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Studies on Lipid Production of Microalgae under Mixotrophic Growth, utilizing Glycerol as a Carbon Source, combined with Nitrogen Starvation

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Résumé

L'augmentation effrénée des prix du pétrole et les effets néfastes des carburants fossiles sur l'environnement sont les raisons principales pour la recherche et le développement de nouvelles sources d'énergie durables et écologiques. Parmi de grands nombres de possibilités, les micro-algues sont proposées comme une source alternative d'énergie aux carburants fossiles, étant donné que leur croissance est durable et écologique. Les micro-algues sont des organismes unicellulaires et photosynthétiques détenant comme pigment essentiel la chlorophylle *a*. Ces organismes sont capables de produire de grandes quantités d'huile, parfois excédant celles des cultures agricoles traditionnellement utilisées pour les biocarburants. Ces huiles peuvent être transformées en biocarburants, tel que le biodiésel et le bio-essence, par certains procédés chimiques. La recherche actuelle est basée sur la découverte de souches d'algues capables de produire un haut rendement de lipides, l'optimisation de milieux de croissance pour accroitre la production lipidique et la manipulation génomique afin de créer des souches de micro-algues dont les rendements peuvent rivaliser avec l'agriculture tradionnelle et même les carburants fossiles.

Dans ce contexte, notre recherche se concentre sur l'utilisation d'un mode de croissance mixotrophe afin d'induire une augmentation dans la production lipidique de certaines souches de micro-algues. De plus, des études sur la carence en azote combinée à la croissance mixotrophe ont été entreprises pour évaluer l'effet de ces paramètres sur la production lipidique. La mixotrophie est un mode de croissance qui utilise en parallèle deux modes trophiques différents, tel que l'hétérotrophie et l'autotrophie. De ce fait, 12 souches d'algues ont été examinées pour leur capacité à croitre dans un milieu mixotrophe. Le glycérol

est un produit secondaire de l'industrie du biodiésel actuelle. Cette substance est à bas prix, abondante et peut être utilisé comme substrat dans plusieurs voies métaboliques. Du criblage initial, plusieurs souches ont été choisies pour des expériences subséquentes impliquant la carence en azote. La carence en azote à été démontrer comme un déclencheur de l'accumulation de lipide chez les micro-algues dans des recherches antérieures. Les résultats obtenus démontrent que la croissance mixotrophe permet d'augmenter la production de lipide chez certaines souches. De plus, la carence en azote combinée à la croissance mixotrophe a permis d'augmenter la production lipidique. Cependant, celle-ci dépendait du temps passer en carence et des concentrations initiales de source d'azote.

Mots-clés: Biodiésel, Glycérol, Micro-algue, Mixotrophie, Carence en Azote

Abstract

Rampant increases in oil prices and detrimental effects of fossil fuels on the environment have been the main impetus for the development of environmentally friendly and sustainable energy sources. Amongst the many possibilities, microalgae have been proposed as a new alternative energy source to fossil fuels, as their growth is both sustainable and ecologically safe. By definition, microalgae are unicellular photosynthetic microorganisms containing chlorophyll *a*. These organisms are capable of producing large quantities of oils, surpassing that of traditional oil-seed crops, which can be transformed, through chemical processes, into biofuels such as biodiesel or bio-gasoline. Thus, recent research has gone into discovering high lipid producing algal strains, optimising growth media for increased lipid production and developing metabolic engineering to make microalgae a source of biofuel that is competitive to more traditional sources of biofuel and even to fossil fuel.

In this context, the research reported here focused on using a mixotrophic growth mode as a way to increase lipid production for certain strains of microalgae. In addition, nitrogen starvation combined with mixotrophy was studied to analyse its effects on lipid production. Mixotrophy is the parallel usage of two trophic modes, in our case photoautotrophy and heterotrophy. Consequently, 12 algal strains were screened for mixotrophic growth, using glycerol as a carbon source. Glycerol is a waste product of the current biodiesel industry; it is a cheap and abundant carbon source present in many metabolic pathways. From this initial screening, several strains were chosen for subsequent experiments involving nitrogen starvation. Nitrogen starvation has been shown to induce lipid accumulation. The results obtained show that a mixotrophic growth mode, using glycerol as a carbon source, enhances

lipid production for certain strains. Moreover, lipid enhancement was shown for nitrogen starvation combined with mixotrophic growth mode. This was dependant on time spent under nitrogen starvation and on initial concentrations of the nitrogen source.

Keywords: Biodiesel, Glycerol, Microalgae, Mixotrophy, Nitrogen Starvation

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List of Abbreviations

ACCase	Acetyl-CoA carboxylase
ATP	Adenosine Triphosphate
BBM	Bold's Basal Medium
CO_2	Carbon Dioxyde
DAG	Diacylglycerol
DAGAT	DAG acyltransferase
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTAB	Dodecyltrimethylammonium bromide
EIA	Energy Information Administration
FAEE	Fatty Acid Ethyl Esters
FAME	Fatty Acid Methyl Esters
FAs	Fatty Acids
FAS	Fatty Acid Synthesis
Fe	Iron
g	Grams
G-3-P	Glyceraldehyde-3-Phosphate
GC-MS	Gas Chromatography-Mass spectrometry
H_2	Dihydrogen
L	Liter
M	Molar
μL	Microliter
mL	Milliliter
mM	Millimolar
N	Normal
NaCl	Sodium chloride
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
$NaIO_4$	Sodium Periodate

$NaNO_3$	Sodium Nitrate
Ni	Nickel
N-J	Neighbour-Joining
nm	Nanometers
NR	Nile Red
O_2	Dioxygen
OD	Optical Density
PCR	Polymerase Chain Reaction
PDAT	Phospholipid Diacylglyceol Acyltransferase
PFOR	Pyruvate ferrodoxin oxido-reductase
PFL	Pyruvate Formate Lyase
PGA	Phosphoglycerate
PUFA	Polyunsaturated Fatty Acids
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
RUBISCO	Ribulose-1,5-bisphosphate carboxylase oxygenase
Sp	Species
TAG	Triacylglycerol
W/W	weight/weight

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Footnotes

During my M. Sc. studies, I authored a book chapter on algal biofuels. This book chapter was used for the introduction of this master thesis.

It should be noted that this book chapter is going to be published by Taylor&Francis Group in May of 2015. Taylor&Francis Group has allowed me to use this book chapter for my master thesis:

"The book "Marine Bioenergy: Trends and Developments" is scheduled to be publish in May 2015. I'll send everyone a complimentary eBook once the book publishes and Kiran Paranjape will be able to use the chapter for his master thesis"

Michele

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Thus, the introduction in this master thesis is adapted from my contribution in Paranjape, K., and Hallenbeck, P. C. "Microalgal Production of Hydrogen and Biodiesel." *Marine Bioenergy: Trends and Development.* ED. Lee/Kim. CRC Press Taylor&Francis Group. In press.

Preface to Introductory Chapter

Fossil fuels are natural fuels, rich in hydrocarbons, produced from the decomposition of dead organisms over long geological periods of time. Their consumption and production has many drawbacks, such as an increase in pollution and atmospheric CO_2 which both have negative environmental and economical impacts. Consequently, research has been focused on the discovery of new alternative fuels that are more sustainable and are not linked to Global Warming. A possible solution is the cultivation of microalgae for biodiesel or hydrogen production. Thus, microalgae are photosynthetic microorganisms containing chlorophyll *a* and spanning from the eukaryotic domain to the prokaryotic domain. These organisms are capable of producing lipids with the carbon dioxide they fix through photosynthesis. These lipids can be converted to biodiesel through their extraction and conversion by a chemical reaction called transesterification. Additionally, certain species of microalgae are capable of producing hydrogen in certain conditions. Hydrogen can be used as a fuel to power vehicles and electric devices and has the main advantage of being a zero emission fuel, meaning it does not produce any waste products that are considered polluting, such as carbon dioxide.

In this opening chapter, we explore the general aspects of microalgal cultivation and production of biodiesel and bio-hydrogen. The chapter is divided into three sections. In the first section, we examine the problems associated with the usage of fossil fuels and the advantages and disadvantages of biofuels. In addition, microalgal diversity is discussed in this section. In the second section, we pay closer attention to biodiesel production, algal lipid metabolism and current enhancement technics of biodiesel production through algal cultivation and genetic manipulation. The third section examines hydrogen production by

microalgae. Thus, in this section we review hydrogen producing enzymes and pathways leading to hydrogen production by microalgae.

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Chapter I- Microalgal Production of Hydrogen and Biodiesel

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Keywords: Biodiesel, Fossil Fuels, Hydrogen, Lipid Metabolism, Microalgae,

1. Disadvantages of Fossil Fuels and Advantages of Algal Biodiesel

Fossil fuels have been the major energy source since the 19th century. During the First Industrial Revolution (1760 to around 1840), coal was mined at an industrial scale for its high energy content, in Great Britain and around Europe. Coal had replaced wood and other archaic bio-fuels due to its abundance and low-coast. The versatility of coal made it an instrumental asset for various industrial sectors. For instance, coal was used as a heat source in homes and in the metallurgy sector, as fuel for steam engines and as a chemical for different chemical processes. Petroleum or Crude Oil was also consumed during this period, however it was not until the Second Industrial Revolution (1860's to World War I) and the advent of the internal combustion engine that oil started to be produced and consumed at industrial levels. As of 2012, the EIA (U.S. Energy information administration) projected that the highest consumed energy source, in the United States, was Petroleum (36%), followed by natural gas (26%), coal (18%), renewable energy (9%) and nuclear electric power (8%) (*EIA, 2013*).

Many difficulties have come up with fossil fuel consumption. As these fuels are formed from fossilized remains of organisms over long geological periods of time, there is a limited amount of these fuels. Thus, the notion of "Peak Oil" has come about. Peak Oil refers to the point in time when maximum rate of petroleum extraction will be reached, after which the rate of production will diminish to drop down to zero. The point in time when Peak Oil will be reached is highly debated, however most specialists can be grouped in two categories, the late-peak advocates and the early peak advocates (*Chapman, 2013*). Some examples of late peak advocates are the CERA (Cambridge Energy Research Associates), the EIA and Shell Company who, respectively, believe Peak Oil will be reached by 2017, 2035 and around 2020 (*Chapman, 2013*). On the opposite side, early peak advocates believe the peak has already

been reached. For instance, some advocates, such as Deffeyes, have stated that the peak was reached in 2005 and that oil production will never again be surpassed (*Deffeyes, 2010*). Furthermore, fossil fuels have been immensely criticized for their negative effects on the environment. Since fossil fuels are mainly formed of hydrocarbons, their consumption liberates carbon compounds (mainly CO_2) that have been trapped for millions of years in the Earth's crust. Carbon dioxide being a greenhouse gas, the increase of atmospheric CO_2 concentrations has been linked to Climate Change and the phenomenon of Global Warming. In addition, the extraction and purification processes for these fuels are highly polluting and toxic and thus causing damage to nearby ecosystems. Oil spills have been one of the major problems of the oil industry causing much discord within the business. There have been many examples of oil spills through out the past few decades; most notable is the BP oil spill during the summer of 2010. The BP Oil Spill began in April 2010, in the Gulf of Mexico, and lasted until July 15th 2010. This particular spill discharged between 62,500 and 84,000 bopd (barrels of oil per day) for 87 days, making it the largest oil spill yet (*Joye et al, 2013*). The spill mainly affected marine ecology with the discharge of toxic chemical compounds, such as polycyclic aromatic hydrocarbons and methane, harming many marine species. This in turn had a negative ripple effect both on the economy (fishing and tourism) and human health in surrounding countries (mainly the USA).

As a result of the negative effects caused by fossil fuel energies, there has been higher investment for greener and more sustainable energies in recent years. Hydroelectricity, solar power and wind power are all examples of green technologies. Among these new technologies microalgae have been making the spot light in recent years. Microalgae or Microphytes are unicellular photosynthetic organisms found in freshwater or marine systems. Their diversity is

highly debated, as some consider them solely to be in the eukaryotic realm and others consider them to be both in the prokaryotic (cyanobacteria) and the eukaryotic realms. However, commonly they are defined as oxygen producing photosynthetic microorganisms containing chlorophyll *a* (*Leite et al, 2012*). Lately, microalgae have been extremely studied for their capacity to produce lipids and hydrogen. Indeed, microalgae have been known to accumulate large quantities of lipids within their cells under certain conditions rivalling quantities of traditional crops, such as soya or corn. The lipids produced could be harvested and either converted to biodiesel or nutritional supplements, such as omega-3-fatty acids. Microalgae have been known also to produce hydrogen, which could be used as a potential biofuel.

The advantages of using microalgae for biofuel production are numerous. Since algae are phototrophic, they have the capacity to fix CO_2 using energy produced from light. Consequently, consumption of algal biodiesel would recycle current atmospheric CO_2 concentrations and would not increase them, as it is in the case with fossil fuel consumption. This would help to hinder increasing global temperatures, which are linked to the increase in atmospheric CO_2 due to the use of fossil fuels. Furthermore, microalgae have a faster growth rate than traditional crops, with some species having a doubling time of less than 24 hours, and under certain conditions could be grown year round (*Leite et al, 2012; Moody et al, 2014*). Another advantage is their capacity to be grown on non-arable land avoiding competition with food crops (*Moody et al, 2014*). This would help to diminish the high food prices for cultivated plants that are now used for biofuel production, such as corn and soya. More importantly, microalgae have been shown to have higher lipid content than traditional food crops. For instance, several species of *Chlorella*, *Scenedesmus*, *Nitzschia* have shown upwards of 50% lipid content per dry weight (*Chisti, 2007; Abdelaziz et al, 2013*). Certain species of

Schizochytrium have been shown to have a lipid content of more than 70% (*Chisti, 2007*). On the other hand, the lipid content of corn, soya and other crops is much lower. Additionally, microalgae can produce certain valuable products, different from lipids used for biodiesel. For example, certain strains of algae produce Omega-3-fatty acids and can be used in the nutraceutical industry; other species can produce other compounds, such as pigments or ceramides, which can be used in cosmetics.

Moreover, microalgae have the capacity of growing in many different types of media and thus could be used to treat certain polluted waters such as wastewater (*Makareviciene et al, 2013*). Wastewater contains high amounts of nitrogen and phosphorus originating from excessive use of cleaning products and fertilizers; the excess phosphorous and nitrogen has triggered problems such as eutrophication of aquatic systems, causing hypoxia and oxygen depletion of water systems. This in turn has had negative impacts on fish populations and aquatic ecology. Microalgae would in principle be able to use up excess nitrogen, phosphorous and other contaminants, from municipal and industrial wastewaters and other liquid wastes, for their own growth while producing lipids for biodiesel production (*Makareviciene et al, 2013*). Furthermore, since microalgae have an enormous diversity, they can easily grow in different locations. As a result, each city could potentially grow microalgae for biodiesel production or other products. This would help local economies, as it would increase job opportunities and investments in novel technologies. Thus algae have many advantages, however each advantage has one or more drawbacks making algal biofuels still not commercially competitive. One of the biggest disadvantages for microalgal cultivation is the lack of carbon dioxide (*Chisti, 2013*). To produce one ton of algal biomass a minimum of 1.83 tons of carbon dioxide is needed (*Chisti, 2007*). As a result, most pilot scale algal cultures rely

on bought carbon dioxide causing high cost for their cultivation (*Chisti, 2013*). Moreover, the high growth rates, that we mentioned earlier, depend largely on optimal conditions easily produced in laboratories but not necessarily reproducible at an industrial scale. Therefore, the cultivation of microalgae is complex and still in the beginning phase of development. Yet advances have been made and some start-up companies have been attempting to commercialize algal fuel (*Chisti, 2013*). Some examples of such start-up companies are Algenol Biofuels, Aquaflow, LiveFuels Inc, Solazyme Inc., Joule Unlimited Inc. and Solix Biofuels Inc. (*Chisti, 2013*). Thus, advances are being made in the algal biofuel field, yet still much research is needed in order to make biofuels from algae a competitive and productive commodity.

In this chapter, we will try to give some information on general and specific topics that have gained interest in the algal biodiesel and bio hydrogen research field.

2. Microalgae

2.1 What are Microalgae?

As a general rule, microalgae consist of any microscopic photosynthetic organism, usually unicellular, containing chlorophyll *a* and a thallus not differentiated into roots, stems and leaves, such as plants and macroalgae (*Tomaselli, 2008*). This definition being very general, microalgae form an extremely diverse polyphyletic group within the tree of life. Though sometimes controversial, this definition takes into account species from the prokaryotic realm, such as cyanobacteria, and species from the eukaryotic realm, such as the green algae. As a result, most microalgae can be regrouped into five kingdoms: the Protozoan

kingdom, the Plantae kingdom, the Chromista kingdom, the Fungi kingdom and the Cyanobacteria phylum (*Leite et al, 2012; Tomaselli, 2008*).

Microalgae are mainly found in aquatic or highly wet environments, such as riverbeds, lakeshores and fresh or salt waters. However, since their phylogenetic diversity is extremely high, some species can be found in very varied habitats. Thus, it is not uncommon to hear of algae growing in extreme conditions. *Chlamydomonas nivalis*, *Chlamydomonas brevispina* and *Chloromonas granulosa* are all microalgae part of the Chlorophyta phylum (Green algae). These species are also known commonly as ‘‘Snow Algae’’ and are capable of growing on snow at very low temperatures (*Raven et al, 2007*). These species have a high tolerance for extreme temperatures, acidity and exposure to sunlight (*Raven et al, 2007*). They also have the capacity to grow at minimal concentrations for their mineral requirements for growth (*Raven et al, 2007*). *Chlamydomonas nivalis* contains a red carotenoid pigment, which protects the chlorophyll from extreme sunlight exposure, creating a phenomenon known as “Watermelon Snow” or “Red Snow”, when the algae bloom (*Raven et al, 2007*). The great versatility of growth habitats can come as an advantage for algal cultivation. As different locations are restricted with different geographical and meteorological conditions, each location would use specific species or strains tailored to withstand and thrive in those locations for algal cultivation. For example, species capable of growing at freezing temperatures, such *C. navalis*, would be used in very cold locations.

2.2 Microalgal Diversity

As a result of this wide variety, microalgae are extremely diverse in term of physiology, anatomical structures and metabolism. Typically, each species, and even strain,

has its own set of physiological, structural and metabolic systems helping it to survive in specific or general environments.

2.2.1 Cell envelopes

Structurally speaking, microalgae can be grouped according to the prokaryotic realm and the eukaryotic realm. Prokaryotic microalgae are mainly composed of the cyanobacteria phylum, which is further subdivided into four main groups (*Stanier et al, 1977*). Thus, we can find the Chroococcacean group, the Pleurocapsalean group and the Oscillatorian and Heterocysteous groups, which both form filaments, composed of mother and daughter cells, called trichomes (*Stanier et al, 1977*). Apart for the Heterocystous group, the other three taxa are still highly debated and have not yet been validly added in the Bacteriological Code.

Cyanobacteria are composed of several different cellular envelopes. These envelopes can differ from species to species, but in general, species are constituted of an outer membrane, cell wall and inner membrane. The outer membrane is similar to Gram-negative bacteria and is composed of proteins, sugars and lipopolysaccharides (LPS), but present several unique elements. For instance, cyanobacterial LPS are usually deficient in ketodeoxyoctaonate (a common element in Gram-negative bacterial LPS) and have small amounts of bound phosphate (*Hoiczyk et al, 2000*). Additionally, the outer membrane may contain certain compounds generally not observable in Gram-negative bacteria. Thus, it is not uncommon to find atypical fatty acids, such as Beta-hydroxypalmitic, or pigments, such as carotenoids (*Hoiczyk et al, 2000*). Furthermore, sugar composition of the outer membrane can be species specific. As a result, the genus *Synechococcus* contains species with rhamnose and other strains with mannose as main sugar constituent of the outer membrane (*Stanier et al,*

1977). Interestingly, the species of *Synechococcus* with rhamnose usually had a high G+C content, whereas, strains with mannose had low G+C content, in their DNA (*Stanier et al, 1977*). Due to the high diversity of outer membrane composition, certain components can be used as biomarkers or molecular markers for phylogenetic or genetic studies of cyanobacteria. Recently, hoponoids, such as 2-methylhoponoids, have been used as molecular fossil biomarkers for the study of ancient fossilized cyanobacteria and oxygenic photosynthesis (*Garby et al, 2013*). Hopanoids are pentacyclic compounds, which play a role in membrane integrity, akin to the role of sterols in eukaryotes (*Garby et al, 2013*). The hopanoid composition can vary depending on the species of cyanobacteria and the environmental conditions.

The cell wall of cyanobacteria is chemically and structurally homologous to Gram-negative bacteria (*Stanier et al, 1977; Richmond, 2004*). It is therefore formed of a peptidoglycan layer (*Stanier et al, 1977; Richmond, 2004; Tomaselli, 2008; Hoiczyk et al, 2000*). However, this peptidoglycan layer is much thicker than in Gram-negative bacteria and is in general around 10nm thick, though certain species can have up to 700nm in thickness such as in *Oscillatoria princeps* (*Hoiczyk et al, 2000*). Additionally, the degree of cross-linking between peptidoglycans in the murein layer is much higher than those found in Gram-negative bacteria and is more reminiscent of Gram-positive bacteria (*Hoiczyk et al, 2000*).

The inner membrane is separated from the cell wall by a space called the periplasmic space. The inner membrane holds the contents of the cell and is usually made up of phospholipid bilayer. This membrane is highly dynamic and in constant communication with the cytoplasmic environment. In the intracellular environment, most species of cyanobacteria

have thylakoid membrane, which contain the pigments and machinery necessary for photosynthesis.

Certain species of cyanobacteria have the capacity to produce external layers to the outer membrane. Thus, a number of species can produce slimes, sheaths, S-layer and capsule that are all layers with different functions, composition and structures, exterior to the outer membrane.

Eukaryotic microalgal cell envelopes have a high degree of variation just like cyanobacteria. As we have mentioned before, eukaryotic algae can be divided between four kingdoms. Thus, the diversity of cell envelopes composition is colossal, however in terms of functionality, eukaryotic cell walls have the main function to protect the cell from the environment. As a result, most cell wall components are formed of very strong and resistant biological molecules. Species found in the Chlorophyta phylum, the Green Algae, typically have similar components to vascular plants, since they share a relatively recent common ancestor. Cellulose is the major component found in these species however xyloglucans, mannans, glucuronan and ulvans are also found (*Popper et al, 2011*). These compounds are all highly resistant polysaccharides. Other groups, such diatoms, contain inorganic molecules, which give even more resistance to the cell wall. Diatoms are a group of microalgae belonging to the Heterokonts. This group has the particularity to contain silica within the cell wall. The biological silica contained in the cell wall is synthesised within the cell in the form of silicic acid monomer (*Scheffel et al, 2011*). The monomers are then forced out and added to the cell wall. More recently, it has been found that some diatom species, such as *Thalassiosira pseudonana*, also contain chitin within their cell walls (*Brunner et al, 2009; Durkin et al, 2009*). Chitin is a polysaccharide closely related to glucose and comparable to cellulose. This

polysaccharide is abundant and can be found in arthropods, such as insects and crustaceans, and is a component of the cell wall of most fungal species.

Thus, the diversity in cell wall structures and composition is very big in microalgae.

2.2.2 Chloroplasts

As microalgae are found in the prokaryotic and eukaryotic realm, their cellular components are organised according to these two categories. Thus, eukaryotic species have a nucleus, containing the genetic material, and several organelles inside the cell; whereas, cyanobacteria don't have any organelles and have a single circular chromosome found at the centre of the cell. We will not go into details about these different organelles and systems of organisation, however, some attention must be paid on the photosynthetic machinery used by these two categories of algae.

