesis, is then produced from pyruvate, and can enter the TCA cycle to produce cellular energy (Fig. 10). Another theory focuses on glycerol, which some microalgae obtain by simple diffusion. Within the cell, glycerol is transformed into glyceraldehyde 3-phosphate, an intermediate of glycolysis pathways, and – similar to glucose – will later yield pyruvate **[15]**. Glyceraldehyde 3-phosphate can also be obtained from the Calvin cycle, one component of photosynthesis, which is active when light is available (Fig. 10). Therefore, identifying and quantifying various metabolites from the microalgae that are characterized by improved lipid productivity under heterotrophic conditions will advance our understanding of lipid production in microalgae.

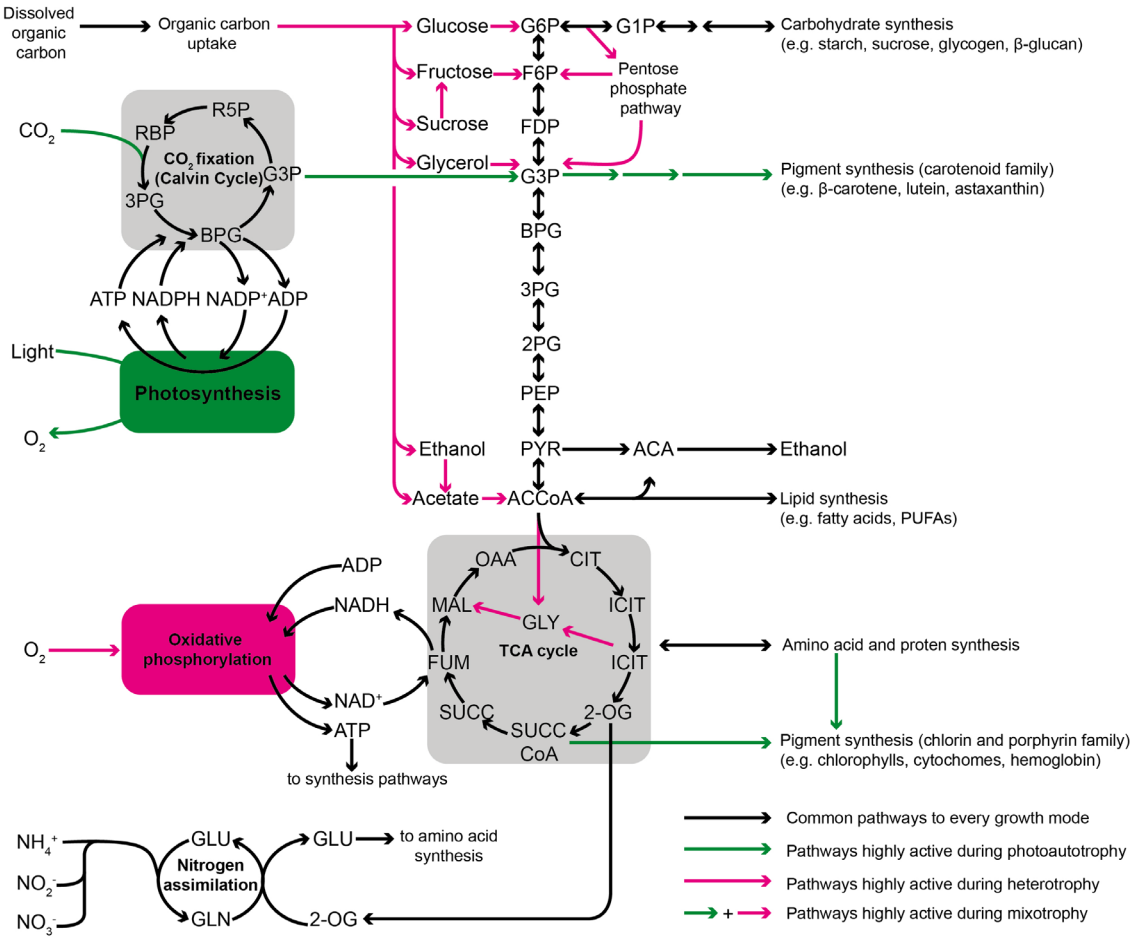

Figure 10: The metabolic pathways active in microalgae under different growth conditions (e.g., photoautotrophy, heterotrophy, mixotrophy). Adapted from **[15]**.

II. Aims

There is a need for a rapid and efficient method to determine lipid contents of microalgae in order to assess (and assist efforts to improve) their suitability for biodiesel production. FTIR spectroscopy fulfills those requirements, and has been recently used, assuming that protein content is constant, to measure lipid (and carbohydrate) contents of microalgae grown in various growth conditions **[42-44]**. However, the assumption that protein content is constant may be false, and result in wrong conclusions **[44]**. A potentially attractive solution is to combine FTIR spectroscopy and and MCR-ALS analysis. **Thus, the aim of the study reported in Paper 1 was to evaluate the ability of FTIR coupled with MCR-ALS to monitor changes in biochemical composition (particularly lipid, carbohydrate and protein contents) of microalgae under different growth conditions. The following growth conditions were used in reported tests: autotrophic, mixotrophic with glucose or glycerol, and heterotrophic with glucose and glycerol.**

Certain growth conditions promote lipid accumulation in microalgae **[15, 77]**. However, lipid production in microalgae is not well understood and recent attempts to increase it by changing the expression of key enzymes have not been successful **[75]**. Several 'omic' approaches have been developed to survey organisms' hierarchical pools of substances involved in biological processes and the transition from genetic information (in the genome), through proteins (the proteome) and myriads of metabolites (the metabolome) to a phenotype **[71, 72]**. **As the metabolome is the closest of these levels to the phenotype, the goal of the study reported in Paper 2 was to identify key metabolites related to high lipid production observed under heterotrophic condition with glycerol.**

Effects of increases in light intensity on microalgae seem to be species-dependent, and may vary from reducing to increasing their lipid contents **[7-9]**. Thus, it is important to study effects of light on the lipid production of each species of potential interest. Moreover, effects of light intensity on relative proportions of three major components of microalgal biomass (lipids, protein and carbohydrates) have received insufficient attention. **Thus, the aim of the study reported in Paper 3 was to investigate effects of varying light intensity (at 50, 150 and 300 µE m^{-2} s^{-1}) on the growth, lipid production and overall biochemical composition of Nordic microalgae.**

Conditions that microalgae have adapted to at high latitudes include long winters with low solar fluxes. Therefore, screening the capacity of locally isolated microalgae for heterotrophic growth is important for large-scale biodiesel production in Nordic regions. While glucose is expensive, glycerol seems to be a better alternative to grow microalgae in heterotrophic conditions as it can be obtained cheaply as a by-product of biodiesel production **[78]. Thus, the aim of the study described in Paper 4 was to identify, by screening, Nordic microalgae capable of growing well in heterotrophic conditions with glycerol.**

In the studies reported in Papers 1, 3 and 4, we analyzed fatty acid profiles of lipids produced under selected growth conditions. We also measured the uptake of nutrients (nitrogen and phosphorus) of microalgae from municipal wastewater used as a growth medium.

