PRODUCTION OF BIOFUELS FROM THE GREEN ALGA *TETRASELMIS*

By

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Abstract

The research described in thesis deals with the production of biofuels and fine chemicals from the green alga *Tetraselmis suecica*. Firstly, the identity of the strain received from the culture collection was confirmed using molecular techniques (18S rDNA sequencing) and electron microscopy. Secondly, a fully defined artificial seawater medium was developed to grow *T. suecica* and then the tolerance of this alga to salinity and pH changes was established. The neutral lipid (triacylglycerol) production was measured using Nile Red dye after stressing *T. suecica* cells with high salinity (up to 1 M NaCl) and pH values (pH 7 to 9). It was established that high salinity and high pH values tended to induce higher levels of triacylglycerol in the algal cells. Then fatty acid profiles of *T. suecica* cells were analyzed by gas chromatography–mass spectrometry (GC-MS) after direct transesterification with hydrochloric acid in methanol. Higher salinity grown cells showed higher levels of monounsaturated fatty acids, which are ideal for biodiesel production. The possibility of growing *T. suecica* on a larger scale was investigated using a 2 L airlift photobioreactor and the response to higher levels of CO_2 was assessed in the airlift bioreactor. The effect of re-using the medium on the growth of *T. suecica* was examined with the aim of developing an integrated algal biorefinery process using *T. suecica* as the feedstock.

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Abbreviations

TAE	Tris-acetate-EDTA
Tris	(hydroxymethyl)aminomethane
V/V	Volume per unit volume
W/V	Weight per unit volume
mg	Milligram (s)
%	Percentage
C^O	Centigrade
µg	Microlitre (s)
mg	Milligram (s)
ml	Millilitre (s)
g	Gram (s)
kb	Kilobase (s)
M	Molar
MW	Molecular weight
min	Minute (s)
OD	Optical density
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
Bp	Base pair (s)
FAME	Fatty Acid Methyl Ester
GCMS	Gas chromatography–mass spectrometry
TAG	Triglyceride

Chapter One

Literature Review

1.1 Introduction to Biofuels

The rapid increase in population and growth in industrialization has led to a much increased global energy demand which has generated a large increase in the use of fossil fuels. According to the United States Energy Information Administration (EIA), the consumption of global energy in the last 20 years has increased from 355 Quadrillon British Thermal Units (QBTU) in 1990 to 510 QBTU in 2010 which is about a 44% increase. The EIA predict that a further 60% increase will take place over the next 20 years (Energy Information Administration, 2005). Most of the energy demand is met from the burning of fossil fuels (petroleum, natural gas and coal) which are easy to use, provide high energy density and are cheap when compared to alternative energy sources. However, the continued use of fossil fuels is inadvisable, because of the acceleration of the accumulation of greenhouse gases (GHG), increases in air pollution and the production of acid rain. Also, the depletion of fossil fuels resources will make their use non-sustainable in the long term (Hook and Tang, 2013).

Therefore, a large amount of research has been carried out with the goal of finding new renewable energy sources that are sustainable and environmentally friendly. Among the alternatives, wind energy, solar energy, geothermal energy, hydroelectric energy and biofuels have attracted significant amounts of research and exploitation. First generation biofuels are produced from food crops or other plants that require good quality arable land and plenty of freshwater (Kikuchi *et al.*, 2009). This has resulted in a food versus fuel debate which has limited the ability of first generation feedstocks to meet the demand for the production of biofuels. In addition the Net Energy Balance (NEB) for corn bioethanol and soybean biodiesel is very small (i.e. only slightly more energy is yielded from the bioethanol/biodiesel than was used to produce it) (Hill *et al.,* 2006). Second generation biofuels are based on so-

called "energy crops" (Miscanthus or Switchgrass), which can grow on marginal land or agricultural wastes left after cropping can be used. The main problem with second generation feedstocks is the difficulty in extracting the lignocellulosic substrates, which make up the bulk of the carbon sources in grasses and agricultural waste like straw (Himmel *et al.,* 2007; Sousa *et al.*, 2009).

The possibilities of developing a new generation of biofuels have increased since the first generation of biofuels run into the problems outlined above. Therefore, there is a growing interest in third generation biofuels using microalgae as the feedstock. Microalgae can be grown in saline water or wastewater and do not compete for arable land and precious freshwater (Gilmour and Zimmerman, 2012). As a result of continuous and increasing burning of fossil carbon, the amount of greenhouse gas CO_2 in the atmosphere has increased. Algae are very efficient at taking up CO_2 from the atmosphere and converting it into organic compounds through the process of photosynthesis. In fact, microalgae can be used to utilise the CO_2 directly from flue gases from steelworks or other industries (Zimmerman *et al.*, 2011). Algal biomass can be used in a number of ways to produce biofuels, but the most likely possibility is using microalgae that produce high levels of neutral lipids (triacylglycerol, TAG) as a basis for biodiesel production (Chisti, 2008). Many microalgae, including *Dunaliella*, *Chlorella*, *Nannochloropsis* and *Tetraselmis* can produce high levels of neutral lipids and can be grown in saline media (Chisti, 2008). The key breakthroughs required to make algal biodiesel a commercial reality are: a) finding a highly productive strain that will produce high levels of neutral lipid during growth and not just in stationary phase, b) finding a good method to harvest small microalgal cells efficiently and c) efficient recovery of the lipids from the algal cells (Gilmour and Zimmerman, 2012). One key idea is

the formation of an "algal biorefinery" which will utilise microalgal biomass to produce biodiesel, protein for animal feed, health supplements and fertiliser (Chisti, 2008).

1.2 Introduction to Microalgae

1.2.1 Classification of Microalgae

Algae are thallophytes (i.e. photosynthetic non-vascular plants which lack roots, stems and leaves, but contain chlorophyll a as their primary photosynthetic pigment) (Vonshak and Maske, 1982). The mechanism of photosynthesis in algae is similar to that of higher plants and involves the evolution of oxygen as a by-product. Their simple cellular structure gives them the ability to convert solar energy more efficiently than plants. Moreover, they have more efficient access to CO_2, water, and other nutrients as the majority of algae grow in aqueous suspension. Nevertheless, algae can be found in both aquatic and soil ecosystems in all geographic zones. Some algal species have the ability to adapt to harsh environmental conditions. Estimates of the number of algal species vary widely with an upper limit of 1 million species (Guiry, 2012). The taxonomic database AlgaeBase (www.algaebase.org) lists 140,661 species names with some 53,000 published documents relating to algal species cited (accessed June 2015). A conservative estimate of the number of algal species made by Guiry (2012) was 72,500.

Algae are morphologically extremely variable and range from macroalgae (seaweeds) up to 70 m long to the microscopic algae (microalgae), which can be single cells a few µm in diameter. Macroalgae are multicellular algae with defined tissues and specialized cells, but they never develop phloem or xylem and are thus differentiated from plants. Microalgae are divided into two major groups (eukaryotic and prokaryotic), all with a unicellular or simple

multicellular structure. Eukaryotic microalgae include a wide range of groups including green algae and diatoms, while prokaryotic microalgae are grouped as cyanobacteria (Cyanophyceae), formerly known as blue-green algae. Eukaryotic microalgae have a nucleus containing genetic material and several other organelles (chloroplast, mitochondria etc.) surrounded by membranes and they have a high degree of internal organization, whereas in the prokaryotic cyanobacteria there is no distinct nucleus and no membrane bound organelles (van den Hoek *et al*., 1995).

Traditionally microalgae have been classified based on to their pigment type, cell wall constituents and the chemical nature of storage products. Also, cytological and morphological characters such as the process of nuclear and cell division, the presence and spatial organization of flagella, as well as the presence of an envelope of endoplasmic reticulum and if there any connection with the nuclear membrane. This traditional classification gives rise to the green algae (chlorophytes), red algae (rhodophytes) and diatoms (van den Hoek *et al*., 1995). However, since the advent of rDNA sequencing (see section 1.7) and other molecular methods, a more detailed classification of algae within the eukaryotic domain has been produced (Figure 1.1). The key information in this figure is the wide separation of different algal groups throughout the eukaryotic domain. The green and red algae are grouped with the higher plants, but diatoms, dinoflagellates and euglenoids are widely separated and have little in common at the molecular level with green algae (Simpson and Roger, 2004). The work described in this thesis was carried out on *Tetraselmis suecica*, which is a green (chlorophyte) alga (Figure 1.1).

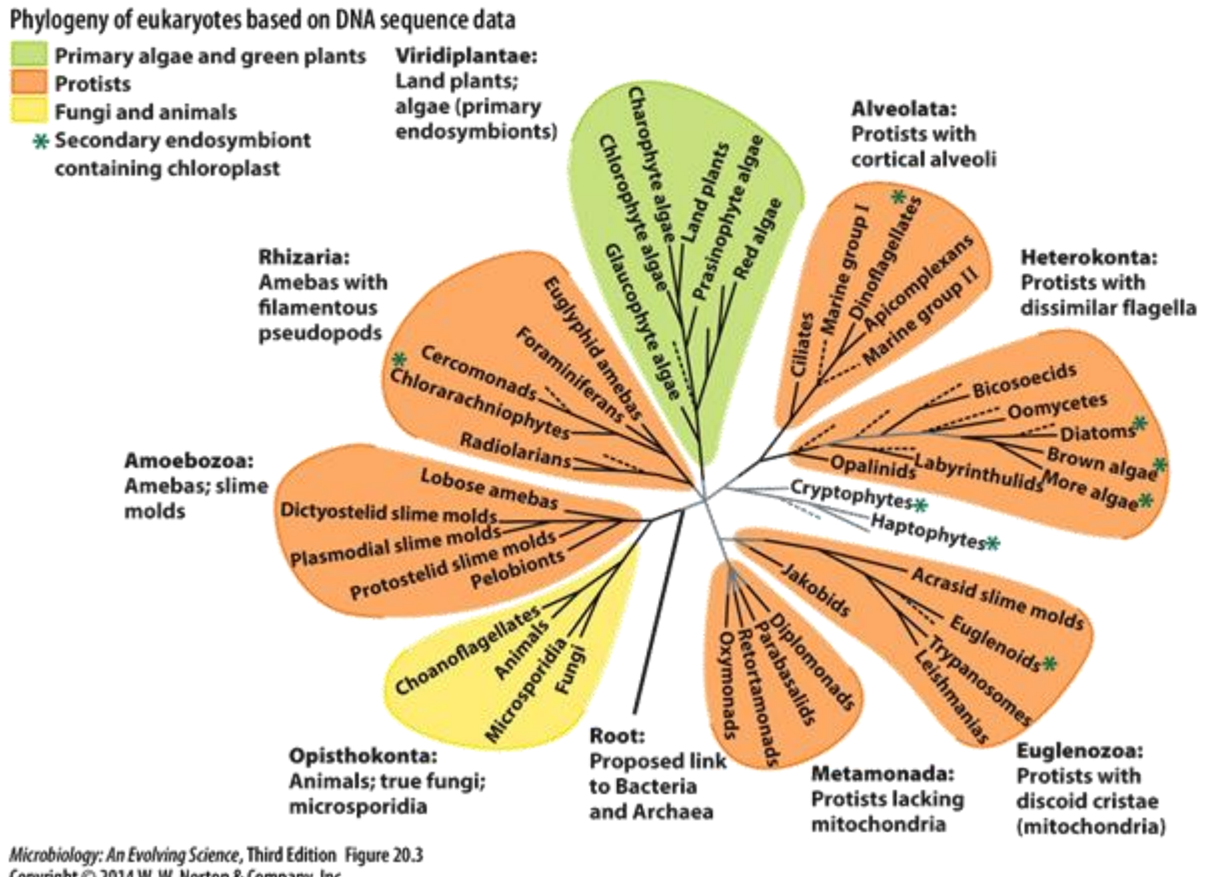


Figure 1.1. Phylogeny of Algae within the Eukaryotic Domain. All the groups in the green bubble are algal groups closely related to plants. The other algal groups (marked with a green asterisk) are widely dispersed around the different groups showing that algae are a very phylogenetically diverse group (Taken from Slonczewski and Foster, 2014).

1.2.2 Photosynthesis in Microalgae

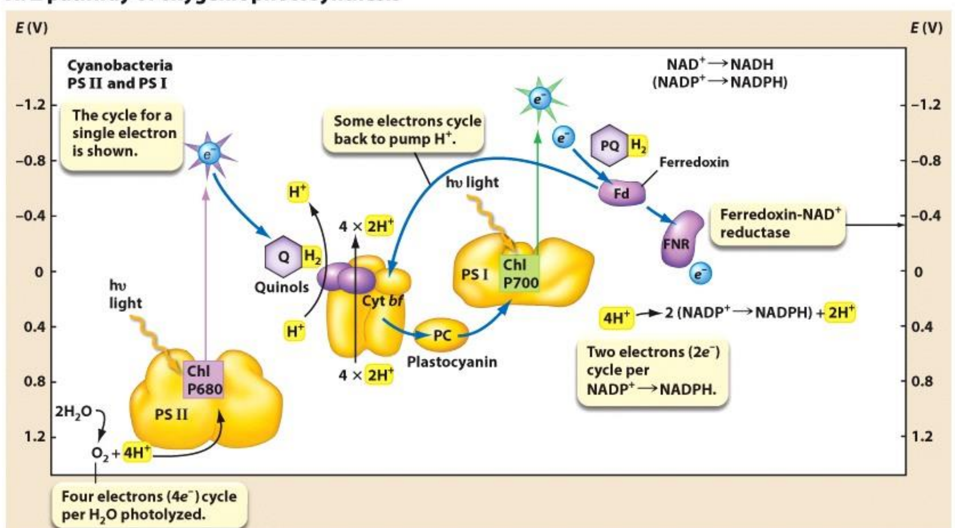
The process of converting CO_2 and H_2O using sunlight energy to glucose and oxygen is called photosynthesis. This process serves as a source of energy for metabolism and growth for almost all forms of life either directly or indirectly (Masojidek *et al.*, 2004). Almost half of the total photosynthesis taking place on Earth is associated with algal cells that form the marine phytoplankton (Camacho *et al.*, 2003). Chlorophyll a works as the key

photochemically active compound, and receives light in order for photosynthesis to occur. Therefore, the content of this pigment in microalgae influences photosynthetic activity (MacIntyre *et al.*, 2002). In addition the absorption of this light has an impact on biomass production in microalgae and on the accumulation of target products so the performance of photosynthesis is affected by the availability of light (Su *et al.*, 2007). In addition to chlorophyll a, algae contain a number of other pigments that contribute to the harvesting of light and include chlorophyll b, chlorophyll c and carotenoids. In the cyanobacteria and red algae, carotenoids are replaced by phycobilins as accessory light harvesting pigments (van den Hoek *et al.*, 1995).

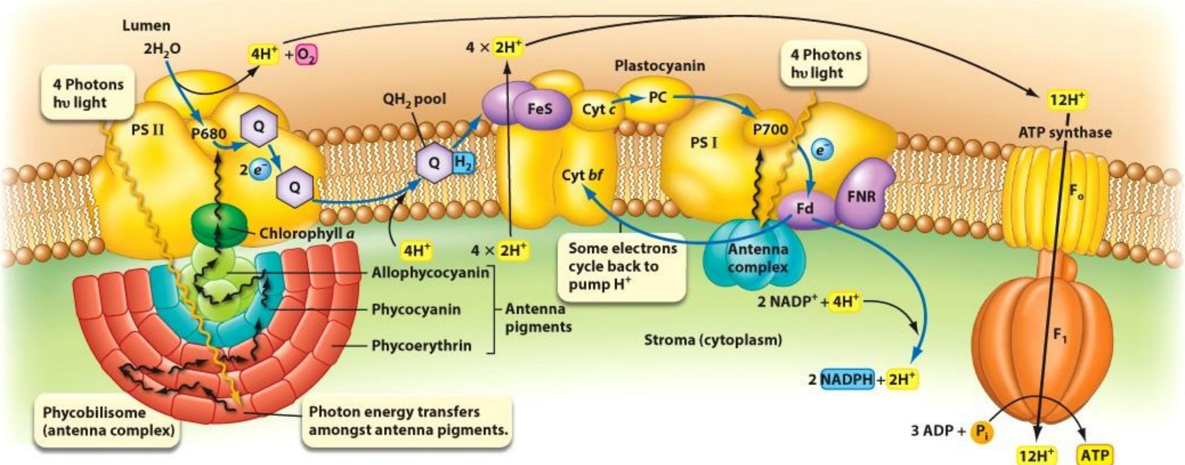
Figure 1.2 provides a detailed overview of photosynthesis taken from a recent textbook of microbiology (Slonczewski and Foster, 2014). The upper panel shows that the light energy absorbed by chlorophyll a is used to promote electrons to a more negative redox potential and that two photosystems work together to produce electrons that can directly reduce NAD(P) to NAD(P)H which is used throughout metabolism. The other key requirement for metabolism is ATP and this is produced via a proton motive force build up across the membrane due to spatial orientation of the components in the membrane.

Oxygenic photosynthesis in cyanobacteria and chloroplasts

A. Z pathway of oxygenic photosynthesis



B. Z pathway reaction complexes and ATP synthase



Microbiology: An Evolving Science, Third Edition Figure 14.36

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Figure 1.2. An overview of photosynthesis showing the key steps in the electron transport chain that allow the reduction of NAD(P) in the upper panel. The lower panel shows the spatial orientation of the electron transport components which pump protons across the membrane and set up a proton motive force which drives ATP production (Modify from Slonczewski and Foster, 2014).

1.2.3 Physical and Chemical Conditions for Algal Growth

The growth and productivity of microalgae can be affected by different factors including light, pH, salinity, temperature, nutrients (nitrogen, phosphorus, potassium, magnesium) and heavy metals (e.g. copper). However the effect of those factors varies from one species to another. Therefore knowledge of those factors can lead to a better growth rate and biomass production which will improve the potential for algal biofuel production.

1.2.3.1 Light

Light is one of the most important factors that affect overall biomass productivity in photoautotrophic cultures i.e. cultures that depend on CO_2 as their sole source of carbon. Light provides the energy source for the growth in microalgae. Go *et al.* (2012) reported that the biomass production and lipid accumulation in *Tetraselmis suecica* were affected by light intensity and nitrate concentration and the optimized conditions were 108.9 μmol m^{-2} s^{-1} and 18.6 mg L^{-1}, respectively. Moreover, *Tetraselmis* sp. showed a decrease in growth rate under a 12:12 h light: dark regime conditions and the highest growth rate was observed under 24 h continuous light regimes (Alsull and Omar, 2012). A key point is that at high cell density the light intensity must be increased to penetrate through the culture, since self shading becomes a limiting factor for growth (Jain *et al.,* 2015).

1.2.3.2 Carbon and pH

Light, water and CO_2 are essential elements for the process of photosynthesis and lack of available CO_2 can result in less photosynthesis which affects the biomass productivity. However, the presence of CO_2 in seawater is less than 1% at pH 8 and more than 90 % occurs in the form of HCO_3^- (Borowitzka, 1982). Nevertheless, the pH in the medium can be increased as a result of conversion of bicarbonate to CO_2 and that has been reported by Moheimani when an increase was observed in the pH of the medium in unregulated CO_2 culture of *T. suecica* (Moheimani, 2013). Also, his working indicated that significantly higher biomass productivity was achieved at pH 7.5 in *T. suecica*.

1.2.3.3 Salinity

It is well known that the biomass productivity and lipid composition are influenced by conditions like salinity (Renaud and Parry, 1994). Microalgae are also known for their tolerance to changes in salinity. Tawfiq *et al.* (1999) reported that *Tetraselmis* had an optimum salinity range between 20 and 35‰ (0.34 to 0.6 M NaCl). A similar conclusion was drawn by Alsull and Omar (2012), who found that *Tetraselmis* sp. achieved the highest growth and cell density over the same salinity range (0.34 to 0.6 M NaCl).

1.2.3.4 Temperature

The response of microalgae chemical compositions to high and low growth temperatures varies from species to species (Chen *et al.*, 2012). Tawfiq *et al.* (1999) reported that optimum temperature for *Tetraselmis* ranged between 19 and 21°C. However, Montaini *et al.* (1995) reported that the optimum temperature for *T. suecica* is 27±1°C.

1.2.3.5 Nitrogen

Nitrogen sources (normally ammonium and nitrate) are essential inorganic salts for cell growth and metabolism. Nevertheless, the most favourable conditions for nitrogen concentration vary from species to species (Huang *et al.*, 2013). Furthermore, the accumulation of lipids has been increased in *Tetraselmis* as a response to limiting the culture nitrogen concentration. A similar conclusion was drawn by Li *et al.* (2008) who suggested that nitrogen reduction in microalgae culture led to rapid accumulation of oil.

1.3 Microalgae Cultivation Systems

From reading the scientific literature, it is clear that there are many different types of systems for microalgae cultivation. The choice of cultivation system depends on cost, final product, nutrients source and CO_2 capture. In general, microalgae cultivation systems can be classified as open or closed systems (Suali and Sarbatly, 2012). The advantages and disadvantages of these two systems are compared in Table 1.1. Open pond system has been used for large scale algae cultivation since the 1950s. This system can be classified into natural waters (lakes, lagoons, and ponds) and artificial ponds or containers. While closed systems, known as photobioreactors (PBRs), have been developed to address some of the issues with open pond systems like contamination and improved regulation and control of nearly all of the biotechnologically important parameters (Bahadar and Khan, 2013). Moreover, flue gases can be used from power plant providing additional environmental benefits to the use of closed system PBRs (Zimmerman *et al.,* 2011). Photobioreactors come in a different range of designs, principally tubular, flat plate, airlift or column photobioreactors (Table 1.1). However, the overheads of closed systems are considerably higher than open pond systems and there are concerns about the feasibility of scaling up PBRs (Kiran *et al.*, 2014).

Culture Systems	Advantages	Limitations
Open systems	Relatively economical Easy to clean up Easy maintenance Utilization of non-agricultural land Low energy inputs	Little control of culture conditions Poor mixing, light and CO_2 utilization Difficult to grow algal cultures for long periods Poor productivity Limited to few strains Cultures are easily contaminated
Tubular PBR	Relatively cheap Large illumination surface area Suitable for outdoor cultures Good biomass productivities	Gradients of pH, dissolved oxygen and CO_2 along the tubes Fouling Some degree of wall growth Requires large land space Photoinhibition
Flat PBR	Relatively cheap Easy to clean up Large illumination surface area Suitable for outdoor cultures Low power consumption Good biomass productivities Good light path Readily tempered Low oxygen build-up Shortest oxygen path	Difficult scale-up Difficult temperature control Some degree of wall growth Hydrodynamic stress to some algal strains Low photosynthetic efficiency
Column PBR	Low energy consumption Readily tempered High mass transfer Good mixing Best exposure to light-dark cycles Low shear stress Easy to sterilize Reduced photoinhibition Reduced photo-oxidation High photosynthetic efficiency	Small illumination surface area Sophisticated construction materials Shear stress to algal cultures Decrease of illumination surface area upon scale-up Expensive compared to open ponds Support costs Modest scalability

Table 1.1: Advantages and limitations of open and closed culture systems (Adapted from Dragone *et al.*, 2010).

1.4 Harvesting Techniques Applied to Microalgae

Microalgal harvesting is the process of concentrating algal biomass from the cultivation medium to a concentration that is appropriate for the economic downstream processing (Bilad *et al*., 2014). Harvesting of algae can be done by different methods such as flotation, filtration, centrifugation and sedimentation. A summary comparison of various harvesting techniques applied to microalgal biomass is given in Table 1.2 (Barros *et al*., 2015). At laboratory scale, centrifugation is the method of choice for harvesting microalgal cells, but once the process is scaled up centrifugation quickly becomes uneconomic due to energy (electricity) costs. Filtration and flocculation methods, as shown in Table 1.2, have been tried with varying degrees of success and there is no doubt that finding an economical way to harvest algal cells is one of the main requirements for building a commercially successful business based on biofuels from microalgae (Gilmour and Zimmerman, 2012).

Harvesting method	Advantages	Disadvantages
Chemical coagulation/flocculation	• Simple and fast method. • No energy requirements.	• Chemical flocculants may be expensive and toxic to microalgal biomass. • Recycling of culture medium is limited.
Auto and bioflocculation	• Inexpensive method. • Allows culture medium recycling. • Non-toxic to microalgal biomass.	•Changes in cellular composition. • Possibility of microbiological contamination.
Gravity sedimentation	• Simple and the inexpensive method.	• Time-consuming. • Possibility of biomass deterioration. • Low concentration of the algal cake.
Flotation	• Feasible for large scale applications. • Low cost method. • Low space requirements. • Short operation times.	• Generally requires the use of chemical flocculants. • Unfeasible for marine microalgae harvesting.
Electrical based processes	• Applicable to a wide variety of microalgal species. • Do not require the addition of chemical flocculants.	• Poorly disseminated. • High energetic and equipment costs.
Filtration	• High recovery efficiencies. • Allows the separation of shear sensitive species.	• The possibility of fouling/clogging increases operational costs. • Membranes should be regularly cleaned. • Membrane replacement and pumping represent the major associated costs.
Centrifugation	• Fast method. • High recovery efficiencies. • Suitable for almost all microalgal species.	• Expensive method. • High energy requirements. • Suitable only for the recovery of high-valued products. • Possibility of cell damage due to high shear forces.

Table 1.2. Comparison of different harvesting techniques applied to microalgae biomass (Adapted from Barros *et al*., 2015).

1.5 Microalgae as a Source of Biodiesel

The proposal to use microalgae as a renewable source of fuel has a long history (Borowitka and Moheimani, 2013 and references therein). In 1942 Harder and von Witsch proposed that microalgae could be a suitable source of lipids which might be used as food or for the production of biofuels. By 1951, the possibility of the producing biofuels using algal oils was suggested by Milner. Shortly afterwards in 1952, Aach reporting on the growth of *Chlorella pyrenoidosa* in a photobioreactor found that under nitrogen deficiency cells accumulated up to 70% of dry weight as lipids in stationary phase. Around the same time it was also reported that the actual lipid productivity is low (Geoghegan, 1951). This paradox of high lipid per cell versus versus low productivity on a volume of medium basis has persisted to the present day (Liu *et al.,* 2013). The potential of algae as food source was a hot topic after the second world war because of the need to feed a growing world population (Spoehr and Miliner, 1949). With this driving force, the first large scale algal cultures were set up at MIT in Boston and at the Stanford Research Institute, USA in 1948 - 1950. During the running of large scale culture experiments, some important fundamental advances were made in our understanding of microalgae biology and these studies laid the ground work for subsequent research into the large scale production of algae for biofuels production (Hunter and Provasoli, 1964). The idea of using algae as human food was overtaken by the great increases in conventional agriculture that took place in the 1950s. Only in the Far East (Japan, Thailand) did microalgae become established as a human food (Gilmour *et al.,* 2012).

1.5.1 Advantages of Using Microalgae for Biodiesel Production

The advantages of using microalgae over the other available feedstocks (first and second generation biofuels) for biodiesel production have been described in many research articles (Chisti, 2007). Microalgae are easy to grow with little or even no attention and they can be grown on non-arable land, brackish water and waste water. Moreover, microalgae have the ability to grow anywhere using water and sunlight to reproduce through photosynthesis which is simply converting energy from the sun into chemical energy with high growth rate comparing to conventional forestry, or agricultural crops. Estimates of the maximum photosynthetic efficiency for algae vary from 5 – 10%, which contrasts with crop plants showing efficiencies of 1 to 2% (Walker, 2009). Some microalgae species can live in harsh environmental conditions such as high salinity and extremes of temperature (Gilmour and Zimmerman, 2012).