In order to carry out photosynthesis, eukaryotic algae use a specific organelle called a chloroplast. These organelles conduct photosynthesis with the help of specific pigments and an electron transport chain found in the membranes of these units. They also carry out most of the fatty acid synthesis. During photosynthesis, molecular pigments called chlorophyll found in the membrane of the thylakoid of the chloroplast are excited, when light is shined on them. This excitation causes the pigment to loose an electron (*Horton et al, 2006*). The electron then passes through a series of complexes in the membrane to ultimately reduce NADP to NADPH (*Horton et al, 2006*). This is the electron transport chain. This transport chain also creates a proton gradient across the chloroplast membrane. The proton gradient is used to drive phosphorylation of ADP to produce ATP thanks to ATP synthase (*Horton et al, 2006*). The NADPH and ATP are then used as energy sources to fix CO_2 through the Calvin cycle and

produce carbohydrates, such as sugars and lipids, either for direct consumption or energy storage.

On the other hand, cyanobacteria and other prokaryotic algae do not have any organelles and carry out photosynthesis directly in cell membrane. The inner cell membrane forms complex folds and is called the thylakoid membrane (*Horton et al, 2006*). This thylakoid membrane contains the different complexes of the electron transport chain used in photosynthesis, such as ATP synthase and photosystem I and II (*Horton et al, 2006*). The folds created in the thylakoid membrane help to increase the surface area of the membrane. As a result, cyanobacteria have a higher concentration of photosynthetic complexes than other photosynthetic bacteria, such as purple sulphur bacteria, and so have a better efficiency of converting light to chemical energy (*Horton et al, 2006*). Interestingly enough, the current formed by the flow of electrons through the electron transport chain has been studied for its use in microbial fuel cell. Thus, it is possible to use cyanobacteria and other photosynthetic bacteria to produce electricity. A study by Madiraju has shown that some strains of *Synecchocystis* could produce up to 6.7 $mW.m^{-3}$ (*Madiraju et al, 2012*).

Moreover, the molecular pigments used for photosynthesis are very variable according to species. Though we mentioned that microalgae are photosynthetic microorganisms containing chlorophyll *a*, many species have more than just this one pigment. For example, diatoms contain chlorophyll *a*, *c* and fucoxanthin as molecular pigments while green algae contain chlorophyll *a, b* and zeaxanthin (*Brennan et al, 2010*). Cyanobacteria also contain chlorophyll *a* however they have a higher concentration of phycobilins, such as the blue pigment phycocyanin or the red pigment phycoererythrin (*Horton et al, 2006; Tomaselli 2008; Chang et al, 2012*). Cyanobacteria get their green-bluish colour from the phycocyanin

pigment. Thus, the diversity in photosynthetic pigments is great and helps to generate a multitude of functions. As a primary function, most molecular pigments are used to capture different wavelength of light. For instance, phycocyanin will absorbs light in the orange range at around 620nm, whereas chlorophyll *a* has an absorption maximum at around 664nm and 430nm (*Chang et al, 2012*). Secondary functions of molecular pigments can include photoprotection (example carotenoids), singlet oxygen scavenging, structure stabilization and excess energy dissipation (*Frank et al, 1996*).

Finally, the origin of chloroplasts is thought to arise from a primary endosymbiosis between the ancestral eukaryote and cyanobacteria (*Alberts et al, 2007*). In this scenario, an ancestral eukaryote would have engulfed a cyanobacterium through phagocytosis. However, due to unknown circumstances, the cyanobacteria was not degraded and survived inside its host, producing energy by photosynthesis. This endosymbiotic theory is supported by many facts. Indeed, chloroplasts detain their own genome, however reduced it may be, this genome has many genes resembling those of cyanobacteria (*McFadden, 2001*). Furthermore, this genome is also organised in a similar manner as prokaryotes, leading to believe that chloroplasts originate from prokaryotes. An even more interesting phenomenon is that of secondary endosymbiosis. In this event some chloroplasts originated from the endosymbiosis between a heterotrophic eukaryote and an algal species, which already had performed a primary endosymbiosis (*McFadden, 2001*). This secondary endosymbiosis would explain why some chloroplasts in some species have up to four cell walls (Heterokonts and Dinoflagellates) and nucleomorphs (remnants of a nucleus). Interestingly, some species of Protists, *Hatena arenicola*, and animals, such as sacoglossan slugs, have the capacity to sequester plastids from algae and use them for energy production through photosynthesis (*McFadden, 2001*).

2.2.3 Cellular Division

For the most part, cyanobacteria reproduce asexually through binary fission. In this process either all cellular envelopes (wall and membranes) or just the inner membrane create an invagination in the middle of a mother cell (*Tomaselli, 2008*). These invaginations then constrict to split the cell into two daughter cells of equal size. Binary fission is the most common method of reproduction but some species have been known to have other forms of reproduction, such as reproduction by fragmentation to form hormogonia, reproduction by budding (*Chamaesiphon*) and production of akinetes (*Tomaselli, 2008*). Though sexual reproduction is not known in cyanobacteria, conjugation and horizontal gene transfer exist. Recent studies on the *Leptolyngbya* genus, the *Fischerella* genus and the *Chlorogloeopsis* genus have shown that foreign genes, such as GFP reporter transgene, can be introduced through conjugation, electroporation and biolistic DNA transfer methods (*Stucken et al, 2012; Taton et al, 2012*).

In contrast, eukaryotic algae can reproduce asexually but also have the capacity to reproduce sexually. For asexual reproduction, just like cyanobacteria, eukaryotic microalgae will reproduce by cell division, fragmentation and production of spores (*Tomaselli, 2008*). In the case of sexual reproduction, individuals of the same species will combine their gametes to produce off springs. Thus, there are five major types life cycles found in eukaryotic microalgae (*Tomaselli, 2008*). The first would be a predominantly diploid life cycle wherein meiosis occurs before the formation of the gametes (*Tomaselli, 2008*). Opposite to this would be a predominantly haploid life cycle where meiosis occurs right after the zygote forms (*Tomaselli, 2008*). Thirdly, an alternation of generation life cycle can exist in some species

(*Tomaselli, 2008*). In this life cycle, individuals of a species will alternate between being haploid (gametophytes) and diploid (sporophytes) every other generation. The fourth life cycle is called heteromorphic alternation of generation (*Tomaselli, 2008*). In this life cycle, the individual goes through an alternation of generation life cycle, however, one of the phases (haploid or diploid) will be dominant over the other one, making either the haploid individuals depend on the diploid individual or vice versa. Finally, some species, such as red algae, have a tri-phasic life style, also called a tri-phasic alternation of generation (*Tomaselli, 2008*). In this life cycle, individuals will have one haploid phase and two distinct diploid phases.

2.2.4 Growth Modes and Metabolism

Microalgae are extremely diverse and as a result can grow under variety of different conditions. Most microalgae are photoautotrophic, using photosynthesis to fix ambient carbon dioxide to create their own carbohydrates for direct consumption or for energy storage. Additionally, some species have the capacity to grow in a heterotrophic growth mode, using reduced carbon sources found in their environment, such as sugars. Consequently, a variety of carbon sources, such as glucose, xylose, glycerol and acetate, can be used to grow some species of microalgae in a heterotrophic manner. Many experiments have shown *Chlorella vulgaris* and *Chlorella protothecoides* to be species capable of using sugars, such as glucose, and glycerol for growth when grown heterotrophically (*Liang et al, 2009; Perez-Garcia et al, 2010; O'Grady et al, 2011*). Recently, mixotrophic growth has been studied in some species. For algae, mixotrophy, as the name implies, is the capacity to mix photoautotrophic growth with heterotrophic growth. Hence, *Chlorella vulgaris* has been shown to grow on glucose in a mixotrophic manner and so being able to fix CO_2 through photosynthesis while consuming glucose (*Ogawa et al, 1981*).

These different growth modes are a result of the diversity in metabolic networks seen across the different groups of microalgae. Indeed, the metabolic diversity is quite large in microalgae and can be seen at the species level and even at the strain level. For example, some strains of *C. vulgaris* have been shown to grow only heterotrophically whereas other can only grow autotrophically (*Liang et al, 2009*). Moreover, this diversity has come as an advantage for the algal industry since each species can offer one or more interesting characteristic (such as protein production, growth medium, growth rate or productivity) for cultivation. Microalgae have been known to have different intracellular lipid profiles according to species and to environment. Depending on the lipids produced, different species of microalgae could be of interest for the biofuel industry.

Thus, microalgae are a highly diverse group spreading over the eukaryotic realm and the prokaryotic realm. Their diversity can be seen at the anatomical level, the physiological level and the metabolic level.

3. Microalgae and Biofuel

3.1 What are Biofuels?

Biofuels are fuels produced from non-fossilized energy rich organic compounds usually produced from plants or microalgae. First generation biofuels utilized vegetable oil, starches and sugars from cultivated plants, such as corn and soya, to produce different types of fuels. Bio-alcohols (such as biologically produced ethanol, propanol and butanol), biodiesel and biogases (such as methane) are all examples of biofuels. On the other hand, second generation biofuels have started to look more at the sustainability of the feedstock. The

sustainability of a feedstock would in principal depend on its abundance and its obtainability, the consequences on cultivable land usage, the consequences on greenhouse gas emissions and the general impact on the environment. The major efforts in producing second-generation biofuels are concentrated on creating a feedstock that does not compete with food crops and has a very low impact on the environment. The major weakness with first generation biofuels is that they use arable land, which could otherwise have been used for food crops, or they used food crops, which could have been used to meet nutritional demands instead of producing biofuels. Consequently, first generation biofuels will, if not already, increase food prices. This is seen in the bioethanol industry. Bioethanol is produced through the fermentation of sugar collected from corn and it is said that ethanol plants will burn up to half of U.S domestic corn supplies within a few years (*Runge et al, 2007*). Already in March of 2007, corn prices rose over 4.38$ a bushel which was one of the highest prices recorded in ten years (*Runge et al, 2007*). This is bad news for consumers and especially in poor developing countries where marginal increases in the cost of staple grains could be devastating (*Runge et al, 2007*). Therefore, the use of second-generation biofuels would help to solve this problem by using non-cultivable lands, as with microalgae, or using inedible waste products of agriculture, such lignocellulose.

Biodiesel produced from microalgae fits the description of a second-generation fuel. Biodiesel is usually constituted of a mix of fatty acids that have been transesterified with an alcohol (usually methanol but ethanol and propanol can be used) to produce fatty acid alkyl esters. As we have mentioned before, algae fix carbon to produce carbohydrates and other energy rich compounds, which can be directly consumed or stored for later use. Among the different stored energy rich compounds, lipids are the most crucial for biodiesel production.

Microalgae have the capacity to produce triacylglycerol molecules or TAGs, a type of lipid. These TAGs are three fatty acid molecules attached to a glycerol molecule. The fatty acid composition can be different for different TAG molecules. For the production of biodiesel, fatty acid (FA) composition is crucial since FA length and degree of saturation will greatly influence the resulting fuel properties (*Leite et al, 2012*). Fatty acids are carboxylic acids with a long aliphatic tail (long chain composed mainly of hydrogen and carbon) which can either be saturated or unsaturated. Fatty acids differ from one another in the length of their hydrocarbon tails, the number of carbon-carbon double bonds, the positions of the double bonds in the chains, and the number of branches (*Horton et al, 2006*). Thus over a 100 different FA molecules have been identified in living organisms (*Horton et al, 2006*). In the case of biodiesel, FAs are evaluated for their hydrocarbon tails and their degree of unsaturation. These properties of the FAs will affect a number of general parameters of the biodiesel produced, such as the neutralization number, the cold filter plugging point (CFPP, which reflects a fuels performance in cold weather), cetane number (ignition characteristics), viscosity or the storage stability (*Meher et al, 2006*). Consequently, FAs used for gasoline would need hydrocarbon chains with a length of 6 to 12 carbons with a mixture of saturated chains, whereas FAs used for biodiesel would need chains of 12 to 18 carbons (*Leite et al, 2012*). Luckily, several species of microalgae are capable of producing FAs with tails around the size mentioned previously. An experiment done by *Gouveia et al* showed that some species of microalgae (*Spirulina maxima, Chlorella vulgaris, Scenedesmus obliquus*) could produce from 17% to 40% in biomass of palmitic acid, a FA with 16 carbons in its tail (*Gouveia et al, 2009*). Another study by *Griffiths et al* showed that different species change their FA composition in nitrogen replete and nitrogen depleted conditions (*Griffiths et al, 2012*). Thus, the Chlorophyta

species studied showed an increase in C18:1 fatty acids with nitrogen limitation, along with a decrease in C16:0, C18:2 and C18:3 fatty acids (*Griffiths et al, 2012*). Due to the variety of fatty acids produced in nature, certain standards have been put in place by industrial and governmental organisations detailing the exact requirements biodiesels must have in order to be acceptably used. In Europe, the EN 14214 gives specific requirements and test methods for FAME (Fatty Acid Methyl Esters) quality. Examples of requirements are the acceptability or not of dye or marker usage, of additives, of stabilizing agents and even percentage of different fatty acids in the biodiesel (*European Standard, 2008*).

3.2 Lipid Metabolism

3.2.1 Photosynthetic Efficiency

As microalgae are photosynthetic organisms, one of the limits to biodiesel production is the efficiency with which each species is capable of capturing light and using the energy captured to fix carbon dioxide, also known as the photosynthetic efficiency. Microalgae contain chlorophyll *a* as their major pigment and thus species can only capture up to a maximum of 45% of the whole solar spectrum (*Leite et al, 2012*). However, additional amounts of energy are said to be lost due to the reflection of light on the surface of the reactor (about 10% of total light is lost), to transfer of light energy to chemical energy at the reaction centre, to respiration, to photosaturation and to photoinhibition (*Leite et al, 2012*). Thus, the theoretical maximum photosynthetic efficiency is said to be between 4.6% and 6% for microalgae (*Leite et al, 2012; Ort et al, 2011*).

Numerous ideas, from cultivation techniques to genetic engineering, have been proposed in order to improve photosynthetic efficiency. Among some of these ideas, the optimization of the light harvesting complex or chlorophyll antenna to improve photosynthetic efficiency through genetic manipulation is one of the more interesting ones. The light-harvesting complex is a group of proteins and chlorophyll molecules fixed in the thylakoid membrane, which capture light and transfer the energy to a chlorophyll *a* molecule in the reaction centre of a photosystem. In the case of microalgal cultivation, the density of cells and the intensity of light in a culture affect the photosynthetic efficiency of the species cultured. Thus, if the cell density in a culture is to high, the efficiency will be lowered, increasing the light intensity might help, but the algae at the surface might become photo-saturated. Reducing the size of the light-harvesting complex through genetic manipulation has been shown to be quite effective in lessening photosynthetic inefficiency related to the over absorption of light in mass culturing (*Ort et al, 2011*). Microalgal strains with reduced chlorophyll antenna sizes have been shown to have improved solar to biomass conversion efficiencies in mass cultures (*Ort et al, 2011*). Recently, research has discovered *tla* (*Truncated light-harvesting antenna size*) mutants were formed in *Chlamydomonas reinhardtii* (*Kirst et al, 2012*). The team of Kirst et al were able to produce a *tla3* mutant for *C. reinhardtii* upon deletion of the *TLA3-CpSRP43* gene. The deletion of the *CpSRP43* gene showed a reduction of the light harvesting Chl antenna size and a two-fold increase for the light saturation maximum from the wild type (*Kirst et al, 2012*). Thus, photosynthetic efficiency is a major concern for biofuel production and must be overcome to produce sustainable biodiesel in an effective manner.

3.2.2 TAG Synthesis

Microalgae are extremely variable in their lipid composition. Many species can produce a plethora of different types of lipids of value for the biodiesel industry, the pharmaceutical industry, the food industry and the cosmetic industry. Thus algae can produce a number of polyunsaturated fatty acids or PUFAs (such as eicosapentaenoic acid, docosahexanoid acid and arachidonic acid), new fatty compounds (long chain hydrocarbons with 35 to 40 carbons, unusual hydrocarbons, such as *n*-alkadienes and trienes, galactolipids, triterpenoids, tetraterpenoids and lycopadienes) and oxylipins (*Guschina et al, 2006*). Consequently, fatty acid and TAG synthesis is highly complex and is under extensive study. However, recent research has shown that some species, mostly green algae, have homologous enzymes to key enzymes used in TAG synthesis in higher plants and fungi (*Merchant et al, 2012; Khozin-Goldberg et al, 2011*). For example, Acetyl coenzyme A carboxylase (ACCase), an enzyme used in the first step of fatty acid synthesis, found in most Green Algae and some red algae species are similar to those found in higher plants (*Khozin-Goldberg et al, 2011*). ACCase is responsible for the carboxylation of acetyl-CoA to malonyl-CoA, which goes into the fatty acid synthesis pathway (*Khozin-Goldberg et al, 2011*). As very little is known about microalgal TAG synthesis, one approach for identifying genes involved in TAG synthesis involves analysis of orthologues of known enzymes from yeast and other animals. In yeast, TAG synthesis has been comprehensively studied in *S. cerevisiae*. The pathway is separated into three parts: (1) the generation of diacylglycerol (DAG), (2) the esterification of DAG to generate TAG and (3) the degradation of TAG (*Merchant et al, 2012*). Acetyl-CoA and NADPH are required for TAG synthesis. To this effect, citrate is accumulated in the mitochondria via the tricarboxylic acid cycle and excreted out into the cytoplasm. The citrate

is then converted to Acetyl CoA and oxaloacetate by the key enzyme acyl-CoA ligase, which uses ATP (*Hu, et al, 2008; Hallenbeck, 2012*). The oxaloacetate is converted to malate and enters the mitochondria, where it is converted to pyruvate, CO_2 and NADPH by the malic enzyme (*Hallenbeck, 2012*). The NADPH will help fuel the production of fatty acids. After the production of acetyl-CoA, ACCase (Acetyl-CoA Carboxylase) will transform acetyl-CoA to Malonyl-CoA, which will be used in the Fatty Acid synthesis (FAS). The products of FAS will be fatty acids with a coenzyme A attached, forming molecules of acyl CoA. After this, a first acyl CoA molecule will be attached to a glycerol-3-phosphate (G-3-P) molecule by an acyltransferase via esterification and so produce a lysophosphatidic acid (*Merchant et al, 2012*). A second acyltransferase then attaches, on the C2 position of the glycerol molecule of the lysophosphatidic acid, a second acyl-CoA molecule. This produces a phosphatidic acid. The phosphate group from the G-3-P is then removed by a phosphatase to produce a diacylglycerol molecule (DAG).

This brings us to the second step: the conversion of DAG to TAG. Two routes of conversion are known: the acyl-CoA-dependant route (also known as the Kennedy pathway) and by trans-esterification. In the acyl-CoA-dependant route, a DAG acyltransferase (DAGAT) will esterify a third acyl-CoA onto the C3 position of the DAG to produce a TAG. In the trans-esterification route a membrane phospholipid is transferred to the C3 position of a DAG to form a TAG (*Merchant et al, 2012; Hildebrand et al, 2013; Bensheng et al, 2013, Hu et al, 2008*). The trans-esterification is catalyzed by a phospholipid diacylglycerol acyltransferase or PDAT. Interestingly enough, in *S. cerevisiae* the DGAT route for TAG synthesis will be undertaken in the stationary phase whereas the trans-esterification route will be undertaken in the exponential phase of a growth (*Merchant et al, 2012*).

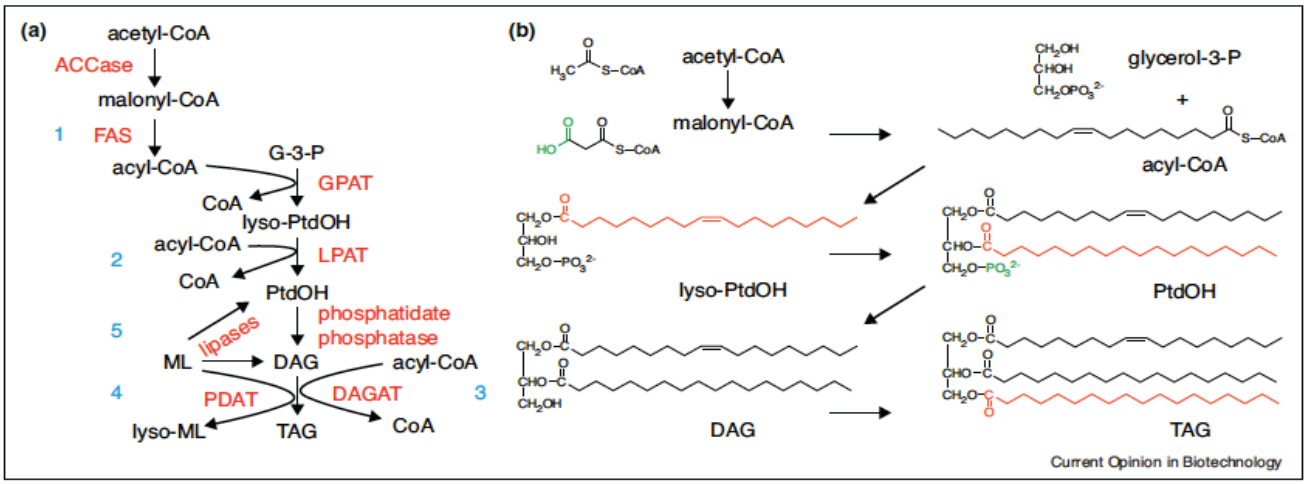

Figure 1: Lipid metabolism in Chlamydomonas. Figure (a) shows TAG synthesis from acetyl-CoA to the esterification of the acyl-CoA molecules on the glycerol molecule to form TAGs. Figure (b) shows the formation of TAGs and the structure of each molecular compound (*Merchant et al, 2012*).

This general scheme for TAG synthesis is applicable to fungal species and plants. In the case of microalgae, this network seems to be applicable, however it is still up for debate. Recent studies have shown that Chlamydomonas tends to have single copy genes for proteins used in fatty acid synthesis, leading one to believe that lipid metabolism in this genus is much simpler than that of higher plants, which tend to have multiple copy genes for fatty acid synthesis (*Liu et al, 2013*). However in the case of TAG synthesis, this same organism has been found to contain six genes coding for DGAT enzymes suggesting a more complicated metabolic network for TAG synthesis than plants, which only have two genes coding for the same enzyme (*Liu et al, 2013*). Moreover, other significant differences are thought to occur in microalgal TAG synthesis. For instance, studies on Chlamydomonas have hypothesized that

TAG synthesis occurs in the chloroplasts, whereas for higher plants, TAGs are assembled both in the Endoplasmic Reticulum and the chloroplasts (*Liu et al, 2013*). Other studies have shown that lipid droplet production inside Chlamydomonas uses a "major lipid droplet protein" (MLDP), which is specific to green algae (*Khozin-Goldberg et al, 2011*). Thus, there is still much research to be done on TAG synthesis in microalgae.

3.2.3 Lipid Accumulation

Environmental stress is known to affect lipid metabolism in microalgae (*Guschina et al, 2006; Liu et al, 2013; Merchant et al, 2012; Khozin-Goldberg et al, 2011; Longworth et al, 2012; Msanne et al, 2012; Hu et al 2008*). Numerous studies have shown that nutrient limitation, temperature changes, light intensity changes, salinity changes and pH changes contribute to modify the quantity and composition of fatty acids.