III Results and discussion

Paper 1

The main aim of this paper was to combine FTIR and MCR-ALS to examine changes in the biochemical composition of *Chlorella sp.* and determine how this method performs in comparison to standard quantification methods. *Chlorella sp.* was grown in municipal wastewater under autotrophic, mixotrophic and heterotrophic conditions with either glucose or glycerol provided as the organic carbon source.

The microalgal strain used in this study was isolated from samples collected at a community wastewater treatment plant in Umeå. The isolation of a local microalgal strain was expected to provide a species that is adapted to wastewater and performs better than other species from a different location. The isolated microalgal strain was identified using primers that were originally developed for cyanobacteria **[79]**. The cyanobacteria-specific primers for 16S rRNA have been shown to also target 16S rRNA gene fragments from microalgae **[80, 81]**. The amplified sequence was compared to sequences from the NCBI database using BLAST software, and the isolated microalgal strain was identified as *Chlorella sp.*

We first examined the total biomass of *Chlorella sp.* after eight days of growth under autotrophic, mixotrophic and heterotrophic conditions. The *Chlorella sp.* biomass under mixotrophic conditions with glycerol as the carbon source was slightly higher (1.29g/l) than what was observed under autotrophic conditions (1.12 g/l) and mixotrophic conditions with glucose as the carbon source (1.17 g/l). After only two days, the biomass measured in microalgae grown under mixotrophic conditions – regardless of carbon source – significantly exceeded the biomass measured in microalgae grown under autotrophic conditions. **[82]** reported similar results for microalgal strains grown under mixotrophic conditions. Additionally, similar to our results, in the cited study **[82]**, four out of 10 strains showed high growth rates that did not correspond with increased biomass – relative to autotrophic conditions – at the end of the experiment. Our study demonstrated that carbon source - either glucose or glycerol – did not noticeably affect the biomass of microalgae grown under heterotrophic conditions. Previous estimations have placed the optimal glucose concentration for microalgal growth between 10 to

60g/l **[83]**, whereas we reported a figure of 6.7g/l. This may explain why microalgal growth at heterotrophic conditions with glucose as the carbon source was not as at the level that was expected.

We tested how efficient the strain of *Chlorella sp*. is at removing nutrients from municipal wastewater by measuring nitrogen and phosphorus concentrations from wastewater samples after eight days of cultivation. The initial nitrogen concentration of 25 mg/l decreased to 6.1 and 5.6 mg/l in heterotrophic conditions with glycerol and glucose as the carbon source, respectively, to 2.83 and 3.1 mg/l in mixotrophic conditions with glycerol and glucose as the carbon source, respectively, and 4.2 mg/l in autotrophic conditions. The lower nitrogen removal efficiency noted for heterotrophic conditions may be linked to the low *Chlorella sp*. growth rates observed in heterotrophic conditions relative to the other tested conditions. The initial phosphorus concentration of 6 mg/l decreased to less than 1 mg/l under all growth conditions. Recent reports of higher nitrogen and phosphorus removal efficiencies than what was observed in this study can be explained by longer cultivation periods and higher microalgal biomass accumulation **[84]**. The results indicate that *Chlorella sp.* is a promising species for microalgae-based municipal wastewater treatment.

We also investigated whether certain biochemical changes in *Chlorella sp.* could be attributed to different growth conditions. FTIR spectroscopy data acquisition and MCR-ALS FTIR data analysis were combined in this stage of the research. We attributed FTIR spectral bands to molecular groups based on descriptions from **[42]**. More specifically, the three bands between 900-1100 cm^{-1} were assigned to *v*C–O–C stretching in polysaccharides, the two bands at *1650 cm^{-1}* and 1540 cm^{-1} were assigned to proteins (amide I and amide II, explained by *v*C=0 and *δ*N–H amide stretching, respectively), and the band at 1740 cm^{-1} was considered to represent lipids (C=O stretching). In this type of FTIR analysis, it is critical to accurately determine the number of components, i.e., signals from either single compounds or groups of compounds that cannot be resolved further **[45]**. The FTIR data gathered in this study could be interpreted using two components, and MCR ALS analysis revealed that component one was predominantly composed of carbohydrates while component two mainly included lipids. Microalgae with the highest content of component 2 (mainly lipids) represented heterotrophic conditions with glycerol as the carbon source, while microalgae with the highest content of component 1 (mainly carbohydrates) represented autotrophic and mixotrophic conditions with glycerol as the carbon source. Both mixotrophic and

heterotrophic conditions with glucose as the carbon source resulted in slightly lower levels of component 1 than the other treatments, with the lowest share of component 1 observed in microalgae grown under heterotrophic conditions with glycerol as the carbon source.

The results observed from the coupling of FTIR and MCR-ALS prompted us to use standard methods for lipid and carbohydrate quantification (i.e., gas chromatography for lipids and anthrone assay for carbohydrates) to check whether both approaches provided similar results. Based on the second analyses, microalgae representing autotrophic conditions showed slightly higher carbohydrate contents (53.12%) than microalgae representing mixotrophic conditions (50.3%), with microalgae cultivated under heterotrophic conditions with glycerol as the carbon source accumulating by far the lowest amount of carbohydrates (8.2%). Moreover, microalgae cultivated under mixotrophic conditions with glucose as the carbon source and heterotrophic conditions with glucose as the carbon source showed carbohydrate contents of 42.9% and 34.9%, respectively. Interestingly, microalgae cultivated under heterotrophic conditions with glycerol as the carbon source demonstrated the highest lipid content (39.5%), with microalgae grown under autotrophic and mixotrophic conditions with glycerol as the carbon source displaying significantly lower lipid contents (13.4% and 10.5%, respectively). In contrast to previous findings that lipid content increases under mixotrophic conditions with glycerol as the carbon source, we found that these conditions actually increase the carbohydrate content of microalgae. It should be noted that no CO_2 was added in the previous experiments while the present study included experiments in which microalgae were grown with 5% CO_2. It has been shown that higher CO_2 levels may favor carbohydrate production, as was observed in the current study **[85]**.

A comparison of the results from standard methods and the reported combination of FTIR and MCR-ALS revealed a high degree of correlation between the two methods. Regarding lipid and carbohydrate measurements, a comparison of the results yielded R^2 values of 0.99 and 0.94 for lipids and carbohydrates, respectively. Hence, our results demonstrate that MCR-ALS can be utilized to efficiently analyze FTIR data for the purpose of monitoring changes in the biochemical composition of microalgae. The combination of MCR-ALS and FTIR can solve the problem of relying on protein amide I and II bands to quantify lipids and carbohydrates, as this approach assumes that protein contents are constant, which is not always the case **[43, 44, 86]**.