Other indirect advantages of using microalgae for biofuel production are as follows (Breenan and Owende, 2010; Borowitzka and Moheimani, 2013):

- Microalgae can mitigate greenhouse gases from the atmosphere and industrial flue gases through carbon capture and use it in photosynthesis.
- Microalgae can be used to remove heavy metals from the environment coupled with the production of potentially valuable biomass.
- The residual biomass fraction after oil extraction can be used as a high protein feed for farm animals. Also, its contains carbohydrates that can be used for bioethanol production.

- Microalgae have the ability to grow in harsh conditions and can grow in unsuitable areas for agriculture purposes regardless of the seasonal weather change, therefore they don't compete with food production.

- Using wastewater as a medium for growing microalgae can lower the cost of biofuel production.

- Other commercial potential form microalgae are human nutrition, biofertiliser, polyunsaturated fatty acids, and recombinant proteins.

1.5.2 Algal Lipids

Algal lipids can be divided into two main groups: the non-polar lipids (fatty acids, hydrocarbons, wax, sterols and steryl esters) and polar lipids (phosphoglycerides, glycosylglycerides) (Gunstone *et al*, 2007). They are essential constituents of all living cells where they perform important functions. Phosphoglycerides, glycosylglycerides and sterols are essential structure components of biological membranes. These lipids maintain specific membrane functions and provide the permeability barrier surrounding cells and between organelles within cells, as well as providing a matrix for various metabolic processes. The non–polar lipids mainly triacylglycerols (TAG) are abundant storage compounds which can be easily catabolised to provide metabolic energy (Gurr *et al,* 2002). As already described in section 1.2.1, algae comprise a large group of photosynthetic organisms from different phylogenetic groups representing many taxonomic divisions. They are distributed worldwide inhabiting predominantly freshwater and seawater ecosystems. The ability of algae to adapt

to environmental conditions is reflected in their exceptional variety of lipids. Many algae accumulate substantial amounts of non-polar lipids, mostly in the form of triacylglycerol (TAG) or hydrocarbons, and these levels may reach up to 20-50% of dry cell weight (Brennan and Owende, 2010). These oleaginous species have been considered as promising sources of oil for biofuels. The potential advantages of algae as a source of oil for biofuels include their ability to grow at high rates exhibiting a rapid biomass doubling time usually from 1-6 days and producing 10-20 times more oil than any crop plants (Chisti, 2007). Algae can grow in saline brackish and coastal seawater with little competition. They may utilize growth nutrients from seawater sources and sequester carbon dioxide from emitted flue gases, thereby providing additional environmental benefits. Moreover, algae can produce valuable co-products including β-carotene, pigments, fatty acid, vitamins, and proteins (Markou and Nerantzis, 2013). Thus, algae exhibit superior attributes to terrestrial crops plants as bioenergy sources. Moreover, in most cases algae will not compete for habitats used to produce food crops (Chisti, 2008).

1.5.3 Induction of Neutral Lipid Production

One of the advantages of using microalgae for biofuels particularly for biodiesel is that they can grow very fast doubling their numbers every few hours under ideal growth conditions, with the ability to produce a large volume of biomass. However, the amount of lipids in their biomass is low which made it less ideal for biofuel (Sharma *et al.*, 2012). From the literature, enhancing of the lipid productivity can be made using one or more of lipid induction techniques such as high light intensity, high salinity, high CO_2 concentrations and nutrient limitations – normally nitrogen or phosphorus. Table 1.3 shows the effect of different environmental stresses on microalgae biomass accumulation and biochemical composition

for biofuels. It's believed that microalgae act in response to the stress conditions by changing their metabolic pattern to maintain their growth rates or to increase the chance of surviving under these harsh environmental conditions (Markou and Nerantzis, 2013).

Microalgae	Biomass productivity (mg l⁻¹ d⁻¹)		Biochemical content (% of dry cell weight)			Environmental stresses
	Before stress	After stress		Before stress	After stress	
Arthrospira (Spirulina) platensis	193	87	Carbohydrate	11	67	Phosphorous limitation
Nannochloropsis sp.	633	457		8	11	High carbon dioxide
Scenedesmus obiquus CNW-N	441	841		16	38	High light
	841	732		38	52	Nitrogen limitation
Spirulina sp.	-	-		14	21	High temperature
Tetraselmis subcordiformis	-	-		<10	32	Nitrogen limitation
Chaetoceros muelleri	70	-	Lipid	19	36	Silicon limitation
Chlorella vulgaris	138	133		6	15	Nitrogen limitation
Cyclotella cryptica	-	-		18	38	Silicon limitation
Dunaliella tertiolecta ATCC 30929	-	-		60	70	High salinity
Nannochloropsis oculata	127	73		8	14	High temperature
	127	103		8	16	Nitrogen limitation
Nannochloropsis sp.	633	457		7	9	High carbon dioxide
Nannochloropsis sp. F&M-M24	360	300		32	60	Nitrogen limitation
Navicula saprophila	-	-		24	49	Silicon limitation
Neochloris oleoabundans	630	400		16	34	Nitrogen limitation
Scenedesmus obiquus CNW-N	841	732		12	22	Nitrogen limitation
Scenedesmus sp. LX1	37–64	27		23–28	53	Phosphorous limitation

Table 1.3. The effects of different environmental stresses on accumulation and composition of microalgal biomass for biofuel production. Adapted from Cheng and He (2014).

1.5.4 Extraction of Algal Lipids

Various methods for extraction of lipids from microalgae have been examined. Those methods can be divided mostly into mechanical extraction such as oil expeller or press, ultrasound assisted, microwave assisted and chemical extractions such as solvent extraction, supercritical CO_2, and ionic liquid extraction. A summary of the advantages and disadvantages of those methods is given in Table 1.4 (Mubarak *et al*., 2009). Selecting a method for lipid extraction needs to show a high level of specificity to algal lipid in order to reduce the co-extraction of non-lipid contaminants, like protein and carbohydrates. Also, the method has to be more selective to triacylglycerols (neutral lipids) more than other lipids such as polar lipids, free fatty acids as only triacylglycerols are suitable for biodiesel production (Halim *et al*., 2012 : Ugoala *et al*., 2012).

	Method	Advantages	Disadvantages
Mechanical methods	Oil expeller	• Easy to use • No solvent required	• Large amount of biomass required • Slow process
	Ultrasonication assisted	• Reduced extraction time • Reduced solvent consumption • Improved release of cell content due to greater penetration of solvent into the cellular materials	High power consumption, difficult to scale up
	Microwave assisted	• More economical • Environmental friendly, • Reduced extraction time • Reduced solvent usage • Improved extraction yield.	• Filtration or centrifugation is necessary to remove the solid residue • Efficiency of microwaves can be very poor when either the target compounds or the solvents are non-polar, or volatile.
chemical methods	Solvent extraction	• Very simple and cheap • Good for small scale • High efficiency.	• Extraction time is long; • Large volume of solvent required, toxic and highly flammable • Solvent recovery is energy intensive.
	Supercritical CO_2	• Reduced time, • Tunable solvating power due to flexibility of changing pressure and temperature for higher selectivity, • Low toxicity solvents, • Favorable mass transfer equilibrium due to intermediate diffusion/viscosity properties of the fluid, • Production of solvent-free extract.	• High process cost associated with its infrastructure and operation.
	Wet extraction	• Saving in energy required for drying the biomass, • Reduced solvent usage.	• Quality of lipid extracted may not be as good as lipid extracted from dried biomass.

Table 1.4. Advantages and disadvantages of different methods of oil extraction. Adapted and modified from Mubarak *et al.* (2009).

1.5.5 Conversion of Algal Lipids to Biodiesel

The production of biodiesel from microalgae has been discussed at length in the literature and it is one of the most desirable biofuel products from microalgae (Suali and Sarbatly, 2012). Following the extraction procedure, the resultant microalgal lipids can be converted into biodiesel through transesterification which is a chemical reaction where triglycerides are converted into fatty acid methyl esters (FAME) and glycerol as a by product in the presence of an alcohol such as methanol or ethanol and a catalyst either alkali or acid (Figure 1.3) (Dragone *et al.*, 2010). For the complete replacement of conventional liquid fuels by microalgae-produced biodiesel, the following would need to be achieved: (1) as much as necessary biomass produced to make fuel at a commercial scale; (2) the production cost should be less than fossil fuel production cost; (3) the algal biodiesel produced should meet all US and Euopean standards for fuel quality (Harun *et al.*, 2010; Stansell *et al.*, 2012).

$$\begin{array}{llllllll} CH_2OCOR_1 & & & & CH_2OH & & R_1COOCH_3 \\ | & & & & | & & \\ CH\,OCOR_2 & + & 3HOCH_3 & \xrightarrow{\text{catalyst}} & CH\,OH & + & R_2COOCH_3 \\ | & & & & | & & \\ CH_2OCOR_3 & & & & CH_2OH & & R_3COOCH_3 \\ \text{Triglycerides} & & \text{Methanol} & & \text{Glycerol} & & \text{Methyl esters} \end{array}$$

Figure 1.3. The chemical reaction of the transesterification procedure (Suali and Sarbatly, 2012). Triglycerides = triacylglycerols = TAGs. The methyl esters are biodiesel.

1.6 Algal Biorefinery Concept

The concept of algal biorefining is analogous to current petroleum refineries in which various fuels and chemicals are produced from crude oil. In the same way, algal biorefining is seen as an integrated facility, for sustainable processing of algal biomass into marketable products and energy through various processes and equipment based on biomass conversion. The variation in the components of microalgal biomass led to a range of products being produced that maximize the value of biomass utilization (Trivedi *et al.*, 2015). The biorefinery concept can meet several environmental deliverables such as mitigating sustainability issues with respect to greenhouse gas emissions, fossil fuel usage, potential food shortages and land use change for fuel production. The biorefinery of the future will carry out several applications aiming to optimize overall process economics and minimize overall environmental impact, Those applications include: (1) Energy options from microalgae (biodiesel, bioethanol, biogas, bio -jet fuel); (2) Non energy options from microalgae (feed industry, food industry, pharmaceutical industry, chemical industry, and cosmetics industry); (3) Environmental applications (bio-mitigation of CO_2 emissions using microalgae and bioremediation of waste water and polluted soil using microalgae). Figure 1.4 shows the future schematic flow sheet of an algae based biorefinery (Hughes *et al.*, 2013; Trivedi *et al.*, 2015). The majority of these applications are being developed individually, but have the potential to be extra efficient and economical when joined together in multi-process intersecting regimens using by-products or waste materials from one process to feed another application.

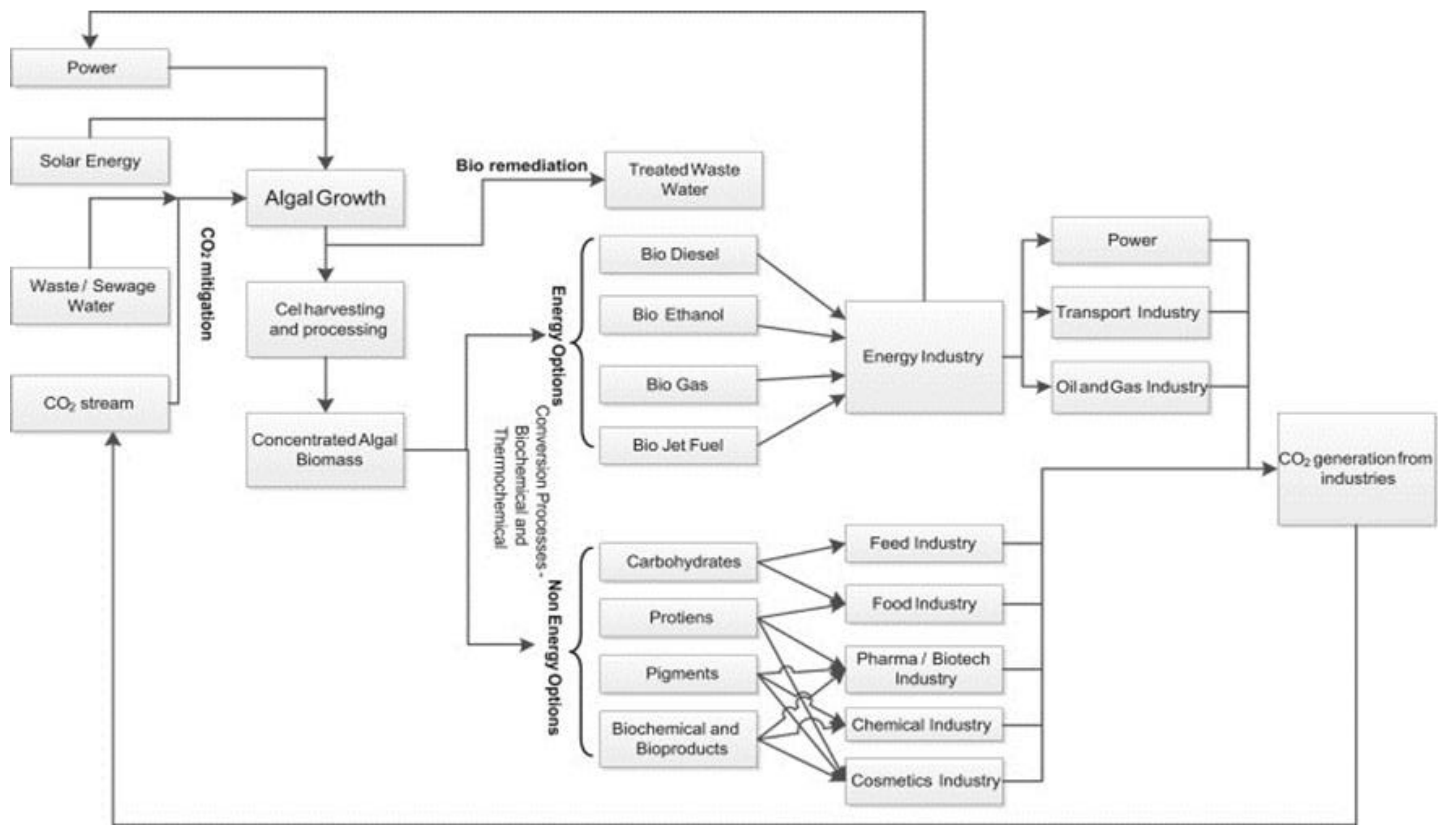


Figure 1.4. The future schematic flow sheet of the algae based biorefinery. Adapted from (Trivedi *et al.*(2015)

1.7 Polymerase Chain Reaction (PCR)

Karry Mullis was awarded a Nobel Prize in 1993 for his work on fine-tuning the polymerase chain reaction (PCR) technique. PCR works by utilising an enzyme called DNA polymerase. This enzyme makes a copy of a selected region of DNA that needs to be amplified. This way, "the amount of the selected DNA region doubles over and over with each cycle - up to millions of times the starting amount - until enough is present to be seen by gel electrophoresis" (Weaver 2005). Two predetermined sites in a region of a DNA are amplified with the help of oligonucleotides (primers) that are complementary to these sites. The latter serve as primers for synthesis of copies of the DNA region. Each cycle of PCR doubles the number of DNA molecules synthesized until a huge amount is produced (Gibbs 1990). PCR can provide molecular biologists and other researchers with almost unlimited amounts of the exact genetic material that they want to study. The PCR reaction consists of three steps: denaturation, synthesis and reannealing (Weaver 2005). These three steps are repeated around 30 times and each cycle only takes 3 to 5 minutes to complete using an automated thermal cycler. In addition, amplification of PCR is both simple and elegant. Oligonucleotide primers are used to complement the ends of a DNA sequence to be amplified. Deoxyribonucleotides are used with an appropriate buffer. Healing and cooling are used to denature the original DNA strands and allow annealing of the primer. This process is continued repeatedly and extended in order to create new copies of the original DNA fragment (Arnheim and Erlich, 1992). Usefulness of PCR lies in its capability to amplify DNA coming from very small amounts of the target DNA or from the DNA of a single cell. Due to this characteristic, PCR has become an intrinsic part in molecular biology laboratories.

In the field of microalgal identification, the 18S ribosomal (r)RNA gene is used and specific primers are produced for this gene. After PCR, the 18S rDNA can be sequenced and then put into the BLAST database to identify the algal species.

1.8 Aims and Objectives

- To confirm the identity of the *Tetraselmis* strain received from the Culture Collection using 18S rRNA gene sequencing.
- To optimise the growth medium for culturing the *Tetraselmis* species and if possible produce a fully defined seawater medium for growing *Tetraselmis.*
- To use methods refined in the Gilmour laboratory to measure the amount of TAG in *Tetraselmis* cells grown under a variety of salinities and pH.
- To use methods refined in the Gilmour laboratory to measure the fatty acid profile of *Tetraselmis* using GC-MS.
- To examine the possibility of using *Tetraselmis* as the basis of an algal biorefinery.

Chapter Two

Materials and Methods

2. Materials and Methods

This chapter describes chemicals, enzymes and other materials used in carrying out the experiments. Also, a description of protocols including molecular biology methods is included.

2.1. Chemicals

Chemical or Kit	Provider
PCR Master Mix	Thermo-Fisher
6x DNA Loading Dye	Fermentas Life Sciences
Genomic DNA Buffer Set	QIAGEN
Genomic-tip Kit	QIAGEN
QIAquick PCR Purification Kit	QIAGEN
Agarose	ICN Biomedical
DNA Hyper ladder	BIOLINE
Ethidium Bromide	BIO-RAD
Percoll™	Sigma-Aldrich
Nile Red	Sigma-Aldrich
Grams Iodine	Camlab
Triolein	Thermo-Fisher
Tridecanoic acid (C13 lipid)	Fluka
Methyl nonadecanoate (C19 FAME)	Fluka

Table 2.1: Non-Standard Chemicals Used.

2.2. Enzymes

Enzymes	Provider
Ribonuclease (RNase)	Sigma
Lysozyme	Sigma
Proteinase	Sigma

Table 2.2: Enzymes

2.3. Algae Strain

Algae strain	Provider
Tetraselmis suecica CCAP 66/4	Culture Collection of Algae and Protozoa

Table 2.3: Algae strain

2.4. Buffers, Vitamins and Trace Elements

Buffer, Vitamin or Trace Element	**Composition**
50X TAE Buffer	Per litre 242g Tris base, 57.1 mL glacial acetic acid, 100 mL of 500 mM EDTA (pH 8.0).

EDTA Buffer	For 500 ml stock of 0.5 M (pH 8.0): 93.05g EDTA disodium salt in 400 ml deionised water.
Trace elements	Per Litre: Na_2EDTA 4.16 g, $FeCl_3.6H_2O$ 3.15 g, $CuSO_4.5H_2O$ 0.01 g, $ZnSO_4.7H_2O$ 0.022 g, $CoCl_2.6H_2O$ 0.01 g, $MnCl_2.4H_2O$ 0.18 g, $Na_2MoO_4.2H_2O$ 0.006 g.
Vitamin Solution F/2 Medium	Per 100 ml: Cyanocobalamin (Vitamin B12) 0.1g and Biotin (Vitamin H) 0.1g.
Trace metal solution BG11 (Blue-Green Medium).	Per litre: H_3BO_3 2.86g, $MnCl_2.4H_2O$ 1.81g, $NaSO_4.7H_2O$ 0.22g, $Na_2MoO_4.2H_20$ 0.39g, $CuSO_4.5H_2O$ 0.08g $Co(NO_3)_2.6H_2O$ 0.05g.

Table 2.4: Buffers, Vitamins and Trace Elements.

2.5. Cleaning and Sterile Techniques

To ensure sterile conditions, all culturing equipment used in experiments was autoclaved. Also, inoculation and sub-culturing were carried out with a flame after cleaning the bench with 70% ethanol. Moreover, the glassware was soaked for 2 hours in concentrated sulphuric acid to ensure that all residual material from previous cultures was removed.

2.6. Medium Preparation

2.6.1 Dunaliella Medium

The medium composition was obtained from Hard and Gilmour (1996). Composition – NaCl (solid) 23.4 g l^{-1}, 0.4 M; KCl: 10 mM; $MgCl_2$: 20 mM; $CaCl_2$: 10 mM; $MgSO_4$: 24 mM; $NaNO_3$: 5 mM; Na_2SO_4: 24 mM; NaH_2PO_4: 0.1 mM; FeEDTA pH 7.6: 1.5 µM; HEPES pH7.6: 20 mM; $NaHCO_3$ (solid): 1 g l^{-1}. Trace elements – H_3BO_3: 185 mM; $MnCl_2.4H_2O$; 7 mM, $ZnCl_2$: 0.8 mM; $CoCl_2$: 20 µM; $CuCl_2$: 0.2 µM.

2.6.2 F/2 Medium

F/2 Medium is an artificial seawater medium and was prepared as described in Guillard and Ryther (1962). Composition – 33.6 g l^{-1} of artificial seawater salts (Ultramarine Synthetica); $NaNO_3$: 0.882 mM; $NaH_2PO_4.H_2O$: 36.2 µM. Trace elements – $FeCl_3.6H_2O$: 11.7 µM; $Na_2EDTA.2H_2O$: 11.7 µM; $MnCl_2.4H_2O$: 0.91 µM; $ZnSO_4.7H_2O$: 0.0765 µM; $CoCl_2.6H_2O$: 0.042 µM; $CuSO_4.5H_2O$: 0.0393 µM; $Na_2MoO_4.2H_2O$: 0.026 µM. Vitamins – Thiamine.HCl (vitamin B1): 0.296 µM; Biotin (vitamin H): 2.05×10^{-9} M; Cyanocobalamin (vitamin B12): 3.69×10^{-10} M.

2.6.3 TS Medium

This was a medium derived in the Gilmour laboratory, but it was based on the seawater composition from the work of Castro and Huber (1997). Levels of Dunaliella and F/2 media components were adjusted to give more similar amounts to seawater composition. F/2 trace elements were used as these were more representative of seawater values. Composition – NaCl (solid): 24.4 g l^{-1}; $MgSO_4$: 53.27 mM; $MgCl_2$: 53.27 mM; Na_2SO_4: 28.11 mM; $CaCl_2$: 10.38 mM; KCl: 9.97 mM; H_3BO_3: 443.89 µM; $NaNO_3$: 4 M.

2.6.4 BG11 Medium

BG11 medium is commonly used for growing cyanobacteria and the composition was obtained from Stanier *et al.* (1971). Three stock solutions are prepared. Stock solution 1: $NaNO_3$ 15 g l^{-1}. Stock solutions 2 to 8: each of the following were added to separate 500 ml volumes of distilled water: K_2HPO_4 2 g, $MgSO_4.7H_2O$ 3.7 g, $CaCl_2.2H_2O$ 1.80 g, citric acid 0.30 g, ammonium ferric citrate green 0.30 g, $EDTANa_2$ 0.05 g, Na_2CO_3 1 g. Stock solution 9 (trace metal solution) per litre: H_3BO_3 2.86 g, $MnCl_2.4H_2O$ 1.81 g, $ZnSO_4.7H_2O$ 0.22 g, $Na_2MoO_4.2H_2O$, 0.39 g, $CuSO_4.5H_2O$ 0.08g, $Co(NO_3)$ $2.6H_2O$ 0.05g. To prepare medium, 29.2 g of NaCl were added to 829 ml of distilled water and 100 ml of stock solution 1 were added, with 10 ml each of stock solutions 2 to 8 and 1 ml of stock solution 9 was added.

2.7 Growth of *Tetraselmis suecica*

2.7.1 Culture Methods

Tetraselmis suecica (CCAP 66/4) was obtained from the Culture Collection of Algae and Protozoa, Oban, UK (Butcher 1959). A primary stock culture (in a 100 ml flask) was prepared using the liquid *T. suecica* samples received from the CCAP and allowed to grow in the culture room at 25 ± 1°C with continuous light (50 – 70 µmol m^{-2} s^{-1}) supplied by daylight fluorescent lights. When the culture reached the stationary phase after around 2 weeks incubation, four different types of medium (TS, F/2, Dunaliella and BG11) were inoculated from the primary stock culture and allowed to grow to allow for adaptation to the medium before setting up growth curves.

2.7.2 Comparison of Different Media for Growing *T. suecica*

Tetraselmis suecica was cultured in four different media (TS medium, F/2 medium, Dunaliella medium and BG11 medium) to observe which medium is the most appropriate based on the highest growth potential. Growth was measured using a Unicam Helios alpha spectrophotometer at 595 nm. OD_{595} readings (using medium as the blank) were taken every day to measure the growth rate of *T. suecica*.

2.7.3 Development of F/2 Defined Medium

One of the advantages of using algae for biofuel production is the ability to control their accumulation and secretion of biofuels by changing their growth conditions or by metabolic engineering. This led to the development of a defined F/2 medium where the quantity of the chemicals added to the media such as N, P, and NaCl can be adjusted. In addition, F/2

medium prepared using the commercial seawater salt mixture is not suitable for use at higher salinities than seawater, because the medium precipitates after autoclaving. The defined F/2 medium is an important outcome of this work.

2.7.4 Comparison of F/2 Medium and Defined F/2 Seawater Medium

After the development of F/2 defined medium in the laboratory a growth comparison for *T. suecica* was done by culturing the alga in both F/2 medium and F/2 defined medium. Growth (OD_{595}) was measured every 24 hours over a 12 day growth period.

2.7.5 Effect of Salinity on Growth of *T. suecica*

A common observation is that an increase in salinity can increase the lipid content of microalgae, but lowers the growth rate of a species. Therefore, the effect of salinity on the growth and lipid content of microalgae species was investigated.

2.7.6 Effect of pH on Growth of *T. suecica*

In this experiment the effect of pH on algal growth was evaluated every day using OD_{595} measurements.

2.8. Relationship between OD at 595 nm and Dry Weight (Biomass) for *T. suecica* cells grown at different salinities

After growing the green alga *T. suecica* for two weeks in defined F/2 medium containing different salinities to ensure adaptation to each salinity medium, three replicate cultures for each medium to be tested were inoculated from the appropriate adapted culture. Growth was determined by measuring OD_{595} every 24 hours.

A set of well grown cultures from 0.4, 0,8 and 1 M NaCl media (approximately $OD_{595} = 1$) was used to prepare dilutions according to the scheme shown in Table 2.5 with a final volume of 30 ml for each dilution. Then the OD_{595} reading of 1 ml from each dilution was taken and the samples returned to each tube. After that, all 12 × 50 ml tubes were centrifuged (3000 *g*) and supernatant was discarded. The pellets were resuspended in 5 ml of distilled water and transferred to 12 × 15 ml Falcon tubes which were then centrifuged (3000 *g*), the supernatants were removed and the pellets were resuspended in 1 ml of distilled water and transferred to pre-weighed 1.5 ml Eppendorf tubes. Eppendorf caps were removed from another set of tubes and used to seal the sample Eppendorf tubes instead of their own caps which remained attached. A hole was made in each cap to allow moisture to escape and the samples were than frozen over night at -80°C and then freeze dried (lyophilised) for 48 hours until they were completely dry. The caps with the hole were removed and discarded and the original caps were used to reseal the tubes. All tubes were then reweighed using the fine balance and the calculation of dry weight for each sample was made by subtracting the initial weight from the final weight. A concentration curve of OD_{595} versus dry weight was created using Excel.

Tube Number	Concentration (%)	Culture (ml)	Medium (ml)
12	0.0	0	30
11	8.3	2.5	27.5
10	16.6	5	25
9	33.3	10	20
8	41.6	12.5	17.5
7	50	15	15
6	58.3	17.5	12.5
5	66.6	20	10
4	75	22.5	7.5
3	83.3	25	5
2	91.6	27.5	2.5
1	100	30	0.00

Table 2.5. Dilution scheme used to prepare each set of samples for dry weight versus OD_{595} comparison.