Nutrient limitation is the reduction of a nutrient in the growth medium to the point where it hinders growth of a species. This has led to a two-system growth method where a strain is grown in a normal medium, containing all of the nutrients necessary, until it reaches a certain biomass. Then, the species is left to grow in a second medium with a limiting nutrient. The limiting nutrient can be any nutrient in the medium however the most common ones are usually nitrogen, phosphorus, sulphur and silicon for diatoms. Depending on the nutrient, different effects may occur on the lipid composition and quantity in the cell of a given species. Thus, nitrogen starvation has shown to increase the lipid content in many species of microalgae. *S. oblique, N. oleoabundans, C. vulgaris C. zofingiensis* are all species that have shown up to 46% lipid content with respect to dry weight (*Breuer et al, 2012*). Phosphorus starvation has been linked to an increase in lipid content in *P. tricornutum, Chaetoceros sp*

and in *P. lutheri*, however it showed a decrease in lipid content for the green flagellates, *N. atomus* and *Tetraselmis sp* (*Guschina et al, 2006*). Interestingly enough, very high levels of nutrients have been shown to increase lipid content. For example, at 15mM of nitrogen source (high amount of nitrogen) *Ulva pertusa* showed an increase in crude lipid content as a percentage of biomass (*Guschina et al, 2006*). Also, when nitrogen levels where augmented, PUFA (polyunsaturated fatty acids, which cannot be used for biodiesel production) concentrations decreased and palmitate and lineolate (fatty acids acceptable for biodiesel production) levels increased for the same species (*Guschina et al, 2006*). Moreover, silicon starvation for *S. minutulus*, a freshwater diatom, was found to increase the TAG content and decrease the polar lipid content (*Guschina et al, 2006*). Finally, different studies have shown that physical environmental conditions, such as light intensity, pH and temperature can have different effects on lipid accumulation when microalgal species are grown under nitrogen starvation (*Breuer et al, 2013*). As a result, *S. obliquus* was shown to have an optimal biomass productivity at pH 7, at 27.5°C. When grown at 20 or 35°C, the total fatty acid and TAG content grew with increasing pH under nitrogen starvation (*Breuer et al, 2013*) Thus, nutrient starvation is an effective way to accumulate TAGs.

Efforts have been made to understand lipid accumulation under starvation at the cellular level but there is still much to be learnt. Most of the research has focused on nitrogen starvation. Nitrogen is important in the synthesis of proteins due to its implication in the protein backbone composition. With the absence of nitrogen, protein synthesis is thought to be hindered as well as cell growth as a consequence of the latter (*Msanne et al, 2012*). As a result of this, cells in a nitrogen-deprived environment are thought to channel excess fixed carbon from photosynthesis into storage molecules, such as TAGs and starch, instead of using the

carbon and energy for growth. Recently, a study with *C. reinhardtii* showed that in the first two days of nitrogen starvation, cells accumulate starch, up to 14 times. However, after 10 days the fatty acid content increases significantly while starch levels decline (*Msanne et al, 2012*). These findings suggest that fatty acid synthesis is a *de novo* process and that fatty acid and TAG synthesis, in a nitrogen-depleted environment, are unlikely to originate only from newly fixed carbon, since most likely most proteins used in carbon fixation, such as RUBISCO, will be greatly reduced (*Msanne et al, 2012; Breuer et al, 2012*). Thus, it is hypothesized that TAG accumulation under nitrogen deprivation comes from carbon already assimilated in other cellular components, such as proteins, ribosomal units and starch. Furthermore, as we mentioned earlier, *C. reinhardtii* contains six different genes coding for DAGAT enzymes grouped in two families, type one (*DGAT*) and type two (*DGTT*). *DGTT* are found in five forms in Chlamydomonas, noted *DGTT 1* to *5* and coded by five different genes. On the other hand, there is only one form of *DGAT* discovered up to now and coded by single gene. *DGAT1* and *DGTT1* are thought to play vital roles in TAG accumulation under nitrogen starvation. Studies have shown that the expression levels for these two genes increases when cells are put in nitrogen starvation conditions and even other types of starvation, such as sulphur, phosphorus, zinc and iron (*Liu et al, 2013; Boyle et al, 2012*). Thus, *DAGAT* genes are thought to play a crucial role in TAG accumulation but the mechanisms involved are still not well understood. For instance, in a knockout and overexpression study of *DGTT 1, 3* and *4*, *C. reinhardtii* showed changes in TAG concentrations. However another study involving the individual overexpression of *DGTT1, 2* and *3* showed no changes in TAG accumulation (*Liu et al, 2013*). Thus, different acetyltransferases must play a crucial role in lipid accumulation under nitrogen starvation, but the exact mechanisms have yet to be elucidated.

Interestingly, research on different species in the green algae taxon have shown similar trends in starch and TAG accumulation (*C. reinhardtii* and *Coccomyxa sp.*) and therefore suggesting that the metabolic response to lipid accumulation under nitrogen starvation may be shared by a large range of green microalgae, since most species are spread over large portion of the tree of life (*Msanne et al, 2012*). Thus, the genetics of lipid accumulation in microalgae is still in its infancy and needs further research.

3.3 Algaculture and Genetic Manipulation

One of the major factors delaying practical algal culture for biofuel production is productivity. Productivity in terms of biodiesel consists in the amount of lipids produced per unit volume of growth media per unit time for a particular species. Consequently, productivity depends on three factors: the growth rate of the species, the lipid quantity produced and the volume of growth media needed. Generally, the best microalgal strain to select for biofuel production would be the species or the strain with the highest productivity. Cultivation methods and genetic manipulation of species have also been considered in order to improve productivity.

The photosynthetic growth of algae necessitates water, carbon dioxide, inorganic salts and light. The essential inorganic salts required for algal growth must contain nitrogen, phosphorus, iron, and, for diatoms, silicon (*Chisti, 2007*). Large algal scale production uses a continuous method of culture. In this method fresh medium is fed at the same rate as it is withdrawn from the culture. Importance is given to agitation as it prevents settling of the biomass, which can lead to photosynthetic inactivity, and in some cases helps to aerate the culture. Thus, there are two typical cultivation methods for algaculture. The first are the open

pond systems and the second are the use of photo-bioreactors. Open pond systems are bodies of water where algal species can grow (*Leite et al, 2012; Chisti, 2007; Abdelaziz et al, 2013*). These bodies of water can either be artificial or can be developed from natural lakes or ponds (*Ugwu et al, 2008*). Maintenance and energy requirements are low for this type of system of cultivation. Generally, open pond systems involve tanks, circular ponds, raceway ponds and shallow big ponds with machinery used for mixing (*Ugwu et al, 2008; Leite et al, 2012; Chisti, 2007; Abdelaziz et al, 2013; Huang et al, 2010*). Raceway ponds are channels organized in closed loop systems typically about 0.3m deep (*Chisti, 2007*). Paddlewheels are used for recirculation and mixing of the culture and the channels are usually made of concrete or compacted earth (*Chisti, 2007*).

Open pond systems can use a wide variety of media however special attention has been focused on the use of wastewater as a growth medium and thus coupling open pond growth operation with wastewater plants (*Abdelaziz et al, 2013; Leite et al, 2012*). Other ideas have been to couple algal growth operations with industrial complexes emitting CO_2 and thus using the CO_2 emitted directly to feed algal cultures (*Chisti, 2007; Huang et al, 2010*). The advantages to using open pond systems are the general low cost with respect to construction and maintenance, the ease of scaling up growth operations, the relatively low energy requirements and the easy maintenance of these systems (*Leite et al, 2012*). However a number of disadvantages are associated with this type of growth operation, including low productivity. Contamination is one of the major concerns with open pond systems. Predatory or commercially uninteresting competitive organisms can overrun a culture and thus destroy cultivation. Water loss through evaporation, poor mixing of CO_2, light intensity, pH and non optimal temperatures are all major factors capable of halting growth and inhibiting lipid

production in cultures. Consequently, the numerous disadvantages pointed out are major obstacles to overcome in order to make biodiesel production a competitive resource.

Photobioreactors have solved some of the problems associated with open pond systems; however, the major drawbacks to these systems are their high cost and high maintenance level. Photobioreactors are closed transparent containers designed to increase control over a culture. Thus, factors such temperature, light intensity, pH, contamination from other species and aeration can be controlled to a certain degree not seen with pond systems. A wide variety of different styles of bioreactors have been invented, which are highly suitable for laboratory or small-scale industries. Photobioreactors include flat plate, tubular, vertical column, internally illuminated and Taurus shaped vessels (*Ugwu et al, 2008*). Amongst all these vessels, tubular photobioreactors are one of the more appropriate types for outdoor mass culture (*Ugwu et al, 2008; Chisti, 2007*). These reactors are generally made of a network of transparent tubes usually made of glass or plastic. The medium inside is pushed by a series of pumps, which helps to create mixing and aeration. These systems generally use continuous culture methods (*Chisti, 2007*). Thus, the use of photobioreactors increases control, productivity, biomass production and restricts contamination. However, the expensiveness, the difficulty in scaling up operations, the fragility of systems (bioreactors can be made of glass) and the high maintenance requirements are all major disadvantages in using these systems.

A second option to increasing productivity is the use of genetic manipulation on selected species. Cellular function can be redirected to synthesize desirable products through metabolic engineering. However, very little is known on algal genetics and therefore, except for *C. reinhardtii* and *V. carteri*, few species, if any, have been used for genetic manipulation experiments. It has been hypothesized that the difficulty of transformation might be due to an

innate defense mechanism that algae posses to suppress transposons or viral invasion (*Rosenberg et al, 2008*). However, a few techniques exist to transform cells, some of these techniques are taken from yeast or plant transformation methods. Electroporation, agitation with glass beads and DNA, biolistic particle delivery systems (such as with a gene gun) and transfection are all effective ways to transform cells (*Rosenberg et al, 2008*). Moreover, some advances in algal genetic engineering have been made in the past and recent history. In 1994, overexpression of acetyl-CoA carboxylase was achieved in *C. cryptica* in order to augment lipid production. The end result showed no difference in lipid production but helped to develop transformation protocols and expression vectors (*Rosenberg et al, 2008; Radakovits et al 2010; Hu et al, 2008; Courshesne et al, 2009*). *V. carteri* was the first microalgal species to successfully incorporate, by transformation, the *Hup1* gene, hexose/H+ symporter gene, from Chlorella in its genome and similar experiments have been done with *C. reinhardtii* and *P. tricornutum* (*Rosenberg et al, 2008; Beer et al, 2009*). As mentioned earlier, experiments were undertaken in order to improve photosynthetic efficiency via truncation of the light-harvesting complex. Improvement in gene silencing strategies has been one of the most significant advances in algal genetics (*Beer et al, 2009*). New amiRNA technology will most likely emerge and thus help to elucidate metabolic pathways utilized for the production of TAGs in microalgae. Other experiments have been focused on overexpression of enzymes used in fatty acid synthesis, fatty acid catabolism and lipid modification to suit biodiesel composition requirements (*Radakovits et al, 2010*). Finally, one of the more interesting modifications would be to create strains capable of directly producing biodiesel (*Hallenbeck, 2012; Radakovits et al 2010*). As of now, most microalgal species produce lipids, which then need to be processed by chemical reaction to give biodiesel. Thus, producing genetically modified

organisms, which directly produce biodiesel, would increase competiveness of algal biodiesel, lower costs of production and be a general boon to the biodiesel industry. As mentioned earlier, TAGs are the type of lipids required for biodiesel production. However, biodiesel is mainly composed of Fatty Acid Methyl Esters (FAMEs). A simple reaction, called transesterification, is used to convert TAGs to FAMEs. Transesterification separates the fatty acids from the glycerol backbone and attaches a methyl group to the freed fatty acids. This reaction usually uses methanol in the presence of an acid or base, but ethanol and propanol can also be used.

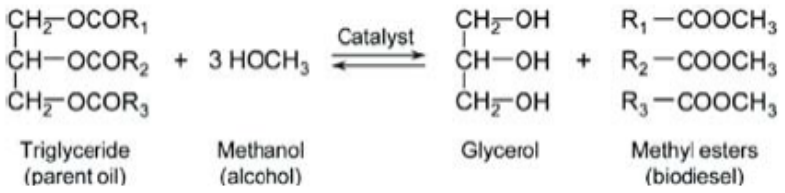

Figure 2: A simplified version of the transesterification reaction. In this reaction methanol is used separate the three fatty acid molecules from the glycerol backbone and produce FAMEs. The catalyst can either be an acid or a base (*Chisti, 2007*).

Thus, the creation of algal strains capable of directly producing FAMEs and circumventing TAG production altogether would be highly interesting. Most organisms do not produce methanol, as it is highly toxic, but produce ethanol, as a result, FAEE (Fatty Acid Ethyl Esters) will have to be produced for biodiesel production (*Hallenbeck, 2012*). This might be an extra advantage as FAEEs have better low temperature characteristics than FAMEs (*Hallenbeck, 2012*). This concept might seem highly imaginative, however, experiments in *E. coli* have already been able to produce the FAEE ethyl oleate through genetic modification (*Radakovits et al 2010; Hallenbeck, 2012*). A non-specific

acetyltransferase from *Acinetobacter* and a pyruvate decarboxylase and alcohol dehydrogenase from *Zymomonas mobilis* were inserted into *E. coli*; the end result was the production of ethyl oleate when glucose and oleic acid was supplied (*Radakovits et al 2010; Hallenbeck, 2012*). FAEE yields from this manipulation were not very impressive, so it would be interesting to see if higher production can be attained and how fatty acid ester accumulation works in microalgae.

Thus, the combination of algal cultivation and genetic manipulation will most certainly help increase productivity in algal grow-ops for the production of biodiesel, however, more research is needed in order to make algal biodiesel competitive with fossil fuels operation and other first generation biodiesel operations.

4. Microalgae and Biohydrogen Production

4.1 Hydrogen producing Enzymes

Hydrogen production by microalgal species has been known since the beginning of the 20th century. In the late 1930's, it was noticed that certain species of microalgae were capable of producing hydrogen under anaerobic conditions. Hans Gaffron was one of the first scientists to discover this phenomenon, with certain species of *Scenedesmus* (*Rathore et al, 2013*). Since then many other species were found to produce hydrogen, both in the prokaryotic and eukaryotic realms. Hydrogen originating from living organisms can be used as a fuel for the transportation industry, as it is highly combustible and flammable. A mix of hydrogen and oxygen is used for rocket fuel in spaceship engines. In addition, hydrogen can be used for a number of different applications, such as in the chemical industry, as coolant, as energy carrier

and in the semiconductor industry.

Hydrogen is produced by hydrogen producing enzymes which bond electrons and protons, generated either directly from the splitting of water by the photosystem II in photosynthesis or indirectly from the degradation of organic molecules such as starch (*Kruse et al, 2010; Hallenbeck et al, 2002*). Up to now three types of protein are known to produce hydrogen: nitrogenase, [FeFe]-hydrogenase and [NiFe]-hydrogenase (*Hallenbeck et al, 2002; Ghirardi et al, 2007*).

Firstly, nitrogenases are enzymes that fix atmospheric N_2 to produce ammonium and molecular hydrogen, as a by-product (*Koku et al, 2002; Zehr et al, 2003; Hallenbeck et al, 2002*). An enormous quantity of energy in the form of ATP is needed to reduce the triple bond of N_2 molecules and so the amount of hydrogen produced is relatively low. Furthermore, the turnover rate, the rate at which a protein is produced and degraded, is very low for this enzyme, making them inefficient candidates for hydrogen production operations (*Hallenbeck et al, 2002*).

On the other hand, hydrogenases are more effective in hydrogen production because of their relatively higher efficient use of energy. Two types of hydrogenases exist, the first being [FeFe]-hydrogenase and the second being [NiFe]-hydrogenase. [FeFe]-hydrogenase is mainly found in green algae, fungi, protist and anaerobic species and is widely conserved. [FeFe]-hydrogenase contains a metallo-cluster, where catalytic reactions occur (binding of a proton with an electron to form molecular hydrogen), constituted of a [4Fe-4S] cubane linked through a cysteine residue to a 2Fe subcluster (*Ghirardi et al, 2007*). Thus, these enzymes contain iron and sulphur in their metallo-cluster. A number of eukaryotic hydrogenases have already been sequenced and are readily available for genetic studies. [FeFe]-hydrogenase is highly sensitive

to oxygen and its presence destroys irreversibly the enzyme. The mechanism of inhibition is not clearly understood, but it is thought that oxygen binds with the metallo-cluster and therefore prevents protons from binding competitively to the same site (*Ghirardi et al, 2007*). Finally, the last class of hydrogenase is the [NiFe]-hydrogenase, found mainly in cyanobacterial groups. Two types of [NiFe]-hydrogenase are found, an uptake and a bidirectional protein (*Ghirardi et al, 2007; Rashid et al, 2013*). Uptake [NiFe]-hydrogenase is usually found in nitrogen fixing cyanobacteria and oxidizes hydrogen produced by nitrogenase. On the other hand, bidirectional [NiFe]-hydrogenase can oxidize and produce molecular hydrogen. The oxidation of hydrogen is a well-understood process where hydrogen is transported by a hydrophobic channel to the [NiFe] cluster. The H_2 brought by the channel is thought to bind with the Ni atom of the cluster. The hydrogen cleavage produces protons and electrons, which are transferred to the protein environment and a redox partner respectively (*Ghirardi et al, 2007*). The production of hydrogen is not well understood, however, the catalytic mechanism for bidirectional [NiFe]-hydrogenase is assumed to operate in inverse sequence of the oxidation of hydrogen.

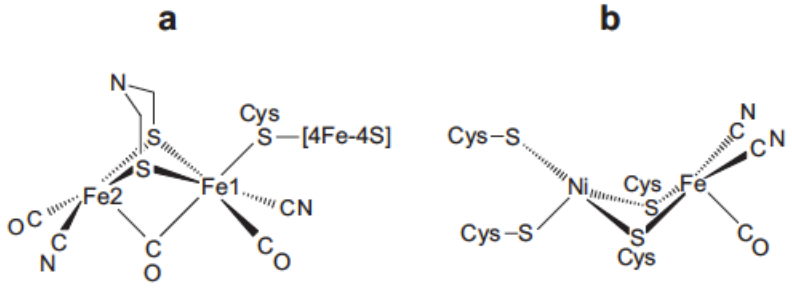

Figure 3: Chemical structure of the metallo-structure of the [FeFe]-hydrogenase (a) and [NiFe]-hydrogenase (b) (*Ghiardi et al, 2007*).

As oxygen irreversibly inactivates the enzyme, different processes have been created to

protect hydrogen-producing enzymes from this phenomenon. A number of different species have produced separate anaerobic compartments preventing oxygen from destroying hydrogen-producing enzymes. For instance, filamentous cyanobacteria produce heterocysts, which are anaerobic cells, where nitrogenase can fix N_2 and produce hydrogen (*Hallenbeck, 2012; Bergman et al, 1997*). Moreover, some species will either have a mutualistic or symbiotic relationship with other organisms helping to create a suitable environment for hydrogen production. Certain cyanobacteria are known to produce mats or biofilms with other species. These mats are known to produce an anaerobic environment where nitrogenase can fix nitrogen and possibly produce hydrogen (*Bergman et al, 1997*). Finally, certain species will activate hydrogen production during night-time or in dark periods, when photosynthesis and oxygen production are halted. This is seen for *C. reinhardtii* (*Tsygankov, 2012*).

4.2 Hydrogen production Mechanisms

Hydrogen production is mediated through various metabolic pathways. In microalgae and cyanobacteria, photosynthesis is the main driver to hydrogen production. During photosynthesis, photons, captured by photosystem II, are used to split water. This consequently produces protons, electrons and molecular oxygen. The electrons are transferred through a series of proteins in the membrane (either thylakoid in chloroplast or cellular membrane of cyanobacteria) to end up with the reduction of ferrodoxin (*Hallenbeck, 2012; Rathore et al, 2013*). This is known as the electron transport chain. Two systems are known to produce hydrogen from reduced ferrodoxin. In the first system, reduced ferrodoxin can either directly give electrons to nitrogenase, which will use ATP and the protons produced from the splitting of water to produce molecular hydrogen. In the second system, reduced ferrodoxin

will reduce NADP to NADPH, with the help of FNR (*Hallenbeck, 2012*). The reduced NADPH will donate electrons to a reversible hydrogenase (Hox system), which will use produce molecular hydrogen with the use of protons (*Hallenbeck, 2012*). Thus, microalgae have a complex way of producing hydrogen.

One major problem is that the generation of H_2 is low since hydrogenase and nitrogenases are inactivated by oxygen (*Rashid et al, 2013; Tsygankov, 2012*). Furthermore, production of NADP+ (a product of photosynthesis) by reduction of protons requires less energy than production of H_2 by reduction of protons, lowering H_2 production levels (*Tsygankov, 2012*). Consequently, two stage growth methods are used to produce hydrogen in order to separate in space or time hydrogen production and the water splitting reactions (*Rathore et al, 2013*). In the first stage microalgal cells are grown photosynthetically to produce carbohydrates and oxygen. The first aerobic stage depends on good growth conditions, such as good lighting conditions, optimal pH, ideal temperatures and so on, to produce biomass. In the second stage, the algal cells are grown or incubated under conditions suitable for hydrogen production, such as sulphur deprivation (*Rathore et al, 2013; Rashid et al, 2013; Melis et al, 2001*). Sulphur starvation helps to augment hydrogen production in microalgae by creating an anaerobic environment for the cell. The photosystem II contains a D1 protein, which is easily photodamaged during illumination period. Methionine and Cysteine, amino acids rich in sulphur are required to repair this protein. Sulphur deprivation causes methionine and cysteine biosynthesis to halt and thus during lighted periods the D1 protein gradually damages (*Kruse et al, 2010; Melis et al, 2001; Rashid et al, 2013*). Consequently, oxygen levels are diminished in the culture and hydrogenase activity can increase inside the cells to produce higher quantities of hydrogen. (*Kruse et al, 2010*).

Thus, hydrogen production relies on a number of different metabolic pathways and nutrient limitation process. Further research must be followed in order to make competitive biohydrogen industry.

5. Conclusion

In conclusion, the many drawbacks of fossil fuels have made microalgae a promising energy source for the near future. Microalgae are photosynthetic organisms containing chlorophyll a and spanning from the eukaryotic realm to the prokaryotic realm. These organisms are capable of producing a number of valuable products, chief of which are biodiesel and biohydrogen. Their cultivation for biodiesel production and hydrogen has numerous advantages, such as sustainability and ecological friendliness. However, as any green energy, algal productivity is still very low and thus much research is being funded to improve productivity. Research in productivity improvement is either funnelled to genetic engineering or research in cultivation technology. Advances have been made in the recent years and this has led to the establishment of a number of growth operations. Thus, we hope that advances in genetic manipulation and algal cultivation will help increase the affordability of algal biofuels in the near future.

Objectives of the Present Study

The objectives of the present study were numerous. The first objective was to uncover algal strains capable utilizing glycerol for enhanced lipid production. To do this, 12 algal strains were screened for mixotrophic growth using glycerol as a carbon source. The strains were compared for growth, lipid production, lipid productivity and glycerol consumption under mixotrophic conditions and photoautotrophic conditions.

Two strains (PCH02 and PCH05) were found to be good lipid producers under mixotrophic conditions. As a result, the second objective was to study lipid production under mixotrophic conditions and nitrogen depleted conditions. Thus, PCH02 and PCH05 were grown so that a substantial amount of biomass could be produced and then transferred to a nitrogen deficient medium. The earlier parameters were measured to evaluate lipid production and glycerol usage.

A third objective was to evaluate lipid production with different concentration of nitrogen source, in our case sodium nitrate. Certain reports have shown that microalgae have higher lipid productivity when they are given the chance to deplete nitrogen naturally. Thus, algal strains were grown in growth media with different concentration of nitrate under mixotrophic and photoautotrophic conditions.

Finally, since the algal strains were isolated from different rivers and lakes in Quebec, Canada, the last objective was to identify them. The *rbcL* and the *18S rRNA* genes were sequenced and phylogenetic analyse was undertaken to identify the wild strains.

Preface to Article

Microalgal oils are an upcoming substitute for fossil fuel. However, high costs and the need for sophisticated equipment make oil production by microalgae still a technology of the future. In this study, lipid production was evaluated for twelve algal strains isolated from different lakes and rivers in Quebec, Canada, using a mixotrophic growth mode with glycerol as the carbon source. Furthermore, lipid production was evaluated under mixotrophic growth and nitrogen starvation. The results indicated that glycerol enhanced biomass production, growth rates and lipid production, sometimes by a 6-fold increase. Furthermore, nitrogen starvation combined with mixotrophic growth, using glycerol, enhanced lipid production. This was dependant on time spent under nitrogen starvation, on nitrogen concentration and on the type of algal strain studied.