We also examined the fatty acid profiles of extracted lipids using gas chromatography. The most prominent fatty acids in microalgae representing all of the growth conditions were 16:0, 16:1, 18:0, 18:1, 18:2 and 18:3. Furthermore, all of the analyzed samples showed 18:3 contents lower than 12%, which is the maximum content of 18:3 allowed in biodiesel according to European standard EN14214 **[87]**. Polyunsaturated fatty acids, such as 18:3, are susceptible to oxidation during long-term storage or at high temperatures, which leads to significant biodiesel degradation **[88]**. Importantly, microalgae cultivated under heterotrophic conditions with glycerol as the carbon source – which showed the highest lipid content – also showed the lowest 18:3 content relative to other growth conditions. The most abundant fatty acids in microalgae representing autotrophic conditions - 16:0 and 18:2 - decreased in microalgae cultivated in heterotrophic condition while 18:1 became the most abundant fatty acid. This supports previous research in which *C. zofingiensis* was shown to have lower 16:0 and higher 18:1 contents in heterotrophic than in autotrophic conditions **[89]**. Saturated fatty acids such 16:0 decrease cold flow properties, which means that a lower content of saturated fatty acids improves biodiesel quality **[54]**. On the other hand, an increase in polyunsaturated fatty acids such as 18:1 improves oxidative stability and cold flow properties, both of which enhance biodiesel performance **[90]**. Therefore, our results suggest that growing *Chlorella sp.* under heterotrophic conditions with glycerol increases lipid content, as well as the quality of the biodiesel that can be produced from these lipids relative to microalgae from other growth conditions.

Paper 2

We have shown in paper 1 that *Chlorella sp.* accumulate more lipids under heterotrophic conditions with glycerol provided as the carbon source than in other growth conditions. Hence, Paper 2 extended the research presented in Paper 1 **[91]** by using GC-MS-based metabolomics analysis to identify key metabolites of *Chlorella sp.* that contribute to the increased lipid production observed under heterotrophic conditions. The metabolite profiles of microalgae grown under different conditions served as the controls against which the metabolomics analysis of microalgae exposed to heterotrophic conditions could be compared. These growth conditions were autotrophic, mixotrophic and heterotrophic with glucose as the carbon source, and mixotrophic with glycerol as the carbon source.

A total of 85 metabolites - including sugars, amino acids and fatty acids – were annotated. We were interested in studying how the metabolite profile of microalgae grown under heterotrophic conditions differed from the metabolite profiles representing other growth conditions. A PCA score plot revealed a clear separation between sample clusters based on growth conditions, which suggests that microalgae grown under different conditions have distinct metabolite profiles, especially heterotrophic condition with glycerol.

Glucose-6-phosphate, fructose-6-phosphate and glyceric acid-3-phosphate were present at high levels in microalgae representing all of the growth conditions except heterotrophic conditions with glycerol as the carbon source. These three sugars are substrates of glycolysis (glucose degradation) and gluconeogenesis (glucose synthesis) **[92]**. Hence, *Chlorella sp.* cultivated under growth conditions other than heterotrophy with glycerol as the carbon source accumulated high levels of carbohydrates associated with glucose metabolism. We theorize that the high levels of certain carbohydrates (glucose-6-phosphate, fructose-6-phosphate and glyceric acid-3-phosphate) were associated with increased gluconeogenesis, which increased the carbohydrate content of microalgae. This finding is supported by previous reports of fructose-1,6-bisphosphatase upregulation, which transforms 1,6-biphosphate into fructose-6-phosphate during gluconeogenesis, in microalgae characterized by low lipid content and high carbohydrate content **[93]**.

Microalgae grown under heterotrophic conditions with glycerol as the carbon source demonstrated heightened levels of the amino acids alanine, lysine, ornithine and glutamine relative to microalgae grown under other conditions. Amino acid recycling can be used to generate acetyl-CoA, which is a precursor of fatty acid synthesis. For example, previous research has shown that microalgae growing under stress conditions recycle nitrogen from amino acids, with the nitrogen initially stored in the form of certain amino acids, but later used to drive lipid synthesis **[94]**. In the current study, microalgae growing in heterotrophic conditions with glycerol as the carbon source showed lower biomass than microalgae grown under other conditions, which could be considered a sign of exposure to stress (paper 1) **[91]**. Moreover, the formation of certain amino acids under stress conditions has been linked to the synthesis of specific phosphorus-free membrane lipids that are required to cope with these conditions. Recent studies have identified two of these phosphorus-free membrane lipids to

contain ornithine and glutamine. Therefore, the high levels of these two amino acids in the microalgae grown under heterotrophic conditions observed in the current study may be associated with the formation of phosphorus-free membrane lipids **[95, 96]**.

In the current study, microalgae grown under heterotrophic conditions with glycerol as the carbon source showed heightened levels (relative to microalgae cultivated under other conditions) of three out of the four investigated fatty acids. These three fatty acids were margaric acid (17:0), pelargonic acid (9:0) and arachidic acid (20:0). This is consistent with our previous data, i.e. high lipid content was observed in microalgae grown under heterotrophic conditions with glycerol as the carbon source **[91]**. In the previously study, we also showed that oleic acid increased (18:1) – at the expense of linolenic acid (18:3) – when microalgae were exposed to heterotrophic conditions with glycerol as the carbon source **[91]**.

Paper 3

The four microalgal strains used in the study were isolated from the Northern Hemisphere. Microalgae were then grown under three different light intensities (50, 150 and 300 µE m^{-2} s^{-1}) to investigate how light intensity influences biomass productivition, total fatty acid content and biochemical composition. The four microalgal strains were cultivated in municipal wastewater.

All four microalgae species were first grown for eight days. *Desmodesmus sp.* showed the highest biomass (1.1g/l) when grown at a light intensity of 300 µE m^{-2} s^{-1}, with biomass in this species increasing as light intensity increased. In contrast, biomass productivition in *C. vulgaris* and *S. obliquus* was not significantly influenced by light intensity, as biomass in these species only increased slightly when light intensity was increased from 150 µE m^{-2} s^{-1} to 300 µE m^{-2} s^{-1}. Therefore, from a biomass productiion point of view, 150 µE m^{-2} s^{-1} was the optimum light intensity for cultivating *C. vulgaris* and *S. obliquus*. Our results agree with previously published research, namely, the light intensity at which biomass begins to decrease varies between microalgae species **[97, 98]**. The two species that showed high biomass (*Desmodesmus sp.* and *S. obliquus*) were selected to study whether a longer cultivation period (15 days) will have a noticeable effect on biomass and fatty acid content. Apart from *E. pseudoalveolaris,* all

of the other microalgae species demonstrated satisfactory growth rates under the tested conditions, which warrants further research.

We used gas chromatography to investigate fatty acid content in the four studied microalgal strains under different light intensities. This methodology is highly relevant to biodiesel research because it can be applied to only quantify the fatty acids in a sample **[37]**. After eight days, *Desmodesmus sp.* showed the highest fatty acid content (6.2%), with *S. abundans* demonstrating a slightly lower content of 5.8%. Our data are in line with previous findings that microalgae grown at higher light intensities produce more lipids than microalgae grown at lower light intensities. For example, it was previously observed that the lipid content of *S. abundans* increases when the light intensity increases from 55 to 110 µE m^{-2} s^{-1} **[99]**. Moreover, another study found that various *Chlorella* species produced more lipids at 600 µE m^{-2} s^{-1} than at lower light intensities **[100]**. This increase in lipid production under high light intensities may be partly attributed to the conversion of excess photoassimilates into fatty acids. However, several recent studies have also reported that lipid content in microalgae decreases as light intensity increases, even though total biomass has increased **[101, 102]**. Cited authors theorize that – in microalgae - the energy from light may be used for cell division rather than the production of storage lipids **[101, 102]**. Our data for *C. vulgaris* and *E. pseudoalveolaris* show that lipid content decreased when light intensity increased from 150 µE m^{-2} to 300 µE m^{-2} s^{-1}. Thus, it seems that light intensity exerts variable effects on lipid production among microalgae species. We decided to cultivate the two species which showed the highest biomass productivities for 15 days (instead of the initial cultivation period of eight days). Between days 8 to 15, the lipid content in *S. obliquus* doubled (5.8 to 11.6%) when grown at 300 µE m^{-2} s^{-1}, while lipid content did not change significantly when microalgae were grown at 50 and 150 µE m^{-2} s^{-1}. High lipid production has been partly associated with nitrogen starvation. However, in the current study, nitrogen and phosphorus levels were not depleted during the 15-day cultivation period.