2.9. Relationship between OD at 595 nm and Cell Number for *T. suecica* Cells Grown in F/2 Medium

To check whether cell number correlates strongly with optical density (OD) measurements of algae cultures, an OD versus direct cell count calibration curve was performed based on the work by Madigan *et al.* (2003), Reed (1998) and Skoog *et al.* (2007). The OD_{595} of a well grown algal culture was adjusted to 1 and then dilutions from 5% to 100% were made using the cell culture and fresh medium in 15 ml Falcon tubes as shown in Table 2.6.

Tube Number	Concentration (%)	Culture (ml)	Media (ml)
11	5.0	0.25	4.75
10	10.0	0.50	4.50
9	20.0	1.00	4.00
8	30.0	1.50	3.50
7	40.0	2.00	3.00
6	50.0	2.50	2.50
5	60.0	3.00	2.00
4	70.0	3.50	1.50
3	80.0	4.00	1.00
2	90.0	4.50	0.50
1	100.0	5.00	0.00

Table 2.6. Dilution scheme used to produce a range of cell concentrations.

Then 1 ml from each dilution was transferred to a plastic cuvette and the OD_{595} measurements were taken using the UNICAM Helios Alpha spectrophotometer with fresh medium as the blank. Following the OD readings, 900 µl from each dilution was transferred to 1.5 ml Eppendorf tube and 100 µl of Gram's Iodine was added and mixed well (to stop cell motility). After that, 20 µl was placed into the counting chamber of a Neubauer improved haemocytometer and viewed using a Nikon microscope with the x40 objective (x400 magnification). Five replicates were carried out for each dilution. The number of cells in each dilution was calculated using the following equation:

$$\text{Depth} = 0.02 \text{ mm, area of small square} = 1/400 \text{ mm}^2 = 2.5 \times 10^{-3} \text{ mm}^2$$

$$\text{Volume of small square} = 2.5 \times 10^{-3} \times 0.02 = 5.0 \times 10^{-5} \text{ mm}^3$$

$$\text{Cell no. per small square} \times \frac{1}{5\times10^{-8}} = \text{cell mL}^{-1} \text{ sample}$$

$$\frac{\text{cells mL sample}}{9} \times 10 = \text{cells mL}^{-1} \text{ original culture}$$

The data from the OD_{595} and cell count were compiled to give OD at 595 nm versus cell count graph.

2.10. Examination of Cell Morphology Using the Scanning Electron Microscope

The cell morphology of *T. suecica* was examined in culture using a scanning electron microscope (SEM). Additionally, the cultures were examined by light microscopy to ensure that the cultures used for experiments were axenic. This method was developed in the Gilmour laboratory based on the work by Stephen Marshall (Marshall, 2013).

2.10.1 Chemicals and Equipment

- Percoll™ (Sigma-Aldrich)
- 15ml Falcon tubes
- Ice
- 1ml plastic cuvettes
- Glass coverslips
- 50ml Falcon tubes
- 0.1% (or higher) polylysine solution
- Fresh growth medium
- 2% osmium tetroxide solution
- Ethanol
- Filter sterilised water
- Self-adhesive SEM sample mounts
- Lab tissue

2.10.2 Sample Preparation

For each strain to be tested 1 ml of culture (OD_{595} of approximately 0.8) was used. The cultures need to be relatively clean and free of bacteria before the slides are made. If cleaning was required, running the sample through a Percoll™ discontinuous gradient was recommended. To make the gradient the following steps need to be performed:

1. Six 10 ml stocks of 10%, 20%, 30%, 40%, 50% was created and 60% Percoll by combining growth medium with the appropriate amount of 100%.
2. Stocks were cooled on ice before slowly layering them in a 15 ml Falcon tube (60% at bottom up to 10% at top).
3. When the fractions had been added and had settled, 1 ml of culture was added to the top of the gradient.
4. The Percoll™ gradients were then centrifuged at 1500 *g* for 15 mins.
5. When completed, the top layers of Percoll were removed gently to reach the green algal band using a 1 ml automatic pipette and discard.
6. Carefully, the green band was removed and used to inoculate a freshly autoclaved flask of sterile media. The contaminating bacteria should be found at or near the bottom of the Falcon tube.

2.10.3 Slide Preparation

1. Standard glass coverslips was coated into 5x5 mm squares using a glass cutting pen.
2. Coverslips was soaked in 0.1% polylysine solution within a 50 ml Falcon tube or trough overnight (or longer).
3. 1 ml of clean culture was taken and adjusted to OD_{595} of 0.8 in a plastic cuvette using the spectrophotometer using the appropriate medium as a blank).
4. 1.5 ml Eppendorf tube were transferred and centrifuged in the microfuge at full power (10000 *g*) for 3 mins and supernatant were discarded.
5. 200 µl of fresh medium were added and agitated gently to bring the pellet slightly out of suspension.
6. 40 µl of 2% osmium tetroxide were added to the pellet. The tube was left to sealed at ambient temperature for 30 mins.
7. The poly-lysine coated coverslips were washed with filter sterilised dH_2O, dry at 40˚C.
8. The Eppendorf tube was agitated gently to bring the cultures into suspension.
9. The coated coverslips was taken and stacked to a self-adhesive SEM sample plate.
10. 40 µl of the cells were added to the centre of one of the coated coverslips and left to stand for 10 min to partially dry.
11. Samples were washed by dropping 60 µl of 50% ethanol solution onto the coverslip. Step was repeated with 60 µl of 75% ethanol.
12. Samples were washed again using 95% and 100% washing gradients (60 µl for each), instead air dry each time in a fume cupboard.
13. When the last gradient is completely dry, sputter coat with gold (2 nm thick) and analysed with a scanning electron microscope (Accelerating voltage: 20.0 kV, 1000-10000 magnification).

2.11 Molecular Identification of *T. suecica*

2.11.1 DNA Extraction

Genomic DNA extraction was performed using two extraction methods (Qiagen Kit and CTAB) as described below.

2.11.1.1 DNA extraction using Qiagen Kit

The extraction was done according to the manufacturer's protocol. Briefly:

Sample preparation for lysis:

- *5 ml of algae culture was centrifuged in Falcon tube for 10 mins at 3000 g*
- *The supernatant was then discarded and the pellet was resuspended in 1 ml of buffer B1 with 2 µl RNase and mixed by vortexing the tube.*
- *20 µl of lysozyme stock solution and 45 µl of proteinase stock solution were added and then tube was incubated at 37°C for 30 min.*
- *0.35 ml of buffer B2 was added and tube was mixed by inverting the tube several times and then incubating at 50°C for 30 min.*

Genomic- tip protocol:

- *QIAGEN- tip was equilibrated with 2 ml of QBT buffer*
- *Sample was vortexed and applied to the equilibrate QIAGEN- tip.*
- *The tip was then washed with 3 x 1 ml of buffer QC.*
- *Genomic DNA was then eluted with 2 x 1 ml of buffer QF.*
- *DNA was precipitated by adding 1.4 x isopropanol to the eluted DNA and tube was inverted 20 times, sample was then centrifuged at 5000 g for 15 mins and supernatant was discarded.*

- *DNA pellets were washed with 1 ml of cold 70% ethanol. The tube was then centrifuged at 5000 x g for 10 min and supernatant was discarded.*
- *Air-drying was performed for 10 mins on the bench.*
- *DNA was then resuspended in Tris-Buffer overnight.*

2.11.1.2 DNA extraction using CTAB

DNA extraction using CTAB was performed based on a method described by Li *et al.* (2002).

- 5 ml of algae culture was centrifuged in a 15 ml Falcon tube for 10 min at 3000 *g*.
- Supernatant was discarded and the pellet was resuspended in 500 µl CTAB and then sonicated for 30 seconds at full power.
- Sample was then incubated at 65°C for 1 hour.
- Phenol- Chloroform extraction was then carried out as follows:
    - 500 µl of phenol-chloroform isoamylacohol (24:25:1) was added to the sample.
    - Sample was then vortexed and centrifuged for 5 mins at full speed in a microfuge.
    - Top layer was removed into a fresh Eppendorf tube and 500 µl of chloroform was added.
    - Sample was then centrifuged for 5 min at full speed in a microfuge and the top layer was transferred into a fresh Eppendorf tube.
    - 1/10 total volume of 3 M sodium acetate pH 5.2 was added. This was followed by adding 2.5 volumes cold 100% ethanol.
    - Sample was then incubated on ice for 30 mins and then centrifuged at 6000 xg for 15 mins.

- Supernatant was discarded immediately and 1 ml of ethanol was added.
- Eppendorf tube was then centrifuged for 5 mins at full speed and supernatant was discarded.
- Centrifugation was carried out again for the same time and speed.
- Air drying was then performed for 10 mins.
- Pellet was resuspended in MilliQ water.
- DNA pellet was left on bench to resuspend overnight and incubated for 90 mins at 50°C in the morning.

2.11.2 DNA Quantification

DNA was quantified using a Nanodrop spectrophotometer, following the manufacturer's protocol using the wavelength of 260 nm.

2.11.3 Polymerase Chain Reaction (PCR):

The PCR amplifications were performed in a Mycycler thermal cycler (Bio-Rad) and the cycle parameters included an initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, elongation at 72°C for 1 min and a final 10 min elongation step at 72°C (see Table 2.7). Two 18S rRNA gene primers were used in the identification of *T. suecica* (Table 2.8) and Table 2.9 provides details for the PCR mix.

Initial Denature	94 °C	5 min
Denature Anneal Elongation	95 °C 58 °C 72 °C	30 sec 30 sec } 30 cycles 1 min
Final Elongation	72 °C	10 min

Table 2.7: PCR cycle conditions for 18S rRNA gene.

Primer Name	Sequence
18S rRNA For Lim	5'-gcg gta att cca gct cca ata gc-3'
18S rRNA Rev Lim	5'-gac cat act ccc ccc gca acc -3'
18S For Sheehan	5'-aat tgg ttg atc ctg cca gc-3'
18S Rev Sheehan	5'-tga ttc tgt gca ggt tca cc-3'

Table 2.8: Sequence for 18S Lim and 18S Sheehan primers.

Sample	18S Lim	Replicate	18S Sheehan	Replicate	Lim Negative Control
Distilled water	17	17	17	17	22
For Primer	4	4	4	4	4
Rev Primer	4	4	4	4	4
Master Mix	20	20	20	20	20
Genomic DNA	5	5	5	5	0
	Total volume is 50 for all samples				

Table 2.9: Preparation for PCR using Master Mix.

2.11.4 PCR Purification

The amplified PCR products were purified using QIAquick PCR Purification kit (Qiagen). Also, the yield of the purified PCR product was measured in quartz cuvette at 260 nm using a spectrophotometer.

2.11.5 Gel Electrophoresis

DNA quality and PCR products were determined using 1% agarose gel which was made up using 0.7 g of agarose powder and 70 ml of distilled water with 1.4 ml of 50x TAE buffer. A 5µl aliquot of ethidium bromide was added to the gel mix after heating the mix in a microwave oven at power 6 for 90 seconds. Ten µl of the sample was mixed with 2 µl of 6x DNA loading dye and then loaded on to the agarose gel. Also, 7 µl of DNA ladder was loaded onto the agarose gel and the electrophoresis was run at 80 V for 60 min. Bands were visualized with a Uvidoc UV visualiser and photographs taken to capture the image.

2.11.6 Sequencing

DNA samples were sent out for sequencing to Eurofins/MWG. The obtained sequences were then compared against sequences in the GenBank nucleotide collection using the Basic Local Alignment Search Tool (BLAST).

2.12 Measurement of Respiration and Photosynthesis in *T. suecica* using Oxygen Electrode

The effects of salinity, pH and temperature on the photosynthesis and respiration of *T. suecica* were assessed in the laboratory using the Oxygen Electrode system as described by Delieu and Walker (1972). Before starting the experiment, 2 ml of distilled water was added to the reaction chamber for 15 minutes with the lid off to calibrate the oxygen electrode. Then a small amount of sodium dithionite was added to the chamber which results in a chemical reaction that removes the oxygen from the chamber and that means 100% oxygen saturation point before addition of dithionite and 0% oxygen point after dithionite addition. The number of chart recorder units between 0 and 100% is known as the range. Then the following equation is used to calculate the respiration rate:

Respiration Rate (µmoles O_2 mg chlorophyll^{-1} h^{-1}) =

$$\boldsymbol{Standard/Range \times Number\ of\ units/Time \times 60/mg\ chloro\ in\ sample}$$

- **Standard:** Amount of oxygen soluble in 2 ml medium (sample) = 0.660 µmoles ml^{-1} at 30°C or 0.722 µmoles ml^{-1} at 25°C
- **Range:** Units taken from calibration (0 – 100%)
- **Number of units:** Number of units are read directly from the chart recorder, it is normal to draw best fit straight line over 5 min
- **Time:** The length of time in minutes for which the sample was measured
- **60:** This converts the time from minutes to hours
- **Chloro = Chlorophyll present in sample (mg):** this relates to amount of chlorophyll in a sample of 2 ml of cells.

2.12.1 Effect of Salinity on Respiration and Photosynthesis Rate

Effect of salinity on respiration and photosynthesis rates was assayed using an oxygen electrode. Cells of *T. suecica* were adapted to stress before measuring photosynthesis and respiration. After adapting the cells to three different salt concentrations 0.4, 0.6 and 0.8 M NaCl, the OD_{595} was measured for each sample. Then 10 ml were taken and centrifuged at 3000 *g* for 10 mins, after that algae pellets were resuspended in 2 ml fresh medium with the same salt concentration and left on the lab bench for 1 hour. Two ml from each sample were added into the reaction chamber and respiratory oxygen uptake was detected in the dark for 3 mins then photosynthetic oxygen evolution was measured with a lamp as light source for 5 mins. The rate of each process was recorded on the chart recorder. The OD_{595} was used in place of chlorophyll content and respiration (oxygen uptake in the dark) was calculated using the equation shown above. To calculate the photosynthesis rate, the oxygen evolution in the light was added to the oxygen uptake in the dark (i.e. respiration rate was assumed to be the same in the light and the dark).

2.12.2 Effect of Temperature on Respiration Rate

Effect of temperature (25, 30, 35, 40°C) on respiration rate in *T. suecica* was assayed using an oxygen electrode. The cells were subjected to the stress immediately before measurement. OD_{595} was measured for each sample. Then 10 ml were taken and centrifuged at 3000 *g* for 10 mins, and after that alga pellets were resuspended in 2 ml of fresh medium with the same salt concentration and left on laboratory bench for 1 hour. Two ml from each sample were added into the reaction chamber and respiratory oxygen uptake and photosynthetic oxygen evolution were measured as described in section 2.12.1.

2.12.3 Effect of pH on Respiration and Photosynthesis Rate

Effect of pH 7, 8, and 9 on respiration and photosynthesis rates in *T. suecica* was assayed using an oxygen electrode. Cells of *T. suecica* were adapted to stress before measuring photosynthesis and respiration. After adapting the cells to three different pH levels (pH 7, pH 8 and pH 9), OD_{595} was measured for each sample. Then 10 ml were taken and centrifuged at 3000 *g* for 10 mins, after that algae pellets were resuspended in 2 ml fresh medium with the same pH concentration and left in lab bench for 1 hour. Two ml from each sample were added into the reaction chamber and respiratory oxygen uptake and photosynthetic oxygen evolution were measured as described in section 2.12.1.

2.13 Neutral Lipid Content and Fatty Acid Composition of *T. suecica*

2.13.1 Lipid Determination of *T. suecica* Cells by Gravimetric Methods

For gravimetric measurements, lipid extraction was performed based on the methods first described by Bligh and Dyer (1959). A well grown culture was taken (approximately OD_{595} = 1) and centrifuged for 10 mins at 3000 *g* and the pellets were resuspended in 5 ml of distilled water and transferred to 15 ml Falcon tubes. The samples were centrifuged again for 5 mins at 3000 *g* and the pellets were resuspended in 5 ml of distilled water and centrifuged once more, and then the pellets were resuspended in 1 ml of distilled water. Six pre-weighed Eppendorf tubes were labelled and 1 ml from each sample was transferred. Samples were frozen at -80°C overnight and then freeze dried (lyophilized) for 48 hours. The weight of biomass was estimated after re-weighing the Eppendorf tubes and the 500 µl of methanol /

chloroform (2:1 v/v) was added. The samples were sonicated for 1 min on ice and then centrifuged at full speed in the microfuge for 5 mins. The supernatants were discarded and chloroform and 1% NaCl (1 g NaCl in 100 ml) were added to give 2:2:1 methanol: chloroform: water. Samples were then centrifuged for 2 mins at full speed in the microfuge and the chloroform phase (top phase) was transferred into pre-weighed Eppendorfs and left with the tops open in the fume cupboard until dry. The weights of lipids recovered were calculated after re-weighing the Eppendorf tubes.

2.13.2 Lipid Determination by Nile Red Fluorescence

A number of experiments have been done to measure neutral lipid concentration in *T. suecica*. Firstly, droplets of neutral lipids were visualised using a fluorescence microscope. Secondly, optimisation of Nile Red Fluorescence emission using the 96 well microplate method involved optimisation of Nile Red concentration, cell concentration and time of staining. Finally, the Nile Red fluorescence method was quantified using triolein as the standard. Figure 2.1 shows schematically the plan of the main Nile Red fluorescence experiments carried out.

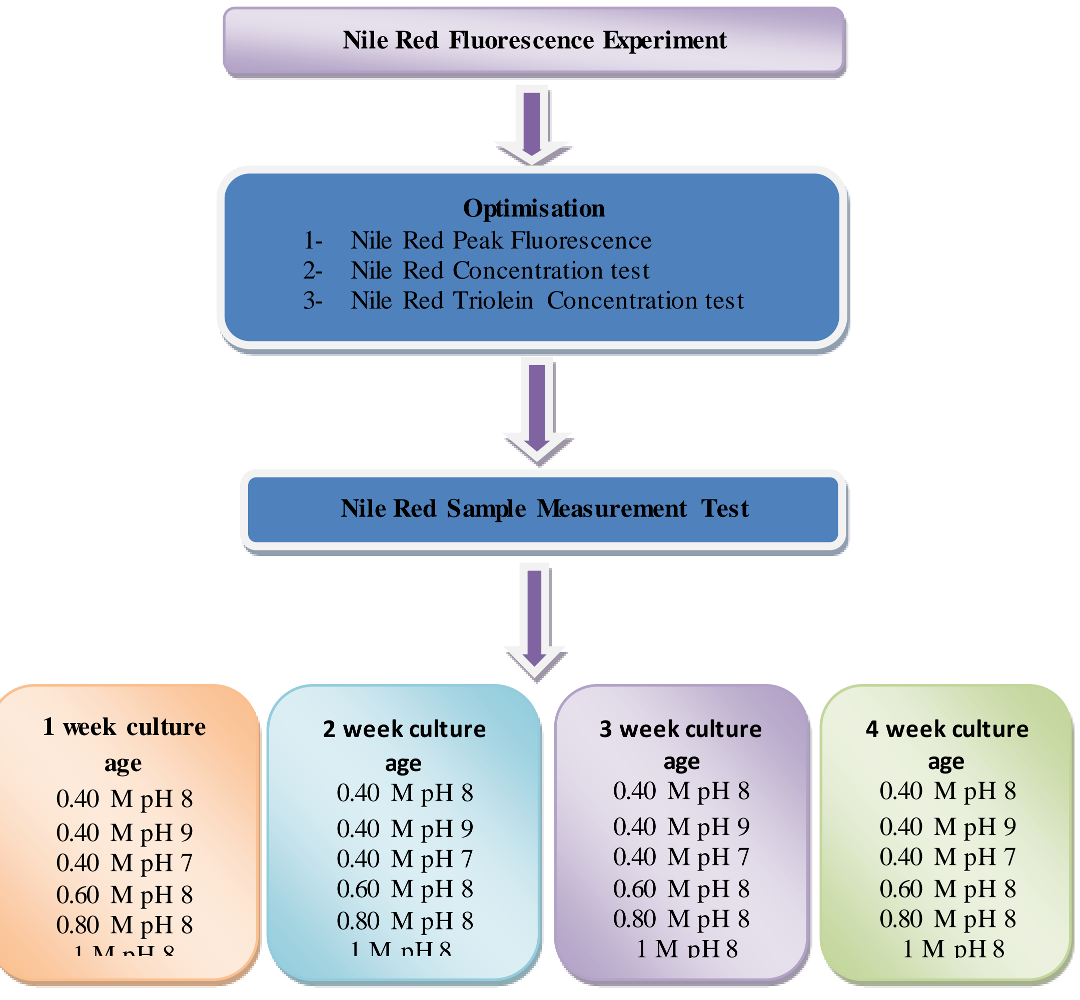


Figure 2.1. Schematic diagram of the plan for Nile Red fluorescence experiments.

2.13.2.1 Lipid body visualisation using a fluorescence microscope

Microalgae accumulate and store natural lipids in lipid bodies which appear as oil droplets inside their body. For visualisation of those lipid bodies Nile Red lipophilic dye was used for staining and a Fluorescence Microscope with a Nikon Digital camera attached was used to capture the images. This approach is based on the work by Cooksey *et al.* (1987). Samples were prepared as follows:

- 5 ml of culture was taken into a 15 ml Falcon tube and the OD_{595} was adjusted to 0.2 using fresh medium.
- 1 ml was then transferred into 1.5 Eppendorf tube and 5 µl of Grams Iodine was added.
- 20 µl of Nile Red (NR) in acetone was added and the sample was vortexed.
- 10 µl of the sample was added to the slide and a cover slip was added.
- The slide was placed in the microscope and a drop of oil was added.
- Images were then taken in two sets with fluorescence and without fluorescence (Just normal microscope light).

2.13.2.2 Nile Red peak fluorescence

This experiment was performed based on the work by Alonzo and Mayzaud (1999), Bertozzini *et al.* (2011) and Chen *et al.* (2009).

Materials used:

- Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one)
- Acetone
- Standard 96 Well plate (Black walled preferably)

- 2 ml Eppendorf tubes (x8)
- 1 ml plastic cuvettes (x2)

  All other chemicals and solvents of analytical grade were purchased from Sigma or other commercial suppliers.

The concentration of Nile Red dye needed in the final culture was 1 µmol/ml from 20 µl, to get this concentration two stocks needed to be made:

1- 0.0025g of Nile Red is added to 10ml of acetone, making the first stock of 0.25 mg/mL (or 250 µg/ml)

2- 63.7 µl of the previous stock was added to 936.3 µl of acetone, making a second stock of 15.9 µg/mL. 20 µl of this stock is equal to 0.318 µg/mL or 1 µmol/ml.

At each step, the mixture was whirlimixed and after the stock solutions were prepared, they need to be wrapped in aluminium foil to stop photodegradation.

To determine the optimum cell concentration to use, a 10 ml sample of a well grown culture was transferred to a 15 ml Falcon tube and the OD_{595} was adjusted to 1 using an appropriate medium as blank. The sample was centrifuged for 5 min at 3000 *g* and the supernatant was discarded. An equivalent volume of fresh medium was added as replacement for the discarded supernatant. The sample was then mixed until the pellet was re-suspended and then pipetted into 2 ml Eppendorf tubes to set up a range of cell concentrations as shown in Table 2.10.

Percentage	100	87.5	75	62.5	50	37.5	25	12.5	Total(ml)
Culture(µl)	2000	1750	1500	1250	1000	750	500	250	9
Medium(µl)	0	250	500	750	1000	1250	1500	1750	7

Table 2.10. Dilution scheme to produce a range of cell concentrations for Nile Red peak fluorescence test.

The details of the plate reader settings and the layout of the plate are shown in Tables 2.11 and 2.12, respectively.

Procedure Details				
Plate Type	96 WELL PLATE			
Read	Fluorescence Endpoint			
	Full Plate			
	Filter Set 1			
	Excitation: 485/20, Emission: 580/50			
	Optics: Top, Gain: 60			
	Read Speed: Normal			

Table 2.11. Plate reader settings for Nile Red peak fluorescence experiments.

Dilution %		100	87.5	75	62.5	50	37.5	25	12.5	Empty Wells			
		1	2	3	4	5	6	7	8	9	10	11	12
A	R1	staine1	staine5	staine9	staine13	staine17	staine21	staine25	staine29	BLK	BLK	BLK	BLK
B	R2	staine2	staine6	staine10	staine14	staine18	staine22	staine26	staine30	BLK	BLK	BLK	BLK
C	R3	staine3	staine7	staine11	staine15	staine19	staine23	staine27	staine31	BLK	BLK	BLK	BLK
D	R4	staine4	staine8	staine12	staine16	staine20	staine24	staine28	staine32	BLK	BLK	BLK	BLK
E	R1	unsta1	unsta5	unsta9	unsta13	unsta17	unsta21	unsta25	unsta29	BLK	BLK	BLK	BLK
F	R2	unsta2	unsta6	unsta10	unsta14	unsta18	unsta22	unsta26	unsta30	BLK	BLK	BLK	BLK
G	R3	unsta3	unsta7	unsta11	unsta15	unsta19	unsta23	unsta27	unsta31	BLK	BLK	BLK	BLK
H	R4	unsta4	unsta8	unsta12	unsta16	unsta20	unsta24	unsta28	unsta32	BLK	BLK	BLK	BLK

Table 2.12. Plate layout for Nile Red Peak fluorescence experiments.

(Note: Row R1 to R4 is replicates from the same concentration)

To carry out the experiment, 4 x 200 µl were transferred from each 2 ml Eppendorf tube to the unstained cell wells (E to H, see Table 2.12) within the relevant cell concentration column. An additional 200 µl was discarded from each 2 ml Eppendorf to make the remaining volume 1 ml. Then 20 µl of the 15.9 µg/mL Nile red stock solution was added to each Eppendorf tube and a timer was started after each tube was whirlimixed. After that the Nile Red stained cells were transferred to the relevant wells (A to D, see Table 2.12) and the plate was then placed in the plate reader machine. Readings were taken 5, 10, 15 and 20 mins after adding Nile Red stain using the Peak finder protocol. The result will give the cell concentration needed for optimal staining along with the optimal time for peak fluorescence.

2.13.2.3 Nile Red concentration test

This experiment was performed based on the work by Alonzo and Mayzaud (1999), Bertozzini *et al.* (2011) and Chen *et al.* (2009). This protocol will give the optimum stain concentration needed for the *T. suecica* strain to produce the clearest fluorescence signal achievable.

Materials used:

- Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one)
- Acetone
- Standard 96 Well plate (Black walled preferably)
- 2 ml Eppendorf tubes (x8)
- 1 ml plastic cuvettes (x2)

All other chemicals and solvents of analytical grade were purchased from Sigma or other commercial suppliers.

In this procedure six stock solutions were made, one primary stock and five secondary stocks which gave the Nile red concentration range from 0.25 to 3 µmol/ml from a 20 µl aliquot.

Primary stock was made by adding 0.0025g of Nile Red to 10ml of acetone, making the first stock of 0.25 mg/mL (or 250 µg/ml). Then the secondary stock solutions from 0.25 to 3 µmol/ml were made as shown in Table 2.13.

Nile red µmol/ml	From primary (µl)	Acetone (µl)
0.25	15.9	984.1
0.5	31.8	968.2
1	63.7	936.3
2	127.3	872.7
3	191	809

Table 2.13. Nile Red stock solutions used in Nile Red concentration test.

From the result of Nile Red peak fluorescence procedure (section 2.13.2.2), the optimal cell concentration for *T.suecica* was found to be OD_{595} = 0.818 which was turbid enough to produce a good fluorescence signal, whilst avoiding self shading. A well grown culture of *T. suecica* was centrifuged for 10 mins at 3000 *g* and the supernatant was discarded and replaced with an equivalent volume of fresh medium and then whirlimixed until the algae pellet was re-suspended. The optimal OD_{595} was then adjusted to the optimal cell concentration. Two ml aliquots of the culture were transferred to 5×2 ml Eppendorf tubes and then 4×200 µl were removed from each 2 ml Eppendorf tube and added to a 96 well plate as unstained cells at the relevant concentration as shown in Table 2.14.