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Author's contribution: All three authors contributed to the work of this article. Gustavo B. Leite created the algal collection and helped establish certain of the biochemical assays. Kiran Paranjape designed the experimental set up with the supervision of Dr. Patrick C. Hallenbeck. He also undertook the PCR, sequencing and phylogenetic analysis and most of the biochemical assays. All the data was collected and analysed by Kiran Paranjape. Dr. Patrick C. Hallenbeck contributed to the analysis of the work and carried out the FAMEs analysis. All the work was done under the supervision of Dr. Patrick C. Hallenbeck.

Studies on Lipid Production of Microalgae under Mixotrophic Growth, utilizing Glycerol as a Carbon Source, combined with Nitrogen Starvation

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1. Introduction

Fossil fuels are remnants of buried organisms anaerobically decomposed over long geological periods of time. Their consumption has been found to date back as far as the Antiquity, but it is only since the beginning of the 20th century that fossil fuel usage has boomed. Fossil fuels are a very useful energy source, fuelling a wide range of industries, such as the transport industry and the petro-chemical industry. However, this usefulness has come at a price. Our society has become completely dependent on them. As of 2012, the EIA (U.S. Energy information administration) projected that the highest consumed energy source, in the United States, was Petroleum at 36% (*EIA, 2013*). This is bad news since fossil fuels are limited in quantities. A complete halt in petroleum oil production would be disastrous for world economies and societies. Furthermore, adverse effects on the environment have caused serious criticism of the petroleum industry. Fossil fuels contain large amounts of carbon, which are released with their consumption. This has increased atmospheric CO_2 concentrations and thus has resulted in the phenomenon known as Climate Change or Global Warming, since CO_2 is a greenhouse gas. Additionally, extraction, transportation and refinement of fossil fuels have caused serious harm to ecological systems, with the most famous being the British petroleum spill in 2010. Thus, recent research has been focused on finding more sustainable and ecological alternatives to fossil fuels. Amongst the numerous possibilities, microalgae have surfaced as a new hope for biodiesel production. Microalgae are unicellular photosynthetic organisms containing chlorophyll ‘‘a’’ (*Tomaselli, 2008*). These organisms span from the Eukaryotic realm to the Prokaryotic realm, and are capable of producing lipids (Triacylglycerides or TAGs), which can be converted to biodiesel through a chemical process known as transesterification. TAGs are lipids composed of three fatty acid

molecules attached to a glycerol molecule. Transesterification is a chemical process of switching the organic group R' of an alcohol with the organic group R" of an ester. Consequently, the reaction separates the fatty acids from the glycerol backbone and attaches a methyl group to the freed fatty acids. This reaction usually uses methanol in the presence of an acid or base, but ethanol and propanol can also be used. Microalgae present many advantages for biodiesel production. Since microalgae are photosynthetic, the biodiesel produced would directly come from sequestered atmospheric CO_2. Thus, no additional carbon would be added to the atmosphere from their use, such as in the case with oil. This would deter increasing global temperatures due to increased CO_2 emission. Furthermore, the use of microalgae for biodiesel production would discontinue the usage of traditional food crops, such as corn or soya, for biofuel production. This would imply lower food costs and direct agriculture towards increased food production. Additionally, since microalgae are generally aquatic, arable land is not required for production. Thus, cultivation of microalgae would have little competition with traditional agriculture. This would also lower food costs. More technical advantages of microalgae are that they have higher lipid content and faster growth periods (doubling time can be less than 24hours) than traditional crops used for biofuel production. Thus, microalgae seem like the perfect solution for biodiesel production. However, for all the advantages, several drawbacks make microalgae still not competitive to fossil fuels. One of the major disadvantages is the cost of production. Thus, microalgae require high demands of certain key resources. For instance, carbon dioxide is needed for cultivating algae for biofuel. Production of one ton of algal biomass requires 1.83 tons of carbon dioxide (*Chisti, 2013*). Consequently, many companies rely on bought CO_2 to increase carbon concentrations, which in effect augments production cost (*Leite et al, 2014; Chisti, 2013*). Furthermore, sophisticated and

costly growth operations are needed for increased productivity, requiring higher input than output. Moreover, the high growth rates, that we mentioned earlier, depend largely on optimal conditions easily produced in laboratories but not necessarily reproducible at an industrial scale. Therefore, the cultivation of microalgae is complex and still in the beginning phase of development. Potential solutions are being investigated for these problems. One solution would be the use of mixotrophic growth mode. Mixotrophy is the coupled use of two or more trophic modes by an organism. Originally, microalgae were thought to be strictly photoautotrophic, using photosynthesis to fix carbon dioxide and produce carbon reserves for growth. However, recent findings have shown that many species are actually capable of photoautotrophic and heterotrophic growth (*Burkholder et al, 2008; Miao et al, 2006; Scott et al, 2010; Brennan et al, 2010*). Thus, species would be able to simultaneously fix carbon dioxide and assimilate a heterotrophic carbon source through the parallel use of photosynthesis and respiration. Recently, mixotrophic growth has shown to enhance lipid production in certain species of algae and thus has renewed interest for different algal research programs (*Miao et al, 2006; Heredia-Arroyo et al, 2011; Garcia et al, 2000; Leite et al, 2014*). Since cost and sustainability is a major component of the equation, a carbon waste product must be used. Glycerol is a major waste product of the current biodiesel industry, with more than a billion kilograms of crude glycerol produced annually (*Leite et al, 2014*). This makes glycerol a cheap and abundant carbon source. Furthermore, glycerol is a ubiquitous molecule that is needed for TAG synthesis, as it composes the backbone of TAG molecules. Research has shown several species of microalgae capable of enhancing lipid production when grown with glycerol. Thus, *Chlorella protothecoides*, *Chlorella vulgaris* and *Chlorella pyrenoidosa* were

all species capable of growing on glycerol and producing higher lipid yields (*Chen et al, 2011; Kong et al, 2013; Rai et al, 2013*).

On the other hand, nutrient depletion has also shown to increase lipid content in certain strains. Nitrogen starvation is one of the most common ways to induce lipid accumulation (*Breuer et al, 2012; Mujtaba, et al, 2012*). Thus, a number species have been shown to increase lipid content up to 46%, such as *S. obliquus, N. oleoabundans, C. vulgaris* and *C. zofingiensis* (*Breuer et al, 2012*). Since nitrogen sources are used for DNA and protein synthesis, the lack of it stops growth. Algal cells will then try to conserve nitrogen stocks by recycling amino acids and nucleic acids. If light is present, algal cells will still fix carbon and channel the fixed carbon to storage compounds, such as TAGs, for later usage. Although nitrogen starvation induces lipid accumulation, a major drawback is that it halts biomass production. Consequently, a trade off must be made, where growers will have to decide if they want high quantity of biomass with lower lipid content or low quantity of biomass with high lipid content. To induce nitrogen starvation, most growers will use a two-stage approach where cells are grown to a certain biomass and then transferred into a nitrogen-depleted growth medium. However, a study by *Stephenson et al* found that *C. vulgaris* was more effective at producing high quantities of lipids when cells depleted their nitrogen source naturally instead of being transferred into a nitrogen depleted medium directly (*Stephenson et al, 2010*).

Thus, the present research focused on examining lipid production for twelve algal strains, collected from several rivers and lakes in Quebec, using a mixotrophic growth mode, with glycerol as a carbon source. Further investigation was focused on evaluating lipid production under mixotrophy (glycerol as the carbon source) and nitrogen starvation for

strains showing interesting results, from the previous experiment. Thus, three algal strains were selected and grown under nitrogen-deplete conditions and mixotrophy. Moreover, as mentioned before, certain algal strains have better lipid productivity when strains are let to deplete their nitrogen reserves naturally. Accordingly, lipid production was assessed for growth under mixotrophy coupled with varying concentrations of nitrogen source for certain strains. The results indicated that glycerol is an acceptable carbon source for mixotrophic growth. Furthermore, mixotrophic growth, using glycerol, was able to enhance lipid production for certain strains but not all, suggesting that glycerol metabolism directed to lipid accumulation is strain specific process. On the other hand, mixotrophic growth combined with nitrogen starvation enhanced lipid production, however, this depended on time spent under nitrogen starvation, on concentration of nitrogen and on the algal strain used. Finally, phylogenetic studies were undertaken using the *18S rRNA* and *rbcL* genes for identification of the algal isolates. Phylogenetic trees were produced from the sequenced genes of the algal isolates and revealed that the strains studied were representative of several diverse genera within the same phylum. Thus, the algal strains were genetically diverse.

2. Materials and Methods

2.1 Algal Cultivation

Twelve algal strains were selected for the work presented. These strains are part of a collection from the Department of Microbiology at the University de Montréal. These strains were isolated from different lakes and rivers in Quebec, Canada. Bold's Basal medium (BBM) was used for cultivation, described by Andersen (*Andersen, 2005*). This medium is

photoautotrophic. BBM with added 25mM of glycerol (reagent grade) was produced for mixotrophic cultivation.

For the mixotrophic screening (first experiment), strains were cultivated in 3.5ml of BBM with 25mM of glycerol in 12 well plates. This was done in triplicates. Plates were placed in an incubator with continuous light at 40W/m^2 intensity at room temperature. For photoautotrophic conditions, strains were grown in BBM without glycerol under the same conditions and in triplicates. Growth curves were measured by measuring optical density (OD) at 630nm using EL-800 universal microplate reader from Bio-tek instruments, Inc., for ten days.

The subsequent experiments used the same procedures but with slight differences. Strains were grown in 125ml Erlenmeyer flasks, with 50mL of BBM, in a shaker. Mixotrophic conditions had 25mM of glycerol whereas photoautotrophic BBM had none. Varying amounts of sodium nitrate (nitrogen source in the recipe) were used. Thus in the second experiment, sodium nitrate was omitted from the recipe. In the experiments with algal strains PCH02 and PCH05, varying amounts of sodium nitrate were added to make BBM (mixotrophic and photoautotrophic) with 0mg/L, 20mg/L, 40mg/L, 60mg/L, 80mg/L, 100mg/L and 250mg/L of final nitrate concentration. Media with a nitrate concentration of 250mg/L represented nitrogen-replete conditions. The strains were grown in constant light at 40W/m^2 intensity at room temperature and shaken at 160rpm. Each day, 200ml of culture was taken as sample and placed in 96-well microplate for OD reading at 630nm. The same machine and settings were used as previously stated. OD was plotted as a function of time and growth curves were produced.

Pre-inoculums were grown under photoautotrophic conditions before each experiment. This would ensure that cultures would be healthy and that growth would start at the same level.

2.2. Biomass Quantification

Biomass was measured using a Biomass standard curve from our laboratory. The standard curve was a plot of Biomass measurements in function of respective OD measurements. Thus, after growth, final ODs of algal cultures were compared to the standard curve and the final Biomass was calculated according to curve equation of the standard curve.

2.3. Lipid Quantification

Nile red was used to quantify intracellular lipids. Nile Red is a fluorescent dye capable of staining neutral lipids used for biodiesel production (*Bertozzini et al, 2011; Chen et al, 2009; Elsey et al, 2007; Huang et al, 2009; Kou et al, 2013; Lee et al, 1998*). A protocol from Chen et al, 2009 was modified and used for lipid quantification. In a 96 well plate, algal samples were diluted with BBM to obtain 0.06 OD for each sample. 143.33ml was transferred into black flat-bottom 96 well plates. To this, 50ml of DMSO was added and 6.66ml of Nile red solution (15mg/ml Nile red in acetone) was added to the algae/DMSO mixture. The plate was incubated and shaken (with a microplate shaker) at room temperature and in a relatively dark place (not in direct sunlight) for 10 minutes. The plates were read with a spectrofluometer at 525nm excitation and 580nm emission. A standard curve made out of triolein was created each time samples were measured.

2.4. Glycerol Assay

A protocol by *Bondioli et al, 2005*, was used to quantify glycerol consumption. The assay uses a reaction known as Hantzsch's reaction, wherein glycerol is converted to formaldehyde using sodium periodate. The formaldehyde is converted to 3,5-diacetyl-1,4-dihydrolutidine by two molecules of acetylacetone in the presence of ammonium acetate. The end product gives a yellow colour, which has a specific absorption at 410nm (*Bondioli et al, 2005*). The protocol was adapted for 96 well micro-plates. Thus, 1ml of algal samples were centrifuged in micro-tubes, at the highest speed. 100ml of the supernatant was placed in 96 well plates, mixed with 60ml of 10mM of ammonium periodate solution (20mM $NaIO_4$ in 1.6M acetic acid and 4M ammonium acetate, 1:1). The plate was shaken with a microplate shaker for 30 seconds. Then 60ml of 0.2M acetylacetone solution (2% v/v acetylacetone in 1.6M acetic acid and 4M ammonium acetate, 1:1) was added to the mixture and heated at 70°C for one minute. The plate was then quickly cooled in water at room temperature and the optical density was read at 405nm with a Bio-Tek EL800 microplate reader. The optical density measures were compared to standard curves with known glycerol concentrations. The analysis was done in triplicates and the values are represented as the average of the triplicates.

2.5. FAME Characterisation

Algal strains were characterised for their Fatty Acid Methylated Esters (FAME) composition. A wet lipid extraction was performed, which extracted and transesterified TAGs in one step. In this protocol, 0.5ml of algal culture was put into 4ml screw cap vials. To this we added 1ml of Saponification Reagent (5ml of 0.8g/ml of KOH with 95ml of methanol). The mixture was vortexed for 20 to 30 seconds and incubated at 100°C for 90 minutes in a

Bloc heater. After this time, the mixture was let to cool to room temperature and 1.5ml of Methylation Reagent (17.5ml of 12N HCL with 282.5ml of methanol) was added. The solution was incubated at 60°C overnight. The next day, 1.25ml of Hexane was added, after solutions had cooled down. The vials were vortexed for 30 minutes and then let stood for 10 minutes. A volume of 150ml was taken from the top layer and run through an Agilent GC-MS (Gas Chromatography- Mass Spectrometry) for FAME characterisation.

2.6. Growth Rate, Lipid Percentage and Lipid Productivity

Growth rates were calculated according to Woods et al, 2005 (*Wood et al, 2005*):

$$\mu = \frac{\ln(Xt1) - \ln(Xt2)}{t1 - t2} \quad \text{(Eq. 1)}$$

Where μ is the growth rate in days^{-1}, X_{t1} and X_{t2} are the optical densities at time t1 and t2. X_{t1} and X_{t2} correspond to the points where cells enter and complete exponential growth. Exponential growth can be seen as a straight line when log scale is used in the Y-axis of the growth curve graph.

Lipid content was calculated as the ratio between the total amounts of lipids produced by the total amount of biomass produced in a culture:

$$Lipid\ percentage = \frac{Total\ amount\ of\ lipids\ produced\ (in\ mg)}{biomass\ (in\ mg)} \quad \text{(Eq. 2)}$$

Lipid productivity is defined as the lipid yield per volume of growth medium per unit time and is often expressed as mg/L/hour or mg/L/Day. Thus, to calculate lipid productivity the following formula was used:

$$Lipid\ Productivity = \frac{Total\ lipid\ (mg)}{(volume\ of\ growth\ media\ (L)) * (days\ of\ growth)} \quad \text{(Eq. 3)}$$

2.7 Phylogenetic Analysis

Algal strains were identified using phylogenetic analysis. Each strain was sequenced for the *18S rRNA* gene and for the *rbcL* gene (Rubisco large subunit). The *18S rRNA* and *rbcL* genes are common markers used for phylogenetic studies due to the presence of highly conserved regions within the sequences of the genes. Genomic DNA was extracted using a modified protocol from *Fawley et al* (*Fawley et al, 2004*). A volume of one to 2ml of algal culture was centrifuged at maximum speed for 1 minute. The supernatant was discarded and 200ml of extraction buffer (1M NaCl, 70mM Tris, 30mM Na_2EDTA, pH 8.6) was added and vortexed briefly. This mixture was centrifuged to pellet cells again and the supernatant was discarded. To the pellet 200ml of fresh extraction buffer was added, along with a quantity of glass beads to fill the conical part of the centrifuge tube, 25ml of 10% DTAB and 200ml of chloroform. The tubes were vortexed for 20 seconds at the highest speed and the mixture was centrifuged at 2000*g* for two minutes. Two phases were separated with a cell layer at the interface (if procedures were well performed). The top phases was the aqueous phase and contained the DNA. One hundred microliters was taken from the aqueous phases and added to a 1.5ml microtube. The genomic DNA was then purified using a Gel/ PCR DNA Fragments Extraction kit from Geneaid (Lot No. JL33414).

With the genomic DNA, polymerase chain reactions (PCR) were conducted using *18S rRNA* internal universal primers and *rbcL* internal universal primers:

18S Forward primer 5'-GTGGTAACGGGTGACGG-3' and *18S reverse* primer 5'-GTGCGGCCCAGAACATC-3'

rbcL forward primer 5'-CTCCTCAACCAGGTGTTCC-3' and *rbcL* reverse primer 5'-CTGGCATGTGCCATACGTG-3'

The PCRs were performed with a Phusion High fidelity DNA Polymerase kit from Finnzymes, part of Thermo Fischer Scientific (product codes: F-530S, 100 U and F-530L, 500 U). The PCR mixture and cycling conditions were selected according to the manufacturer. PCR products were migrated through an agarose gel electrophoresis at 0.8% and at 100V for 45 minutes. The gel was exposed to UV light to detect DNA bands of 1Kbp. The PCR products were purified using the same kit as mentioned previously and the purified PCR products were sent for sequencing at the sequencing centre of the IRIC (Institut de Recherche en Immunologie et Cancérologie) affiliated to the University of Montreal.

The sequenced genes were aligned with different *18S rRNA* and *rbcL* sequences using the BLAST database from NCBI to find the closest organisms related to the algal strains. BLAST (Basic Local Alignment Search Tool) is a program that compares query sequences to a database of sequences in order to find a sequence from the database, which most resembles the query sequence, above a certain threshold of identity. Subsequently, algal strains were aligned along with nearest neighbour sequences and outlier sequences (outlier sequences have further phylogenetic relation to the nearest neighbour sequences and the algal query sequences) using the program MEGA (*Tamura et al, 2011*). The MUSCLE program was used to align sequences after which neighbour joining (N-J) tree were created using the Jukes-Cantor model at a 1000 bootstraps. The phylogenetic relationships of the algal strains were modelled after the algal classification done by Guiry et al (*Guiry et al, 2014*).

3. Results and Discussion

3.1 Mixotrophic screening of Algal Strains

3.1.1. Screening Strains for enhanced Growth and/or Lipid Production in the Presence of Glycerol.

Growth rates and lipid production of twelve algal strains were compared under mixotrophic and photoautotrophic conditions in order to identify strains capable of enhanced growth and/or lipid production in the presence of glycerol (Table 1). Measurement of glycerol consumption showed that all strains were capable of taking up glycerol from the growth medium. Maximum growth rates (μ (day-1)) were determined by measuring growth daily (Figure S1) under both mixotrophic and photoautotrophic growth conditions and volumetric biomass production under the two conditions was determined after ten days of growth at which point most of the algal strains examined had entered stationary phase.

With either the calculated maximum growth rates (Table 1) or the individual growth curves (Figure S1) it can be seen that different strains produced different responses. There was an appreciable variation in maximum growth rate observed depending upon the strain (Table 1), with some, PCH05, PCH21Y, and PCH32, showing quite high specific growth rates (from 0.283 to 0.372 day^{-1}), whereas others, MA1A08 and MA1A21, gave appreciably lower maximum growth rates (0.105 and 0.141 day^{-1}). Regardless of other factors, this measure would be important in choosing strains to develop for a practical production system, as higher growth rates would increase productivity.

Maximal growth rates are also varied for mixotrophic or photoautotrophic growth for each algal strain. As a result, strains could be categorized into three groups. Four strains, PCH02, PCH05, PCH32 and MA1A21 had much higher maximal growth rates under

mixotrophy (1.53, 1.62, 1.55 and 2.44 fold increase) than photoautotrophy. On the other hand, the maximal growth rates of six strains, PCH11, PCH20, PCH21Y, PCH28, PCH29 and MA1A08, was indifferent to the presence of glycerol, differing by twenty per cent or less in both conditions. Finally, two strains, PCH21G, and PCH30 had 25-50% decrease in maximal growth rates in the presence of glycerol. There are probably several different possible explanations for different effects of the presence of glycerol on the maximum growth rate of different strains. However, without a doubt, carbon assimilation and partitioning must play important roles. It is known that many unicellular organisms need to reach a critical size, in order to start cellular division (*Millar et al 1995; Fogg et al, 1973; Tomaselli, 2008*). For example, certain types of yeast will only start expressing certain genes necessary for DNA replication when the critical size is reached (*Millar et al, 1995).* Thus, if one assumes that the rate of carbon fixation, which indirectly depends upon the rate of photosynthesis, is the major growth-limiting factor, then glycerol assimilation would help to overcome this bottleneck, thus possibly explaining the increased growth rates in strains where this was observed. Inversely, strains where the presence of glycerol was without effect on growth rate could in fact be incapable of assimilating this compound. However, analysis of glycerol concentrations showed that it was consumed, suggesting that it was assimilated but converted into materials, such as storage compounds, that do not directly lead to cell growth. Finally, two strains actually grew slower in the presence of glycerol (PCH21G and PCH30), suggesting that in some cases this compound could act as an inhibitor, possibly by acting to unbalance metabolism after its assimilation.

Some of these aspects were investigated further. In terms of the effect of the presence of glycerol on biomass production, again, the strains could be divided into two different

groups. Two strains, PCH02 and MA1A21, had final biomass yields under mixotrophic conditions that were much higher (1.92 to 1.30 fold) than under photoautotrophic conditions. The other strains presented indifferent biomass yields to the presence of glycerol, with less than twenty percept difference in the two growth conditions. Two different mechanisms might be at play to produce higher biomass under mixotrophic conditions. In one scenario, assimilated glycerol could be primarily channelled into storage molecule production, such as lipids or sugars, and consequently, algal cells would be "fattened up". In a second scenario, assimilated glycerol could be directed into synthesis of essential cell components, thus directly working to increase the total number of cells observed at the end of growth. Similarly to what was observed with maximum growth rates, total biomass production varied widely with different strains. By the time they had reached stationary phase, almost all strains had produced more than 0.5g/L biomass, with strain PCH29 showing the highest production under the mixotrophic growth conditions used here (1.02g/L). On the other hand, strains MA1A08 and MA1A21 were the lowest biomass producers, giving only 0.16g/L and 0.497g/L. Thus, although, strain MA1A21 had a high increase in biomass production under mixotrophic conditions (1.3 fold), its final biomass production is relatively low and consequently this strain would not be very suitable for mass cultivation.

Interestingly enough, an increased maximum growth rate under mixotrophic conditions does not necessarily correlate with increased biomass production under the same conditions (Table 1), i.e. strains with high growth rates under mixotrophic conditions do not necessarily show higher biomass production. For example, PCH05 had a 62% higher growth rate under mixotrophic conditions, but had similar biomass production under both mixotrophic and photoautotrophic conditions. Similarly, PCH02 and MA1A21 were the only strains to have

both significantly high biomass and high maximum growth rates under mixotrophic conditions. When cultivated with glycerol, PCH02 showed a 1.92 fold increase in biomass and a 1.53 fold increase growth rate, and MA1A21 had a 1.30 fold increase in biomass production and a 2.44 fold increase in growth rate. Consequently, these findings suggest that with these strains glycerol played a significant role in biomass increase, either through increased cell production or through increased production of storage molecules, such as lipids.

Thus, glycerol seemed to have different effects on biomass production and growth rates. Two strains, PCH02 and MA1A21, showed increased growth rates and biomass when grown with glycerol. However, MA1A21 presented one of the lowest growth rates and biomass production. Furthermore, these results show that, at least with some strains, glycerol addition can be used to increase growth rates and biomass production. Hence, it was of interest to determine if glycerol addition could enhance lipid production.