Previous research has tended to focus on how light intensity affects biomass production and lipid content, while changes in carbohydrate and protein contents have been overlooked. Therefore, we investigated the effect of light intensity on lipid, carbohydrate and protein contents using the FTIR method presented in paper 1 **[91]**. In *Desmodesmus sp*. and *S. obliquus,* an increase in light intensity was associated with an increase in lipid content at the expense of protein content, while carbohydrate levels

stayed almost constant. Our results agree with previous data that *Dunaliella tertiolecta* grown at high light intensity shows high lipid content and low protein content **[103]**. On the other hand, recent studies have shown that the high lipid content observed during nitrogen starvation conditions corresponds to a decrease in carbohydrate content – with protein levels staying constant **[104-106]**. One of these studies revealed that increased lipid production was associated with starch degradation, which is based on the hypothesis that blocking starch synthesis may increase lipid production because lipid and carbohydrate pathways compete for the same carbon precursors **[106]**. However, our observation that lipid content increases at the expense of protein content suggests that lipid synthesis depends on protein degradation. Therefore, the increased lipid synthesis observed under high light intensities and nitrogen starvation (which was not applied in this study) may depend on distinct pathways, resulting in either protein or carbohydrate catabolism.

We also examined fatty acids composition among the four microalgal strains using gas chromatography. An increase in light intensity changed the fatty acid composition in all of the microalgal strains except for *E. pseudoalveolaris*, which showed a constant fatty acid profile at all three light intensities. In the other three strains, the fatty acids 16:0 and 18:3, and – to an extent - 18:2, dominated the profile at lower light intensities. As light intensity increased, the prevalence of 18:3 decreased while 18:1 became more pronounced in the fatty acid profile. This agrees with the results of experiments in which *C. prototheco*ides grown at a high light intensity showed low 18:3 content and high 18:1 content **[107]**. As described above (Paper 1) **[91]**, feedstocks characterized by low 18:3 content and abundant 18:1 will yield high-quality biodiesel. Therefore, we provide evidence that increasing the light intensity will change the lipid composition of microalgae in a way that benefits biodiesel production.

Paper 4

The research presented in Paper 4 aimed to identify algal strains that could grow in heterotrophic conditions with glycerol (20mM or 40mM) as the carbon source. The experiments were performed in municipal wastewater.

Screening conducted on agar plates identified six strains that can grow under heterotrophic conditions and use glycerol as carbon source. The four strains with the highest growth rates, more specifically, *Desmodesmius* (2-6), *Coelastrella* (3-4), *Scenedesmus obliquus* (SP) and *Chlorella vulgaris* (LNY), were selected for further experiments. *Ettlia pseudoalveolaris* (FNY-2) was used as a control. The selected strains were grown under heterotrophic conditions with either 20mM or 40mM of glycerol, after which the results were compared to what had been observed under autotrophic conditions. LNY demonstrated the highest biomass, which was observed under heterotrophic conditions with 40 mM glycerol. Under autotrophic conditions, all of the four tested species showed almost the same biomass of around 0.3g/l. The low biomass productivities observed under autotrophic conditions may be explained by the decision to not provide the microalgal cultures with external CO_2, which usually increases biomass production by stimulating photosynthesis. Under heterotrophic conditions, the two strains (LNY and 2-6) with the highest biomass productivities showed higher biomass accumulation when provided with 40mM glycerol than 20 mM glycerol. **[16]** recently showed that the biomass productivities of nine out of 10 strains were lower when algae were grown under heterotrophic conditions than when algae were grown under autotrophic conditions. The algal cultures in the study were provided with 20mM glycerol as the carbon source. In the current study, algae grown under heterotrophic conditions with 20mM glycerol also showed lower biomass productivities than algae grown under autotrophic conditions. In summary, despite the low observed biomass productivities across all of the tested strains, 2-6 and LNY were nevertheless identified as good candidates for biomass production under heterotrophic conditions with 40mM glycerol.

The fatty content analysis of all the strains grown under heterotrophic conditions with 40 mM glycerol showed that 2-6, LNY and SP had the highest fatty acids contents of around 7.6%. When grown under heterotrophic conditions with 40mM glycerol provided as the carbon source, the fatty acid content of LNY increased relative to autotrophic conditions. 3-4 and FNY had the lowest fatty acid contents. Since 2-6 and

LNY had shown the highest biomass of the tested strains under heterotrophic conditions, and the same lipid content, 2-6 can be selected for lipid production since 2-6 had higher 18:1 content and lower 18:3 content than 3-4. Thus, 2-6 was also superior in terms of providing a feedstock that could be used to produce high-quality biodiesel as described in paper 1 **[91]**. This research is relevant because using crude glycerol, which is far more affordable that glucose, in a heterotrophic microalgae biomass production system could significantly reduce the price of operating such a system.

IV. Summary and future perspectives

We showed that the combination of FTIR and MCR-ALS is a powerful analytical method for monitoring changes in the biochemical composition of microalgae grown under different conditions. This method could have applications for the daily monitoring of various classes of compounds (lipids, carbohydrates and proteins) in a microalgal cultivation system through a single experiment. Even though microalgae may show high lipid content, the extraction of these compounds may be complicated by the rigidity, and high structural diversity, of the cell wall **[108]**. Therefore, lipid extraction in the context of microalgae should be extensively studied so that researchers can confidently determine whether microalgae-based production of biodiesel is feasible. The research underlying this thesis revealed that some microalgae are able to use glycerol as a carbon source in heterotrophic conditions. Furthermore, some of these microalgae also accumulated significant amounts of lipids under these conditions; it would be interesting to quantify precisely how much glycerol was taken up by the microalgae. In addition, other cheap carbon sources, such as molasses and food waste, could be studied for their relevance in microalgae biomass production systems operating under heterotrophic conditions. We have also demonstrated that microalgal lipid production is positively correlated with light intensity, although the threshold for this effect varies between species.