NR Concntration		3	2	1	0.5	0.25	Empty wells						
		1	2	3	4	5	6	7	8	9	10	11	12
A	R1	Con1	Con5	Con9	Con13	Con17	BLK	BLK	BLK	BLK	BLK	BLK	BLK
B	R2	Con2	Con6	Con10	Con14	Con18	BLK	BLK	BLK	BLK	BLK	BLK	BLK
C	R3	Con3	Con7	Con11	Con15	Con19	BLK	BLK	BLK	BLK	BLK	BLK	BLK
D	R4	Con4	Con8	Con12	Con16	Con20	BLK	BLK	BLK	BLK	BLK	BLK	BLK
E	R1	unstin1	unstin5	unstin9	unstin13	unstin17	BLK	BLK	BLK	BLK	BLK	BLK	BLK
F	R2	unstin2	unstin6	unstin10	unstin14	unstin18	BLK	BLK	BLK	BLK	BLK	BLK	BLK
G	R3	unstin3	unstin7	unstin11	unstin15	unstin19	BLK	BLK	BLK	BLK	BLK	BLK	BLK
H	R4	unstin4	unstin8	unstin12	unstin16	unstin20	BLK	BLK	BLK	BLK	BLK	BLK	BLK

Table 2.14. Plate layout for Nile Red concentration test.

(Note: Row R1 to R4 is replicates from the same concentration)

An additional 200 µl was discarded from each 2 ml Eppendorf tube making the remaining volume 1 ml. From the secondary Nile Red stock solutions, 20 µl of each Nile Red concentration was added to the appropriate Eppendorf tube and mixed well. Then 4 x 200 µl stained cells were transferred to the appropriate wells (Table 2.14). The 96 well plate was then placed in a plate reader machine for measurement. See Table 2.15 for the plate reader settings.

Procedure Details				
Plate Type	96 WELL PLATE			
Read	Fluorescence Endpoint			
	Full Plate			
	Filter Set 1			
	Excitation: 485/20, Emission: 580/50			
	Optics: Top, Gain: 60			
	Read Speed: Normal			

Table 2.15. Plate reader settings for Nile Red concentration test.

2.13.2.4 Nile Red triolein concentration test

This experiment was performed based on the work by Alonzo and Mayzaud (1999), Bertozzini *et al.* (2011) and Chen *et al.* (2009). This protocol will allow the Nile Red fluorescence signal to be converted to triolein equivalents.

Materials used:

- Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one)
- Acetone
- Triolein (TO) or 1,2,3-Tri-[(cis)-9-octadecenoyl]glycerol, $C_{57}H_{104}O_6$ ~99%) (44895-U Supelco) – Neutral Lipid
- Isopropanol
- Standard 96 Well plate (Black walled preferably)
- 2 ml Eppendorf tubes (x8)
- 1 ml plastic cuvettes (x2)

All other chemicals and solvents of analytical grade were purchased from Sigma or other commercial suppliers.

The concentration of Nile Red dye needed in the final culture was 1 µmol/ml from 20 µl, to achieve this concentration two stocks needed to be made:

1- 0.0025g of Nile Red is added to 10 ml of acetone, making the first stock of 0.25 mg/mL (or 250 µg/ml).

2- 63.7 µl of the previous stock was added to 936.3 µl of acetone, making a second stock of 15.9 µg/mL. 20µl of this stock is equal to 0.318 µg/mL or 1 µmol/ml.

At each step, the mixture should be whirlimixed and after the stock solutions are prepared, the containers need to be wrapped in aluminium foil to stop photodegradation.

From the result of the Nile Red peak fluorescence procedure, the optimal cell concentration for *T. suecica* was found to be OD_{595} = 0.818 which was turbid enough to produce a good fluorescence signal, whilst avoiding self shading. A well grown culture of *T. suecica* was centrifuged for 10 mins at 3000 *g* and the supernatant was discarded and replaced with an equivalent volume of fresh medium then mixed until the algae pellet was re-suspended. The OD_{595} was then adjusted to the optimal value.

In this experiment, 8 different lipid concentration standards were made using triolein (To) as a model neutral lipid dissolved in isopropanol. As recommended by Bertozzini *et al.* (2011), cultured *T. suecica* cells were also added as shown in Table 2.16.

Conc of triolein (mg/ml)	0.05	0.04	0.03	0.02	0.01	0.005	0.0025	0	Total(ml)
Culture(µl)	1980	1980	1980	1980	1980	1980	1980	1980	15.84
Triolein(µl)	20	16	12	8	4	2	1	0	0.063
Isopropanol(µl)	0	4	8	12	16	18	19	0	0.097

Table 2.16. Dilutions for triolein concentration curve.

After whirlimixing 4×200 µl were transferred from each Eppendorf tube and added as the unstained cells at the relevant concentration as shown in the plate layout in Table 2.17.

Triolein Conc	0.05	0.04	0.03	0.02	0.01	0.005	0.0025	0	Empty wells			
	1	2	3	4	5	6	7	8	9	10	11	12
A	stain	stain	stain	stain	stain	stain	stain	stain	BLK	BLK	BLK	BLK
B	stain	stain	stain	stain	stain	stain	stain	stain	BLK	BLK	BLK	BLK
C	stain	stain	stain	stain	stain	stain	stain	stain	BLK	BLK	BLK	BLK
D	stain	stain	stain	stain	stain	stain	stain	stain	BLK	BLK	BLK	BLK
E	unsta	unsta	unsta	unsta	unsta	unsta	unsta	unsta	BLK	BLK	BLK	BLK
F	unsta	unsta	unsta	unsta	unsta	unsta	unsta	unsta	BLK	BLK	BLK	BLK
G	unsta	unsta	unsta	unsta	unsta	unsta	unsta	unsta	BLK	BLK	BLK	BLK
H	unsta	unsta	unsta	unsta	unsta	unsta	unsta	unsta	BLK	BLK	BLK	BLK

Figure 2.17. Plate layout for triolein concentration curve experiment.

An additional 200 µl was discarded from each Eppendorf tube to make the remaining volume 1 ml. Then 20 µl of Nile Red stock solution was added to each Eppendorf tube and mixed well. Four x 200 µl samples of the stained cells were then transferred to the appropriate wells (Table 2.17). The 96 well plate was then placed in the plate reader machine and the plate reader settings shown in Table 2.18 were used.

Procedure Details				
Plate Type	96 WELL PLATE			
Read	Fluorescence Endpoint			
	Full Plate			
	Filter Set 1			
	Excitation: 485/20, Emission: 580/50			
	Optics: Top, Gain: 60			
	Read Speed: Normal			

Table 2.18. Plate reader settings for triolein concentration curve.

2.13.2.5 Nile Red sample measurement test

Nile Red measurements on algal cells grown under different environmental conditions were performed after performing the three optimisation test experiments described in sections 2.13.2.2, 2.13.2.3. and 2.13.2.4 i.e the Nile Red peak fluorescence test, the Nile Red concentration test and the Nile Red triolein concentration test. To find the optimise time for harvesting cells during the induction of lipid accumulation, measurements were performed on cells grown for 1 week, 2 weeks, 3 weeks and 4 weeks. Ten ml samples were taken from each flask at each time point and centrifuged at 3000 *g* for 5 mins. Supernatants were discarded and replaced with the appropriate medium, and algal pellets were re-suspended and the OD_{595} was adjusted to give the optimised cell concentration which was $OD_{595} = 0.818$. Then 2 ml aliquots from the adjusted culture were transferred to 2 ml Eppendorf tubes. After that 4 × 200 µl aliquots were transferred to the 96 well plate in the appropriate wells as unstained cells as shown in the plate layout in Table 2.19.

Samples		0.40 M	PH 7	PH 9	0.60 M	0.80 M	1 M	Empty wells					
		1	2	3	4	5	6	7	8	9	10	11	12
Nile red unstained cell	A	SPL1	SPL2	SPL3	SPL4	SPL5	SPL6	BL	BL	BL	BL	BL	BL
	B	SPL1	SPL2	SPL13	SPL4	SPL5	SPL6	BL	BL	BL	BL	BL	BL
	C	SPL1	SPL2	SPL3	SPL4	SPL5	SPL6	BL	BL	BL	BL	BL	BL
	D	SPL1	SPL2	SPL3	SPL4	SPL5	SPL6	BL	BL	BL	BL	BL	BL
Nile red stained cell	E	SPL1	SPL2	SPL3	SPL4	SPL5	SPL6	BL	BL	BL	BL	BL	BL
	F	SPL1	SPL2	SPL3	SPL4	SPL5	SPL6	BL	BL	BL	BL	BL	BL
	G	SPL1	SPL2	SPL3	SPL4	SPL5	SPL6	BL	BL	BL	BL	BL	BL
	H	SPL1	SPL2	SPL3	SPL4	SPL5	SPL6	BL	BL	BL	BL	BL	BL

Table 2.19. An example plate layout for sample measurements.

200 µl were then discarded from each Eppendorf tube leaving the remaining volume 1 ml. Then 20 µl of Nile red with the concentration of 3 µmol/ml were added to the sample and mixed well. This was followed by transferring 4 × 200 µl of stained cell samples to the appropriate wells (Table 2.19). Protocol was then run to make fluorescence measurements 10, 15 and 20 mins after Nile Red addition (Table 2.20).

Procedure Details				
Plate Type	96 WELL PLATE			
Read	Fluorescence Endpoint			
	Full Plate			
	Filter Set 1			
	Excitation: 485/20, Emission: 580/50			
	Optics: Top, Gain: 60			
	Read Speed: Normal			

Table 2.20. Plate settings for sample measurements.

2.13.3 Lipid Determination by Direct Transesterification Methods

This work was performed based on methods described by Griffiths *et al.* (2010). A well grown culture was transferred to a 50 ml Falcon tube and then centrifuged at 3000 *g* for 10 mins. The supernatant was discarded and the remaining pellet resuspended in 5 ml of distilled water. The culture was centrifuged again at the same speed and for the same time. The supernatant was discarded and the remaining pellet resuspended in 1 ml of distilled water. The sample was then transferred into pre-weighed 1.5 ml Eppendorf tube and then frozen at -80° C for 48 hours. The sample was then lyophilised (freeze dried) for 48 hours and the biomass was estimated. Ten mg of biomass was taken into a fresh glass crimped vial and 190 µl of chloroform/methanol (2:1, v/v) was added to solubilise the lipids. Moreover, 10 µl (0.1

mg) of tridecanoic acid (C13 lipid) dissolved in chloroform/methanol (2:1, v/v) was added as an internal standard to check for reaction efficiency. This was followed by adding 0.3 ml HCl/ MeOH (5%, v/v) catalyst to the vial, which was crimp-sealed quickly. The transesterification process started after the vial was placed in a hot plate at 85°C and it was left for 1 hour to complete the process. The vial was left at room temperature to cool for 10 mins. The vial was then opened and 975 µl of hexane was added for fatty acid methyl ester (FAME) extraction. The vial was then closed again, mixed and left at room temperature for 1 hour. Once the extraction was complete, the vial was opened and 487 µl of the top hexane phase was taken into a new vial. This was followed by adding 12.5 µl (0.125 mg) of 10 mg/ml methyl nonadecanoate (C19 FAME) in hexane as an internal standard to check the efficiency of GC-MS analysis, and the vial was re-closed.

The extracted FAMEs from the transesterification reactions were then identified by gas chromatography mass spectroscopy (GC-MS) using a Perkin Elmer - Auto System XL Gas Chromatograph (CHM-100-790) and Perkin Elmer - Turbo Mass Mass Spectrometer (13657). The machine was fitted with a Zebron - ZB-5MS - 30 m 0.25 mm ID and 0.25 µm FT (7HG-G010- 11) GC Capillary column and ran using the following settings; Autosampler Method: Injection volume: 1 µl, Preinjection solvent washes: 2, Post injection solvent washes: 6. Temperature Program (FAME03_100mins): 100–300°C, Ramp 1: 2ºC/min to 300, 20 ml/min He constant carrier gas flow. MS Scan: EI+, Start mass: 50, End mass: 600, Scan time: 0.5 s, Interscan time: 0.1 s, Start time: 0, End time: 100 min. The resultant peaks were identified and integrated using a Perkin Elmer's Turbomass software linked to a NIST database.

2.14 Nuclear Magnetic Resonance (NMR) Analysis of Compatible Solutes

This work was performed based on the methods described by Derome (1987) with the help of Prof M P Williamson in the MBB department for NMR analysis. Duplicate 20 ml samples from a well grown culture (OD_{595} =1) were taken and centrifuged for 10 mins at 3000 *g* and the supernatant was discarded. The pellets were then resuspended in 1 ml of distilled water and sonicated on ice for 2 × 20 seconds with a 10 second cooling period between sonication steps. After that samples were transferred to Eppendorf tubes and centrifuged for 5 mins in the microfuge at full speed. The supernatants were then transferred to fresh Eppendorf tubes and frozen at -80°C for 48 hours followed by freeze drying (lyophilisation) for 48 hours until the samples were completely dry. The sample was then weighed to estimate the weight of biomass.400 µl ofdeuterated chloroform (CDCl3) and 100 µL of deuterated methanol (CD3OD) were added to each sample for solubilisation of the biomass.The samples were then mixed and transferred to a 5 mm NMR tube, and 5 µl of Chloroform (CHCl3) was added as an internal standard. NMR spectra were obtained on a BrukerAvance 600 equipped with a cryoprobe. Data was recorded into 16k complex data points with simple pulse-acquire pulse program and a 3 s recycle time. Fourier transformation was applied using a 1 Hz line broadening followed by manual baseline correction. All spectra were acquired using 8 scans (with 4 dummy background scans). Processing and integration was performed using Bruker Topspin v1.3 software.

2.15 Re-cycling of Nutrients and its Effect on Growth of *T. Suecica*

A 10 day old *T. suecica* culture was centrifuged and the medium was reused (the supernatant). Incubation of *T. suecica* cells was carried out in the reused medium and also in a control fresh F/2 medium. The OD_{595} was then taken every 24 hours to measure growth. As there were no growth in the recycled medium after 4 days of incubation, enrichment with N and P was carried out by adding N and P stock solutions in to the recycled medium to test if the lack of growth was due to N or P limitation or if both elements were limiting (Table 2.21).

Recycling medium 1	Enrich with N
Recycling medium 2	Enrich with P
Recycling medium 3	Enrich with N and P

Table 2.21 Protocol for enriching recycled medium.

The enrichment cultures were incubated and growth was measured by measuring the OD_{595} every 24 hours. After 6 days of enrichment, flasks with single element enrichment were enriched again with the other element (Table 2.22).

Recycling medium 1 with previous Enrichment with N	Enrich with P
Recycling medium 2 with previous Enrichment with p	Enrich with N

Table 2.22 Protocol for second cycle of enrichment.

Cultures were then incubated and OD_{595} readings were taken every 24 hours.

2.16 Growth of *T. suecica* Using a Two Litre Photobioreactor

Growth of *T. suecica* was measured using a 2 litre airlift photobioreactor with enrichment of CO_2, P and N. Three separate photobioreactor experiments were carried out. The first experiment was the control without addition of N and P and just with normal air flow (0.03% CO_2) to ensure a good mixing in the culture. In the second experiment, the *T. suecica* culture was supplied with 5% CO_2 and additional N and P was added to the culture. In the final experiment atmospheric levels of CO_2 were used, but with the addition of N and P.

Chapter Three

Physiological Characterization of *Tetraselmis suecica*

3.1 Introduction

As described in section 1.5, the idea of using microalgae as a source of renewable fuels has a long history, but recent interest in algal biofuels can be traced to the review published in 2007 by Chisti. In this publication, a list of microalgae was presented containing the likely candidates for biodiesel production (Chisti, 2007) and it is shown in an adapted form as Table 3.1 overleaf. Based on the highest level of lipid produced, *Botryococcus braunii* looks to be a strong candidate with a top value of 75% lipid. In actual fact, this figure represents the amount of hydrocarbon produced by *B. braunii* (Banerjee *et al.*, 2002), which is a better basis for biodiesel production than the neutral lipids normally stored by the other algae listed in Table 3.1. However, commercial exploitation of *B. braunii* is very difficult due its colonial growth form and it naturally grows as sticky mats of biomass and not as suspended cells in liquid medium (Metzger and Largeau, 2005).

The next best candidate from Table 3.1 appears to *Schizochytrium*, which is an unusual alga found within the Heterokonta group (Figure 1.1). This organism has been mainly studied by research groups in Japan and China, and produces the polyunsaturated fatty acid (PUFA) docosahexanoic acid (DHA) in large amounts, which is used as a human dietary supplement (Qu *et al.*, 2013). The recent publication by Qu *et al.* (2013) confirmed that neutral lipid can make up about 56% of the dry weight of *Schizochytrium*. The other organism from Table 3.1 that has been studied in detail is *Nannochloropsis*, which like *Schizochytrium*, is also a heterokont alga belonging to the class Eustigmatophyceae (Van den Hoek, 1995). *Nannochloropsis* has been well studied and high levels of neutral lipids (in excess of 25% of biomass) have been measured in this

alga on a regular basis (e.g. Pal *et al.*, 2011). However, *Nannochloropsis* cells are small (around 1 to 2 µm) and have thick cell walls, which makes the task of harvesting the neutral lipids more difficult (Rios *et al.,* 2013). Of the other algae listed in Table 3.1, species in the *Dunaliella* genus (Ying *et al.*, 2015) and species related to the *Chlorella* genus (Smith *et al.,* 2015) are being actively studied in the Gilmour lab. For this reason, the current project was based on another organism listed in Table 3.1 (i.e. *Tetraselmis suecica*).

Tetraselmis suecica is a marine green alga belonging to the chlorophyte group of algae closely related to higher plants (Figure 1.1). This species has been used in aquaculture (fish and shellfish farming) since the 1970s and it has been widely studied for the effect of salinity and other environmental parameters on the biochemical composition of the cells (e.g Fabregas *et al.,* 1984). In particular changing nutrient concentrations (e.g. nitrate in the medium) has been used to alter the composition of protein, lipid and carbohydrate in *T. suecica* (Fabregas *et al.,* 1995). More recently, *T. suecica* has been studied with respect to production of PUFAs and neutral lipid. Flow cytometry has been used by Guzman *et al.* (2010) to screen for strains that are hyperproductive for PUFAs and the factors affecting neutral lipid production by *T. suecica* have been studied by Bondioli *et al.* (2012) and Go *et al.* (2012).

In the present work, it was decided to re-evaluate *T. suecica* as a source of neutral lipid and to use the CCAP strain 66/4 which has not been previously studied for neutral lipid production.

Microalga	**Lipid or equivalent (% dry wt)**
Botryococcus braunii	25 - 75
Chlorella sp.	28 - 32
Crypthecodinium cohnii	20
Cylindrotheca sp.	16 - 37
Dunaliella primolecta	23
Isochrysis sp.	25 - 33
Monallanthus salina	> 20
Nannochloris	20 - 35
Nannochloropsis sp.	31 - 68
Neochloris oleoabundans	35 - 54
Nitzschia sp.	45 - 47
Phaeodactylum tricornutum	20-30
Schizochytrium sp.	50 - 77
Tetraselmis suecica	15 - 23

Table 3.1. List of algal species with the maximum value of lipid produced from each organism (adapted from Chisti, 2007). Note that the "lipid or equivalent" content may refer to different types of lipids and not all are necessarily suitable for biodiesel production.

3.2 Results

3.2.1 Identification of *T. suecica* using 18S rDNA Sequencing and SEM

On receiving the *T. suecica* liqiuid culture form the culture collection (CCAP, Oban), the cells were checked for bacterial contamination and then the identity of the strain was checked using 18S rDNA sequencing

3.2.1.1 DNA extraction and PCR

Tetraselmis suecica cells were harvested through centrifugation and the genomic DNA was extracted using two methods (CTAB and the Qiagen kit) as described in section 2.11.1. Both methods successfully extracted the genomic DNA. However, the CTAB method showed a clear bright DNA band on the gel (Figure 3.1), therefore this band was selected for PCR amplification.

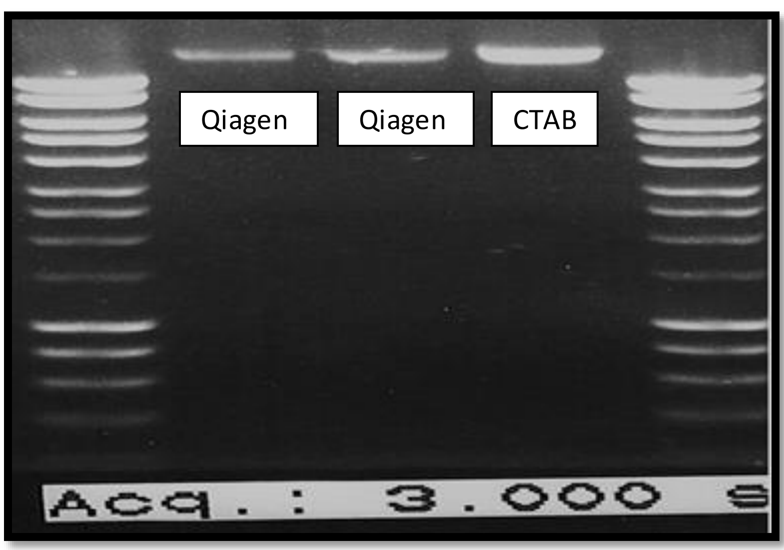


Figure 3.1. Samples of extracted DNA run in a 1% agarose gel. Lanes 1 and 5 are ladders.

3.2.1.2 PCR Amplification

When the 18S rRNA gene was amplified using the Sheehan primers, no amplification was seen and the gel shows only the primers themselves (Figure 3.2A). However, when the Lim primers were used, the correct sized band (approximately 1800 bp) was amplified (Figure 3.2 B). The DNA was cleaned up using a PCR purification kit.

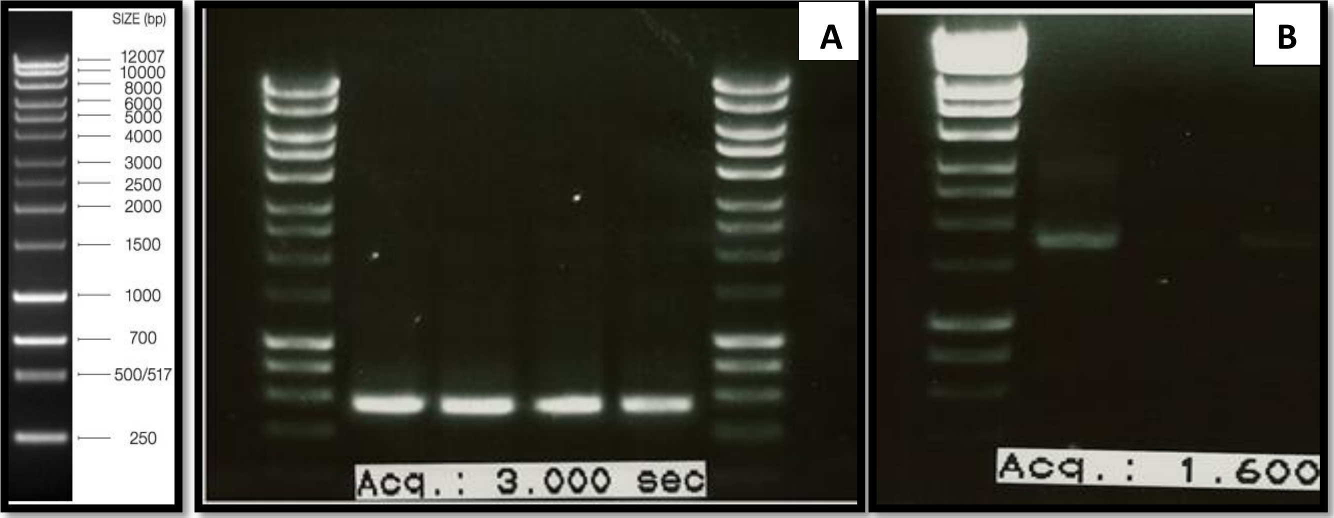


Figure 3.2: PCR amplification results. A: Using 18S Sheehan primers, lanes 1 and 6 are DNA ladders, see scale at left hand side of figure and Lanes 2-5 show only primers. B: Using 18S Lim primers, Lane 1 = ladder and lane 2 shows the 18S rDNA band at around 1800 bp.

3.2.1.3 PCR purification

The amplified PCR products were purified using QIAquick PCR Purification kit (Qiagen) (Figure 3.3), and both purified PCR products were of the correct size (approximately 1800 bp).

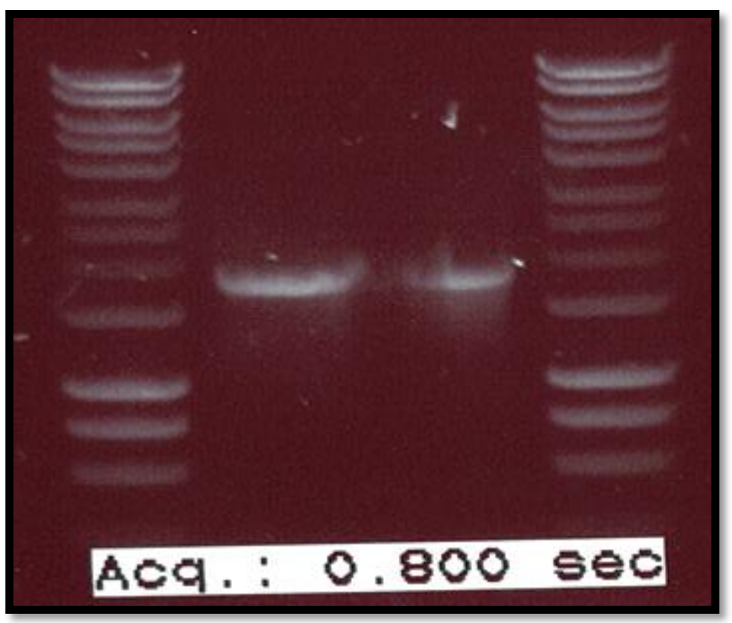


Figure 3.3. Purification of PCR products – it is clear that 1.8 kb bands remained after the purification process (Lanes 2 and 3).

3.2.1.4 Sequencing of 18S rDNA and Identification using BLAST

The sequences returned from Eurofins/MWG are shown in Figure 3.4. Only around 500 bases were successfully sequenced. Using BLAST on the NCBI website indicated that both forward and reverse Lim 18S primer sequences matched the genus *Tetraselmis* (99% identity). With this length of sequence it was not possible to confirm the species identification to *T. suecica.*

Forward sequence (508 letters):

CAGTTACAGCTCGTAGTTGGATTTCGGATGGGATTTGCCGGTCCGCCGTTTCG
GTGTGCACTGGCCAGTCCCATCTTGTTGTCGGGGACTAGCTCCTGGGCTTCAC
TGTCCGGGACTAGGAGCTGACGAGGTTACTTTGAGTAAATTAGAGTGTTCAA
AGCAAGCCTACGCTCTGAATACATTAGCATGGAATAACATGATAGGACTCTG
GCTTATCTTGTTGGTCTGTGAGACCAGAGTAATGATTAAGAGGGACAGTCGG
GGGCATTCGTATTTCATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGACGA
ACTTCTGCGAAAGCATTTGTCAAGGATGTTTTCATTAATCAAGAACGAAAGTT
GGGGGCTCGAAGACGATTAGATACCGTCCTAGTCTCAACCATAAACGATGCC
GACTAGGGATTGGCAGACGTTTTTTTGATGACTCTGCCAGCACCTTATGAGAA
ATCAAAGTTTTTGGGTTGCGGG

Reverse sequence (516 letters):

TATAGGTGCTGGCAGAGTCATCAAAAAAACGTCTGCCAATCCCTAGTCGGCA
TCGTTTATGGTTGAGACTAGGACGGTATCTAATCGTCTTCGAGCCCCCAACTT
TCGTTCTTGATTAATGAAAACATCCTTGACAAATGCTTTCGCAGAAGTTCGTC
TTTCATAAATCCAAGAATTTCACCTCTGACAATGAAATACGAATGCCCCCGAC
TGTCCCTCTTAATCATTACTCTGGTCTCACAGACCAACAAGATAAGCCAGAGT
CCTATCATGTTATTCCATGCTAATGTATTCAGAGCGTAGGCTTGCTTTGAACA
CTCTAATTTACTCAAAGTAACCTCGTCAGCTCCTAGTCCCGGACAGTGAAGCC
CAGGAGCTAGTCCCCGACAACAAGATGGGACTGGCCAGTGCACACCGAAA
CGGCGGACCGGCAAATCCCATCCGAAATCCAACTACGAGCTTTTTAACTGCA
GCAACTTAAATATACGCTATTGGAGCTGGAAAA

Figure 3.4. Sequences of 18S rRNA gene from *T. suecica* using Lim forward and reverse primers.