When the amount of lipid produced as a percentage of total dry weight is examined for the strains grown under both mixotrophic and photoautotrophic conditions, it can be seen that more than half of the strains (eight; PCH02, PCH05, PCH11, PCH20, PCH29, PCH30, MA1A08 and MA1A21) had a significantly higher lipid content when grown under mixotrophic conditions (Figure 2A). While most strains showed increases that were 30 to 50% above the content seen under photoautotrophic conditions, two strains, MA1A08 and PCH02, doubled and tripled their lipid content under mixotrophic conditions (Figure 2A). Since the only difference in the two growth conditions was the presence of glycerol, this suggests that these algal strains largely utilise the assimilated glycerol for lipid production. At any rate, these results show that for the majority of strains examined, lipid content is very significantly enhanced by mixotrophic growth on glycerol. Moreover, in the presence of glycerol, six

strains, PCH02, PCH05, PCH11, PCH20, PCH29 and MA1A21 showed a lipid content of above 20%, making them potentially interesting strains for further development for biofuels production.

Of course, for practical biofuel production, the volumetric lipid production, a function of both per cent lipid content and total biomass production, would be a very important consideration. Some interesting observations in this regard can be made when this is examined with these strains (Figure 2B). For one thing, total lipid production was highly variable, differing greatly between the various strains. Some produced only minimal amounts of neutral lipids under all conditions and thus can be eliminated from consideration for biofuels production. From this point of view, six strains were of some interest since they produced at least 0.1g/L neutral lipid and three strains, PCH05, PCH20 and PCH29, might be of particular interest in that they produced more than 0.2g/L neutral lipids (Figure 2B). Total lipid production in these strains was 40 % to 60 % higher under mixotrophic conditions. It should be pointed out that these levels of production were obtained without special measures, e.g. nitrogen deprivation, being taken to enhance lipid production. Another strain of potential interest is PCH02, which, although having only a modest level of total lipid production (0.14 g/L (mixotrophic)), showed a very remarkable nearly six-fold increase in the presence of glycerol (Figure 2B).

FAME characterisation showed algal strains were capable of producing substantial amounts of interesting lipids for biodiesel production. In general strains produced Oleic acid, palmitic acid and Stearic acid (Table 2). All of these fatty acids are compatible for biodiesel production (*Hoekman et al, 2012*). Oleic acid is a monounsaturated omega-9 fatty acid and palmitic acid is a 16-carbon long heavily saturated fatty acid. Moreover, most strains

produced higher amounts of oleic acid, followed by palmitic acid and then by stearic acid. Overall, oleic acid percentages did not change when strains were grown in mixotophy or photoautotrophic conditions. One exception was found. PCH32 had almost a 2-fold decrease in oleic acid content when grown in mixotrophic conditions (Table 2). On the other hand, palmitic acid content changed depending on growth conditions and algal strain. Thus, PCH20, PCH30 and PCH32 increased palmitic acid production by 16%, 33% and 42% when grown under mixotrophy. Conversely, PCH05, PCH11 and PCH29 increased palmitic acid production by 47%, 20% and 50%, under photoautotrophic conditions. In the case of stearic acid, PCH11 increased stearic acid production under mixotrophic conditions by almost 2-folds, whereas PCH21Y increased by 57%, under photoautotrophic conditions. The other strains had relatively similar content in terms of stearic acid. Unfortunately, not all strains gave results and thus five strains showed no detectable FAMEs. This might have been due to an error in manipulation during the wet lipid extraction procedure.

3.1.2. Phylogenetic Analysis

Since the strains examined showed great differences in their growth and lipid production under photoautotrophic conditions and in their response to the presence of glycerol, it was of interest to determine their phylogenetic relatedness to each other and to known strains. Genomic regions of each strain corresponding to the *18S rRNA* and *rbcL* genes were amplified by PCR, sequenced and phylogenetic trees were constructed using the MEGA program (*Tamura et al, 2011*). The phylogenetic analysis using the determined *18S rRNA* sequences shows that all the strains examined belong to the Chlorophyta phylum, also known as the green algae (Figure 3). This analysis shows that PCH05, PCH11, PCH20 and PCH29

are all similar and closely related to *Chlorella vulgaris* a finding that would appear to agree with the lipid, biomass and growth data, since all of these strains showed similar results. PCH02, which had appeared different from the four strains closely related to *C. vulgaris* in that it showed a very large difference in lipid accumulation under the two conditions, is also a member of the *Chlorella* genus. However, it was more closely associated to *Chlorella sorokiniana*. PCH28 belongs to the *Hindakia* genus, being most closely related to *Hindakia fallax*. The *Hindakia* and *Chlorella* genera are both members of Chlorellaceae (*Guiry, M.D et al, 2014*). Consequently, an outlier group was created in the tree, which shared a common ancestor with the *Chlorella* and *Hindakia* genera. The outlier group contained the *Prasiola* and *Lobosphaera* genera. These two genera along with the *Chlorella* and *Hindakia* genera all belong to Trebouxiophyceae class in the Chlorophyta phylum (*Guiry, M.D et al, 2014*). Three strains, PCH21Y, PCH21G and PCH30, were shown to be relatively poor lipid producers (Figure 2A and B) and the phylogenetic analysis showed them to be very closely related to each other as well as being closely related to *Acutodesmus obliquus,* which belongs to the Scenedesmaceae family of the Chlorophyta phylum. Likewise, strain PCH32 belongs to the related *Scenedesmus* genus and is most closely related to *S. abundans*. The analysis of the *rbcL* gene suggests that MA1A08 is closely related to the *Desmodesmus* genus (results not shown) and thus the most related to strain PCH32 amongst the strains examined here.

The strain MA1A21 is most closely related in this analysis to *Mychonastes rotundus*. The *Mychonastes* and *Monoraphidium* genera represent an outlier group to the Scenedesmaceae family, with *Mychonastes* belonging to the Mychonastaceae family and *Monoraphidium* belonging to the Selenastraceae family, all of which belong to the Sphaeropleales order (*Guiry, M.D et al, 2014*). Since the Sphaeropleales order belongs to the

Chlorophyceae class, it is an outlier group to the Trebouxiophyceae class, which contains PCH02, PCH05, PCH11, PCH20 and PCH28, and thus MA1A21, PCH32, PCH30, PCH21G and PCH21Y are quite different phylogenetically from them.

Thus, screening with 25mM of glycerol showed that it could be used as a carbon-source for mixotrophic growth for most of the algal strains examined here. Phylogenetic analysis demonstrated the high diversity of strains within the Chlorophyta phylum that were used for this initial screen. Furthermore, many strains studied showed enhanced lipid production under these conditions, with PCH02, PCH05, PCH11, PCH20 and PCH29, all within the *Chlorella* genus, seeming to demonstrate the most promise for industrial cultivation due to their high biomass production, high growth rates and high lipid production under mixotrophic conditions.

3.2. Nitrogen Depletion and Accumulation of Neutral Lipids

Many algal species have been shown to accumulate lipids under different stress conditions, in particular under nitrogen deprivation (*Breuer et al, 2013; Boyle et al, 2012; Rios et al, 2014; Recht et al, 2014*). Fixed nitrogen is essential for the biosynthesis of a variety of required cellular components and hence, in its absence cell growth is necessarily arrested. However, in the presence of light, photosynthesis and other metabolism continue to function and fixed carbon is accumulated as storage material since it cannot be used in the absence of nitrogen for cell growth. Therefore, it was of interest to determine the pattern of lipid accumulation in the presence and absence of glycerol when the cells were subjected to nitrogen deprivation.

Three different strains from the previous screen (see above) were selected for further analysis based on their lipid production patterns. Strain PCH02 had previously shown both a good growth rate and significant biomass production when grown with glycerol. It also had, out of all of the strains tested, the largest lipid increase under mixotrophic conditions. Strain PCH05, in addition to also showing an increase in lipid production in presence of glycerol, had shown the highest lipid production for both growth conditions. In addition, phylogenetic analysis had shown that this strain is closely associated with *Chlorella vulgaris* and phylogenetically distinct from strain PCH02. Finally, strain PCH28 was selected as a strain that had shown a different pattern when screened in that it, unlike strains PCH02 and PCH05, had only low lipid production under all growth conditions tested.

Active cultures with appreciable amount of biomass were obtained by growing the selected strains under nitrogen-replete conditions for seven days at which point they had attained ODs ranging from 0.63 to 0.88 (0.622 to 0.884 g/L). The cultures were collected by centrifugation, re-suspended in medium lacking nitrogen with 25mM glycerol (mixotrophic conditions) and without glycerol (photoautotrophic conditions) and incubated for three weeks. When changes in OD (optical density) were followed, two different phases can be observed (Figure 4). There was an initial phase, lasting from inoculation to the first day and a half where additional growth occurred. For example, strain PCH02 had an OD of 0.737 at inoculation, but after 36 hours the OD had increased to 1.336. After this initial period of growth, the strains entered a phase where the OD was stationary and no additional growth occurred. It is reasonable to assume that, since the algal cultures had been grown under nitrogen-replete conditions, sufficient nitrogen stores were available after re-suspension in

nitrogen-free medium to permit one or two cell doublings before growth arrest due to nitrogen depletion.

When total lipid production over the period of incubation under nitrogen-free conditions is examined a number of interesting observations can be made. First, strain PCH28 showed very little increase in lipid production throughout the nitrogen starvation period under both conditions, essentially staying constant (Figure 5). In fact, lipid production with this strain under the nitrogen deprivation conditions used here was only slightly higher than that observed under the conditions used for the initial screening. Thus, it would appear that strain PCH28 did not respond to the lack of nitrogen stress applied here, and, in accordance with this, this strain remained green throughout the incubation period, therefore showing no signs of the chlorophyll degradation, which is usually induced by nitrogen starvation.

On the other hand, by the end of the incubation period, strain PCH02 had shown a marked response to incubation in nitrogen-free medium with increased lipid production over the period of nitrogen starvation for both mixotrophic (13 fold) and photoautotrophic conditions (12 fold) (Figure 5). Lipid production under both conditions increased very little for the first 10 days, dramatically increasing afterwards for mixotrophic growth and photoautotrophic growth conditions and reaching levels as high as 0.5 to 0.7 g/L.

Strain PCH05 also showed a lipid production response to nitrogen limitation. When examined under the same conditions used for strains PCH28 and PCH02, strain PCH05 gave a large difference in total lipid production, with lipids accumulating too much higher levels in the presence of glycerol than in its absence (Figure 5). This is somewhat surprising since this was not the case in the previous experiment where the cultures were not subjected to a sudden nitrogen limitation.

Thus, when these three strains were re-suspended in nitrogen-free medium, subsequent incubation under nitrogen starvation had different effects on lipid production, which seemed to give species-specific responses and to be different for the two different conditions. The direct inoculation of algal samples into nitrogen-depleted medium might have contributed to low lipid yields for some of the strains. As we mentioned before, certain algal species produce greater lipid quantities when nitrogen concentrations are depleted naturally. Thus, our next experiment examined the effect of different nitrogen concentrations under photoautotrophic and mixotrophic conditions on lipid production.

3.3. Lipid Production of PCH02 and PCH05 for different Concentrations of nitrogen under Photoautotrophic and Mixotrophic Growth Modes

A study by Stephenson et al found that C. vulgaris was more effective at producing high quantities of lipids when cells depleted their nitrogen source naturally instead of being transferred into a nitrogen depleted medium directly (*Stephenson et al, 2010*). Thus, a similar experiment was conducted with PCH02 and PCH05, wherein these strains were grown under different concentration of nitrogen and mixotrophy with 25mM of glycerol. Photoautotrophic conditions were also examined as a control to the mixotrophic conditions. Sodium nitrate was used as nitrogen source and the following concentration were used: 0mg/L, 20mg/L, 40mg/L, 60mg/L, 80mg/L, 100mg/L and 250mg/L. Cultures with 250mg/L of nitrate corresponded to nitrogen-replete conditions. Each condition was performed in triplicate.

3.3.1. Biomass Production and Growth Rate Analysis for PCH02 and PCH05

When comparing maximum growth rates and biomass production, both strains presented different responses to growth under mixotrophic and photoautotrophic conditions (Table 3). The maximum growth rate was indifferent for PCH05 grown in mixotrophy and photoautotrophy and was the same for all nitrate concentration. For instance, between a nitrate concentration of 20mg/L and 250mg/L, the average growth rate was around 0.632 $days^{-1}$ and 0.637$days^{-1}$ for mixotrophy and photoautotrophy, with less than ten per cent variation. On the other hand, PCH02 had in general higher growth rates under mixotrophy than photoautotrophy, with increases up to 65% (Table 3). Additionally, growth rates increased as nitrate concentration increased in both growth conditions for this same strains. However, mixotrophic growth had intense increase in growth rates as opposed to photoautotrophic growth.

For both strains, biomass increased as nitrate concentration increased in both conditions. Thus, nitrogen-replete conditions had the highest biomass production. This was expected, as nitrogen sources are generally used for protein and DNA synthesis and, thus, are necessary for cellular division. Interestingly enough, PCH05 consistently produced higher biomass under mixotrophy at all nitrate concentration (increases up to 77%). On the other hand, PCH02 showed different stages in biomass production depending on nitrate concentrations. At low levels of nitrate (below 60mg/L), biomass production was similar (less than 11% difference) in both conditions. However, at higher levels of nitrate (60mg/L and above), biomass production was much higher under mixotrophic conditions than in photoautotrophic conditions.

Since glycerol was consumed, these findings suggest that both strain assimilate glycerol in different ways. Mixotrophic growth seems to increase biomass production and makes growth faster for PCH02. Thus, this would suggest that PCH02 probably uses glycerol for both growth and lipid production. On the other hand, PCH05 had very similar growth rates and only increased in biomass production. This would suggest that glycerol would be used for storage compound production, such as TAGs.

Thus, it was of interest to further investigate neutral lipid production and lipid content for these two strains.

3.3.2. Lipid Production for PCH02 and PCH05

Neutral lipid measurements were conducted using a Nile red assay at different time points of growth for the two strains. These measurements were done in triplicates. Volumetric lipid production represents the total amount of lipid produced per volume of culture, whereas specific lipid production represents the lipid production per volume of culture per density of culture.

In general, volumetric lipid production was higher for mixotrophic growth than photoautotrophic growth for both strains (figure 6 and 9). For instance, in figure 6A, after 4 days of growth, lipid production for PCH02 ranged from 0.01g/L to 0.06g/L under mixotrophic conditions. In contrast, lipid production stayed around 0.01g/L under photoautotrophic conditions at day 4 (figure 6A). Thus, glycerol was most probably used for lipid production.

Furthermore, lipid production increased during the cultivation period for both strains. As a result, strains in the stationary phase had a much higher lipid production than at the beginning of growth. This was seen for total lipid production (figure 6 and 9) and specific

lipid production (figure 7 and 10) for both strains. For instance, PCH02 grown under mixotrophy with 80mg/L of sodium nitrate (Figure 7E) at day 4 had specific lipid production of around 100μg/ml/OD. However, when this strain entered the stationary phase, its specific lipid production increased upwards of 250μg/ml/OD, around 2.5 fold increase (Figure 7E). This was also seen for PCH05 (figure 10). This indicates that algal strains accumulate lipids through out growth and that lipid content increases with time.

Interestingly, lipid production patterns under the varying nitrate concentrations, for both strains, were different. Under mixotrophy, PCH02 increased lipid production as nitrate concentration increased. This was seen at day 4 and when cultures entered the stationary phase (Figure 6A). Thus, the highest production was reached at 250mg/L of nitrate concentration (0.06g/L at day 4 and 0.322g/L in stationary phase). Under photoautotrophy, PCH02 had a different neutral lipid production pattern. At day 4, lipid production was constant, around 0.01g/L for the different nitrate concentrations (Figure 6A). However, in the stationary phase, lipid production was highest at a nitrate concentration of 20mg/L (0.09g/L) and decreased slightly as nitrate concentration increased, down to 0.03g/L neutral lipids produced at 250mg/L of nitrate concentration (Figure 6B). These findings suggest that nitrogen starvation did not induce lipid accumulation under mixotrophy, but, did induce lipid accumulation under photoautotrophy. This is further corroborated with lipid content of PCH02 under mixotrophy and photoautotrophy in the stationary phase (Figure 7 and 8A). Under mixotrophy, lipid percentage was very similar (around 30%), less then ten per cent difference, for each of the different nitrate concentration (Figure 8A). Under photoautotrophy, lipid content was highest at 33% for strains grown at 20mg/L of nitrate concentration (Figure 8A). Following this, lipid content decreased gradually as nitrate concentration increased.

On the other hand, lipid production patterns were very different for PCH05. In the beginning of cultivation (day 7), lipid production levels were highest at lower nitrate concentrations for both growth modes (Figure 9A). Thus, under mixotrophy, lipid production levels were highest at 20mg/L of nitrate concentration, producing 0.07g/L of neutral lipids. After this concentration, lipid production decreased progressively, going as far down as 0.02g/L under nitrogen-replete conditions (250mg/L). The same can be seen under photoautotrophic conditions, however, the highest production peak shifts to 40mg/L and production is much lower, around 0.04g/L (Figure 9A). However, after 7 days of growth, lipid production patterns changed completely. Thus, at 11 days, lipid production reached a maximal production peak (0.12g/L for mixotrophy and 0.10g/L for photoautotrophy) between 40mg/L and 80mg/L for both mixotrophy and photoautotrophy (Figure 9B). Above 80mg/L and below 40mg/L, lipid production diminished gradually. This lipid production pattern was changed again, when strains entered the stationary phase (Figure 9C). Accordingly, under mixotrophy, the highest lipid production was reached at 80mg/L, with 0.369g/L of lipid produced (Figure 9C). At higher or lower concentration of nitrate, lipid production decreased. However, an increase in lipid production is seen from 100mg/L to 250mg/L of nitrate (Figure 9C). This same pattern is seen for photoautotrophic conditions, however, at 250mg/L, lipid production is much higher than at 80mg/L.

Lipid production at stationary phase for PCH05 was further investigated. It is clear that lipid production at 80mg/L of nitrate concentration and at 250mg/L were very similar (Figure 9C), for both conditions. However, lipid content was much higher at 80mg/L than at 250mg/L for both conditions (Figure 11A). This indicates that the increase in lipid production in nitrogen-replete conditions is probably due to the higher biomass production. Lipid production

is a function of lipid content and biomass production. As we mentioned before, biomass production increased with higher nitrate concentrations. Thus, for nitrogen-replete conditions, lipid content was lower but total biomass produced was higher, causing similar production levels as that of 80mg/L. As a general rule, lower nitrogen concentrations, between 0mg/L and 80mg/L, increased lipid content (Figure 11A), but lowered total biomass production (Table 3). This resulted in lower lipid production levels for lower nitrate concentrations. It was only at 80mg/L nitrate concentration that trade off was found, wherein, lipid content and biomass production were high enough to compete with nitrogen-replete conditions.

Finally, lipid productivity supported the findings mentioned previously for both strains Figure 8B and 11B). Mixotrophic growth induced higher productivity levels than photoautotrophic conditions for the two algal strains. Furthermore, lipid productivity was different for mixotrophic growth and photoautotrophic for PCH02, whereas, PCH05 had similar productivity patterns in both conditions. PCH02 increased productivity levels as nitrate levels increased, with the highest productivity (40.26mg/L/day) at 250mg/L (nitrogen replete conditions), under mixotrophic conditions (Figure 8B). However, in photoautotrophic conditions, lipid productivity decreased as nitrate concentrations increased, with the highest productivity at 20mg/L of nitrate at 11.5mg/L/day of lipids produced (Figure 8B). In contrast, the highest productivity was found at 80mg/L of nitrate for both conditions for PCH05 (Figure 11B). Higher and lower concentrations of nitrate generated progressively smaller productivities. Mixotrophic growth induced higher productivity, at 21mg/L/day lipids, whereas, photoautotrophic generated 15.85mg/L/day lipids. These measurements are practical for industrial purposes, as they help set optimal conditions for maximal lipid production.

Conclusion

In conclusion, three different experimental designs were tested to examine the effects of mixotrophic growth for lipid production. The initial screening showed that all strains were capable of consuming glycerol but only certain strains were capable of enhancing lipid production, under mixotrophy. Furthermore, depending on strains, glycerol was assimilated in different ways. Thus, glycerol could either be used for growth, by increasing growth rates and biomass, or it could be used for lipid production. Phylogenetic analysis revealed that the algal strains were very diversified, but still belonging to the *Chlorophyta* phylum. As a result, strains belonged to the *Chlorella* genus, the *Acutodesmus*, the *Scenedesmus*, the *Mychonastes* genus and the *Hindakia* genus. Furthermore, nitrogen starvation under mixotrophic growth induced higher lipid production than under photoautotrophic conditions, for strains PCH02 and PCH05. The lipid accumulation was dependant on the amount of time spent in nitrogen deplete conditions. Thus, three weeks incubation was required for maximal lipid production. This had an effect on lipid productivity. Not all strains tested increased lipid production under nitrogen starvation, and thus, this phenomenon is dependant of the strains tested. Moreover, lipid production of PCH02 and PCH05 was investigated when strains were grown at different nitrate concentration and under mixotrophy. The results indicated biomass production was dependant on nitrate concentration but growth rates were unaffected. Growth rates were affected by the addition of glycerol for PCH02. Lipid production patterns were different for both strains and evolved during growth. Under mixotrophy, PCH02 increased lipid production as nitrate concentration increased, with the highest production level under nitrogen-replete conditions. Under photoautotrophy, this same strain showed highest lipid production at

20mg/L of nitrate concentration, after which, production decreased progressively as nitrate concentration increased. On the other hand, PCH05 had peak in lipid production at 80mg/L of nitrate concentration for both growth conditions. Higher or lower nitrate concentration showed decreased lipid production. These findings indicate that mixotrophy, using glycerol, a waste product of the biodiesel industry, can be considered for industrial production of biodiesel at a cheaper coast than other more expensive carbon sources, such as glucose. Thus, microalgae are a prospective source of biofuel. Several perspectives can be considered to complement the work presented here. As only one concentration of glycerol was used (25mM), it would have been interesting to study the effects of lipid production using different glycerol concentrations (higher or lower). Also, different carbon sources (ideally waste products), such as xylose, acetate or glucose, could have been examined for mixotrophic growth and enhanced lipid production. Finally, very little is known about how the different fatty acids (composing TAGs) are produced or how their production is regulated. Biodiesel requires fatty acids carbon tails containing 12 to 18 carbons atoms. Thus, an interesting project would be to understand certain of the mechanisms that permit production of fatty acids adapted for biodiesel production.

Tables and Figures

Table 1: Final biomass, maximum specific growth rates and glycerol consumption for the strains grown under mixotrophic (with 25mM of glycerol) and photoautotrophic conditions (Mixotrophic screening).