However, since the highest light intensity studied in the research presented in this thesis was 300 $\mu E\ m^{-2}\ s^{-1}$, future research should investigate whether even higher light intensities can stimulate microalgal growth and lipid production. It could be presumed that extremely high light intensities would inhibit microalgal growth; as such, higher light intensities could be applied during the last days of cultivation when cells are in the late exponential growth phase. Under heterotrophic conditions, the observed increase in lipids was associated with a decrease in carbohydrate levels, while high light intensities resulted in decreased protein levels. These changes in the biochemical composition of microalgae, especially those related to an increase in lipid production, need to be studied in more detail so that scientists can better understand which conditions have the largest impact on lipid production. Certain metabolites were present at either high or low concentrations under the growth conditions that stimulated lipid production. The metabolic changes associated with these conditions could be studied through the exogenous introduction of certain metabolites, which

could reveal the pathways that are responsible for the observed changes in biochemical composition. Although the increase in lipid content was associated with better biodiesel quality, we feel that the transmethylation step of biodiesel production from fatty acids warrants more research so that it can be optimized. The utility of introducing microalgae to wastewater for the production of biomass depends on their ability to also contribute to the cleaning of the wastewater. The results presented in this thesis showed that microalgal strains that are promising candidates for biofuel production can also effectively remove nutrients (nitrogen and phosphorus) from wastewater. Nevertheless, these results reflect laboratory-scale experiments, which have to be replicated in pilot studies and full-scale wastewater facilities to determine their applicability. Some of these microalgal strains could also be tested for their ability to assimilate CO_2, which is a by-product of various industrial processes **[109]**. Last but not least, microalgal biomass should be considered as a precursor for diverse high value products. Hence, future research should also investigate whether microalgal biomass can be used to produce nutritional, cosmetics and/or pharmaceutical products **[9]**.

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VI References

1. Chisti, Y., Biodiesel from microalgae. *Biotechnology Advances* **2007,** 25, (3), 294-306.
2. Brennan, L.; Owende, P., Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products. *Renewable and Sustainable Energy Reviews* **2010,** 14, (2), 557-577.
3. Najiha Badar, S.; Mohammad, M.; Emdadi, Z.; Yaakob, Z., Algae and their growth requirements for bioenergy: a review. *Biofuels* **2018**, 1-19.
4. Mata, T. M.; Martins, A. A.; Caetano, N. S., Microalgae for biodiesel production and other applications: A review. *Renewable and Sustainable Energy Reviews* **2010,** 14, (1), 217-232.
5. Chinnasamy, S.; Rao, P.; Bhaskar, S.; Rengasamy, R.; Singh, M., Algae- A Novel Biomass Feedstock for Biofuels. in Microbial Biotechnology Arora, R. **2012**; pp 224-239.
6. Guiry, M. D., How many species of algae are there? *Journal of Phycology* **2012,** 48, (5), 1057-63.
7. Varshney, P.; Mikulic, P.; Vonshak, A.; Beardall, J.; Wangikar, P., Extremophilic micro-algae and their potential contribution in biotechnology. *Bioresource Technology* **2014,** 184.
8. Anbu, R. M.; Valliappan, K.; Li, Z.-Y., Prospect of Marine Algae for Production of Industrially Important Chemicals. **2015**; pp 195-217.
9. Ariede, M.; Candido, T.; Morocho-Jácome, A.; Velasco, M.; Carvalho, J.; Baby, A., Cosmetic attributes of algae - A review. *Algal research* **2017,** 25, 483-487.
10. Montemezzani, V.; Duggan, I.; Hogg, I.; Craggs, R., A review of potential methods for zooplankton control in wastewater treatment High Rate Algal Ponds and algal production raceways. *Algal Research* **2015,** 11, 211-226.
11. Chia, S. R.; Kit Wayne, C.; Pau Loke, S.; Ong, H. C.; Phang, S.-M.; Ling, T.; Nagarajan, D.; Lee, D.-J.; Chang, J.-S., Sustainable approaches for algae utilization in bioenergy production. *Renewable Energy* **2017**,129, 838-852
12. Tang, D.; Han, W.; Li, P.; Miao, X.; Zhong, J.-J., CO2 Biofixation and Fatty Acid Composition of Scenedesmus obliquus and Chlorella pyrenoidosa in Response to Different CO2 Levels. *Bioresource Technology* **2010,** 102, 3071-6.
13. Venkata Mohan, S.; Rohit, M. V.; Chiranjeevi, P.; Chandra, R.; Beeramganti, N., Heterotrophic Microalgae Cultivation to Synergize Biodiesel Production with Waste Remediation: Progress and Perspectives. *Bioresource Technology* **2015,** 184, 169–178.
14. Tanner, W., The Chlorella hexose/H+-symporters. *International review of cytology* **2000,** 200, 101-41.

15. Perez-Garcia, O.; Bashan, Y., Microalgal Heterotrophic and Mixotrophic Culturing for Bio-refining: From Metabolic Routes to Techno-economics. In *Algal Biorefineries: Volume 2: Products and Refinery Design*, Prokop, A.; Bajpai, R. K.; Zappi, M. E., Eds. Springer International Publishing: Cham, **2015**; pp 61-131.
16. Leite, G.; Paranjape, K.; Abdelaziz, A.; Hallenbeck, P., Utilization of biodiesel-derived glycerol or xylose for increased growth and lipid production by indigenous microalgae. *Bioresource Technology* **2014,** 184.
17. Ogbonna, J. C.; Tanaka, H., Light requirement and photosynthetic cell cultivation – Development of processes for efficient light utilization in photobioreactors. *Journal of Applied Phycology* **2000,** 12, (3), 207-218.
18. Wehr, J., Algae: Anatomy, Biochemistry, and Biotechnology by Barsanti, L. & Gualtieri, P. *Journal of phycology* **2007,** 43.
19. Juneja, A.; Ceballos, R. M.; Murthy, G. S., Effects of Environmental Factors and Nutrient Availability on the Biochemical Composition of Algae for Biofuels Production: A Review. *Energies* **2013,** 6, (9), 4607-4638.
20. Markou, G.; Vandamme, D.; Muylaert, K., Microalgal and cyanobacterial cultivation: The supply of nutrients. *Water Research* **2014,** 65, 186-202.
21. Cuellar-Bermudez, S. P.; Aleman-Nava, G. S.; Chandra, R.; Garcia-Perez, J. S.; Contreras-Angulo, J. R.; Markou, G.; Muylaert, K.; Rittmann, B. E.; Parra-Saldivar, R., Nutrients utilization and contaminants removal. A review of two approaches of algae and cyanobacteria in wastewater. *Algal Research* **2017,** 24, 438-449.
22. Ferro, L.; Gorzsás, A.; Gentili, F.; Funk, C., Subarctic microalgal strains treat wastewater and produce biomass at low temperature and short photoperiod. *Algal Research* **2018,** 35, 160-167.
23. Jiang, Y.; Chen, F., Effects of temperature and temperature shift on docosahexaenoic acid production by the marine microalge Crypthecodinium cohnii. *Journal of the American Oil Chemists' Society* **2012,** 77, 613-617.
24. Yen, H.-W.; Hu, I. C.; Chen, C.-Y.; Nagarajan, D.; Chang, J.-S., Chapter 10 - Design of photobioreactors for algal cultivation. In *Biofuels from Algae (Second Edition)*, Pandey, A.; Chang, J.-S.; Soccol, C. R.; Lee, D.-J.; Chisti, Y., Eds. Elsevier: **2019**; pp 225-256.
25. Kaplan, D., Algal nutrition: In: Richmond A (ed) Handbook of microalgal mass culture. CRC Press, Boca Raton, USA: **1986**.
26. Wynn, J., 1.05 - Lipids, Fatty Acids. In *Comprehensive Biotechnology (Second Edition)*, Moo-Young, M., Ed. Academic Press: Burlington, **2011**; pp 41-52.
27. Christie, W. W.; Han, X., Lipid Analysis: Isolation, Separation, Identification and Lipidomic Analysis: Fourth Edition. *Lipid Analysis: Isolation, Separation, Identification and Lipidomic Analysis: Fourth Edition* **2010**, 1-428.
28. Craig, N. L.; Cohen-Fix, O.; Storz, G., *Molecular biology: principles of genome function*. Oxford University Press: **2014**.