3.2.1.5 Scanning electron microscope images of *T. suecica*

The SEM images below confirm that the culture is likely to be a member of the *Tetraselmis* genus due to the presence of four flagella (Figure 3.5) and the haptonema – which is a cavity at the base of the flagellum (Figure 3.5). Only two flagella are clearly visible in the right hand image of Figure 3.5, but flagella are fairly easily lost during SEM preparation.

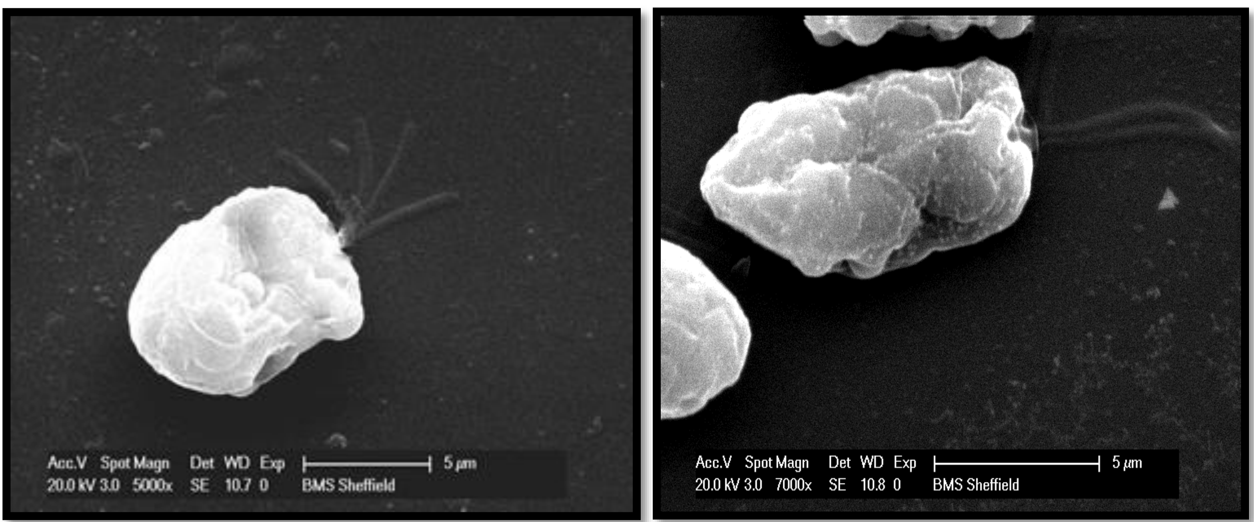


Figure 3.5. Scanning electron microscope images of *Tetraselmis suecica*. Left image shows the four flagella characteristic of this algal genus and right image shows haptonema at the base of the flagella.

3.2.2 Comparison of Different Media for Growing *T. suecica*

Tetraselmis suecica was cultured in four different media (TS medium, F/2 medium, Dunaliella medium, BG11 medium) to observe which medium is the most appropriate based on the highest growth potential. Three replicate OD_{595} measurements for each medium were performed each day, the initial OD_{595} for all cultures was 0.1. From the growth curves shown in Figure 3.6, it is clear that F/2 medium is the best medium for growing *T. suecica*, reaching an OD_{595} of 0.8 after 7 days incubation. For the other media, the best result was with Dunaliella medium with an OD_{595} of 0.6 after 7 days. However, the poorest medium was BG11 with an OD_{595} of 0.275 after 7days.

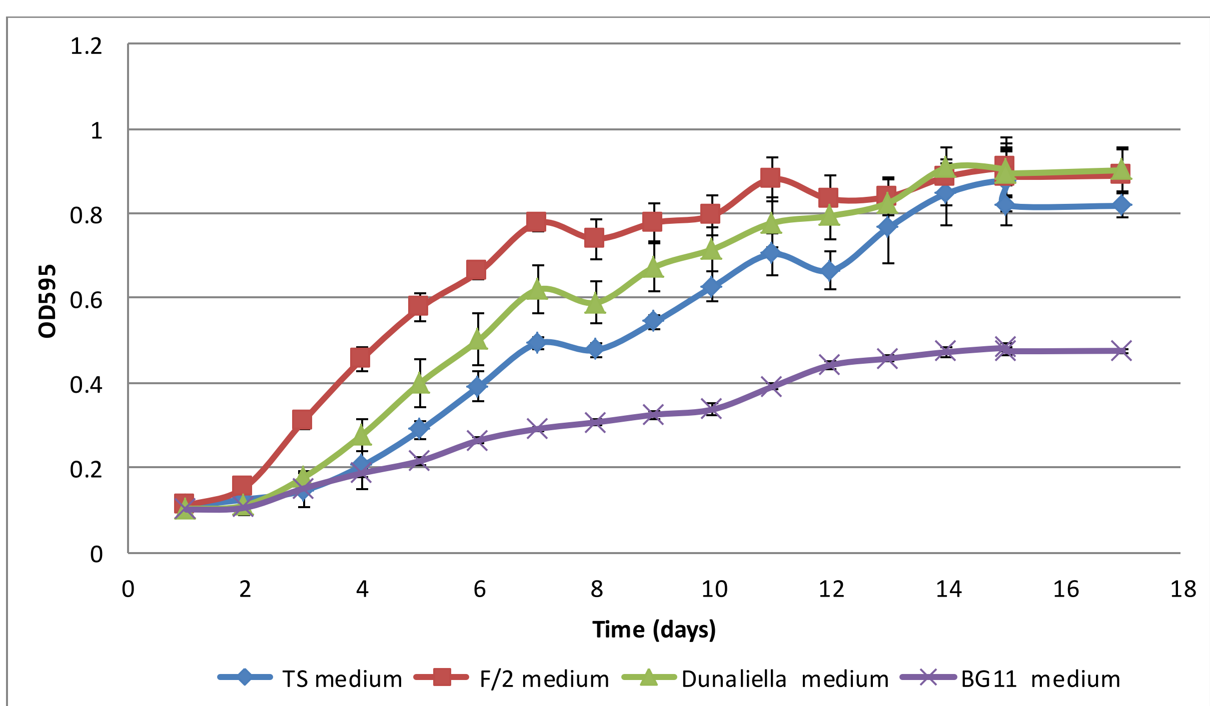


Figure 3.6. Growth curves for *T. suecica* in TS medium, F/2 medium, Dunaliella medium and BG11 medium. X axis is OD_{595} measured against medium blank and Y axis is time in days.

3.2.3 Development of F/2 Defined Medium

One of the advantages of using algae for biofuel production is the ability to control their accumulation and secretion of biofuels by changing their growth conditions or by metabolic engineering. That led to the development of a defined F/2 medium where the quantity of the chemicals that add to the media such as N, P, and NaCl can be control. In addition, F/2 medium prepared using the commercial seawater salt mixture is not suitable for use at higher salinities than seawater, because the medium precipitates after autoclaving. The defined F/2 medium is an important outcome of this work and the composition of defined F/2 medium and how to prepare it is shown below.

Stock Solutions

1. Artificial Seawater (Hydrated Salts)

Magnesium Sulphate ($MgSO_4$)	2.92 g	$MgSO_4 7H_2O$	5.98 g
Magnesium Chloride ($MgCl_2$)	4.66 g	$MgCl_2 6H_2O$	9.95 g
Calcium Chloride ($CaCl_2$)	1.17 g	$CaCl_2 2H_2O$	1.55 g
Strontium Chloride ($SrCl_2$)	0.02 g	$SrCl_2 6H_2O$	0.034 g

Each of the hydrated salts above was added to 400 ml of distilled water and each one was dissolved before adding the next salt. The volume was then made to 500 ml with distilled water before autoclaving.

2. Artificial Seawater (Anhydrous salts)

Sodium Chloride (NaCl)	23.9 g
Potassium Chloride (KCl)	0.62 g
Sodium Bicarbonate ($NaHCO_3$)	0.18 g
Potassium Bromide (KBr)	0.024 g
Boric Acid (H_3BO_3)	0.020 g
Sodium Thiosulphate ($Na_2S_2O_3$)	0.00 4 g

Each of the anhydrous salts above was added to 400 ml of distilled water and each one was dissolved before adding the next salt. The volume was then made to 470 ml with distilled water before autoclaving.

3. Nitrate

Sodium Nitrate ($NaNO_3$) –0.75 g was dissolved in 100 ml of distilled water.

4. Phosphate

Sodium Dihydrogen Phosphate ($NaH_2PO_42H_2O$) – 0.057 g was dissolved in 100 ml of distilled water.

5. Trace Elements

For F/2 medium

6. Vitamin Mix

For F/2 medium

7. Tris Buffer

1 M Tris buffer pH 7.8 was prepared

For 1 litter of medium, aseptically stock solution 1 was added to stock solution 2. Then 10 ml of nitrate stock and 10 ml of phosphate stock was added using filter sterilisation, after that 1 ml of trace element and 1 ml of vitamin mix was added using filter sterilisation. Finally 10 ml of 1 M Tris was added using filter sterilisation. The mixer was mixed well and the pH was checked.

3.2.4 Comparison of Growing *T. suecica* in Defined F/2 medium and normal F/2 medium

After the development of F/2 medium, a comparison with the growth rate in normal F/2 medium was carried out. Figure 3.7 shows the growth over 11 days of incubation where the final OD in defined F/2 medium reached 0.507, but in normal F/2 medium the final OD was

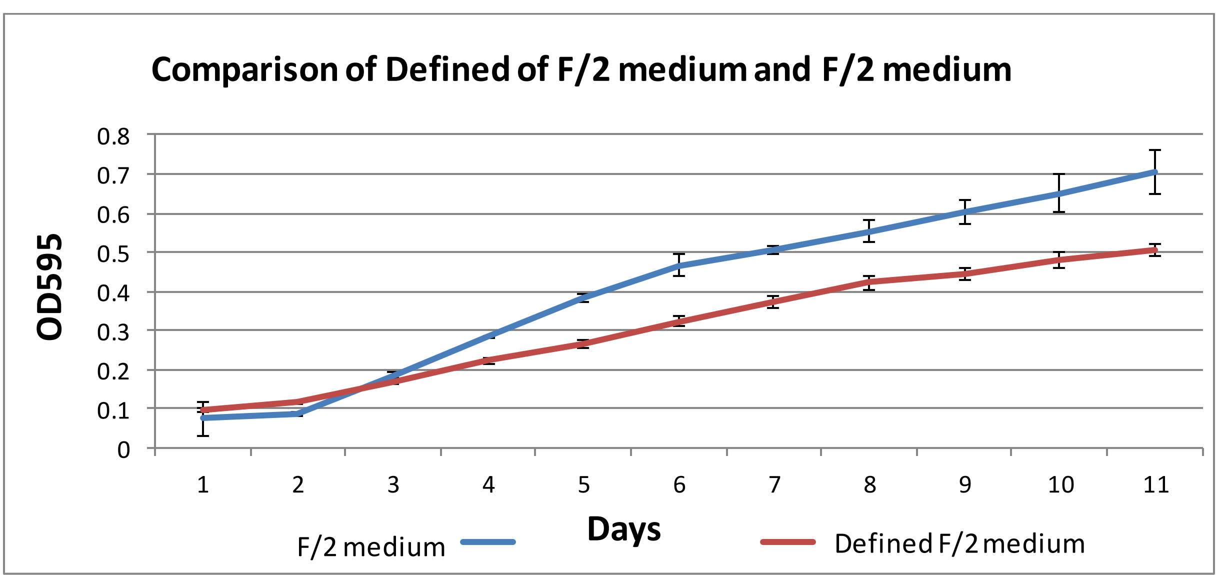


Figure 3.7. Comparison of growth of *T. suecica* in defined F/2 medium and normal F/2 medium. Each point represents the mean of three readings plus/minus the standard error.

about 0.7. It was clear that the normal F/2 medium produced a higher biomass, but the advantages of having a defined medium that supported good growth outweighed the small decrease in biomass. For the rest of the work described in this thesis, defined F/2 medium was used.

3.2.5 Effect of Salinity on Growth of *T. suecica*

Increasing the salinity can increase the lipid content of microalgae, but often lowers the growth rate of a species. Measurements of lipid content will be described in Chapter 4, but in this section the effect of salinity the growth was investigated. As expected, growth rate and final biomass level reached after 11 days were decreased as salinity increased (Figure 3.8).

However, at the highest salinity tested (1 M NaCl), the level of growth observed was not significantly different from that at normal seawater salinity (0.4 M NaCl).

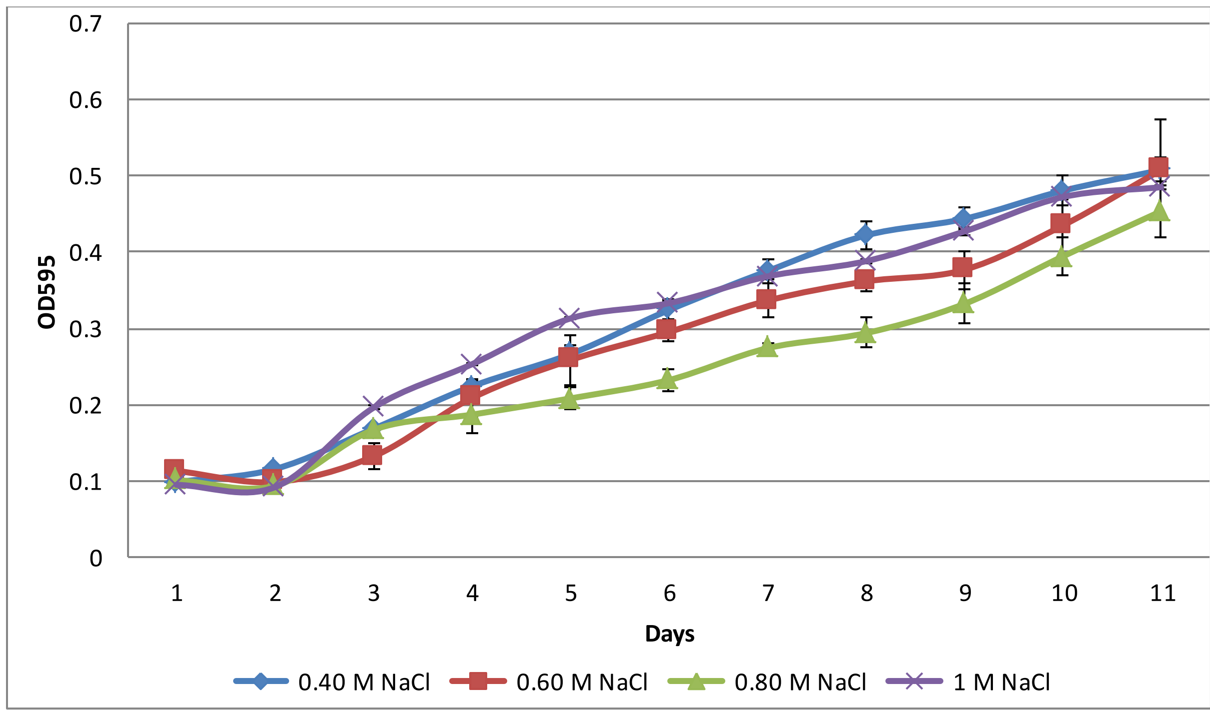


Figure 3.8. The effect of increasing salinity on the growth of *T. suecica*. Each point represents the mean of three readings plus/minus the standard error.

3.2.6 Effect of pH on Growth of *T. suecica*

In this experiment, the effect of pH on algal growth was evaluated every day using the spectrophotometer to measure OD at 595 nm. Increasing the initial pH from 7 to 9 had no negative effect on growth of *T. suecica* (Figure 3.9). In fact the highest biomass was observed for cell cultures grown at pH 9 which reached an OD_{595} of 0.784 compared with 0.616 for pH 7 and 0.507 for pH 8 grown cells.

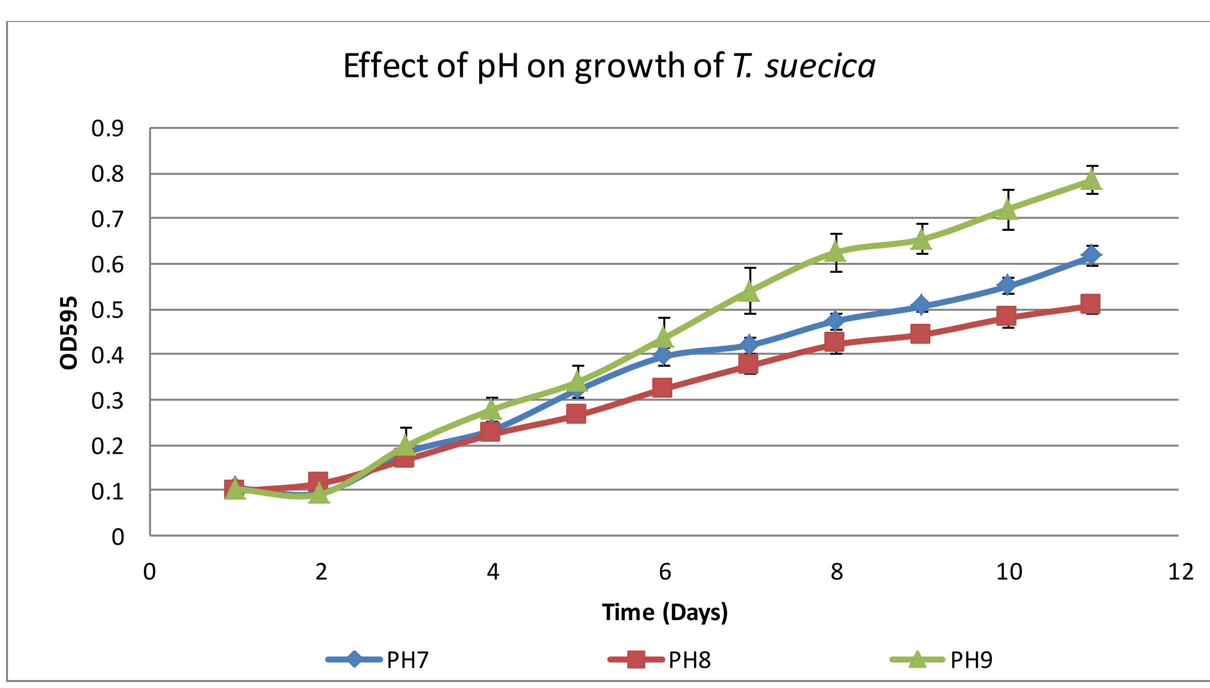


Figure 3.9. The effect of pH on growth of *T. suecica.* Each point represents the mean of three readings plus/minus the standard error.

3.2.7 Effect of Salinity, pH and Temperature on Photosynthesis and Respiration of *T. suecica*

To further investigate the effect of environmental parameters (salinity, pH and temperature) on *T. suecica,* measurements of the rates of photosynthesis and respiration were made using an oxygen electrode system. Cells were adapted to the stresses before measuring photosynthesis and respiration except for temperature; in this case the cells were subjected to the stress immediately before measurement. Table 3.2 shows that the rate of respiration decreased with increasing salinity from 0.4 to 0.6 to 0.8 M NaCl. In contrast the rate of photosynthesis increased slightly at 0.6 M NaCl, but then decreased at 0.8 M NaCl.

NaCl concentration	Oxygen Evolution (µ mol O_2 mg Chl^{-1} min^{-1})	Respiration (µ mol O_2 mg Chl^{-1} min^{-1})	Rate of Photosynthesis (µ mol O_2 mg Chl^{-1} min^{-1})
0.40 M	1.312 ± 0.15	1.849 ± 0.18	3.161 ± 0.33
0.60 M	2.317 ± 0.25	1.158 ± 0.14	3.475 ± 0.39
0.80 M	0.514 ± 0.32	0.771 ± 0.35	1.285 ± 0.67

Table 3.2. Effect of salinity on the rates of photosynthesis and respiration. Photosynthesis rate is calculated by adding together oxygen evolution in the light plus oxygen uptake in the dark. Data are means of three replicates plus/minus the standard error.

Increasing the initial pH from pH 7 to pH 8 decreased the rates of both photosynthesis and respiration, and at pH 9, both photosynthesis and respiration were very significantly decreased (Table 3.3).

Potential of hydrogen (pH)	Oxygen Evolution (µ mol O_2 mg Chl^{-1} min^{-1})	Respiration (µ mol O_2 mg Chl^{-1} min^{-1})	Rate of Photosynthesis (µ mol O_2 mg Chl^{-1} min^{-1})
pH 7	1.570 ± 0.11	2.340 ± 0.25	3.910 ± 0.35
pH 8	1.312 ± 0.15	1.849 ± 0.18	3.161 ± 0.33
pH 9	0.449 ± 0.17	0.533 ± 0.19	0.982 ± 0.36

Table 3.3. Effect of pH on the rates of photosynthesis and respiration. Photosynthesis rate is calculated by adding together oxygen evolution in the light plus oxygen uptake in the dark. Data are means of three replicates plus/minus the standard error.

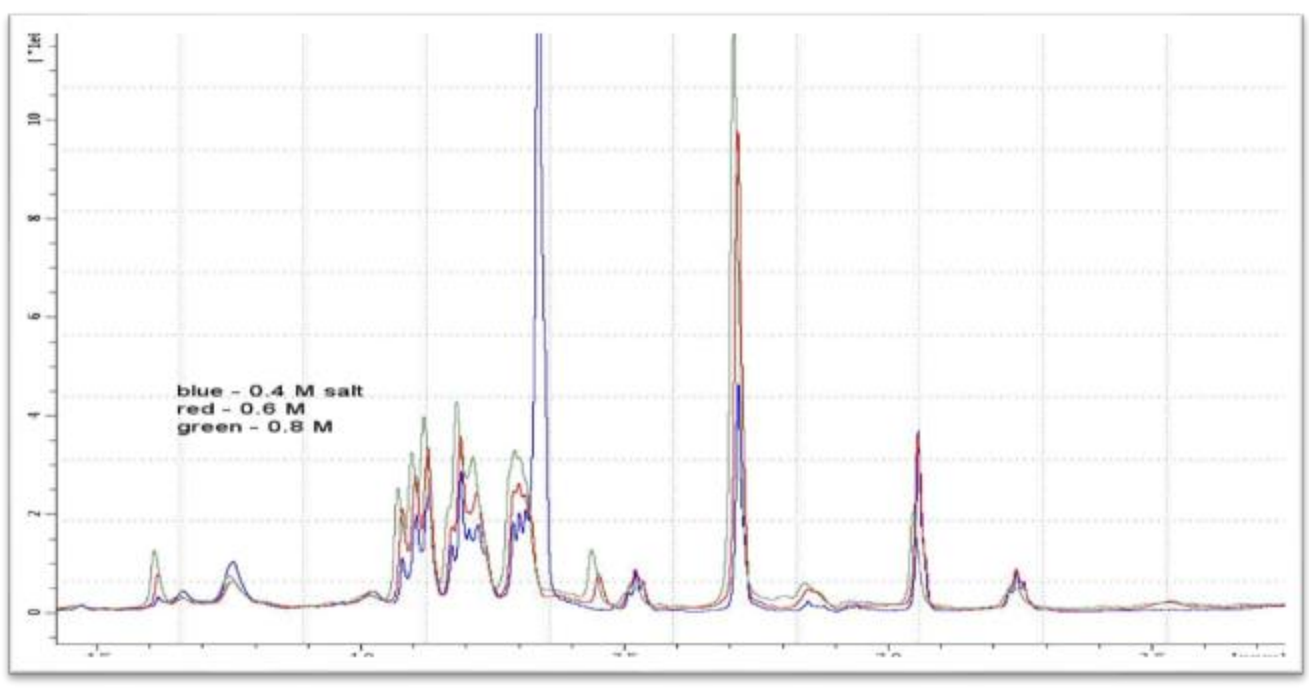
Increasing the temperature above the growth temperature of 25°C led to a slight increase in respiration rate at 30°C, but then a steep decline in respiration rate took place at 35 and 40°C. The rate of photosynthesis also declined as the temperature increased (Table 3.4).

Temperature	Oxygen Evolution (µ mol O_2 mg Chl^{-1} min^{-1})	Respiration (µ mol O_2 mg Chl^{-1} min^{-1})	Rate of Photosynthesis (µ mol O_2 mg Chl^{-1} min^{-1})
$25^{O}C$	1.312 ± 0.15	1.849 ± 0.18	3.161 ± 0.33
$30^{O}C$	0.815 ± 0.14	1.419 ± 0.08	2.234 ± 0.22
$35^{O}C$	0.886 ± 0.31	0.957 ± 0.35	1.814 ± 0.66
$40^{O}C$	0.709 ± 0.41	0.603 ± 0.51	1.312 ± 0.92

Table 3.4. The effect of temperature on photosynthesis and respiration. Photosynthesis rate is calculated by adding together oxygen evolution in the light plus oxygen uptake in the dark. Data are means of three replicates plus/minus the standard error.

3.2.8 Determination of Compatible Solute Production with Increasing Salinity using NMR Analysis

Tetraselmis suecica cells were grown at different salinities (0.4, 0.6 and 0.8 M NaCl) and freeze dried extracts were analysed by NMR. Figure 3.10 (top panel) shows that increasing salt gave rise to higher peaks at 3.7 to 3.9 ppm. Figure 3.10 (bottom panel) shows that there was no corresponding increase in these peaks in response to increasing pH. Therefore, it is likely that the 3.7 to 3.9 ppm peaks correspond to a compatible solute (osmolyte) being accumulated. To identify the compatible solute a 2D NMR spectrum was run with the most likely polyols. Figure 3.11 shows that the unknown compound accumulated as a compatible solute by *T. suecica* is mannitol.