Algal Strain	Biomass (g L^{-1})			Growth rate (day^{-1})			Glycerol consumption (%)
	Mixotrophy	Photoautotrophy	Fold	Mixotrophy	Photoautotrophy	Fold	
PCH02	0.566 +/- 0.033	0.296 +/-0.040	1.92	0.258 +/-0.037	0.169 +/-0.024	1.53	94.19 +/-1.655
PCH05	0.725 +/-0.078	0.719 +/-0.076	1.01	0.283 +/-0.032	0.175 +/-0.037	1.62	94.38 +/-0.265
PCH11	0.708 +/-0.029	0.633 +/-0.005	1.12	0.231 +/-0.001	0.199 +/-0.040	1.16	94.32 +/-0.346
PCH20	0.831 +/-0.006	0.712 +/-0.040	1.17	0.227 +/-0.006	0.266 +/-0.074	0.86	93.98 +/-0.401
PCH21G	0.681 +/-0.009	0.619 +/-0.030	1.08	0.257 +/-0.031	0.505 +/-0.047	0.51	95.10 +/-0.342
PCH21Y	0.903 +/-0.08	0.862 +/-0.042	1.05	0.366 +/-0.043	0.315 +/-0.018	1.16	95.28 +/-0.224
PCH28	0.559 +/-0.066	0.574 +/-0.039	0.97	0.240 +/-0.008	0.230 +/-0.023	1.04	95.15 +/-0.535
PCH29	1.018 +/-0.08	0.908 +/-0.045	1.12	0.251 +/-0.034	0.307 +/-0.035	0.82	95.22 +/-0.146
PCH30	0.632 +/-0.025	0.737 +/-0.024	0.86	0.218 +/-0.025	0.286 +/-0.039	0.76	95.63 +/-0.312
PCH32	0.854 +/-0.018	0.844 +/-0.057	1.01	0.372 +/-0.036	0.240 +/-0.041	1.55	95.27 +/-0.197
MA1A08	0.159 +/-0.005	0.150 +/-0.014	1.06	0.105 +/-0.005	0.099 +/-0.01	1.06	96.45 +/-0.723
MA1A21	0.497 +/-0.018	0.382 +/-0.009	1.30	0.141 +/-0.031	0.058 +/-0.029	2.44	96.17 +/-0.174

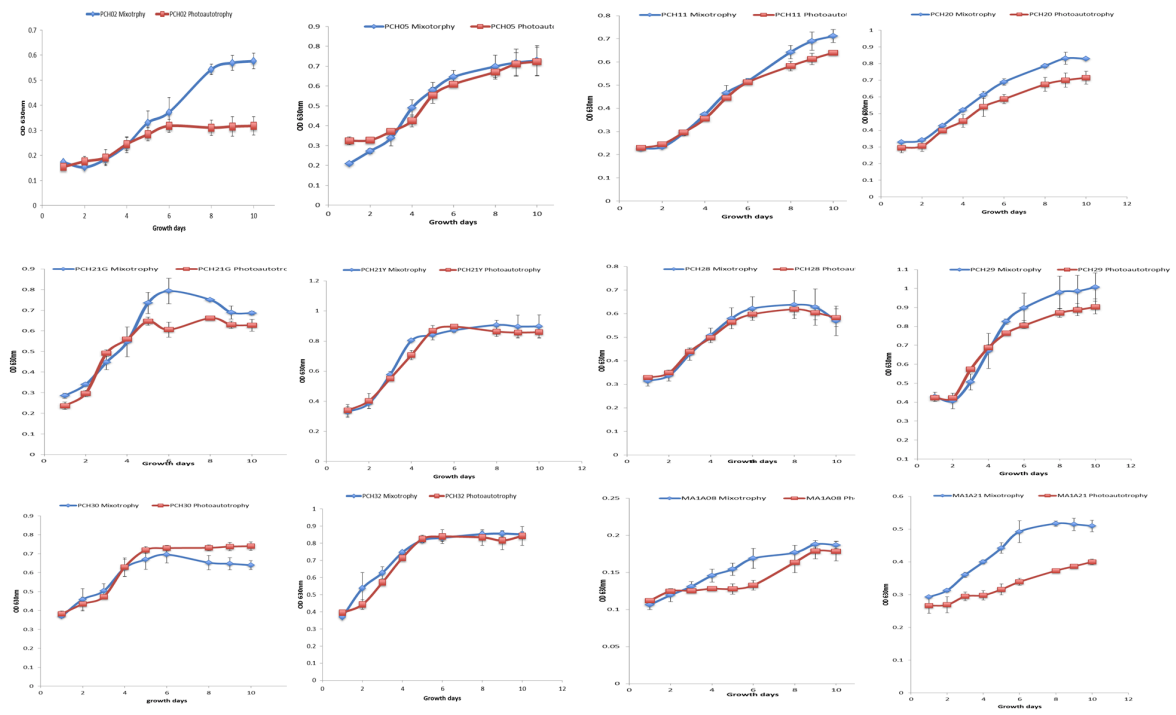

Figure S1: Growth curves of twelve algal strains grown under mixotrophic (blue) conditions, with 25mM of glycerol, and photoautotrophic (red) conditions in Bold's Basal Medium (BBM) under continuous light at 40W/m^2 and at room temperature. From left to right starting from the top, the algal strains are: PCH02, PCH05, PCH11, PCH20, PCH21G, PCH21Y, PCH28, PCH29, PCH30, PCH32, MA1A08 and MA1A21. The y-axis corresponds to optical density at 630nm and the x-axis represents the number of growth days.

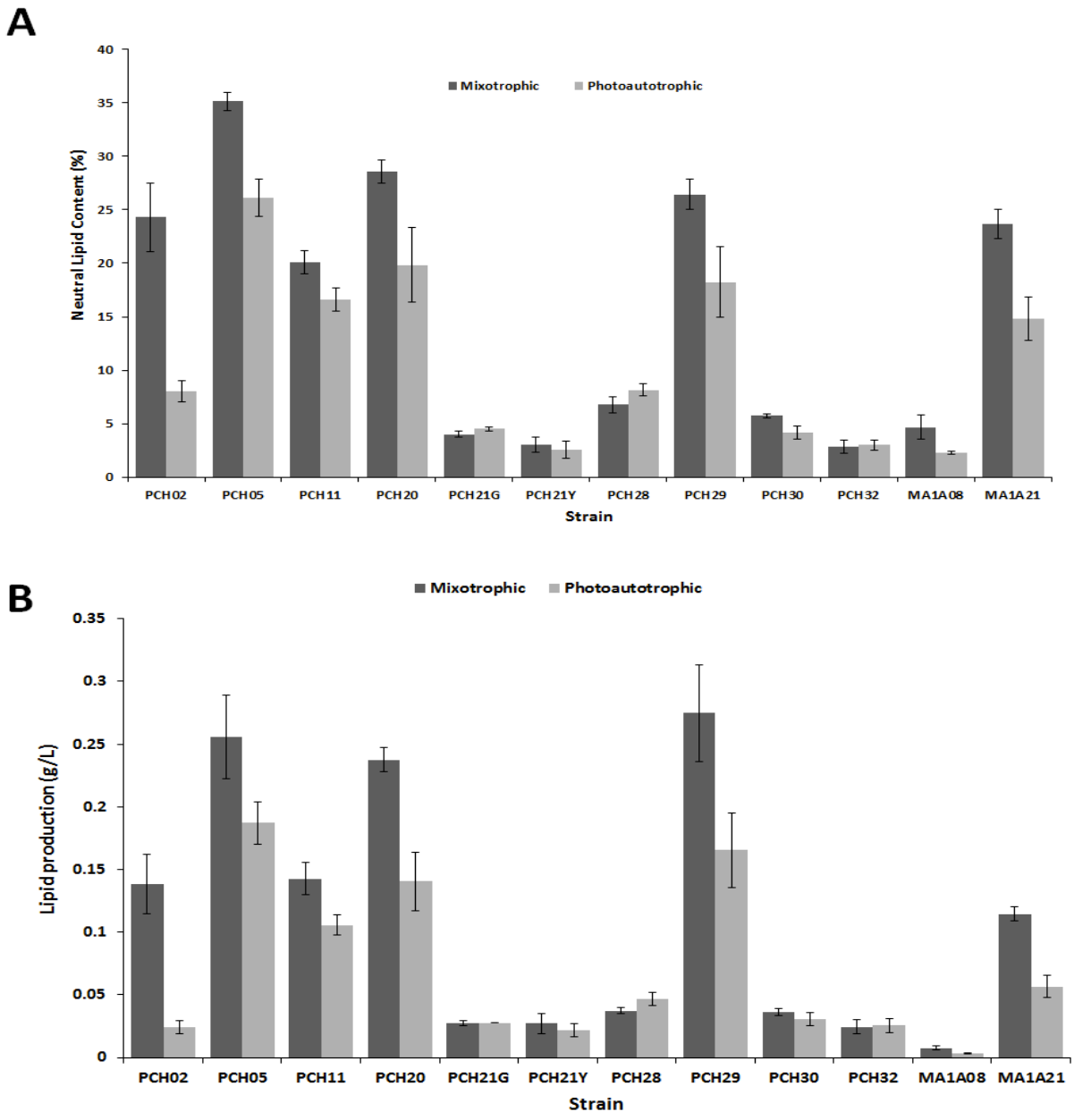

Figure 2: A) Lipid percentage by dry weight for each algal strain grown under mixotrophic conditions, with 25mM of glycerol, and photoautotrophic conditions for ten days under continuous light at 40W/m^2 at room temperature. B) Volumetric lipid production (g/L) for each algal strain grown under mixotrophic conditions, with 25mM of glycerol, and photoautotrophic conditions for ten days under continuous light at 40W/m^2 at room temperature.

Table 2: Fatty Acid Methyl Esters compositions (%) for several strains grown under mixotrophic conditions, using 25mM of glycerol, and photoautotrophic conditions in Bold's Basal medium.

Algal strains	Palmitic acid (16:0)		Oleic acid (18:1)		Stearic acid (18:0)	
	Mixotrophy	Photoautotrophy	Mixotrophy	Photoautotrophy	Mixotrophy	Photoautotrophy
PCH05	19	28	59	56	18	17
PCH11	30	25	52	58	22	12
PCH20	29	25	64	67	7.2	7.8
PCH21Y	34	31	52	58	7	11
PCH29	14	21	30	36	3	3.3
PCH30	60	45	40	55	0	0
PCH32	60	42	22	41	18	16

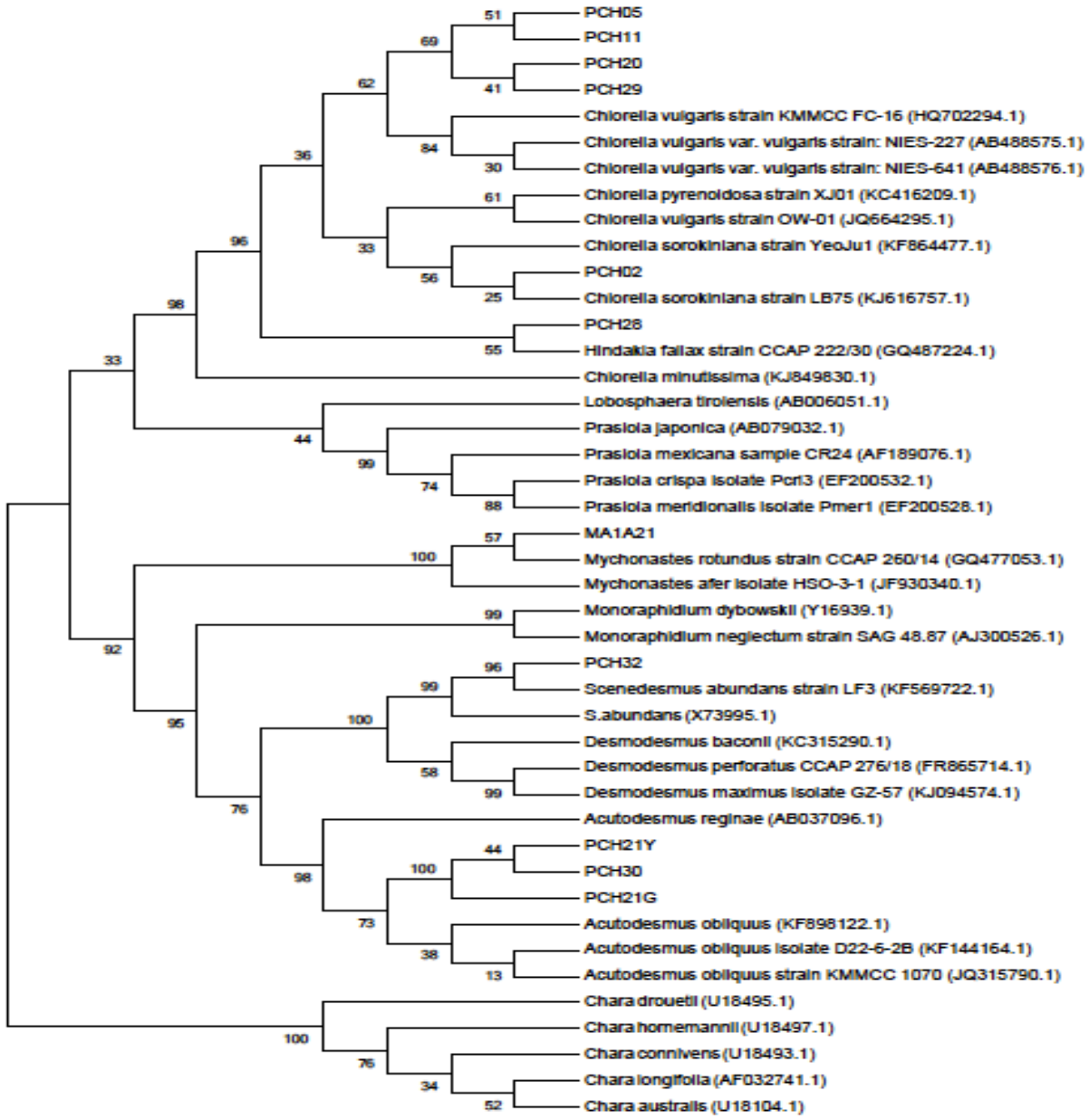

Figure 3: Neighbour joining tree showing phylogenetic relationship between each algal strains. This phylogenetic tree is a neighbour joining tree using Jukes-Cantor model with 1000 bootstraps. Each number on the tree represents the bootstrapping values. Bootstrapping is a resampling method and helps to assign measure of accuracy to sample estimates. The values on the trees are the bootstrapping values and thus represent the accuracy percentages.

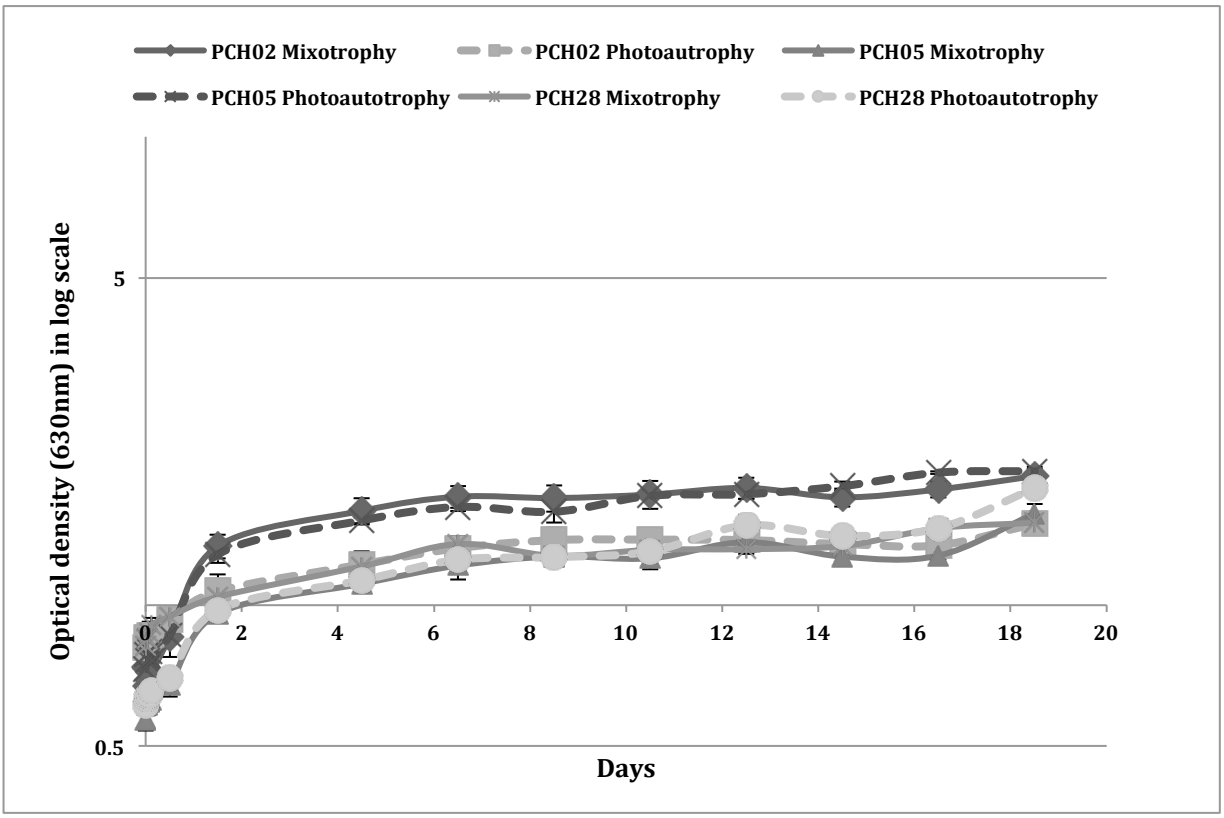

Figure 4: Mixotrophic and photoautotrophic growth under nitrogen deplete BBM at room temperature, with constant shaking at 150RPMs and constant light at 40W/m2, for three algal strains (PCH02, PCH05 and PCH28). Mixotrophic conditions used BBM with 25mM of glycerol whereas photoautotrophic conditions used BBM without any nitrogen source or any glycerol.

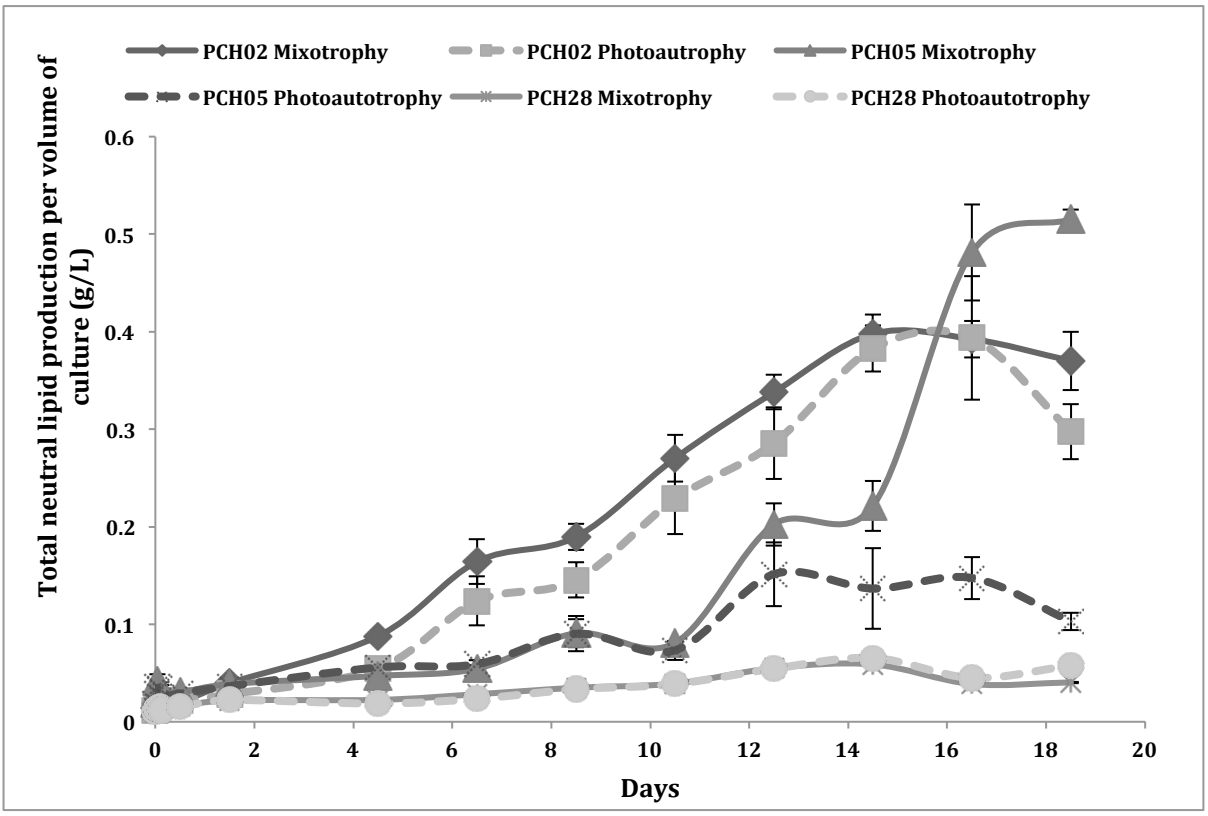

Figure 5: Total lipid production for PCH02, PCH05 and PCH28 under nitrogen starvation. The algal stains were grown in mixotrophic conditions in BBM with 25mM of glycerol and under photoautotrophic conditions, without any glycerol.

Table 3: Biomass production per volume of culture medium (g/L), growth rates and glycerol consumption (%) for algal strains PCH02 and PCH05, grown at different concentration of sodium nitrate (mg/L) and under photoautotrophic conditions and mixotrophic conditions, with 25mM of glycerol.

Strains	Sodium nitrate concentration (mg/L)	Biomass (g/L)			Growth rate ($days^{-1}$)			Glycerol consumption (%)
		Mixotrophy	Photoautotrophy	Fold	Mixotrophy	Photoautotrophy	Fold	
PCH02	0	0.055	0.062	0.89	0.440	0.525	0.84	15.25.
	20	0.272	0.281	0.97	0.817	0.782	1.04	21.39
	40	0.397	0.401	0.99	0.895	0.733	1.22	46.12
	60	0.515	0.444	1.16	1.032	0.756	1.37	42.19.
	80	0.662	0.498	1.33	1.062	0.760	1.40	47.88
	250	1.556	0.864	1.34	1.371	0.832	1.65	70.67
PCH05	0	0.036	0.020	1.80	0.337	0.325	1.037	97.58
	20	0.202	0.160	1.26	0.601	0.612	0.982	97.74
	40	0.320	0.236	1.36	0.611	0.646	0.946	98.26
	60	0.564	0.489	1.15	0.597	0.649	0.920	98.17
	80	0.700	0.676	1.03	0.667	0.663	1.006	98.08
	100	0.829	0.753	1.10	0.663	0.591	1.122	97.97
	250	1.784	1.493	1.19	0.653	0.664	0.983	98.21

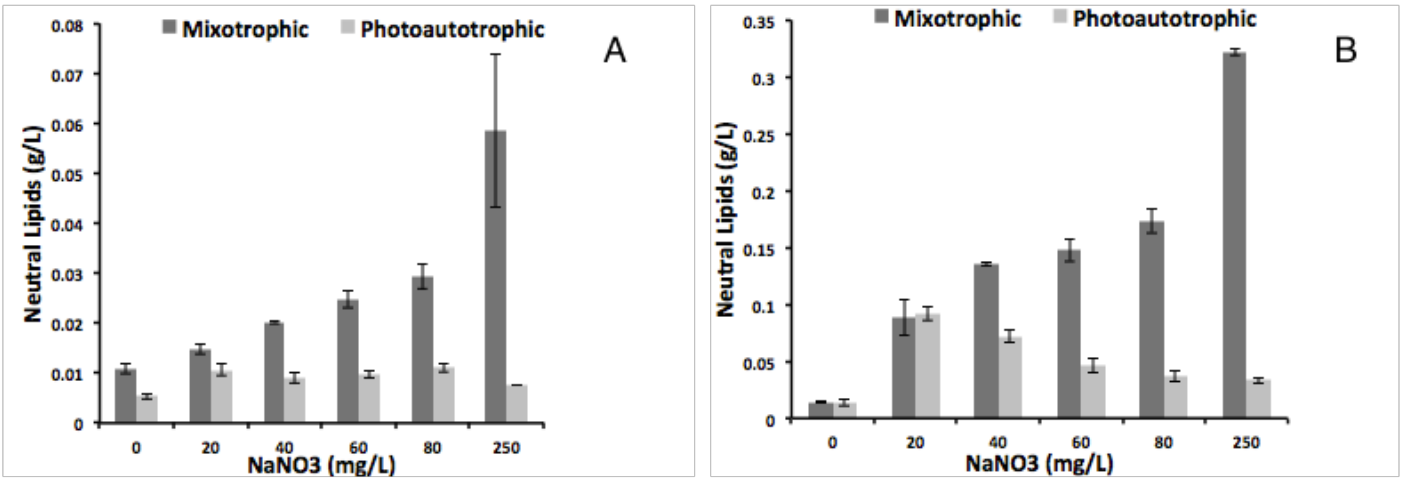

Figure 6: Volumetric lipid production for PCH02 grown at different concentrations of sodium nitrate under mixotrophy (25mM of glycerol) and photoautotrophy, at day 4 (A) and when strains entered stationary phase (B).