29. Connelly, R., Chapter 10 - Second-Generation Biofuel from High-Efficiency Algal-Derived Biocrude. In *Bioenergy Research: Advances and Applications*, Gupta, V. K.; Tuohy, M. G.; Kubicek, C. P.; Saddler, J.; Xu, F., Eds. Elsevier: Amsterdam, **2014**; pp 153-170.
30. Zienkiewicz, K.; Du, Z.-Y.; Ma, W.; Vollheyde, K.; Benning, C., Stress-induced neutral lipid biosynthesis in microalgae — Molecular, cellular and physiological insights. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **2016,** 1861, (9, Part B), 1269-1281.
31. Barsanti, L.; Gualtieri, P., *Algae: anatomy, biochemistry, and biotechnology*. CRC press: **2014**.
32. Topf, M.; Tavassi, M.; Kinel-Tahan, Y.; Iluz, D.; Dubinsky, Z.; Yehoshua, Y., Algal Oils: Biosynthesis and Uses. In *The Science of Algal Fuels: Phycology, Geology, Biophotonics, Genomics and Nanotechnology*, Gordon, R.; Seckbach, J., Eds. Springer Netherlands: Dordrecht, **2012**; pp 193-214.
33. Fang, S.-C., Chapter 3 - Metabolic Engineering and Molecular Biotechnology of Microalgae for Fuel Production. In *Biofuels from Algae*, Pandey, A.; Lee, D.-J.; Chisti, Y.; Soccol, C. R., Eds. Elsevier: Amsterdam, **2014**; pp 47-65.
34. Hu, Q.; Sommerfeld, M.; Jarvis, E.; Ghirardi, M.; Posewitz, M.; Seibert, M.; Darzins, A., Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *The Plant Journal : for cell and molecular biology* **2008,** 54, (4), 621-39.
35. Folch, J.; Lees, M.; Sloane Stanley, G. H., A simple method for the isolation and purification of total lipides from animal tissues. *The Journal of Biological Chemistry* **1957,** 226, (1), 497-509.
36. Bligh, E. G.; Dyer, W. J., A rapid method of total lipid extraction and purification. *Can J Biochemestry Physiology* **1959,** 37, (8), 911-7.
37. Breuer, G.; Evers, W.; de Vree, J.; Kleinegris, D.; Martens, D.; Wijffels, R.; Lamers, P., Analysis of Fatty Acid Content and Composition in Microalgae. *Journal of Visualized Experiments : JoVE* **2013,** 80.
38. Merck Derivatization of Fatty acids to FAMEs. https://www.sigmaaldrich.com/technical-documents/articles/analytical/food-beverage/derivatization-of-fatty-acids-to-fames.html
39. Rumin, J.; Bonnefond, H.; Saint-Jean, B.; Rouxel, C.; Sciandra, A.; Bernard, O.; Cadoret, J.-P.; Bougaran, G., The use of fluorescent Nile red and BODIPY for lipid measurement in microalgae. *Biotechnology for biofuels* **2015,** 8, 42.
40. Ren, H.-Y.; Liu, B.-F.; Ma, C.; Lei, Z.; Ren, N.-Q., A new lipid-rich microalga Scenedesmus sp. strain R-16 isolated using Nile red staining: Effects of carbon and nitrogen sources and initial pH on the biomass and lipid production. *Biotechnology for Biofuels* **2013,** 6, 143.
41. Sequeira, C. A. C., Testing and Evaluation. In *High Temperature Corrosion*, **2018** December 18; pp 391-476.

42. Stehfest, K.; Toepel, J.; Wilhelm, C., The application of micro-FTIR spectroscopy to analyze nutrient stress-related changes in biomass composition of phytoplankton algae. *Plant Physiology and Biochemistry : PPB* **2005,** 43, (7), 717-26.
43. Dean, A. P.; Sigee, D. C.; Estrada, B.; Pittman, J. K., Using FTIR spectroscopy for rapid determination of lipid accumulation in response to nitrogen limitation in freshwater microalgae. *Bioresource Technology* **2010,** 101, (12), 4499-507.
44. Meng, Y.; Yao, C.; Xue, S.; Yang, H., Application of Fourier transform infrared (FT-IR) spectroscopy in determination of microalgal compositions. *Bioresource Technology* **2014,** 151, 347-354.
45. Felten, J.; Hall, H.; Jaumot, J.; Tauler, R.; de Juan, A.; Gorzsas, A., Vibrational spectroscopic image analysis of biological material using multivariate curve resolution-alternating least squares (MCR-ALS). *Nature Protocols* **2015,** 10, (2), 217-40.
46. Nzayisenga, J. C. ; Bergman E. ; Sellstedt A., Evaluating a local Phormidium strain for its capability of carbon dioxide sequestration and nutrient uptake from flue gas and municipal wastewater. **2018,** unpublished data.
47. Liu, B.; Benning, C., Lipid metabolism in microalgae distinguishes itself. *Current opinion in biotechnology* **2013,** 24, (2), 300-9.
48. Johansen, B. E., *The encyclopedia of global warming science and technology*. ABC-CLIO: **2009**.
49. Patel, N. K.; Shah, S. N., 11 - Biodiesel from Plant Oils. In *Food, Energy, and Water*, Ahuja, S., Ed. Elsevier: Boston, **2015**; pp 277-307.
50. Huang, G.; Chen, F.; Wei, D.; Zhang, X.; Chen, G., Biodiesel production by microalgal biotechnology. *Applied Energy* **2010,** 87, 38-46.
51. Silva, K.; Corazza, M.; Raposo, J., Renewable Energy Sources: A Sustainable Strategy for Biodiesel Productions. **2018**; pp 1-31.
52. Rawat, I.; Ranjith Kumar, R.; Mutanda, T.; Bux, F., Dual role of microalgae: Phycoremediation of domestic wastewater and biomass production for sustainable biofuels production. *Applied Energy* **2011,** 88, (10), 3411-3424.
53. Knothe, G., Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. *Fuel Processing Technology* **2005,** 86, 1059-1070.
54. Dwivedi, G., Impact of cold flow properties of biodiesel on engine performance. *Renewable and Sustainable Energy Reviews* **2014,** 31, 650-656.
55. Nelson, D. L.; Lehninger, A. L.; Cox, M. M., *Lehninger principles of biochemistry*. Macmillan: **2008**.
56. Ikehata, K., Hazardous Agents in Wastewater: Public Health Impacts and Treatment Options for Safe Disposal and Reuse. In *Wastewater Reuse and Management*, Sharma, S. K.; Sanghi, R., Eds. Springer Netherlands: Dordrecht, **2013**; pp 165-191.