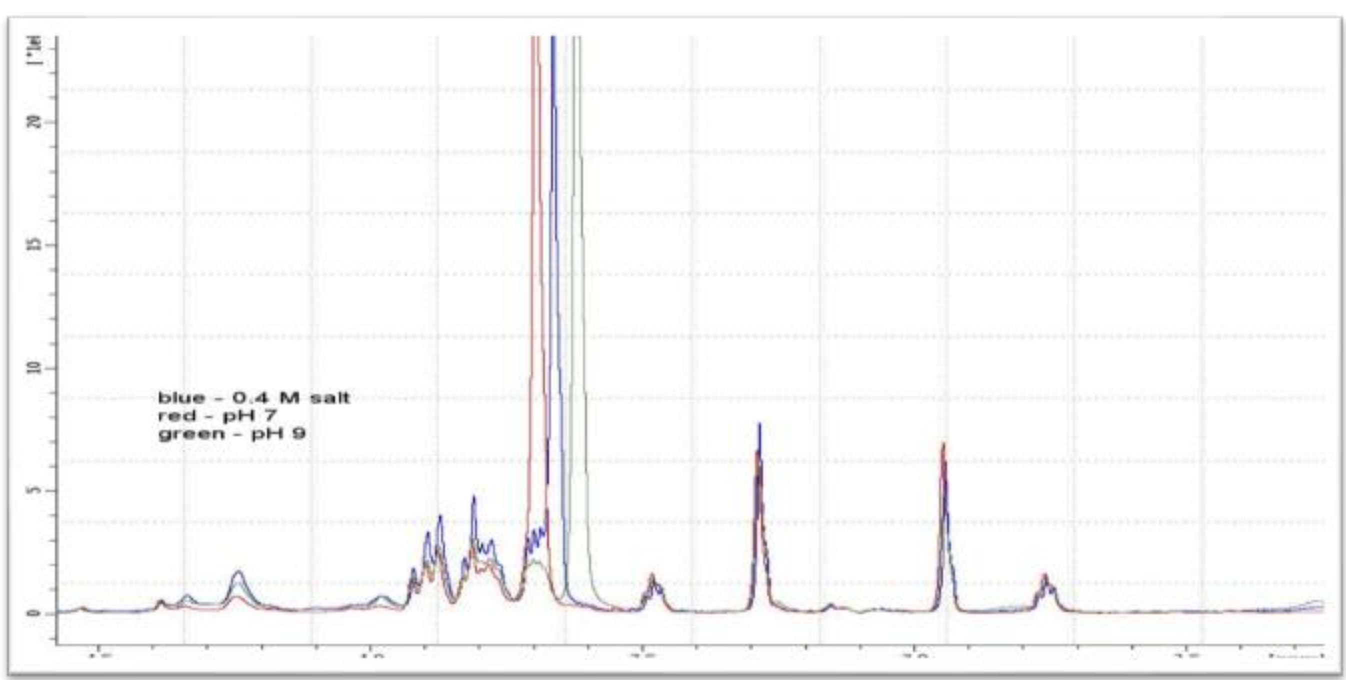


Figure 3.10. NMR spectra for *T. suecica* grown at a range of salinities (top panel) or pH values (bottom panel).

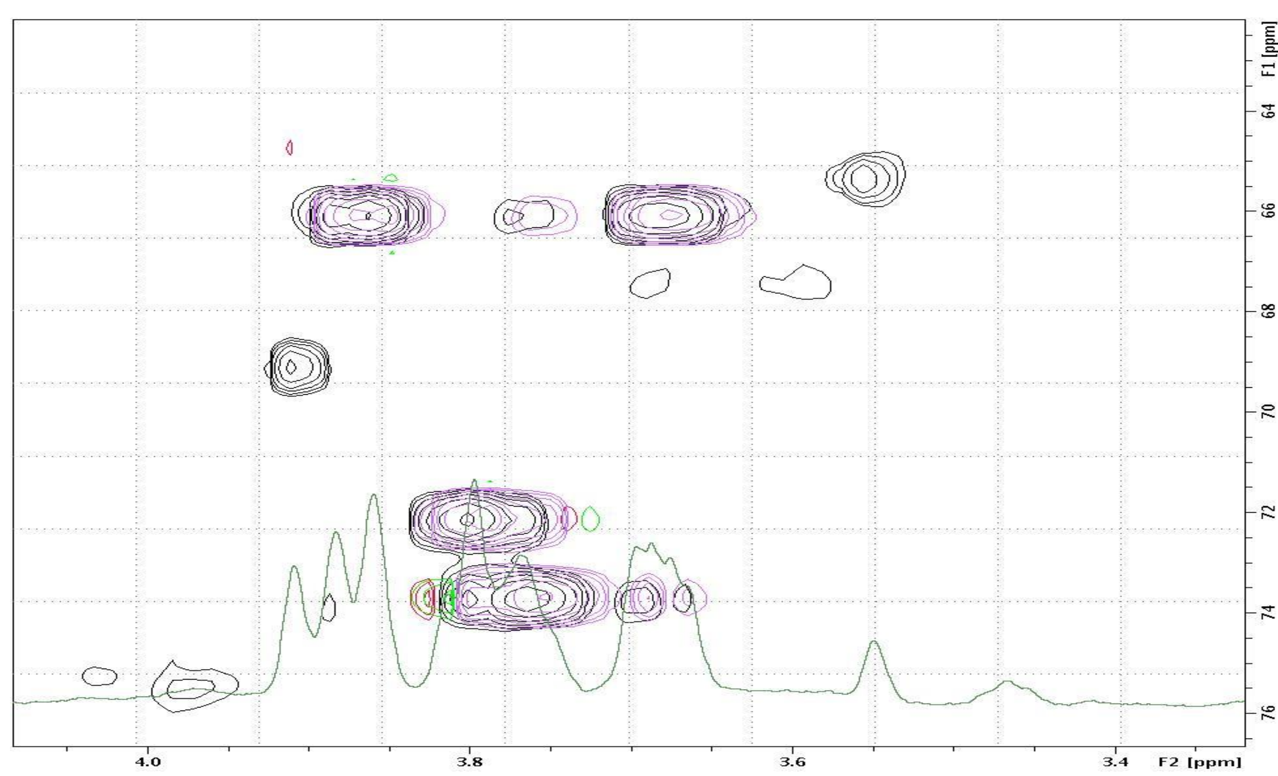


Figure 3.11. 2D 13C HSQC spectra of the 0.8 M NaCl sample from *T. suecica* cells (black) and authentic mannitol (purple). This demonstrates the presence of mannitol in the algal extract.

3.3 Discussion

The rDNA sequencing work confirmed that the algal isolate obtained from the CCAP was indeed a species of the green algal genus *Tetraselmis*. The lengths of the sequences achieved (just over 500 bp) were insufficient to prove conclusively that the isolate was *T. suecica* (Figure 3.4). The SEM images in Figure 3.5 allow the isolate to be distinguished from other green algae grown in the laboratory due to the presence of a haptonema at the base of the four flagella diagnostic for *Tetraselmis*.

The next step was to confirm the optimising growth medium for *T. suecica* and this was shown to be F/2 medium (Figure 3.6), which is a very widely used seawater medium that can be made using filtered natural seawater or (as in the present work) by adding commercially available seawater salt mixtures. In either case, F/2 is not a defined medium, because the concentration of all the salts is not known. On a confidential basis, the manufacturer of the synthetic seawater salt mix provided a recipe and this was adapted to produce the defined F/2 medium described in section 3.2.3. This medium was used successfully to grow *T. suecica* and allowed different salinities and nutrient limitations to be imposed on the cells.

Tetraselmis suecica was shown to be able to grow well across a range of salinities (up to 1 M NaCl) with little difference in final biomass achieved. Earlier work by Fabregas *et al.* (1984) found optimal growth of *T. suecica* between 0.4 and 0.6 M NaCl, broadly in agreement with the results shown in Figure 3.8. Less work has been done with regards to changing the pH, but the results in the present work (Figure 3.9) show a strong pH tolerance for *T. suecica* with optimum biomass accumulation at pH 9. It appears that *T. suecica* can grow well at a range of pH and salinity values.

The experiments carried out in the oxygen electrode do slightly contradict the growth data in as much that both salinity and pH increase significantly decreased the rates of photosynthesis and respiration (Tables 3.2 and 3.3). Although the cells were adapted to the salinities before being placed in the oxygen electrode, the measurements were taken over a very short time (15 mins in total). Thus it is not a fair comparison with the long term growth rates over 11 days. The effect of temperature on the respiration and photosynthesis rates does seem to show that 25°C is the optimal temperature for growth (Table 3.4).

To survive at elevated salt concentrations, microorganisms need to synthesize compatible solutes to balance the increase in osmotic pressure in the external medium (Empadinhas and da Costa, 2008). The NMR results shown in Figure 3.11 identified the polyol mannitol as the compatible solute for *T. suecica*. This agrees with the work published by Craigie *et al.* (1967) where it was shown that prasinophyte green algae like *Tetraselmis* use mannitol, whereas the true green algae (Chlorophyceae) like *Dunaliella* use glycerol. In terms of the energy efficiency using a C6 polyol like mannitiol is less efficient than using a C3 polyol like glycerol, thus *Tetraselmis* grows well up to 1 M NaCl (Figure 3.8), whereas *Dunaliella* grows well up to at least 3 M NaCl (Ying *et al.,* 2015).

In this chapter, *T. suecica* has been shown to be tolerant to a range of salinities and pH values, which is a key requirement for an organism that could commercially produce biofuels and other fine chemicals (Chisti, 2007). In the next chapter, the neutral lipid production by *T. suecica* cells will be assessed when grown at different salinities and pH values.

Chapter Four:

Neutral Lipid Content and Fatty Acid Composition of *Tetraselmis suecica*

4.1 Introduction

As described in section 1.5.2., algal lipids can be divided into polar and non-polar lipids. Within the non-polar lipid fraction, fatty acid chains attached to a glycerol backbone (neutral lipid, triacylglycerol, TAG) are the basis for biodiesel production. Therefore, the suitability of microalgae for the production of biodiesel depends on their TAG content. It is important to understand at what growth stage and under which culture conditions that TAG accumulation is maximised. Since TAG is synthesized as a storage compound, it makes sense to stress the algae using environmental factors like temperature, light intensity, pH and salinity to reduce growth and switch on TAG production by altering the activity of metabolic pathways (Huang *et al.*, 2013). However, this means that growth is halted to induce TAG synthesis, rather than having TAG synthesized as the cells grow. The latter is more desirable since it would lead to higher productivity.

The other aspect of TAG synthesis is the composition of the fatty acid chains. A number of characteristics of the fatty acids can change including length (number of carbons) and presence/absence and number of double bonds (i.e. degree of unsaturation). The properties of the fatty acids affect the properties of the biodiesel produced (Stansell *et al.*, 2012). The composition of diesel fuels is covered by strict standards throughout the world (e.g. EN 14214 is the fuel standard used in Europe). This standard ensures that the ignition properties of the fuel, its viscosity and oxidative stability all fall within acceptable limits (Stansell *et al.*, 2012). Fully saturated fatty acids (no double bonds) tend to add stability to the fuel, but can cause problems in cold weather (Knothe, 2005). Fatty acids rich in monounsaturated fatty acids (one double bond) are likely to be suitable and should make up a high proportion of the biodiesel. Polyunsaturated fatty acids are detrimental to fuel due to their susceptibility to

oxidation (Stansell *et al.*, 2012). The chain length is also important with C16 and C18 fatty acids being most suitable (Knothe, 2005).

In Chapter 3, the tolerance of *T. suecica* to salinity and pH changes was established. In the current chapter, salinity and pH will be used to stress *T. suecica* cells and then the neutral lipid (TAG) production will be measured. There are a number of ways to measure lipids, but the lipid-soluble fluorescent dye Nile Red (9-diethylamino-5H-benzo[α]phenoxa-phenoxazine-5-one) has been widely employed for the quantification of neutral lipid/TAG content in microalgae as a rapid, accurate and specific method (Cooksey *et al.,* 1987; Chen *et al.*, 2009). In this chapter, the intracellular neutral lipid in *T. suecica* was detected and then quantified with Nile Red (NR) staining. Secondly, fatty acid profiles of *T. suecica* were analyzed by gas chromatography–mass spectrometry (GC-MS) after direct transesterification with hydrochloric acid in methanol.

4.2 Results

4.2.1 Visualisation of Lipid Bodies using Fluorescence Microscopy

The accumulation of neutral lipids in *T. suecica* cells was first monitored by examining NR fluorescence under the fluorescence microscope to check that the NR dye dissolved in acetone was able to penetrate into the lipid droplets within the *T. suecica* cells. The microscope images in Figure 4.1 clearly show the yellow fluorescence of NR when it is staining neutral lipids (TAG). The staining shows that the neutral lipids are present as discrete droplets within the cells. There may be some indication that the amount of neutral lipid increased with increasing salinity, middle

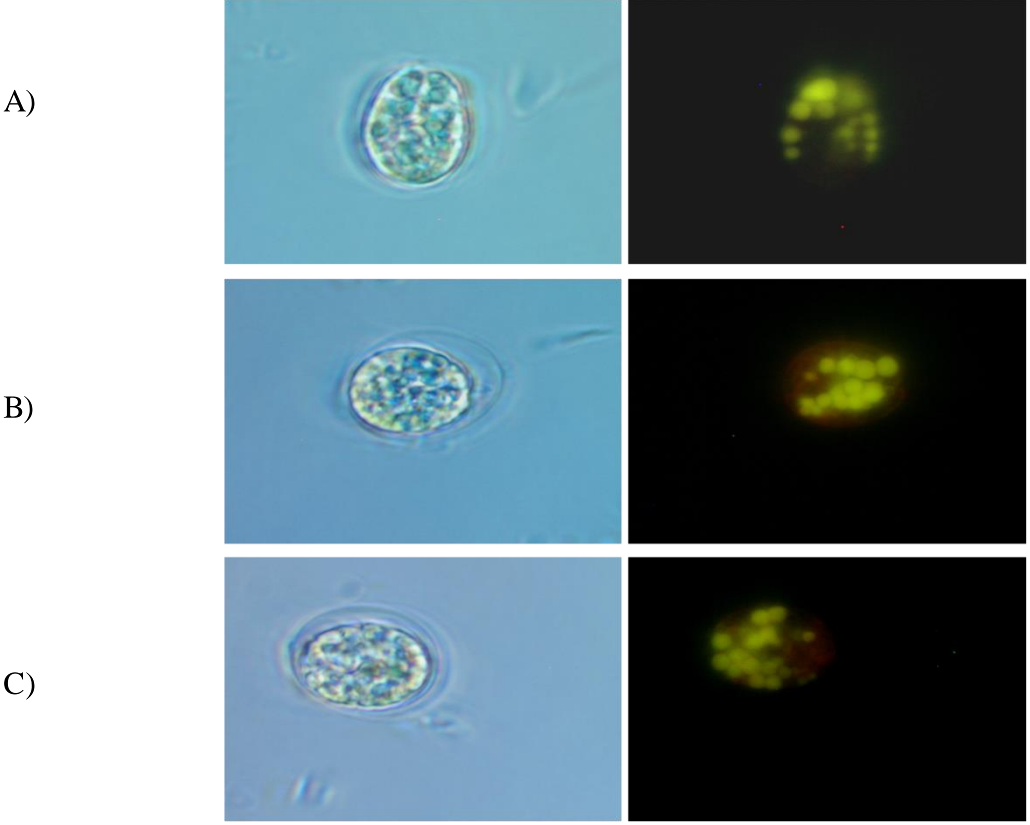


Figure 4.1. Fluorescence microscopy images of *T. suecica* cells stained with NR at a final concentration of 1 µmol/ml. The cells were grown for 4 weeks under different growth conditions. A) Cells grown in 0.4 M NaCl defined F/2 medium. B) Cells grown in 1 M NaCl defined F/2 medium. C) Cells grown in 0.6 M NaCl defined F/2 medium. The images on the left are taken under normal light and the images on the right show the same cell under fluorescent light conditions.

images being from highest salinity (Figure 4.1), but this is not a quantitative method. The key finding is that NR in acetone successfully entered the *T. suecica* cells and that the stained neutral lipid droplets were clearly visualised under the fluorescence microscope.

4.2.2 Optimization of Nile Red Fluorescence Method

To allow the quantification of NR fluorescence, a 96 well plate method using stained and unstained cells was used as described in section 2.13. Several parameters were set as described below before the algal samples were analyzed.

4.2.2.1 Nile Red peak fluorescence

Figure 4.2 shows the fluorescence intensities for different cell concentrations ranging from 12.5% to 100%, where 100% was OD_{595} = 1. The highest fluorescence intensities were with 87.5, 100 and 75, respectively with 20 mins staining time. The other lower cell concentrations gave lower fluorescence intensities which decreased as the cell concentration decreased. The optimal cell concentration is 87.5% which is equivalent to an OD_{595} = 0.818. The measurement of NR after 5 mins staining was excluded as a result of lower fluorescence intensities.

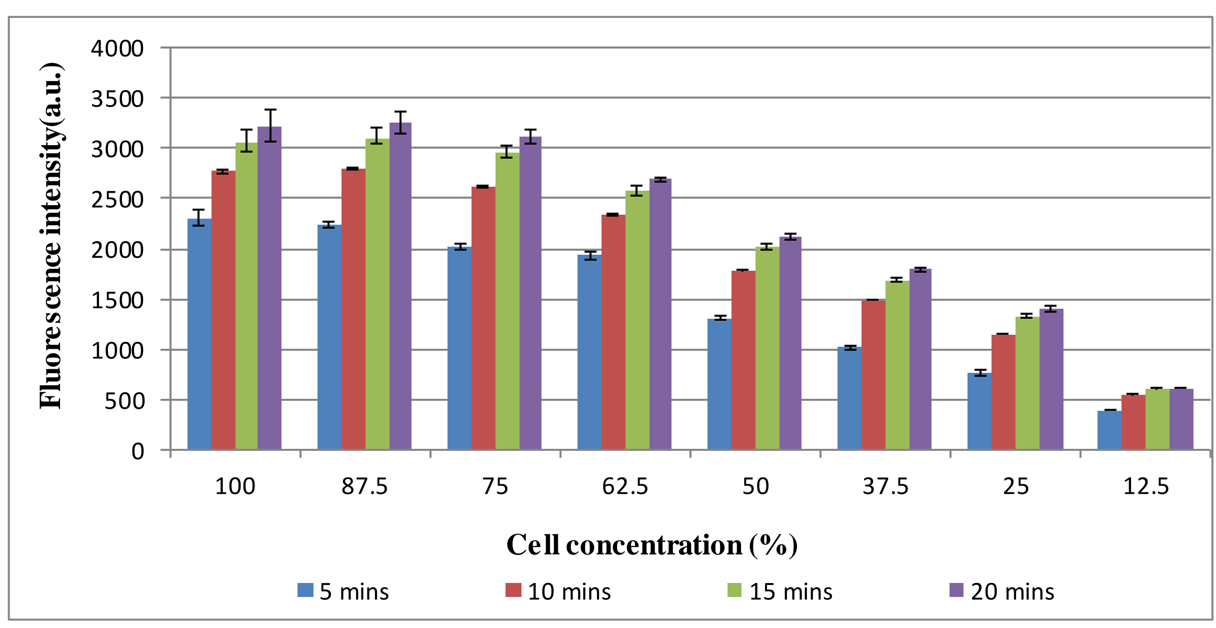


Figure 4.2. Optimization of NR staining time and cell concentration on fluorescence intensity of the green alga *T. suecica*. The optimum conditions were 87.5% after 20 mins staining. Each column represent the mean of four reading plus / minus standard deviation.

4.2.2.2 Nile Red concentration test

Figure 4.3 shows the effect of using different concentrations of NR ranging from 0.25 to 3 μmol/ml (dissolved in acetone) with the 87.5% cell concentration (OD_{595} = 0.818) of *T. suecica* with different staining times (10, 15 and 20 mins). Very similar fluorescence intensities were observed when NR concentration was 3 μmol/ml after 10, 15 or 20 mins. However, the lower concentrations of NR gave low fluorescence intensities compared to 3 μmol/ml after 10 mins staining (Figure 4.3). The optimal concentration of NR dye was 3 μmol/ml.

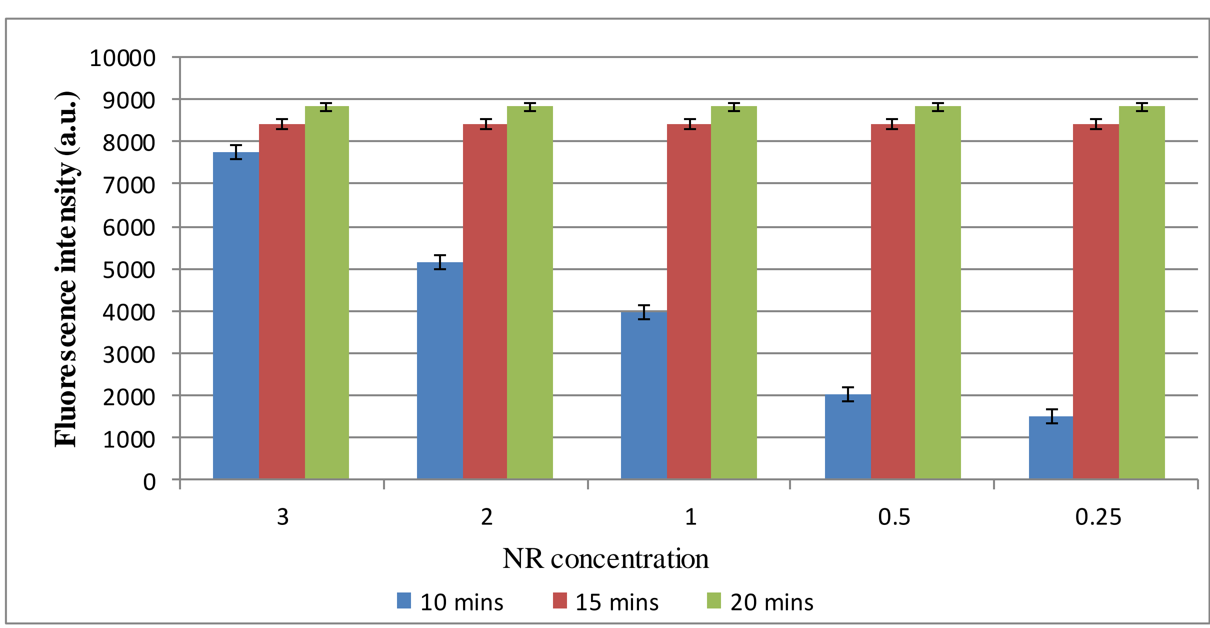


Figure 4.3. Optimization of NR staining concentration for the green alga *T. suecica*. The optimum cell concentration of 87.5% was used, but the time of staining was varied from 10 to 20 mins. Each column represent the mean of four reading plus / minus standard deviation

4.2.2.3 Nile Red Triolein test

The final part of the NR set-up experiments was to produce a concentration curve using triolein as the model neutral lipid. This will allow the conversion of fluorescence in arbitrary units to triolein equivalents. The resulting concentration curve is shown in Figure 4.4.

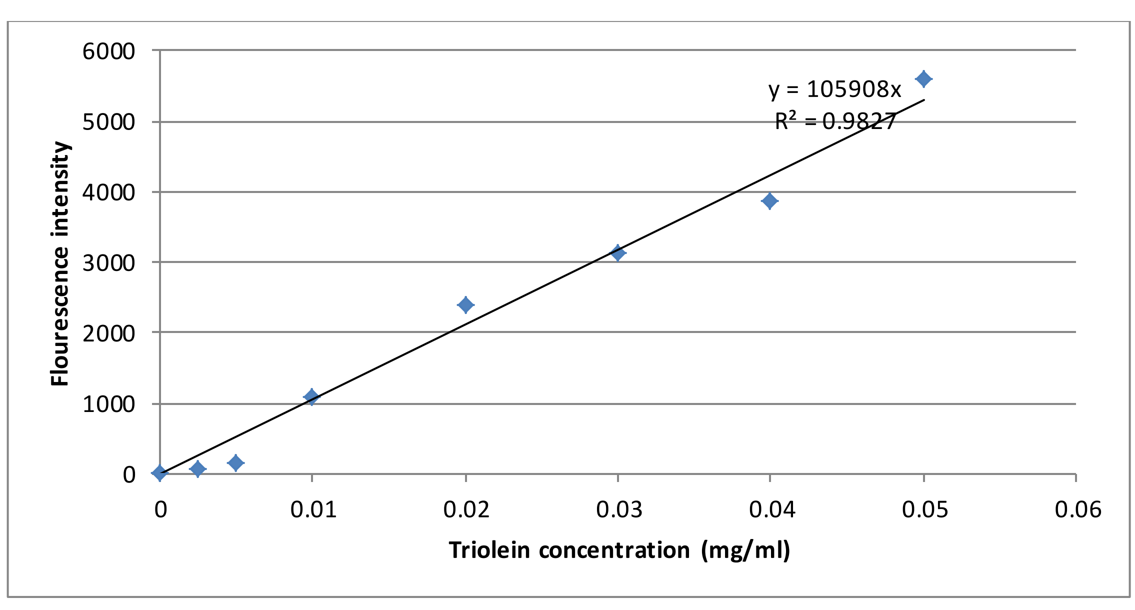


Figure 4.4. Linear correlation between fluorescence intensity and triolein concentration to allow the conversion of fluorescence readings to triolein equivalents.

4.2.3 Neutral Lipid Content of *T. suecica* Cells Grown at Different Salinities

The aim of the experiment was to determine if there was a correlation between increased molarity of NaCl in the medium, and production of neutral lipids (TAG) in *T. suecica*. The accumulation of lipids in *T. suecica* was assessed using NR dye staining as optimised in the previous section using 3 μmol/ml of NR dye concentration with the concentration of *T. suecica* cells set at OD_{595} = 0.818 (87.5% cell concentration) and the measurement of fluorescence intensities 20 mins after staining the algal cells with NR dye. The test was performed every week for four weeks to investigate the effect of salt stress over a significant time period. Figure 4.5 shows that the highest levels of neutral lipids were observed with the highest NaCl concentration (1 M) after 4 weeks culture. The relationship between increasing the salt concentration and TAG

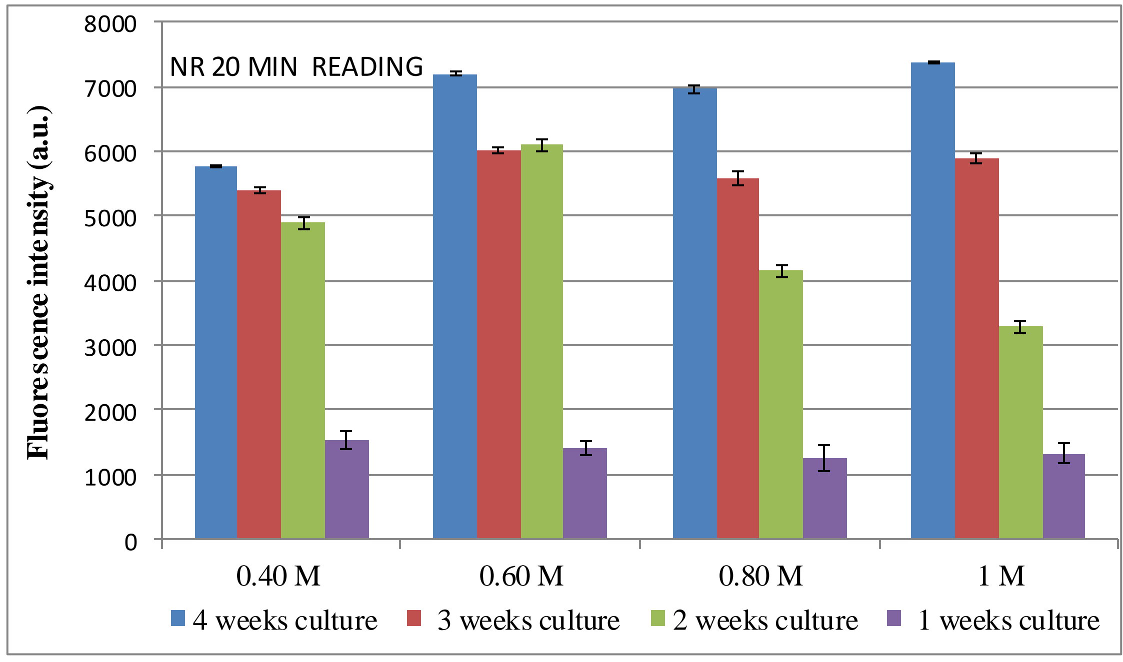


Figure 4.5. Neutral lipid content measured by NR fluorescence of *T. suecica* in defined F/2 medium with different concentrations of NaCl. Each column represent the mean of four reading plus / minus standard deviation

accumulation was not straight forward. After two weeks growth, the lowest levels of TAG were found in the 1 M NaCl grown cells (Figure 4.5). It is clear that age of culture is also important when considering the optimum conditions for TAG accumulation.