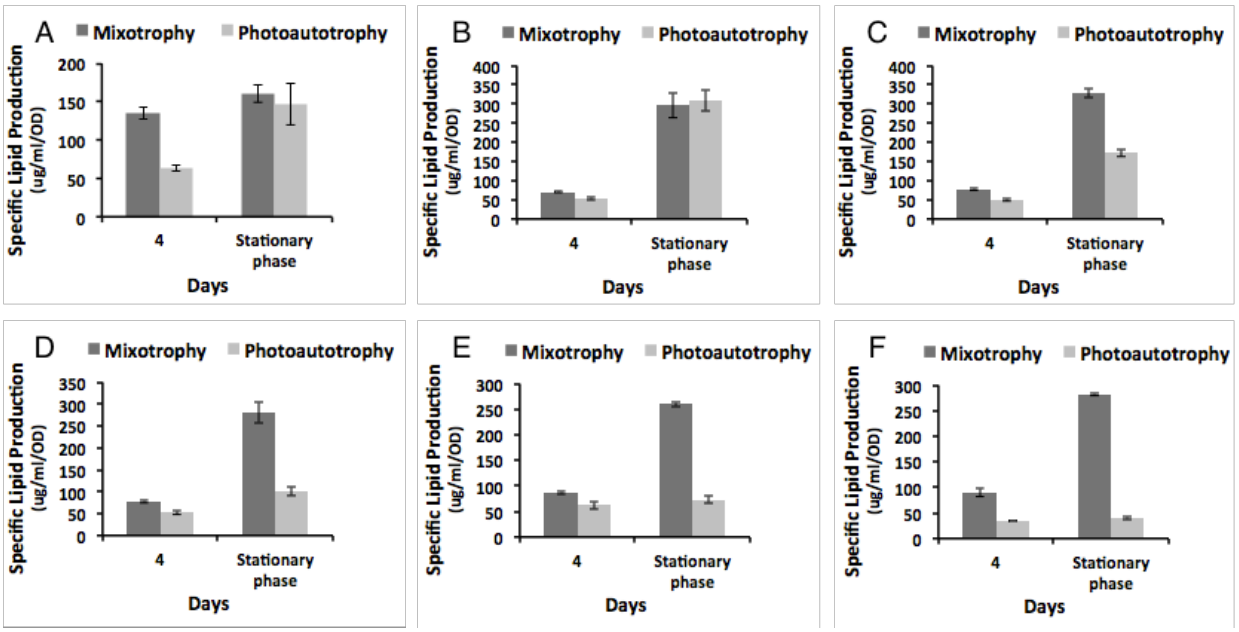

Figure 7: Specific lipid production (µg/ml/OD) for PCH02 after 4 days of growth and when strains entered stationary phase and at (A) 0mg/L, (B) 20mg/L, (C) 40mg/L, (D) 60mg/L, (E) 80mg/L, (F) 250mg/L of sodium nitrate concentration in growth medium.

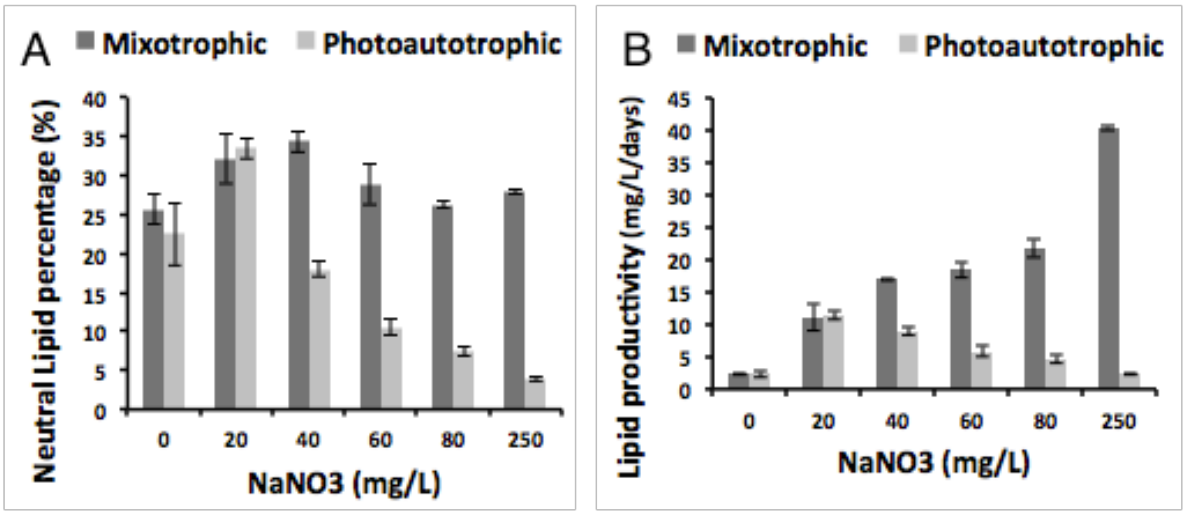

Figure 8: Lipid content (A) and Lipid productivity (B), in mg/L/day, for algal strain PCH02. This strain was grown at different concentration of sodium nitrate (mg/L) and under mixotrophic, with 25mM of glycerol, and photoautotrophic conditions in Bold's Basal Medium. Results were calculated when strains were in stationary phase.

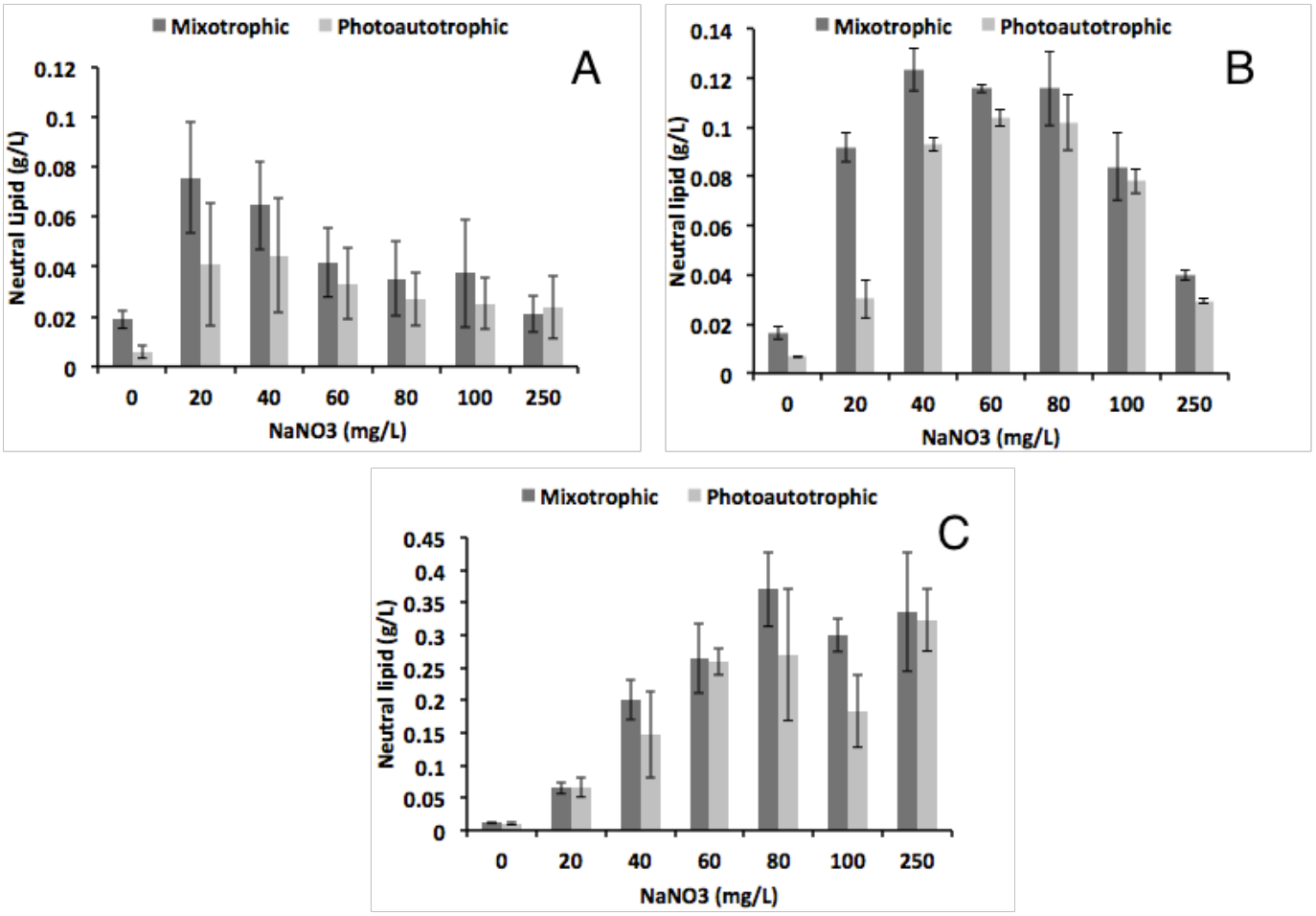

Figure 9: Volumetric lipid production for PCH05 grown at different concentrations of sodium nitrate under mixotrophy (25mM of glycerol) and photoautotrophy, after 7 days of growth (A), after 11 days of growth (B) and in stationary phase (C).

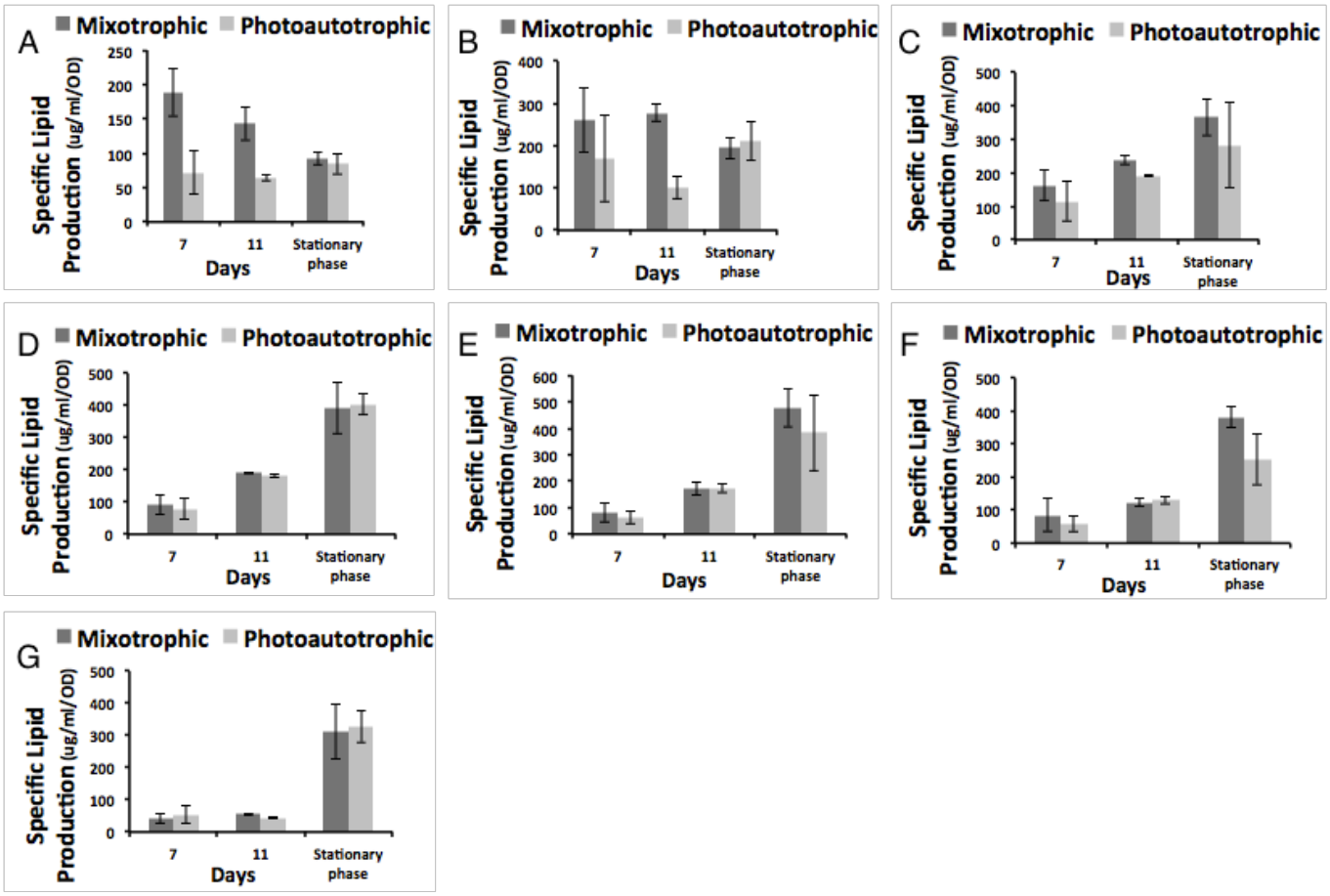

Figure 10: Specific lipid production (µg/ml/OD) for PCH05 at different growth periods and at (A) 0mg/L, (B) 20mg/L, (C) 40mg/L, (D) 60mg/L, (E) 80mg/L, (F) 100mg/L and (G) 250mg/L of sodium nitrate concentration in growth medium.

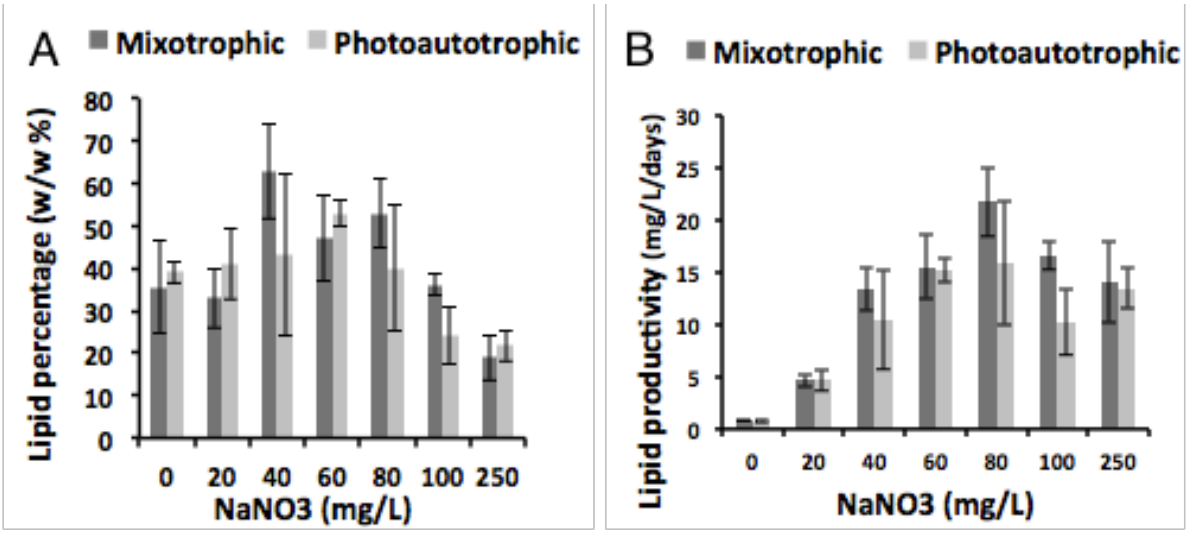

Figure 11: Lipid content (A) and Lipid productivity (B), in mg/L/day, for algal strain PCH05. This strain was grown at different concentration of sodium nitrate (mg/L) and under mixotrophic, with 25mM of glycerol, and photoautotrophic conditions in Bold's Basal Medium. Results were calculated when strains were in stationary phase.

References

1- Abdelaziz, A. E. M, Leite, G. B., and Hallenbeck, P. C. "Addressing the challenges for sustainable production of algal biofuels: I. Algal strains and nutrient supply." *Environmental Technology.* 34 (2013): 1783-1805. Print.

2- Abdelaziz, A. E. M, Leite, G. B., and Hallenbeck, P. C. "Addressing the challenges for sustainable production of algal biofuels: II. Harvesting and conversion to biofuels." *Environmental Technology.* 34 (2013): 1783-1805. Print.

3- Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. *Biologie Moléculaire de la Cellule*. Paris: Médecine-Science Flammarion, 2007. Print.

4- Andersen, R. A., Berges, J. A., Harrison, P. J., and Watanabe, M. M. "Appendix A-Recipes for Freshwater and Seawater Media." *Algal culture techniques.* ED. Andersen, R. A. Burlington, USA: Elsevier Academic Press, 2005. 429-539.

5- Bergman, B., Gallon, J. R., Rai, A. N., and Stal, L. J. "N_2 Fixation by non-heterocystous cyanobacteria." *FEMS Microbiology Reviews*. 19 (1997): 139-185.Print.

6- Bertozzini, E., Galluzzi, L., Penna, A., and Magnani, M., "Application of standard addition method for the absolute quantification of neutral lipids in miccoralgae using Nile Red." *J. Microbiol. Methods.* 87 (2011): 17-23. Print.

7- Beer, L. L., Boyd, E. S., Peters, J. W., and Posewtiz, M. C. "Engineering algae for biohydrogen and biofuel production." *Current Opinion in Biotechnology*. 20 (2009): 264-271. Print.

8- Bondioli, P., and Della Bella, L. "An alternative spectrophotometric method for the determination of free glycerol in biodiesel." *European Journal of lipid Science.* 107 (2005): 153-157. Print.

9- Boyle, N. R., Page, M. D., Liu, B., Blaby, I. K., Casero, D., Kropat, J., Cokus, S. j., Hong-Hermesdorf, A., Shaw, J., Karpowicz, S. J., Gallaher, S. D., Johnson, S., Benning, C., Pellegrini, M., Grossman, A., and Merchant, S. S. "Three Acyltransferases and Nitrogen-responsive Regulator are implicated in Nitrogen Starvation-induced Triacylglycerol Accumulation in *Chlamydomonas*." *Journal of Biological Chemistry*. 287 (2012): 15811-15825. Print.

10- Bensheng, L., and Benning, C. "Lipid metabolism in microalgae distinguishes itself." Current Opinion in Biotechnology. 24 (2013):300-309. Print

11- Brennan, L., and Owende, P. "Biofuels from microalgae- A review of technologies for production, processing, and extraction of biofuels and co-products." *Renewable and Sustainable Energy Reviews*. 14 (2010): 557-577. Print.

12- Breuer, G., Lamers, P. P., Martens, D. E., Draaisma, R. B., and Wijffels, R. H. "The impact of nitrogen starvation on the dynamics of triacylglycerol accumulation in nine microalgae strains." *Bioresource Technology*. 124 (2012): 217-226. Print.

13- Breuer, G., Lamers, P. P., Martens, D. E., Draaisma, R. B., and Wijffels, R. H. "Effect of light intensity, pH and temperature on triacylglycerol (TAG) accumulation induced by nitrogen starvation in *Scenedesmus obliquus*." *Bioresource Technology*. 143 (2013): 1-9. Print.

14- Brunner, E., Richthammer, P., Ehrlich, H., Paasch, S., Simon, P., Ueberlein, S. and Van Pée, K-H. "Chitin-based Organic Networks: An Integral Part of Cell Wall Biosilica in the Diatom *Thalassiosira pseudonana*." *Angewandte Chemie International Edition*. 48 (2009): 9724-9727. Print.

15- Burkholder, J. M., Glibert, P. M. and Skelton, H. M. "Mixotrophy, a major mode of nutrition for harmful algal species in eutrophic waters." *Harmful Algae* 8 (2008): 77-93. Print.

16- Chang, D. W., Hobson, P., Burch, M., and Lin, T. F. "Measurement of cyanbacteria using *in vivo* fluoroscopy- Effect of cyanobacterial species, pigments, and colonies." *Water research*. 46 (2012): 5037-5048. Print.

17- Chapman, Ian. "The end of Peak Oil? Why this topic is still relevant despite recent denials." *Elsevier Energy Policy* (2013). http://dx.doi.org/10.1016/j.enpol.2013.05.010i.

18- Chen, W., Zhang, C., Song, L., Sommerfeld, M., and Hu, Q. "A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae." *Journal of Microbiological Methods*. 77 (2009): 41-47. Print.

19- Chen, Y., and Walker, T. H. "Biomass and lipid production of heterotrophic microalgae *Chlorella protothecoides* by using biodiesel derived crude glycerol."*Biotechnol. Lett.* 33 (2011): 1973-1983. Print.

20- Chisti, Y. "Biodiesel from Microalgae." *Biotechnology Advances* 25 (2007): 294-306. Print.

21- Chisti, Y. "Constraints to commercialization of algal fuels." *Journal of Biotechnology* 167 (2013): 201-214. Print.

22- Courchesne, N. M. D., Parisien, A., Wang, B., Lan, C. Q. "Enhancement of lipid production using biochemical, genetic and transcription factor engineering approaches." *Journal of biotechnology*. 141 (2009): 31-41. Print.

23- Deffeyes, K. S. *When Oil Peaked*. New York: Hill and Wang, 2010. Print.

24- Durkin, C. A., Mock, T. and Armbrust, E. V. "Chitin in Diatoms and Its Association with the Cell Wall." *Eukaryotic Cell* 8 (2009): 1038-1050. Print.

25- EIA. "What are the major sources and users of energy in the United States." Eia.gov. U.S. Energy Information administration. August 1, 2013. Web. September 10, 2013. http://www.eia.gov/energy_in_brief/article/major_energy_sources_and_users.cfm

26- Elsey, D., Jameson, D., Raleigh, B., and Cooney, M. J. "Fluorescent measurement of microalgal neutral lipids." *J. Microbiol. Methods*. 68 (2007): 639-642. Print.

27- European Standard. " EN 14214 – Automotive fuels – Fatty acid methyl esters (FAME) for diesel engines – Requirements and test methods." Brussels: Comité Européen de Normalisation, 2008. Print.

28- Fawley, M. W., and Fawley, K. P. " A simple and Rapid Technique for the isolation of DNA from Microalgae." *J. Phycol*. 40 (2004): 223-225. Print.

29- Fogg, G. E., Stewart W. D. P., Fay, P., and Walsby, A. E. *The Blue-Green Algae*. London: Academic press Inc. LTD. 1973. Print

30- Frank, H. A., and Cogdell, R. J. "Carotenoids in photosynthesis". *Photochemistry and Photobiology*. 63 (1996): 257-264. Print.

31- Garby, T. J., Walter, M. R., Larkum, A. W. D. and Nellan, B. A. "Diversity of cyanobacterial biomarker genes from the stromatolites of Shark Bay, Western Australia." *Environmental Microbiology* 15 (2013). 1464-1475. Print.

32- Garcia, M. C. C., Sevilla, J. M. F., Fernandez, F. G. A., Grima, E. M., and Camacho, F. G. "Mixotrophic growth of *Phaeodactylum tricornutum* on glycerol: growth rate and fatty acid profile." *Journal of Applied Phycology*. 12 (2000): 239-248. Print.

33- Ghirardi, M. L., Posewitz, M. C., Maness, P. C., Dubini, A., Yu, J., and Seibert, M. "Hydrogenases and Hydrogen Photoproduction in Oxygenic Photosynthetic Organisms." *Annual Review of Plant Biology*. 58 (2007): 71-91. Print.

34- Gouveia, L., and Oliveira, A. C. "Microalgae as a Raw Material for Biofuels Production." *Journal of Industrial Microbiology and Biotechnology*. 36 (2009): 269-274. Print.

35- Griffiths, M. J., Van Hille, R. P., and Harrison, S. T. L. "Lipid productivity, settling potential and fatty acid profile of 11 microalgal species grown under nitrogen replete and limited conditions." *Journal of Applied Phycology*. 24 (2012): 989-1001. Print.