57. Don, W. G.; Marylee, Z. S., Indutrial wastewater management 9th edition. ed.; McGraw-Hill Education: New York, **2019**.
58. Wu, Y.-H.; Hu, H.-Y.; Yu, Y.; Zhang, T.-y.; Zhu, S.-F.; Zhuang, L.-L.; Zhang, X.; Lu, Y., Microalgal species for sustainable biomass/lipid production using wastewater as resource: A review. *Renewable and Sustainable Energy Reviews* **2014,** 33.
59. Razzak, S. A.; Hossain, M. M.; Lucky, R. A.; Bassi, A. S.; de Lasa, H., Integrated CO2 capture, wastewater treatment and biofuel production by microalgae culturing—A review. *Renewable and Sustainable Energy Reviews* **2013,** 27, 622-653.
60. Cai, T.; Park, S. Y.; Li, Y., Nutrient recovery from wastewater streams by microalgae: Status and prospects. *Renewable and Sustainable Energy Reviews* **2013,** 19, 360-369.
61. Chislock, M. F.; Doster, E.; Zitomer, R.; Wilson, A. E., Eutrophication: Causes, consequences, and controls in aquatic ecosystems. *Nature Education Knowledge* **2013,** 4.
62. Mareddy, A. R., 6 - Impacts on water environment. In *Environmental Impact Assessment*, Mareddy, A. R., Ed. Butterworth-Heinemann: **2017**; pp 217-248.
63. Peirce, J. J.; Weiner, R. F.; Vesilind, P. A., Chapter 8 - Wastewater Treatment. In *Environmental Pollution and Control (Fourth Edition)*, Peirce, J. J.; Weiner, R. F.; Vesilind, P. A., Eds. Butterworth-Heinemann: Woburn, **1998**; pp 105-123.
64. Archis, A. J., A, N Wastewater Treatment. https://www.britannica.com/technology/wastewater-treatment/Sources-of-water-pollution
65. Sparks, T.; Chase, G., Section 5 - Solid–Liquid Filtration – Examples of Processes. In *Filters and Filtration Handbook (Sixth Edition)*, Sparks, T.; Chase, G., Eds. Butterworth-Heinemann: Oxford, **2016**; pp 297-359.
66. Tarayre, C.; Huu Thanh, N.; Brognaux, A.; Delepierre, A.; De Clercq, L.; Charlier, R.; Michels, E.; Meers, E.; Delvigne, F., Characterisation of phosphate accumulating organisms and techniques for polyphosphate detection: A Review. *Sensors* **2016,** 16, 1-14.
67. Källqvist, T.; Svenson, A., Assessment of ammonia toxicity in tests with the microalga, Nephroselmis pyriformis, Chlorophyta. *Water research* **2003,** 37, 477-84.
68. Mehrabadi, A.; Farid, M., Wastewater treatment high rate algal ponds (WWT HRAP) for low-cost biofuel production. *Bioresource technology* **2014,** 184.
69. Jones, C. S.; Mayfield, S. P., Algae biofuels: versatility for the future of bioenergy. *Current Opinion in Biotechnology* **2012,** 23, (3), 346-351.
70. Khan, V.; Putluri, N. r.; Sreekumar, A.; Mindikoglu, A., Current Applications of Metabolomics in Cirrhosis. *Metabolites* **2018,** 8, 67.

71. Jens, N., Metabolomics in Functional Genomics and Systems Biology. In *Metabolome Analysis*, **2009**; pp 1-14.
72. Roessner, U.; Nahid, A.; Chapman, B.; Hunter, A.; Bellgard, M., 1.33 - Metabolomics – The Combination of Analytical Biochemistry, Biology, and Informatics. **2011**; pp 447-459.
73. Yin, P.; Xu, G., Current state-of-the-art of nontargeted metabolomics based on liquid chromatography-mass spectrometry with special emphasis in clinical applications. *Journal of Chromatography. A* **2014,** 1374, 1-13.
74. Strassburg, K.; Walther, D.; Takahashi, H.; Kanaya, S.; Kopka, J., Dynamic Transcriptional and Metabolic Responses in Yeast Adapting to Temperature Stress. *Omics : a Journal of Integrative Biology* **2010,** 14, 249-59.
75. Bellou, S.; Baeshen, M. N.; Elazzazy, A. M.; Aggeli, D.; Sayegh, F.; Aggelis, G., Microalgal lipids biochemistry and biotechnological perspectives. *Biotechnology Advances* **2014,** 32, (8), 1476-1493.
76. Shafii, B., Profiling of bioactive lipids using ultrahigh performance liquid chromatography and mass spectrometry. Jones, A. D.; Baker, G.; Borhan, B.; Henry, R., Eds. ProQuest Dissertations Publishing: **2012**.
77. Zienkiewicz, K.; Du, Z. Y.; Ma, W.; Vollheyde, K.; Benning, C., Stress-induced neutral lipid biosynthesis in microalgae - Molecular, cellular and physiological insights. *Biochimica et Biophysica Acta* **2016,** 1861, (9 Pt B), 1269-1281.
78. Vivek, N.; Sindhu, R.; Madhavan, A.; Anju, A. J.; Castro, E.; Faraco, V.; Pandey, A.; Binod, P., Recent advances in the production of value added chemicals and lipids utilizing biodiesel industry generated crude glycerol as a substrate - Metabolic aspects, challenges and possibilities: An overview. *Bioresource Technology* **2017,** 239, 507-517.
79. Nübel, U.; Garcia-Pichel, F.; Muyzer, G., PCR primers to amplify 16S rRNA genes from Cyanobacteria. *Applied and Environmental Microbiology* **1997,** 63, 3327-32.
80. Burja, A.; Tamagnini, P.; Bustard, M.; Wright, P., Identification of the green alga, Chlorella vulgaris (SDC1) using cyanobacteria derived 16S rDNA primers: targeting the chloroplast. *FEMS Microbiology letters* **2001,** 202, 195-203.
81. Lakshmikandan, M.; Murugesan, A. G., Enhancement of growth and biohydrogen production potential of Chlorella vulgaris MSU-AGM 14 by utilizing seaweed aqueous extract of Valoniopsis pachynema. *Renewable Energy* **2016,** 96, 390-399.
82. Paranjape, K.; Leite, G.; Hallenbeck, P., Strain variation in microalgal lipid production during mixotrophic growth with glycerol. *Bioresource Technology* **2015,** 204.
83. Bumbak, F.; Cook, S.; Zachleder, V.; Hauser, S.; Kovar, K., Best practices in heterotrophic high-cell-density microalgal processes: Achievements, potential

and possible limitations. *Applied Microbiology and Biotechnology* **2011,** 91, 31-46.