4.2.4 Neutral Lipid Content of *T. suecica* Grown at Different pH Values

The influence of pH on the neutral lipid content of *T. suecica* was examined using NR staining dye as optimised using 3 µmol/ml concentration of NR dye with the concentration of *T. suecica* cells (OD_{595} = 0.818) and the measurement of fluorescence intensities at 20 mins after staining the algal cells with NR dye. The test was performed each week from 1 to 4 weeks after inoculation to examine the effect of pH stress over a long period. Figure 4.6 shows that, interestingly, the highest neutral lipid levels were observed in *T. suecica* cells grown at pH 9 for 2 weeks. However, after four weeks growth, there was no difference in the TAG levels in cells grown at different pH values (Figure 4.6).

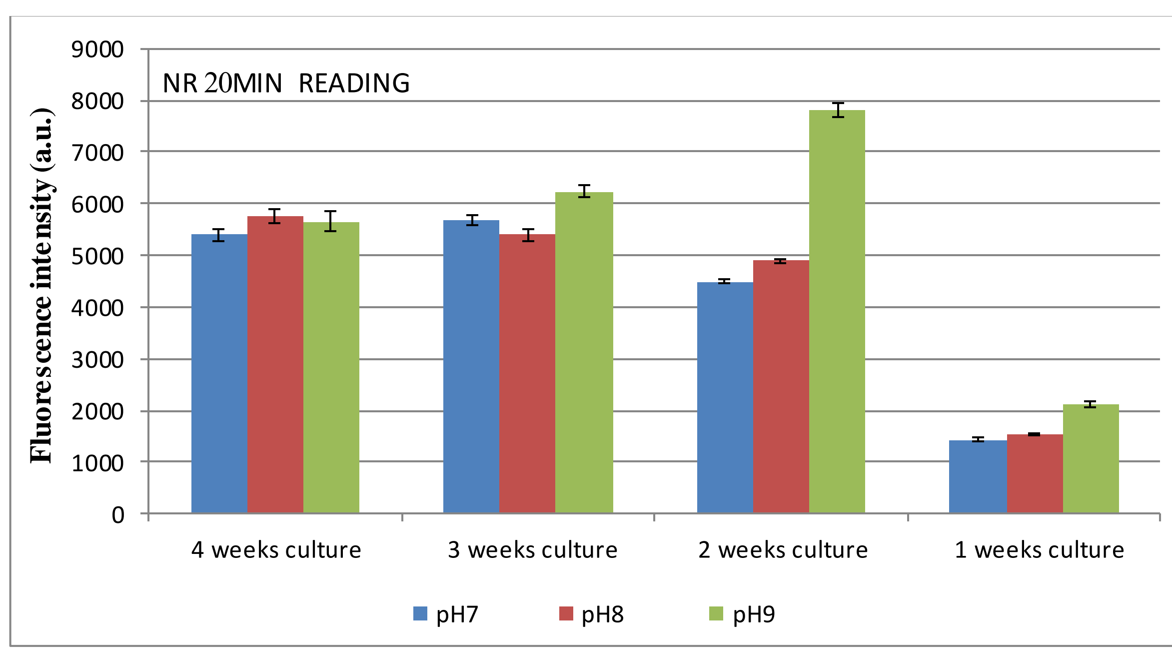


Figure 4.6. Neutral lipid content measured by NR fluorescence of *T. suecica* cells grown in defined F/2 medium at different pH values. Each column represent the mean of four reading plus / minus standard deviation

4.2.5 Total Lipid Determination of *T. suecica* Cells by Gravimetric Method

A one-off experiment was undertaken to look at the effect of pH and salinity stress on the total lipid production by *T. suecica* cells. A large amount of biomass is required for the gravimetric method so the cells were grown for 4 weeks and the whole flask used to generate sufficient biomass (section 2.13.2). Figure 4.7 shows that the same trends were seen as for 4 week old cultures used for NR dye neutral lipid determination (i.e. higher levels of total lipids in cells exposed to 1 M NaCl), but little difference in cells exposed to pH changes.

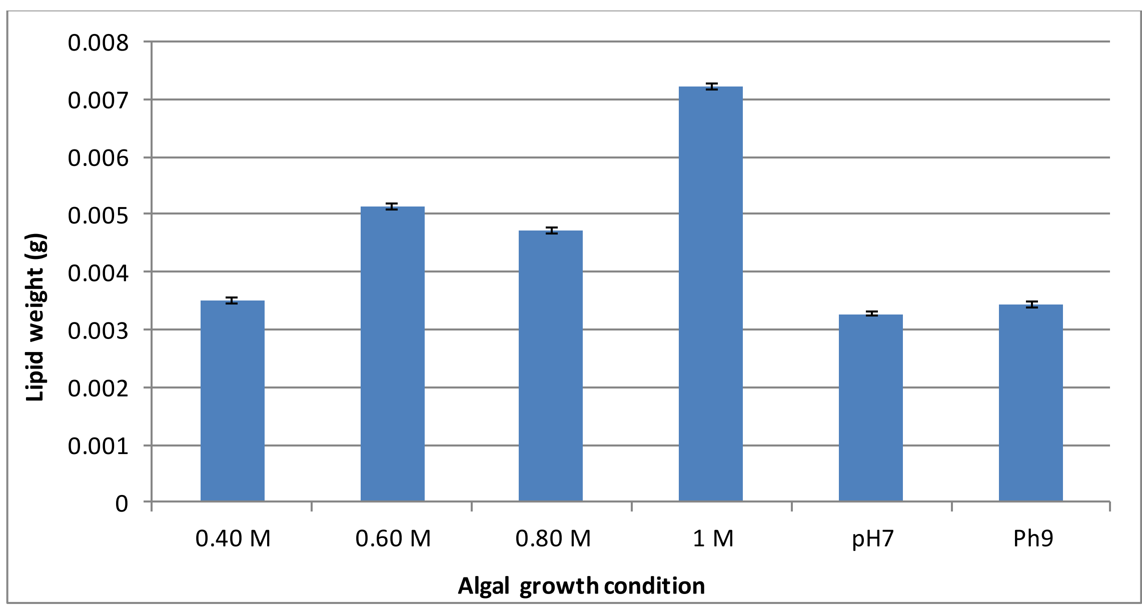


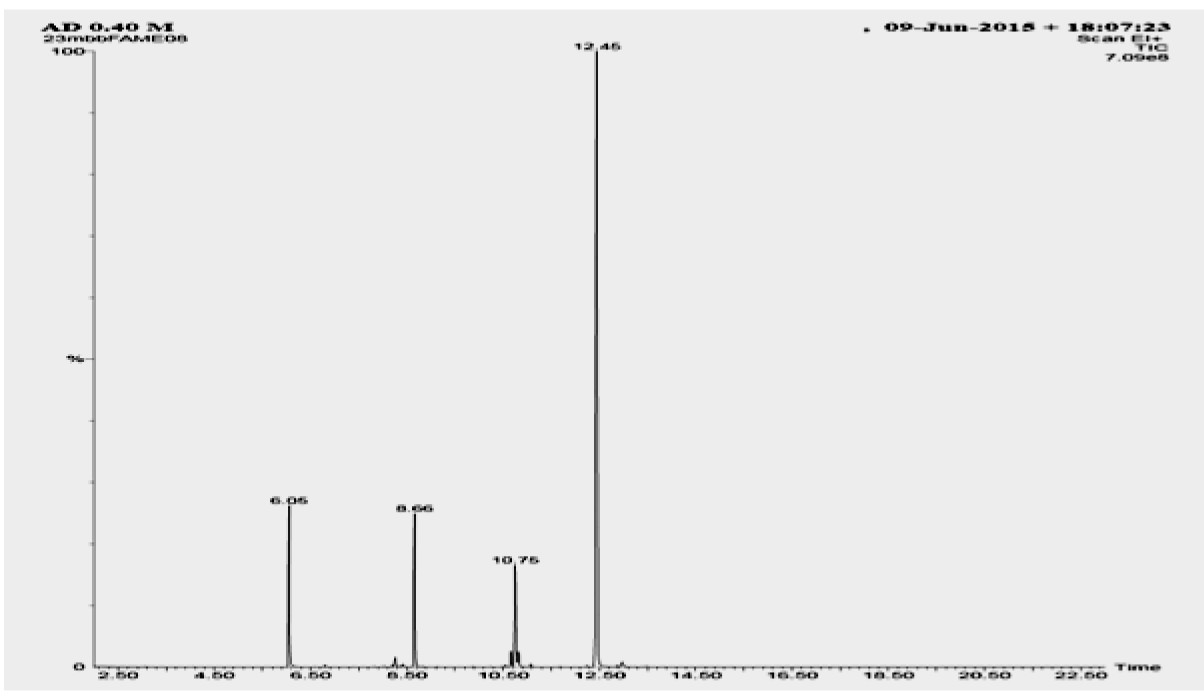
Figure 4.7. Gravimetric measurement of total lipid content for 4 week old *T. suecica* cultures grown at different salinities and pH values. The pH of all salinity cultures was pH 8 and the salinity of the different pH cultures was 0.4 M NaCl. Each column represent the mean of four reading plus / minus standard deviation

4.2.6 Fatty Acid Profiles of *T. suecica* Cells Grown at Different Salinities

Figures 4.8, 4.9 and 4.10 show the spectra of fatty acids found in *T. suecica* cells exposed to increasing salinity. In each figure, the top panel shows the fatty acid spectrum for cells grown in normal salinity (0.4 M NaCl) and two saturated fatty acids dominate the spectrum – hexadecanoic acid (palmitic acid, C16:0) and octadecanoic acid (stearic acid, C18:0). In cells grown in 0.6 M NaCl, several other fatty acids are detected in addition to the two major saturated fatty acids i.e. pentadecanoic acid (C15:0), cis-10-heptadecanoic acid (C17:1), elaidic acid (C18:1) and linolelaidic acid (C18:2). At higher salinities (0.8 and 1 M NaCl), the same spectrum of additional fatty acids was found (Figures 4.9 and 4.10). It seems clear that with increased salinity above 0.4 M NaCl, more unsaturated fatty acids are produced in response to the salt stress.

4.2.6 Fatty Acid Profiles of *T. suecica* Grown at Different pH Values

Figures 4.11 and 4.12 show that stressing the *T. suecica* cells, through changing the external pH to 7 or 9, had a very similar effect on the fatty acid profile as increasing the salinity. Again a number of unsaturated fatty acids were produced in response to the change in pH including linolelaidic acid, which contains two double bonds and is a polyunsaturated fatty acid.



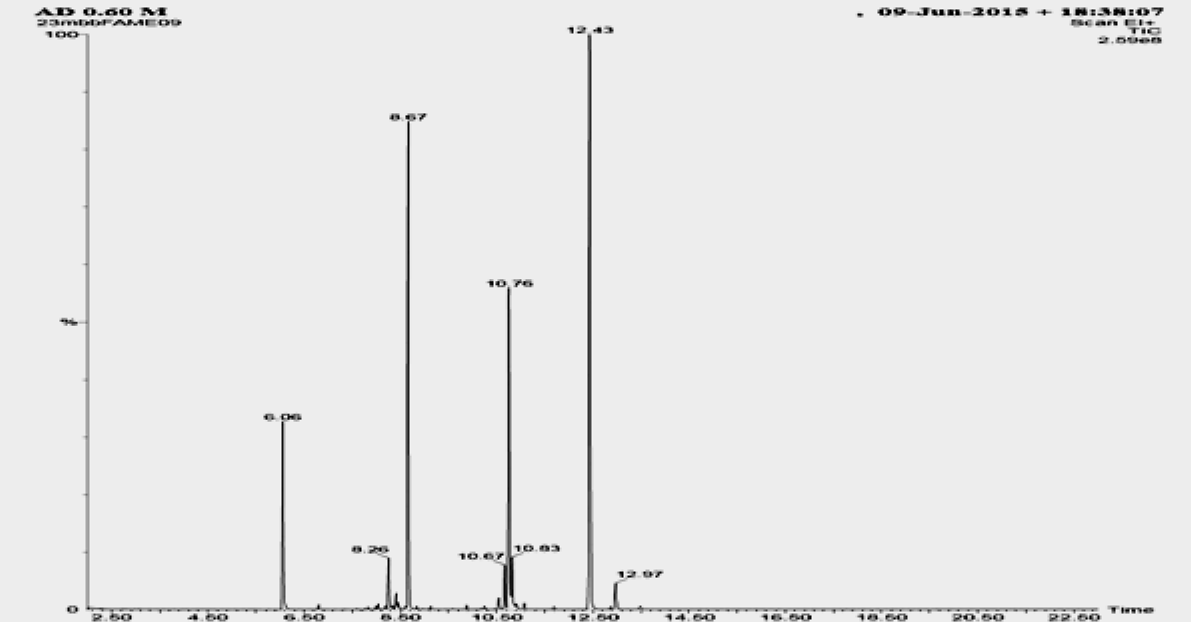
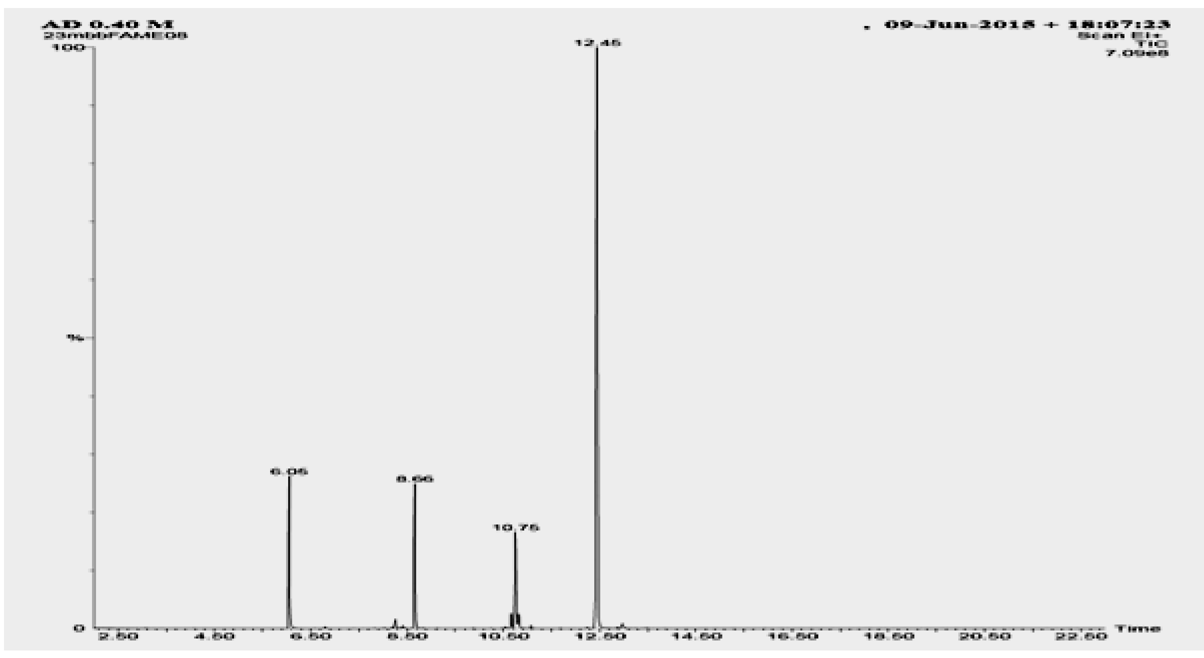


Figure 4.8. Comparison of GC-MS chromatographs for fatty acid accumulation in *T. suecica*; Top: grown in normal conditions with 0.4 M salt concentration and pH 8. The fatty acids accumulated were identified as (1)- hexadecanoic acid, methyl ester C16 (peak 8.66) (2)- octadecanoic acid methyl ester C18:0 (Peak 10.75) and the other two peaks were internal standard peaks 6.05 (C13) and peak 12.45 (C19). Bottom: *T. suecica* cells grown under stress conditions with 0.6 M salt concentration and the fatty acids accumulated were (1) pentadecanoic acid methyl ester c15:0 (Peak 8.26), (2) hexadecanoic acid, methyl ester C16 (Peak 8.67), (3) cis-10-heptadecenoic acid methyl ester C17:1 (Peak 10.67), (4) octadecanoic acid methyl ester C18:0 (Peak 10.76), (5) elaidic acid methyl ester C18:1,9t (Peak 10.83), (6) linolelaidic acid methyl ester C18:2n6t (Peak 12.97). The other two peaks were internal standard peaks 6.06 (C13) and 12.43 (C19).



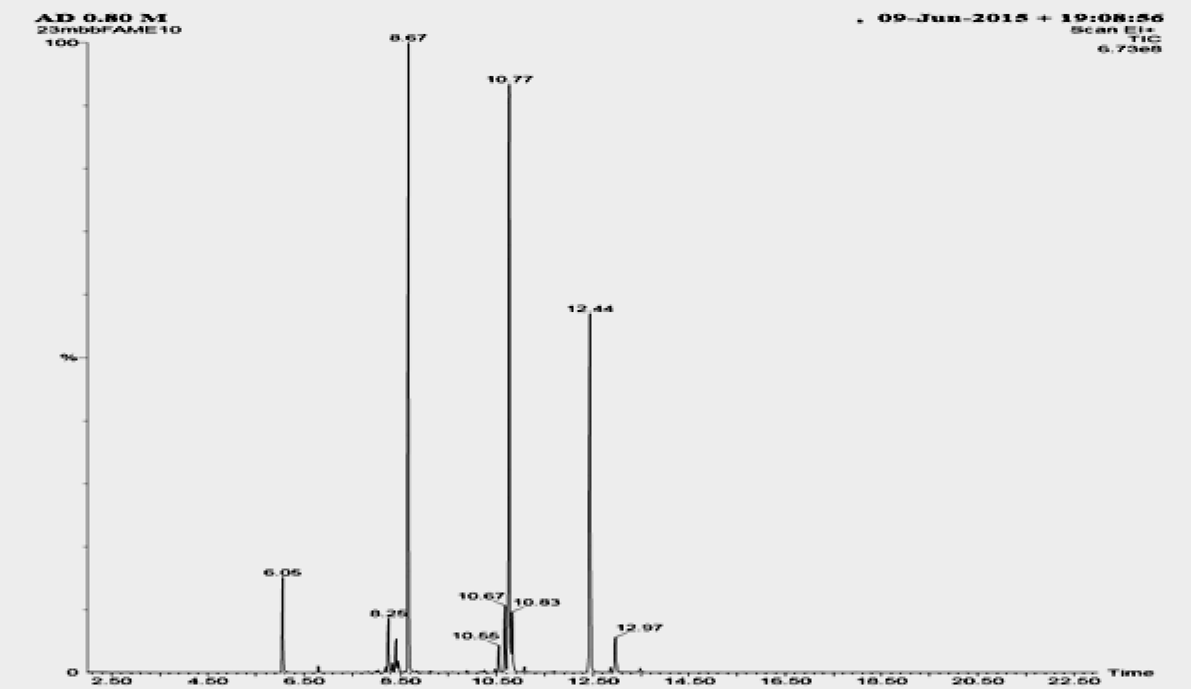
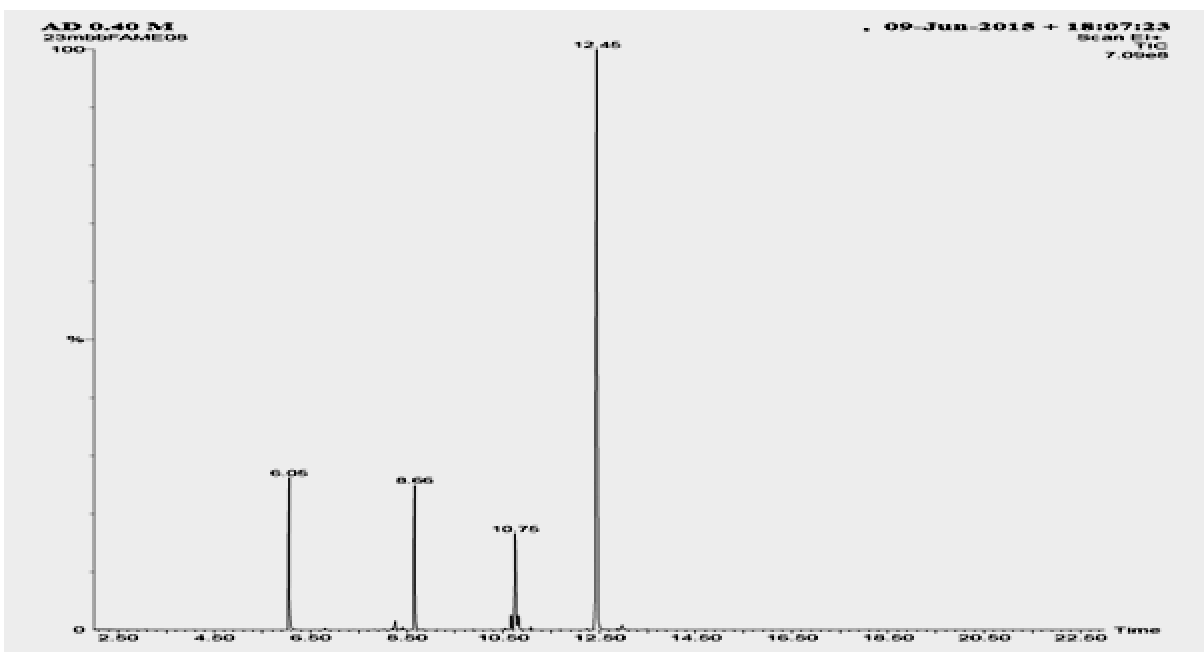


Figure 4.9: Comparison of GC-MS chromatographs for fatty acid accumulation in *T. suecica*; Top grown in normal condition with 0.4 M salt concentration and pH 8. The fatty acids accumulated were (1)- hexadecanoic acid, methyl ester C16 (peak 8.66) (2)- octadecanoic acid methyl ester C18:0 (Peak 10.75) and the other two peaks were internal standard peak 6.05 (C13) and Peak 12.45 (C19). Bottom *T. suecica* cells grown under stress conditions with 0.8 M salt concentration and the fatty acids accumulated were (1) pentadecanoic acid methyl ester c15:0 (Peak 8.26), (2) hexadecanoic acid, methyl ester C16 (Peak 8.67), (3) cis-10-heptadecenoic acid methyl ester C17:1 (Peak 10.67), (4) octadecanoic acid methyl ester C18:0 (Peak 10.77), (5) elaidic acid methyl ester C18:1,9t (Peak 10.83), (6) linolelaidic acid methyl ester C18:2n6t (Peak 12.97). The other two peaks were internal standard peaks 6.05 (C13) and 12.44 (C19).



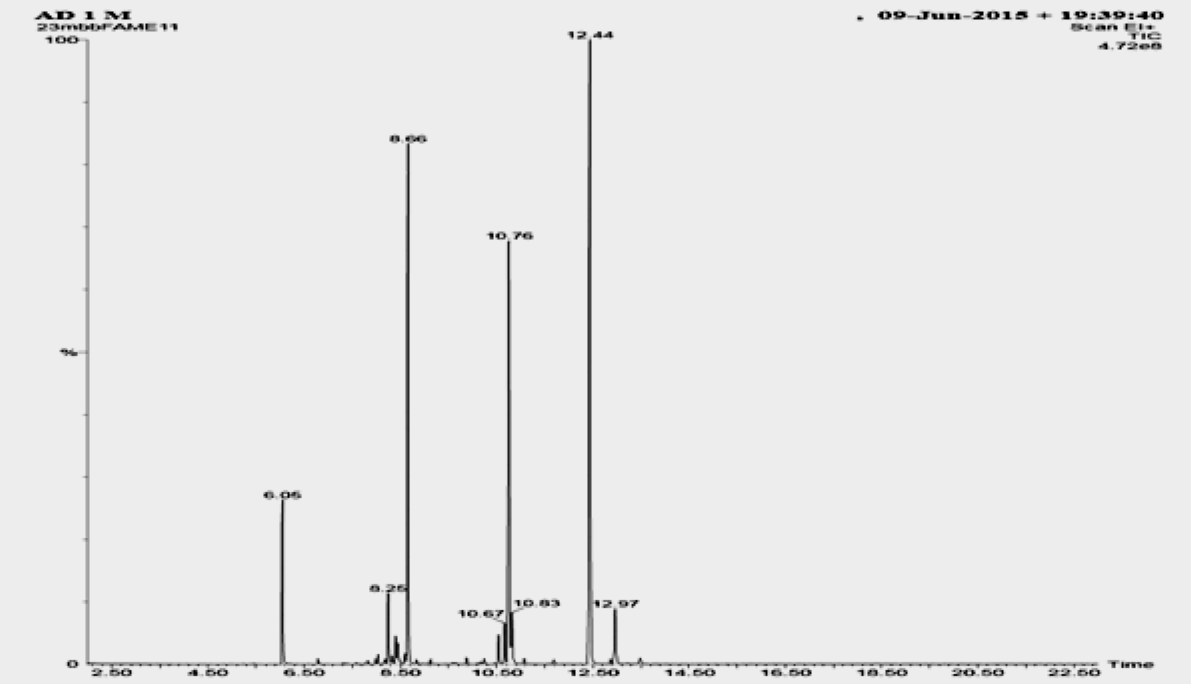
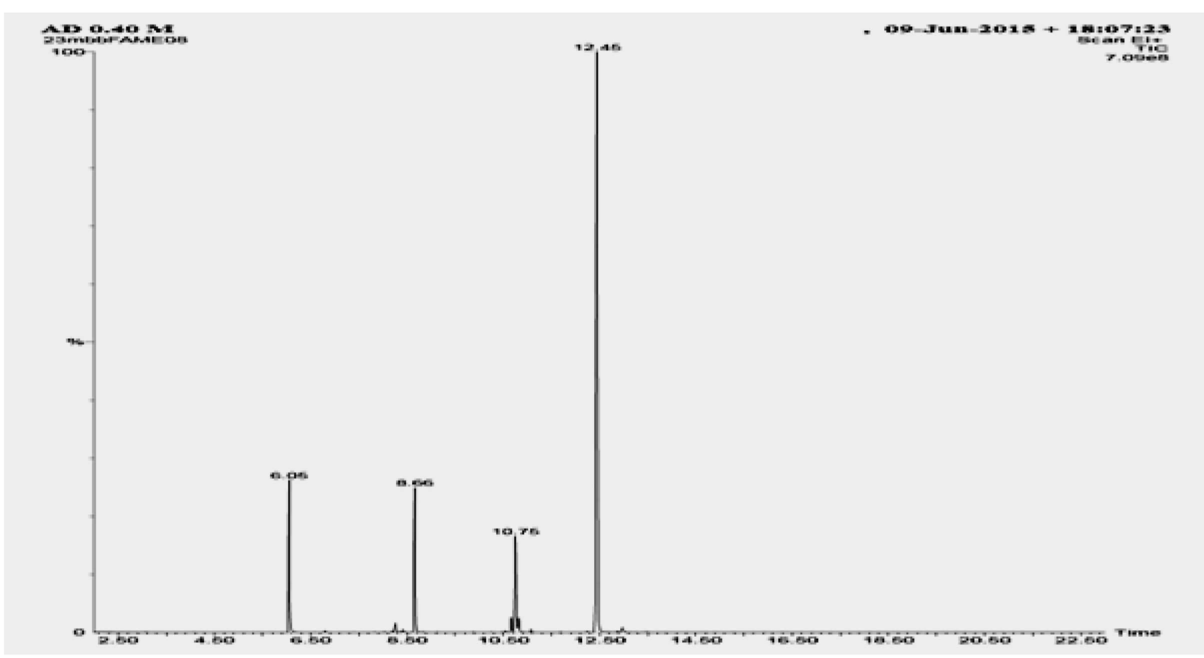


Figure 4.10. Comparison of GC-MS chromatographs for fatty acid accumulation in *T. suecica*; Top: grown under normal conditions with 0.4 M salt concentration and pH 8. The fatty acid accumulated were (1)- hexadecanoic acid, methyl ester C16 (peak 8.66) (2)- octadecanoic acid methyl ester C18:0 (peak 10.75) and the other two peaks were internal standard peaks 6.05 (C13) and12.45 (C19). Bottom: *T. suecica* grown under stress conditions with 1 M NaCl concentration and the fatty acids accumulated were (1) pentadecanoic acid methyl ester c15:0 (peak 8.25), (2) hexadecanoic acid, methyl ester C16 (peak 8.66), (3) cis-10-heptadecenoic acid methyl ester C17:1 (peak 10.67), (4) octadeconoic acid methyl ester C18:0 (peak 10.76), (5) elaidic acid methyl ester C18:1,9t (peak 10.83), (6) linolelaidic acid methyl ester C18:2n6t (peak 12.97). The other two peaks were internal standard peaks 6.05 (C13) and 12.44 (C19).