36- Guiry, M. D., and Guiry, G. M. (2014). "AlgaeBase." World-wide electronic publication, National University of Ireland, Galway. http://www.algaebase.org; searched on July 2014.

37- Guschina, I. A., and Hardwood, J. L. "Lipids and lipid metabolism in eukaryotic algae." *Progress in Lipid Research*. 45 (2006): 160-186. Print.

38- Hallenbeck, P. C. "Microbial Production of Fatty Acid Based Biofuels." *Microbial Technologies in Advanced Biofuels Production*. Ed. Hallenbeck, P. C. New York, New York, USA: Springer Science + Business Media, 2012. 213-230. Print.

39- Hallenbeck, P. C., and Benemann, J. R. "Biological hydrogen production; fundamentals and limiting processes." *International Journal of Hydrogen Energy*. 27 (2002): 1185-1193. Print.

40- Heredia-Arroyo, T., Wei, W., Ruan, R., and Hu, B. "Mixotrophic cultivation of Chlorella vulgaris and its potential application for the oil accumulation from non-sugar materials." *Biomass and Bioenergy*. 35 (2011): 2245-2253. Print.

41- Hildebrand, M., Abbriano, R. M., Polle, J. E. W., Traller, J. C., Trentacoste, E. M., Smith, S. R., and Davis, A. K. "Metabolic and cellular organization in evolutionarily diverse microalgae as related to biofuels production." *Current Opinion in Chemical Biology*. 17 (2013): 506-514. Print.

42- Hoekman, S. K., Broch, A., Robbins, C., Ceniceros, E., and Natarajan, M. "Review of biodiesel composition, properties, and specifications." *Renewable and Sustainable Energy Reviews*. 16 (2012): 143-169. Print

43- Hoiczyk, E. and Hansel, A. " Cyanobacterial Cell Walls: News from an Unusual Prokaryotic Envelope." *Journal of Bacteriology* 182 (2000): 1191-1199. Print.

44- Horton, H. R., Moran, L. A., Scrimgeour, K. G., Perry, M. D., and Rawn, J. D. *Principles of Biochemistry*. Upper Saddle River, New Jersey, USA: Pearson Prentice Hall, 2006. Print.

45- Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., and Darzins, A. "Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances." *The plant Journal*. 54 (2008): 621-639. Print.

46- Huang, G. H., Chen, F., Wei, D., Zhang, X. W., and Chen, G. " Biodiesel production by microalgal biotechnology." *Applied Energy*. 87 (2010): 38-46. Print.

47- Huang, G. H., Chen, G., and Chen, F. " Rapid screening method for lipid production in algal based on Nile Red Fluorescence." *Biomass Bioenergy*. 33 (2009): 1386-1392. Print.

48- Joye, S. B., Macdonald, I. R., Leifer, I., Asper, V. "Magnitude and oxidation potential of hydrocarbon gases released from the BP oil well blowout." *Nature geoscience* 4 (2011): 161-164. Print.

49- Khozin-Goldberg, I., and Cohen, Z. "Unraveling algal lipid metabolism: Recent advances in gene identification." *Biochimie*. 93 (2011): 91-100. Print

50- Kirst, H., Garcia-Cerdan, J. G., Zurbriggen, A., Ruehle, T., and Melis, A. "Truncated Photosystem Chlrophyll Antenna Size in Green Microalga *Chlamydomonas reinhardtii* upon Deletion of *TLA3-CpSRP43* Gene." *Plant Physiology*. 160 (2012): 2251-2260. Print.

51- Koku, H., Eriglu, I., Gündüz, U., Yücel, M., and Türker, L. "Aspects of the metabolism of hydrogen production by *Rhodobacter sphaeroides*." *International Journal of Hydrogen Energy*. 27 (2002): 1315-1329. Print.

52- Kong, W. B., Yang, H., Cao, Y. T., Song, H., Hua, S. F., Xia, C. G. " Effet of glycerol and glucose on the enhancement of biomas, lipid and soluble carbohydrate production by *Chlorella vulgaris* in mixotrophic culture." *Food Technol. Biotechnol*. 51 (2013): 62-69. Print.

53- Kou, Z., Bei, S., Sun, J., Pan, J. "Fluorescent measurement of lipid content in the model organism *Chlamydomonas reinhardtii*." *J. Appl. Phycol.* 25 (2013): 1633-1641. Print.

54- Kruse, O., and Hankamer, B. "Microalgal hydrogen production." *Current Opinion in Biotechnology*. 21 (2010): 238-243. Print.

55- Lee, S. J., Yoon, B. D., Oh, H. M. "Rapid method for the determination of lipid from the green algal *Botryococcus braunii*." *Biotechnol. Tech.* 12 (1998): 553-556. Print.

56- Leite, G. B., and Hallenbeck, P. C. "Algae Oil." *Microbial Technologies in Advanced Biofuels Production*. Ed. Hallenbeck, P. C. New York, New York, USA: Springer Science + Business Media, 2012. 231-259. Print.

57- Leite, G. B., Paranjape, K., Abdelaziz, A. E. M., and Hallenbeck, P. C. "Utilization of biodiesel-derived glycerol or xylose for increased growth and lipid production by indigenous microalgae." *Bioresource Technology*. (2014). In press.

58- Liang, Y., Sarkany, N., and Cui, Yi. "Biomass and Lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions." *Biotechnology Letters*. 31 (2009):1043-1049. Print.

59- Liu, B., and Benning, C. "Lipid metabolism in microalgae distinguishes itself." *Current Opinion in Biotechnology*. 24 (2013): 300-309. Print.

60- Longworth, J., Noirel, J., Pandhal, J., Wright, P. C., and Vaidyanathan, S. "HIIC- and SCX- Based Quantitative Proteomics of *Chlamydomonas reinhardtii* during Nitrogen Starvation Induced Lipid and Carbohydrate Accumulation." *Journal of Proteome Research*. 11 (2012): 5959-5971. Print.

61- Madiraju, K. S., Lyew, D., Kok, R., and Raghavan V. "Carbon neutral electricity production by *Synechocystis sp.* PCC6803 in a microbial fuel cell." *Bioresource Technology* 110 (2012): 214-218. Print.

62- Makareviciene, V., Skorupskaite, V., Andruleviciute, V. "Biodiesel fuel from microalgae-promising alternative fuel for the future: a review." *Reviews in Environmental science and Biotechnology* 12 (2013): 119-130. Print.

63- Mcfadden, G. I. " Primary and Secondary Endosymbiosis and the Origin of Plastids." *Journal of Phycology* 37 (2001): 951-959. Print.

64- Meher, L. C., Vidya, D., Naik, S. N. " Technical aspects of biodiesel production by transesterification- a review." *Renewable and Sustainable Energy Reviews*. 10 (2006): 248-268. Print.

65- Melis, A., and Happe, T. "Hydrogen Production. Green Algae as a Source of Energy." *Plant physiology*. 127 (2001): 740-748. Print.

66- Merchant, S. S., Kropat, J., Liu, B., Shaw, J., and Warakanont, J. "Tag, You're it! Chlamydomonas as a reference organism for understanding algal triacylglycerol accumulation." *Current Opinion in Biotechnology*. 23 (2012): 352-363. Print.

67- Miao, X., and Wu, Q. "Biodiesel production from heterotrophic microalgal oil." *Bioresource Technology*. 97 (2006): 841-846. Print.

68- Millar, J. B., Buck, V., and Wilkinson, M. G. " Pyp1 and Pyp2 PTPases dephosphorylate an osmosensing MAP kinase controlling cell size at division in fission." *Genes and Development*. 9 (1995): 2117-2130. Print

69- Moody, J. W., McGinty, C. M., and Quinn, J. C. " Global evaluation of biofuel potential from microalgae." *PNAS*. 111 (2014): 8691-8696. Print.

70- Msanne, J., Xu, D., Konda, A. R., Casas-Mollano, J. A., Awada, T., Cahoon, E. B., and Cerutti, H. "Metabolic and gene expression changes triggered by nitrogen deprivation in photoautotrophically grown microalgae *Chlamydomonas reinhardtii* and *Coccomyxa sp. C-169*." *Phytochemistry*. 75 (2012): 50-59. Print.

71- Mujtaba, G., Choi, W., Lee, C-G., and Lee, K. "Lipid production by *Chlorella vulgaris* after a shift from nutrient-rich to nitrogen starvation conditions."*Bioresource Technology*. 123 (2012): 279-283. Print.

72- Ogawa, T., and Aiba, S. " Bionergetic Analysis of Mixotrophic Growth in *Chlorella vulgaris* and *Scenedesmus auctus*." *Biotechnology and Bioengineering*. 23 (1981): 1121-1132. Print.

73- O'Grady, J., and Morgan, J. A. " Heterotrophic growth and lipid production of *Chlorella protothecoides* on glycerol." *Bioprocess and Biosystems Engineering*. 34 (2011): 121-125. Print.

74- Ort, D. R., Zhu, X., and Melis, A. " Optimizing Antenna Size to Maximize Photosynthetic Efficiency." *Plant Physiology*. 155 (2011): 79-85. Print.

75- Perez-Garcia, O., De-Bashan, L. E., Hernandez, J. P., and Bashan Y. "Efficiency of growth and nutrient uptake from wastewater by heterotrophic, autotrophic, and mixotrophic cultivation of *Chlorella vulgaris* immobilized with *Azospirillum brasilense*." *Journal of Phycology*. 46 (2010): 800-812. Print.

76- Popper, Z. A., Michel, G., Hervé, C., Domozych, D. S., Willats, W. G. T., Tuohy, M. G., Kloareg, B., and Stengel, D. B. "Evolution and Diversity of Plant Cell Walls: From Algae to Flowering Plants." *The Annual Review of Plant Biology*. 62 (2011): 567-590. Print.

77- Radakovits, R., Jinkersin, R. E., Darzin, A., and Posewitz, M. C. "Genetic Engineering of Algae for Enhanced Biofuel Production." *Eukaryotic Cell*. 9 (2010): 486-501. Print.

78- Rai, M. P, Nigam, S., and Sharma, R. "Response of growth and fatty acid compositions of Chlorella pyrenoidosa under mixotrophic cultication with acetate and glycerol for bioenergy application." *Biomass Bioenergy*. 58 (2013): 251-257. Print.

79- Rashid, N., Rehman, M. S. U., Memon, S., Rahman, Z. U., Lee. K, and Han, J. I. "Current status, barriers and developments in biohydrogen production by microalgae." *Renewable and Sustanable Energy Review*. 22 (2013): 571-579. Print.

80- Rathore, D., and Singh, A. "Biohydrogen Production from Microalgae." *Biofuel Technologies Recent Developments*. ED. Gupta, V. K., and Tuohy, M. G. Berlin, Heidelberg: Springer-Verlag, 2013. 317-333.

81- Raven, P. H., Evert, R. F. and Eichhorn, S. E. *Biologie végétale*. Brussels: De Boeck, 2007. Print.

82- Recht, L., Toepfer, N., Batushansky, A., Sikron, N., Zarka, A., Gibon, Y., Nikoloski, Z., Fait, A., and Boussiba, S. "Metabolite Profiling and Integrative Modeling Reveal Metabolic Constrains for Carbon-Partitioning under Nitrogen-Starvation in the Green Alga *Haematococcus pluvialis*." *Journal of Biological Chemistry* (2014): jbc-M114.

83- Rios, L. F., Klein, B. C., Luz, L. F. Jr., and Maciel Filho, R. "Nitrogen starvation for Lipid Accumulation in the Microalga Species *Desmodesmus sp.*". *Applied biochemistry and biotechnology*. (2014): 1-8. Print.

84- Rosenberg, J. N., Oyler, G. A., Wilkinson, L., and Betenbaugh, M. J. “A green light for engineered algae: redirecting metabolism to fuel a biotechnology revolution.” *Current Opinion in Biotechnology*. 19 (2008): 430-436. Print.

85- Runge, C. F., and Senauer, B. “How Biofuels could Starve the Poor.” *Foreign Affairs*. 86 (2007): 41-53. Print.

86- Scheffel, A., Poulsen, N., Shian, S., and Kröger, N. “Nanopatterned protein microrings from a diatom that direct silica morphogenesis.” *Proceedings of the National Academy of Science*. 108 (2011): 3175-3180. Print.

87- Scott, S. A., Davey, M. P., Dennis, J. S., Horst, I., Howe, C. J., Lea-Smith, D. J., and Smith, A. G. “Biodiesel from algae: Challenges and prospects.” *Current Opinion in Biotechnology*. 21 (2010): 277-286. Print.

88- Stanier, R. Y., and Cohen-Bazire, G. "Phototrophic Prokaryotes: The Cyanobacteria." Ann. Rev. Microbiol. 31 (1977):225-74. Print.

89- Stephenson, A. I., Dennis, J. S., Howe, C. J., Scott, S. A., and Smith, A. G. “Influence of nitrogen-limitation regime on the production by *Chlorella vulgaris* of lipids for biodiesel feedstocks.” *Biofuels*. 1 (2010): 47-58. Print.

90- Stucken, K., Ilhan, J., Roettger, M., Dagan, T., and Martin, W. F. “Transforamation and Conjugal Transfer of Foreign Genes into the Filamentous Multicellular Cyanobacteria (Subsection V) *Fischerlla* and *Chlorogloeopsis*.” *Current Microbiology*. 65 (2012): 552-560. Print.

91- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. “MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods.” *Molecular Biology and Evolution.* 28 (2011): 2731-2739.

92- Taton, A., Lis, E., Adin, D. M., Dong, G., Cookson, S., Kay, S. A., Golden, S. S., and Golden, J. W. “Gene Transfer in *Leptolyngbya* sp. Strain BL0902, a Cyanobacterium Suitable for Production of Biomass and Bioproducts.” *PLoS one*. 7 (2012): e30901. Print.

93- Tomaselli, L. “The Microalgal Cell’’. *Handbook of Microalgal Culture Biotechnology and Applied Phycology*. ED. Richmond, A. Ames, Iowa: Blackwell Publishing, 2008. 3-19. Print.

94- Tsygankov, A. A. “Hydrogen Production: Light-Driven Process-Green Algae.” *Microbial Technologies in Advanced Biofuels Production*. ED. Hallenbeck, P. C. New York, New York, USA: Springe Science+Business, 2012. 29-53.Print

95- Wood, M. A., Everroad, R. C., and Wingard, L. M. “ Measuring Growth rates in Microalgal cultures.” *Algal culture techniques.* ED. Andersen, R. A. Burlington, USA: Elsevier Academic Press, 2005. 269-285.

96- Ugwu. C. U., Aoyagi, H., and Uchiyama, H. “Photobioreactors for Mass cultivation of Algae.” *Bioresource Technology.* 99 (2008): 4021-4028. Print.

97- Zehr, J. P, Jenkins, B. D., Short S. M., and Stewart, G. F. “Nitrogenase gene diversity and microbial community structure: a cross-system comparison.” *Environmental Microbiology*. 5 (2003): 539-554. Print.

Final Conclusion

In conclusion, fossil fuels have a number of important and beneficial applications for our society today. For instance, they are used as fuel for the transportation industry, as an energy source for the production of electricity and as raw materials for the production of certain materials such as plastics. Unfortunately, fossil fuels have a number of drawbacks, which are going to cause serious economical, political and environmental problems in the near future. Increase in atmospheric CO_2, due to the combustion of fossil fuels, has caused average global temperatures to increase exponentially in the last few decades. This in turn will probably have an impact on population demographics. As sea levels are rising, populations near the sea line will have to be displaced to other regions causing economical and political difficulties. Moreover, increases in global temperatures have had negative impacts on the environment and thus will most likely have serious impacts on agrarian societies. Furthermore, fossil fuels are a limited resource, as their production is dependant on large geological time frames. Consequently, there will be a point in time when fossil fuel production will be depleted. This is a serious matter as most societies are completely dependant on fossil fuels. Hence, there is necessity to find solutions.

Amongst the numerous proposed solutions, biofuels have come up as a prospective replacement for fossil fuels. Biofuels are fuels produced from living organisms. Examples of such fuels are ethanol, biodiesel and biogases such as bio-hydrogen or methane. Many advantages are associated with their usage and production, which solve some of the problems associated with fossil fuel combustion. For one thing, biofuels are produced from living organisms and as a result are sustainable. Furthermore, most biofuels are either directly or

indirectly produced by photosynthetic organisms and thus mitigate CO_2 emissions. Consequently, biofuel usage would have drastic effects on Global Warming by reducing CO_2 emissions. Additionally, biofuels are less polluting then most fuels, as they are composed of biological molecules produced by organisms and thus most biofuels are biodegradable. Microalgae have become a potential crop for the practical production of biodiesel.

Microalgae are photosynthetic microorganisms containing chlorophyll *a*. This definition being very general, this polyphyletic group comprises eukaryotic and prokaryotic organisms. These organisms are capable of producing storage compounds, specifically triacylglycerol molecules (TAGs), by fixing carbon dioxide through photosynthesis. These TAGs are capable of being extracted and converted to biodiesel by a chemical reaction called transesterifcation. TAGs are lipids that are composed of a glycerol molecule attached to three fatty acid molecules. In the transesterification reaction, the glycerol is separated from the fatty acid molecules by methanolysis. The fatty acid molecules are converted to a methylated-ester form and are called Fatty Acid Methyl Esters (FAMEs). The glycerol produced from the transesterification reaction is a waste product. Consequently, the market has been flooded with crude glycerol. Microalgae being photosynthetic are usually considered autotrophic. However, many species are known to consume heterotrophic carbon sources, for instance when light intensity is not sufficient enough to permit photosynthesis such as during night-time. Thus, certain species or strains of microalgae are capable of in parallel both use an autotrophic (CO_2 fixation through photosynthesis) and heterotrophic (consumption of an external heterotrophic carbon source) growth modes. This mixing of two different trophic modes is called mixotrophy. Certain strains of microalgae have shown to increase lipid production under

mixotrophic growth. Thus, mixotrophy has been, in recent years, considered for enhancement of lipid production in order to increase biodiesel production by cultivation of microalgae.

In the research presented here, isolated strains of microalgae from different lakes and rivers from Quebec, Canada, were examined for lipid production under mixotrophic growth with glycerol as a carbon source. Glycerol is a cheap and abundant carbon source and can enter several metabolic pathways. Thus, it is an ideal carbon source for mixotrophic growth. The first experimental design screened twelve algal strains, from our collection, for their capacity to consume glycerol and, through this consumption, enhance their lipid production. The results showed that glycerol could be consumed by all of the strains; however, the different strains had different responses to mixotrophic growth. Thus, glycerol had the effect to increase growth rates, biomass production and lipid production for some of the strains. However, certain strains showed no change in these parameters. This leads to believe that strains, which were capable of increasing lipid production, used the glycerol for TAGs production. However, strains showing similar lipid production in both growth conditions used glycerol for other processes than lipid production. Certain strains showed increase in growth rates but not the other parameters suggesting that glycerol could be used to accelerate growth. This would be interesting for practical algal cultivation, as harvesting time would be quicker. Thus, algal strains producing certain valuable products (not necessarily TAGs) could use glycerol to accelerate production of these products. From these findings, glycerol has a number of interesting consequences, which seem to be strain specific. Moreover, the initial screening showed that most of the strains were capable of producing FAMEs compatible with biodiesel production. Most strains produced higher percentages of oleic acid followed by palmitic and then stearic acid. Interestingly enough, palmitic and stearic acid changed

percentages depending on growth conditions, whereas oleic acid percentages were similar in both conditions. This suggests that certain proteins or regulation mechanisms are expressed in either conditions depending on the strain. Consequently, since glycerol was able to enhance lipid production, growth rates and biomass for certain strains, it is an ideal waste product for practical algal cultivation.

Since the strains were isolated from the wild, identification was needed. Thus, *18S rRNA* phylogenetic analysis and bioinformatics alignments of the sequences showed that the strains all belonged to the *Chlorophyta* phylum. Strains belonged to the *Chlorella* genus, the *Hindakia* genus, the *Scenedesmus* genus, the *Mychonastes* or the *Acutodesmus* genus. Hence, the strains were diverse and belonged to several distinct genera.

From the initial screening, strains PCH02, PCH05 and PCH28 were chosen for the following experiment as they showed interesting results in terms of lipid production. Nitrogen starvation is the process wherein algal strains are grown to a certain biomass and then transferred to a medium without any nitrogen source. This initiates lipid accumulation for microalgal cells. Nitrogen is used for DNA and protein synthesis. Thus, a lack of nitrogen halts growth and channels remaining proteins to carbon fixation and lipid accumulation. PCH02 and PCH05 showed high lipid production under mixotrophic growth. Hence, these strains were examined for lipid production under mixotrophic growth and nitrogen starvation. PCH28 showed very little difference in lipid production in both growth conditions, thus this strain was used as a negative control for the experiment. The results suggested that lipid accumulation under nitrogen starvation and mixotrophy was strain specific process. Thus, PCH02 increased lipid accumulation as time progressed in the nitrogen starvation period for both conditions. Mixotrophic conditions had slightly higher production then photoautotrophic

growth for this strain. However, this was not at a significant level. On the other hand, PCH05 did not increase lipid production until ten days of incubation. Furthermore, lipid accumulation was much higher under mixotrophic growth then photoautotrophic growth, suggesting that lipid accumulation based on glycerol assimilation is dependant on nitrogen metabolism and that nitrogen deplete condition triggers lipid accumulation. Finally, PCH28 showed no increase in lipid production in both condition through out the nitrogen starvation period. This suggests that nitrogen starvation or glycerol does not trigger any particular mechanism necessary for lipid accumulation for this strain. Furthermore, the two-step cultivation method requires a lot of time and thus decreases productivity. As a result, recent research has shown that lipid productivity can be increased if algal strains are allowed to deplete their nitrogen source naturally.

Finally, the last experiment examined lipid production under varying concentrations of nitrogen source and mixotrophy for PCH02 and PCH05. Thus, these two strains were grown under varying concentrations of sodium nitrate and under mixotrophic and photoautotrophic conditions. The results indicated that nitrate concentrations and time were both factors in lipid production. Algal strain PCH02 increased lipid production with increasing concentration of nitrate under mixotrophic conditions. Under photoautotrophy, lipid patterns were similar at the beginning of growth and then were highest at 20mg/L of nitrate concentration when strain entered stationary phase. This would indicate that nitrogen depletion has no effect on lipid accumulation under mixotrophy but does under photoautotrophy. On the other hand, PCH05 showed a different lipid production pattern that seemed to evolve with time. Thus, at the beginning of growth, lipid production was highest at lower nitrate concentrations. However, as growth progressed in time for this strain, lipid production increased with nitrate

concentrations. At stationary phase, lipid production was highest at nitrate concentrations above 80mg/L of nitrate, with a peak at that same concentration. Moreover, lipid production seemed to increase with time. Thus, strains had higher levels of specific lipid production and total lipid production at the end of growth than at the beginning of growth.

These experiments suggest that glycerol and nitrogen starvation are potential techniques that could be used for practical cultivation of algae for the production of biodiesel. Additionally, they validate the notion that mixotrophic growth increase growth and lipid production fro certain strains. However, further research needs to be done, in order to increase algal lipid productivity. One possibility would be to investigate protein expression linked to fatty acid and TAG synthesis at different periods of growth (lag phase, exponential phase and stationary phase) and under mixotrophic and photoautotrophic conditions. This would be important, as it would help produce, through genetic manipulation, certain strains of algae capable of producing specific FAMEs compatible with biodiesel production. Another possibility would be to investigate different amounts of glycerol and their effects on lipid production. As only one concentration of glycerol was used in the work presented, different strains would probably respond different to different concentrations. A possible outcome of this experiment would most likely show that each strain would have an optimum concentration of glycerol, where lipid production is highest. Lastly, different parameters, such as pH, temperature, light intensity and nitrogen source could all be tested to examine their effects on lipid production. Different parameters would help to optimise practical cultivation of microalgae for maximal growth and lipid production.