84. Cabanelas, I. T. D.; Arbib, Z.; Chinalia, F. A.; Souza, C. O.; Perales, J. A.; Almeida, P. F.; Druzian, J. I.; Nascimento, I. A., From waste to energy: Microalgae production in wastewater and glycerol. *Applied Energy* **2013,** 109, 283-290.
85. Ji, M.-K.; Yun, H.-S.; Park, Y.-T.; Kabra, A. N.; Oh, I.-H.; Choi, J., Mixotrophic cultivation of a microalga Scenedesmus obliquus in municipal wastewater supplemented with food wastewater and flue gas CO2 for biomass production. *Journal of Environmental Management* **2015,** 159, 115-120.
86. Driver, T.; Bajhaiya, D. A.; Allwood, J.; Goodacre, R.; Pittman, J.; Dean, A., Metabolic responses of eukaryotic microalgae to environmental stress limit the ability of FT-IR spectroscopy for species identification. *Algal Research* **2015,** 11.
87. EN14214, E. S., Automotive fuels-fatty acid methyl esters (FAME) for diesel engines, requirements and test methods. Belgrade, Serbia: Standardization Institute: **2004**.
88. Monirul, I. M.; Masjuki, H. H.; Kalam, M. A.; Zulkifli, N. W. M.; Rashedul, H. K.; Rashed, M. M.; Imdadul, H. K.; Mosarof, M. H., A comprehensive review on biodiesel cold flow properties and oxidation stability along with their improvement processes. *Rsc Advances* **2015,** 5, (105), 86631-86655.
89. Liu, J.; Huang, J.; Sun, Z.; Zhong, Y.; Jiang, Y.; Chen, F., Differential lipid and fatty acid profiles of photoautotrophic and heterotrophic Chlorella zofingiensis: assessment of algal oils for biodiesel production. *Bioresource Technology* **2011,** 102, (1), 106-10.
90. Knothe, G., Improving biodiesel fuel properties by modifying fatty ester composition. *Energy & Environmental Science* **2009,** 2, (7), 759-766.
91. Nzayisenga, J. C.; Eriksson, K.; Sellstedt, A., Mixotrophic and heterotrophic production of lipids and carbohydrates by a locally isolated microalga using wastewater as a growth medium. *Bioresource Technology* **2018,** 257, 260-265.
92. Chen, H. H.; Jiang, J. G., Lipid Accumulation Mechanisms in Auto- and Heterotrophic Microalgae. *Journal of agricultural and food chemistry* **2017,** 65, (37), 8099-8110.
93. Wang, H.; Zhang, Y.; Zhou, W.; Noppol, L.; Liu, T., Mechanism and enhancement of lipid accumulation in filamentous oleaginous microalgae Tribonema minus under heterotrophic condition. *Biotechnology for Biofuels* **2018,** 11.
94. Chen, H.; Zheng, Y.; Zhan, J.; He, C.; Wang, Q., Comparative metabolic profiling of the lipid-producing green microalga Chlorella reveals that nitrogen and carbon metabolic pathways contribute to lipid metabolism. *Biotechnology Biofuels* **2017,** 10, 153.
95. Diercks, H.; Semeniuk, A.; Gisch, N.; Moll, H.; Duda, K.; Hölzl, G., Accumulation of Novel Glycolipids and Ornithine Lipids in Mesorhizobium loti under Phosphate Deprivation. *Journal of Bacteriology* **2014,** 197.

96. Smith, A. F.; Rihtman, B.; Stirrup, R.; Silvano, E.; Mausz, M. A.; Scanlan, D. J.; Chen, Y., Elucidation of glutamine lipid biosynthesis in marine bacteria reveals its importance under phosphorus deplete growth in Rhodobacteraceae. *The ISME Journal* **2019,** 13, (1), 39-49.
97. Chia, S. R.; Ong, H. C.; Chew, K. W.; Show, P. L.; Phang, S.-M.; Ling, T. C.; Nagarajan, D.; Lee, D.-J.; Chang, J.-S., Sustainable approaches for algae utilisation in bioenergy production. *Renewable Energy* **2018,** 129, 838-852.
98. Misra, N.; Panda, P. K.; Parida, B. K., Agrigenomics for microalgal biofuel production: an overview of various bioinformatics resources and recent studies to link OMICS to bioenergy and bioeconomy. *Omics : a Journal of Integrative Biology* **2013,** 17, (11), 537-49.
99. Mandotra, S. K.; Kumar, P.; Suseela, M. R.; Nayaka, S.; Ramteke, P. W., Evaluation of fatty acid profile and biodiesel properties of microalga Scenedesmus abundans under the influence of phosphorus, pH and light intensities. *Bioresource Technology* **2016,** 201, 222-9.
100. Pribyl, P.; Cepak, V.; Zachleder, V., Production of lipids in 10 strains of Chlorella and Parachlorella, and enhanced lipid productivity in Chlorella vulgaris. *Applied Microbiology and Biotechnology* **2012,** 94, (2), 549-61.
101. Cheirsilp, B.; Torpee, S., Enhanced growth and lipid production of microalgae under mixotrophic culture condition: Effect of light intensity, glucose concentration and fed-batch cultivation. *Bioresource Technology* **2012,** 110, 510-516.
102. George, B.; Pancha, I.; Desai, C.; Chokshi, K.; Paliwal, C.; Ghosh, T.; Mishra, S., Effects of different media composition, light intensity and photoperiod on morphology and physiology of freshwater microalgae Ankistrodesmus falcatus--a potential strain for bio-fuel production. *Bioresource Technology* **2014,** 171, 367-74.
103. Cuhel, R. L.; Ortner, P. B.; Lean, D. R. S., Night synthesis of protein by algae. **1984,** 29, (4), 731-744.
104. Breuer, G.; Martens, D. E.; Draaisma, R. B.; Wijffels, R. H.; Lamers, P. P., Photosynthetic efficiency and carbon partitioning in nitrogen-starved Scenedesmus obliquus. *Algal Research-Biomass Biofuels and Bioproducts* **2015,** 9, 254-262.
105. Li, T.; Gargouri, M.; Feng, J.; Park, J. J.; Gao, D.; Miao, C.; Dong, T.; Gang, D. R.; Chen, S., Regulation of starch and lipid accumulation in a microalga Chlorella sorokiniana. *Bioresource Technology* **2015,** 180, 250-7.
106. Zhu, L. D.; Li, Z. H.; Hiltunen, E., Strategies for Lipid Production Improvement in Microalgae as a Biodiesel Feedstock. *BioMed Research International* **2016,** 2016, 8792548.
107. Krzemińska, I.; Nawrocka, A.; Piasecka, A.; Jagielski, P.; Tys, J., Cultivation of Chlorella prototheco̜ides in photobioreactors: The combined impact of

photoperiod and CO2 concentration. *Engineering in Life Sciences* **2015,** 15, (5), 533-541.
108. Lee, S.; Cho, J.; Chang, Y.; Oh, Y.-K., Cell disruption and lipid extraction for microalgal biorefineries: A review. *Bioresource Technology* **2017,** 244.
109. Pau Loke, S.; Chang, J.-S.; Ling, T.; Juan, J. C., Biosequestration of atmospheric CO2 and flue gas-containing CO2 by microalgae. *Bioresource Technology* **2014,** 184.