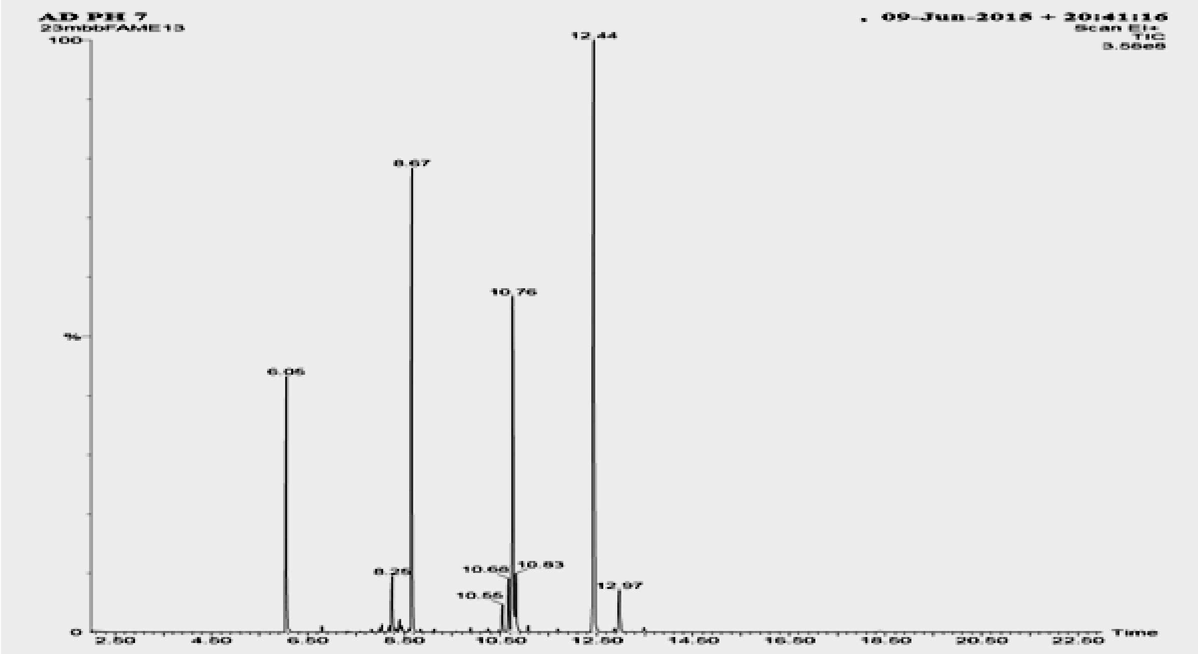
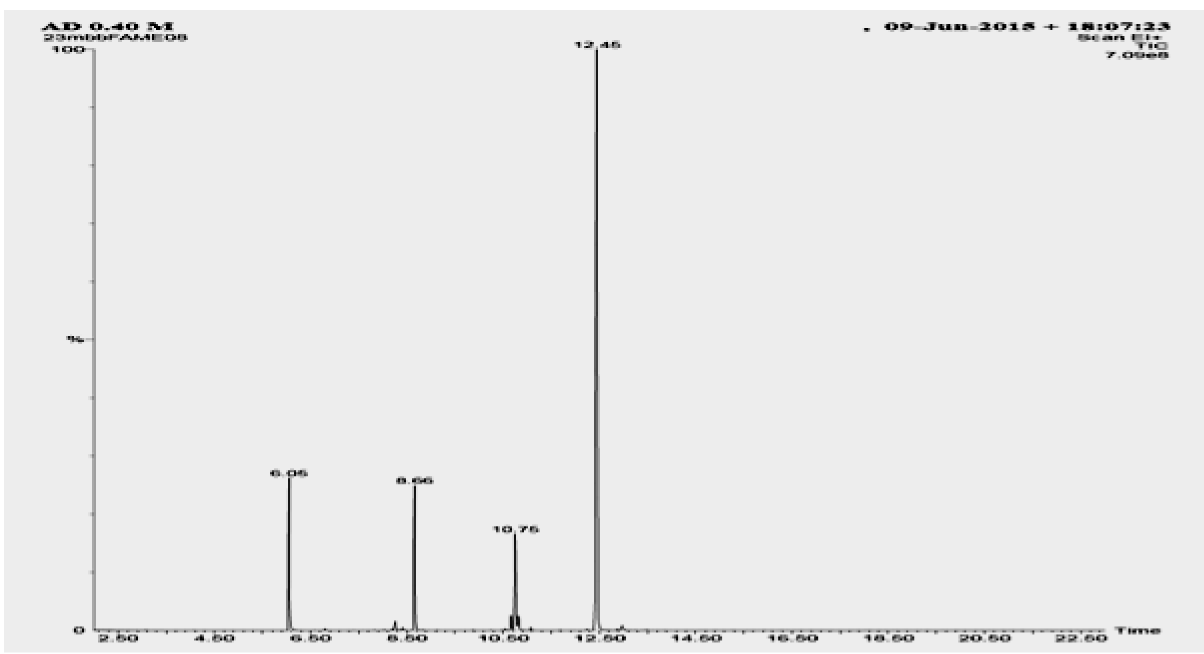


Figure 4.11: Comparison of GC-MS chromatographs for fatty acid accumulation in *T. suecica*; Top: grown in normal conditions with 0.4 M salt concentration and pH 8. The fatty acids accumulated were (1)- hexadecanoic acid, methyl ester C16 (peak 8.66) (2)- octadecanoic acid methyl ester C18:0 (peak 10.75) and the other two peaks were internal standard peaks 6.05 (C13) and 12.45 (C19). Bottom: *T. suecica* grown under stress conditions at pH 7 and the fatty acids accumulated were (1) pentadecanoic acid methyl ester c15:0 (peak 8.25). (2) hexadecanoic acid, methyl ester C16 (peak 8.67), (3) cis-10-heptadecenoic acid methyl ester C17:1 (peak 10.68), (4) octadecanoic acid methyl ester C18:0 (peak 10.76), (5) elaidic acid methyl ester C18:1,9t (peak 10.83), (6) linolelaidic acid methyl ester C18:2n6t (peak 12.97). The other two peaks were internal standard peaks 6.05 (C13) and Peak 12.44 (C19).



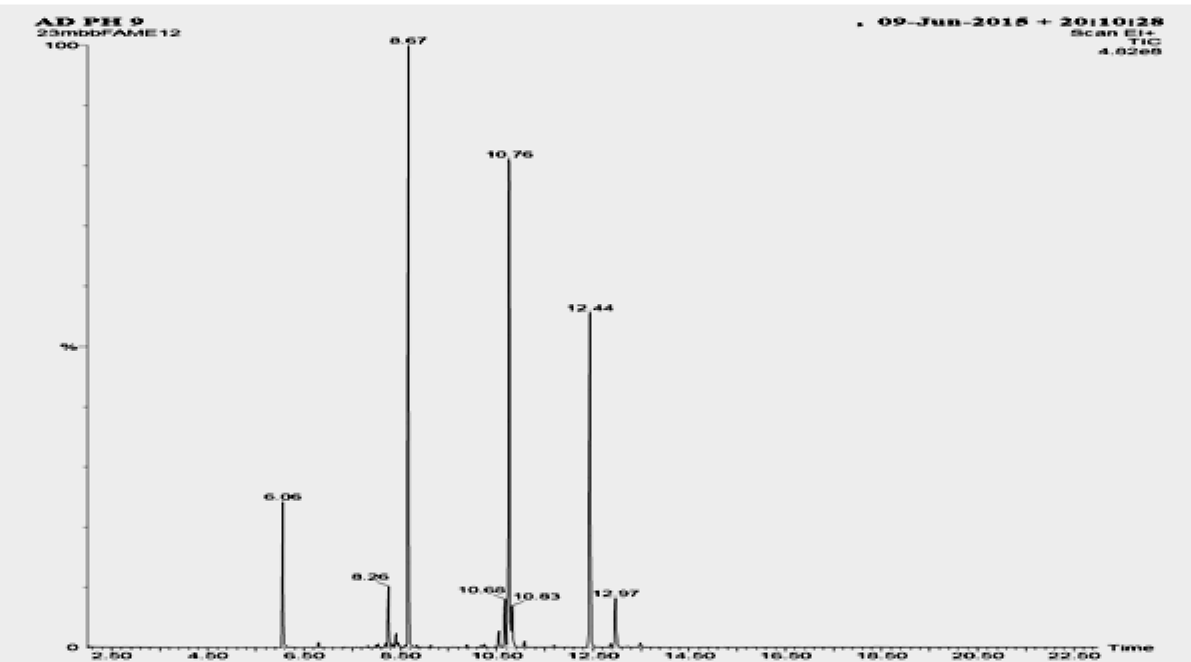


Figure 4.12: Comparison of GC-MS chromatographs for fatty acid accumulation in *T. suecica*; Top: grown under normal conditions with 0.4 M salt concentration and pH 8. The fatty acids accumulated were (1)- hexadecanoic acid, methyl ester C16 (peak 8.66) (2)- octadecanoic acid methyl ester C18:0 (peak 10.75) and the other two peaks were internal standard peaks 6.05 (C13) and 12.45 (C19). Bottom: *T. suecica* grown under stress conditions at pH 9 and the fatty acids accumulated were (1) pentadecanoic acid methyl ester c15:0 (peak 8.26), (2) hexadecanoic acid, methyl ester C16 (peak 8.67). (3) cis-10-heptadecenoic acid methyl ester C17:1 (peak 10.68). (4) octadecanoic acid methyl ester C18:0 (peak 10.76), (5) elaidic acid methyl ester C18:1,9t (Peak 10.83), (6) linolelaidic acid methyl ester C18:2n6t (peak 12.97). The other two peaks were internal standard peaks 6.06 (C13) and 12.44 (C19).

4.3 Discussion

In this chapter, NR fluorescence was shown to be a suitable method for visualising lipid droplets inside *T. suecica* cells (Figure 4.1). The NR fluorescence method was then optimised for a quantitative assay in a 96 well plate reader format by setting the time required for peak fluorescence and the concentration of both cells and NR dye that are optimal (section 4.2.2). Using these parameters, the amount of neutral lipid (TAG) accumulated by *T. suecica* cells under salinity and pH stress was measured (Figures 4.5 and 4.6). At the normal pH of 8, it was found that four weeks incubation at 1 M NaCl gave rise to the highest amount of TAG (Figure 4.5). In contrast at the normal salinity of 0.4 M NaCl, it was found that 2 weeks culture at pH 9 produced the most TAG (Figure 4.6). This established that environmental stresses can turn on TAG synthesis as has been shown previously for N-limited *Tetraselmis* cultures (Xu *et al.*, 2013). It was also interesting that for 4 week old *T. suecica* cultures, total lipid measured gravimetrically showed the highest lipid level in 1 M NaCl grown cells. The conclusion to be drawn is that a large increase in salinity can induce increased TAG accumulation and high salinity may be the best condition to use for TAG synthesis in a commercial biofuel process based on *T. suecica* as the feedstock. This has the additional advantage that contamination of *T. suecica* cultures will be more easily controlled at elevated salinities (Azma *et al.*, 2010; Qin *et al.*, 1999).

The fatty acid profiles of the TAG accumulated under salinity and pH stresses were measured using GC-MS. Any increase in salinity above 0.4 M NaCl induced the production of monounsaturated fatty acids and one polyunsaturated fatty acid. The mixture of saturated, unsaturated and polyunsaturated fatty acids looks very promising as

a base for biodiesel production and should meet the requirements for cold-flow, ignition properties, viscosity and oxidative stability (Stansell *et al.*, 2012).

In the next chapter, attempts to scale up *Tetraselmis* cultures in 2 litre photobioreactors will be described and the possibility of recycling nutrients will be explored.

Chapter Five:

Suitability of *Tetraselmis suecica* for Growth in a Biorefinery

5.1 Introduction

In the previous chapter, the suitability of *T. suecica* cells as a feedstock for biofuel production was confirmed due to their tolerance of high salinity and the fact that TAG production was increased at high salinity and showed a good mix of saturated and monounsaturated fatty acids. The logical next step is to examine scaling up of cultures from 100 ml in 250 ml flasks used in the previous chapters to a 2 litre airlift fermenter. Previous work in the Gilmour laboratory with *T. suecica* (Bangert and Gilmour, unpublished) has shown that the cells may stick to the surfaces of the fermenter which disrupts growth of the culture. However, it is important to examine how well *T. suecica* can grow in an aerated culture rather than a static 250 ml flask culture as a first step towards larger scale cultures.

The second aspect of scaling up is that nutrients are required in much larger amounts. The availability of CO_2 is not a major problem, although transferring CO_2 from an industrial source to an algal pond or bioreactor may not be cost effective and most recent proposals envisage building the algal ponds adjacent to the industrial facility (Rosenberg *et al.*, 2011). However, the availability of nitrogen and phosphorus sources is a potential major limitation to the scale up of algal biodiesel production to a level that provides a significant percentage of the diesel requirement of a major industrial country like the UK or USA (Pate *et al.*, 2011). Two ways of addressing the problem of N and P limitation are a) to recycle the nutrients and/or b) use wastewater rich in nutrients to supplement the algal growth medium. Both ideas can be incorporated into the concept of an algal biorefinery, where medium is recycled, industrial or domestic waste is part of the inputs

and all parts of the algal biomass are used for products or recycled back into the process (Razzak *et al.*, 2013).

The aims of the work presented in this chapter are to examine the possibility of growing *T. suecica* on a larger scale using a 2 L airlift photobioreactor and using higher levels of CO_2. Also, the effect of re-using the medium on the growth of *T. suecica* will be examined with the aim of developing an integrated algal biorefinery process using *T. suecica* as the feedstock.

5.2 Results

5.2.1 Re-using the Medium and its Effect on Growth of *T. suecica*

For this experiment, 10 day-old cultures of *T. suecica* grown in 0.4 M NaCl defined F/2 medium were centrifuged to remove all the algal cells and then the supernatant (used medium) was inoculated with fresh cells along with a control in fresh 0.4 M NaCl F/2 defined medium. Figure 5.1 shows the growth rate in recycled medium over the first four days was very low compared with the control, therefore it is clear that little or no growth took place in all three re-used medium flasks in comparison with the control. To test if this was due to a limitation in N or P, additional N and P was added as shown in Figure 5.1. There was still poor or no growth shown after a single enrichment with N or P (red and green lines, respectively). However, when both N and P was added (purple line, Figure 5.1) then good growth was seen. Figure 5.2 shows pictures of the growth flasks during the experiment. Panel B in Figure 5.2 shows the lack of growth in the re-used medium after 4 days and then Panel C shows that addition of both N and P gave good

growth, but single nutrient enrichment was not effective. After 10 days, N was added to the culture which had only P enrichment previously (green line in Figure 5.1) and P was added to the flask which had previously had only N enrichment (red line in Figure 5.1). In both cases, good growth resulted from this second enrichment step. Panel D in Figure 5.2 shows the flasks after 14 days growth and all have reached reasonable levels of biomass (Figure 5.1). This experiment shows clearly that after 10 days growth in F/2 defined medium, both N and P are becoming limiting so that the used medium cannot support a new cycle of growth from a fresh inoculum of cells. Addition of both N and P was required to restore growth to near the control rate of growth (Figure 5.1). However, it is important to note that no toxic substance was built up in the used medium; it was a lack of N and P that prevented growth.

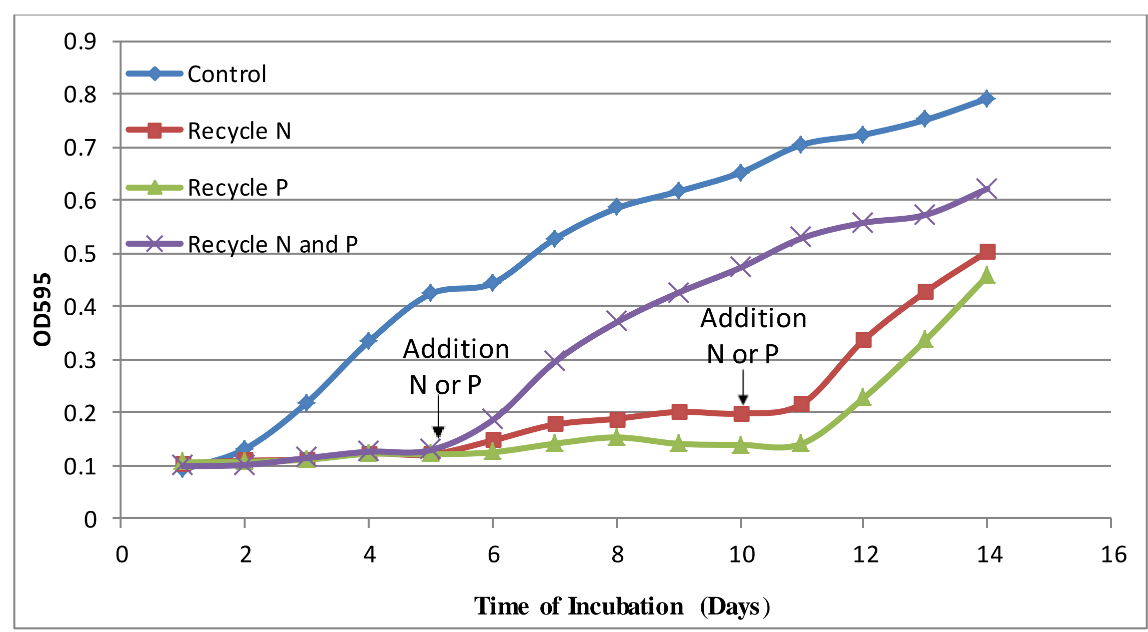


Figure 5.1. Growth curves for *T. suecica* cultures grown in reused 0.4 M NaCl defined F/2 medium (purple, red and green) versus a control flask grown in fresh 0.4 M NaCl F/2 medium. After 5 days additional N (1 ml of defined F/2 medium Nitrate stock solution) was added to the culture depicted in red, additional P (1 ml of defined F/2 medium Phosphate stock solution) was added to the culture depicted in green and both N and P (1 ml of each defined F/2 medium Nitrate and Phosphate stock solution) were added to the culture depicted in purple. After 10 days, N (1 ml of defined F/2 medium Nitrate stock solution) was added to the culture depicted in green and P (1 ml of defined F/2 medium Phosphate stock solution) was added to the culture depicted in red.

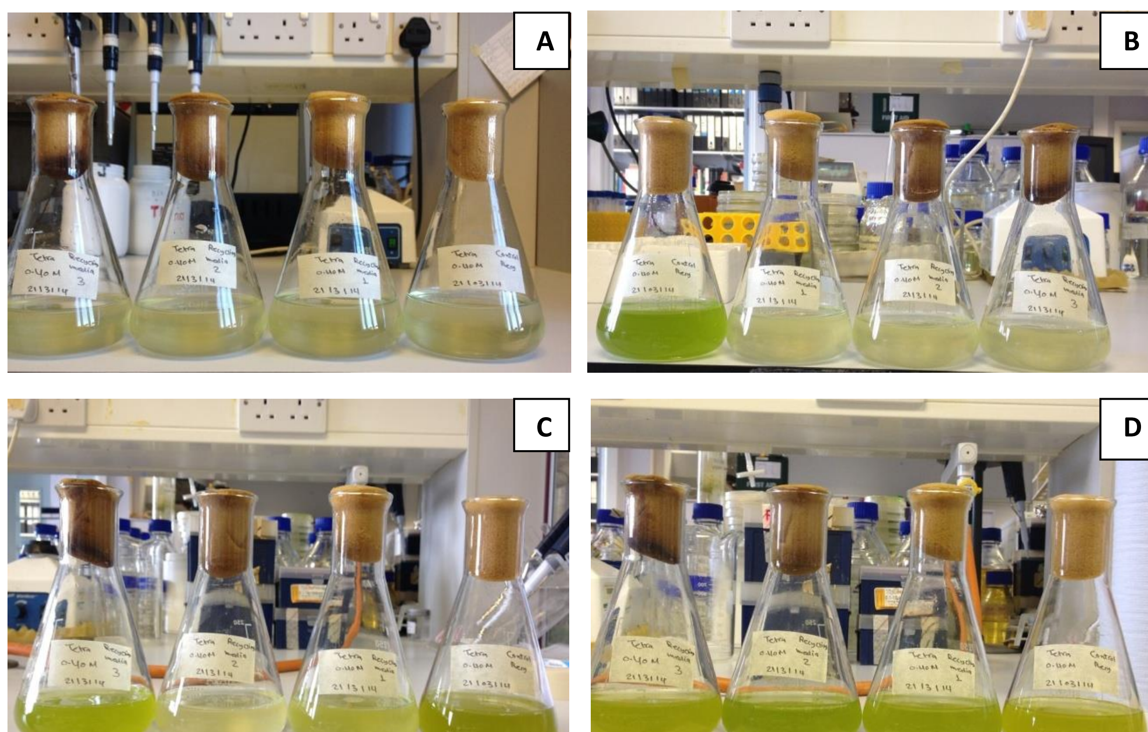


Figure 5.2. Growth flask pictures of *T. suecica* cultures during the experiment to test the re-use of 0.4 M NaCl defined F/2 medium. A) All flasks before the inoculation. B) After four days incubation, growth has only taken place in the control flask. C) After 10 days, good growth has taken place in flask with both N and P enrichment. D) After 14 days, good growth has now taken place in both flasks with single enrichment after the second stage enrichment.

5.2.2 Growth of T. *suecica* Using a Photobioreactor

Three separate experiments were performed using the 2 litre airlift photobioreactor (Figures 5.3 and 5.4). Approximately 1.9 litres of 0.4 M NaCl defined F/2 medium were added to the vessel and then it was sterilized by autoclaving. The inoculum was a well grown 100 ml culture and the temperature was controlled at 25°C. In each experiment, the OD_{595} was measured daily to estimate the growth rate over a 14 day growth period. The first fermenter experiment used normal 0.4 M defined F/2 medium with atmospheric air bubbled through the medium. The second experiment used 0.4 M F/2 medium with extra N and P (19 ml of each defined F/2 medium nitrate and phosphate stock solution, see section 3.2.3) and finally the third experiment had the extra N and P, and 5% CO_2 bubbled through the medium rather than air. Figure 5.5 shows that for the first 7 days there was little difference in the growth for all three conditions. However, in the second 7 days, the N and P became limiting in the control culture and the enriched N and P culture achieved a much higher biomass at the end of the 14 day incubation period. Adding in additional CO_2 provide a further boost to the final biomass (OD_{595} more than 1.2) against a final control OD_{595} value of just above 0.6.

Both experimental sections in this chapter have shown the new F/2 defined medium does become N and P limiting quite soon after inoculation (7 to 14 days) and that N and P additions significantly increase the final biomass levels produced. As expected, increasing the CO_2 from atmospheric levels (0.03%) to 5% also increased the final biomass obtained.

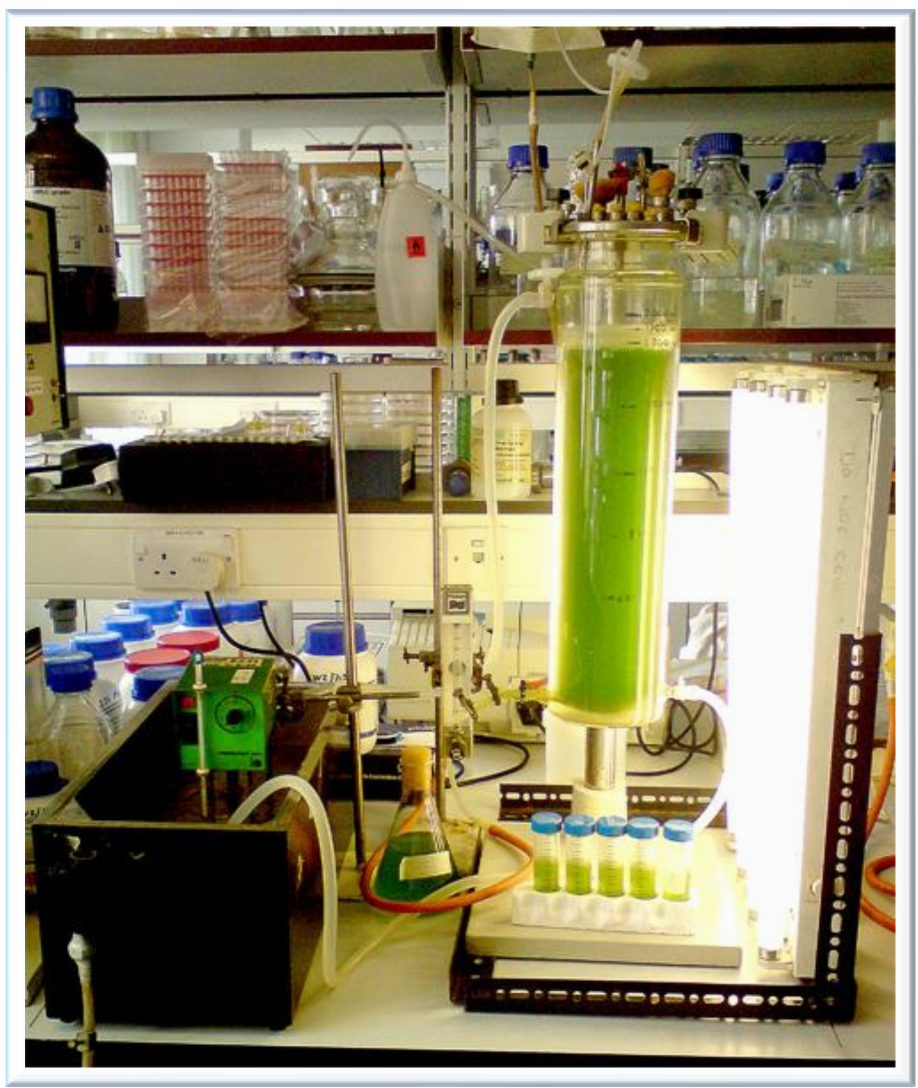


Figure 5.3. Two litre airlift photobioreactor set-up on the laboratory bench. There is a water jacket which is connected to water circulating from the water bath to control the temperature at 25°C. The fluorescence lights are daylight fluorescence tubes and provided a light intensity of 35 µmol m^{-2} s^{-1} (Picture taken by Krys Bangert).

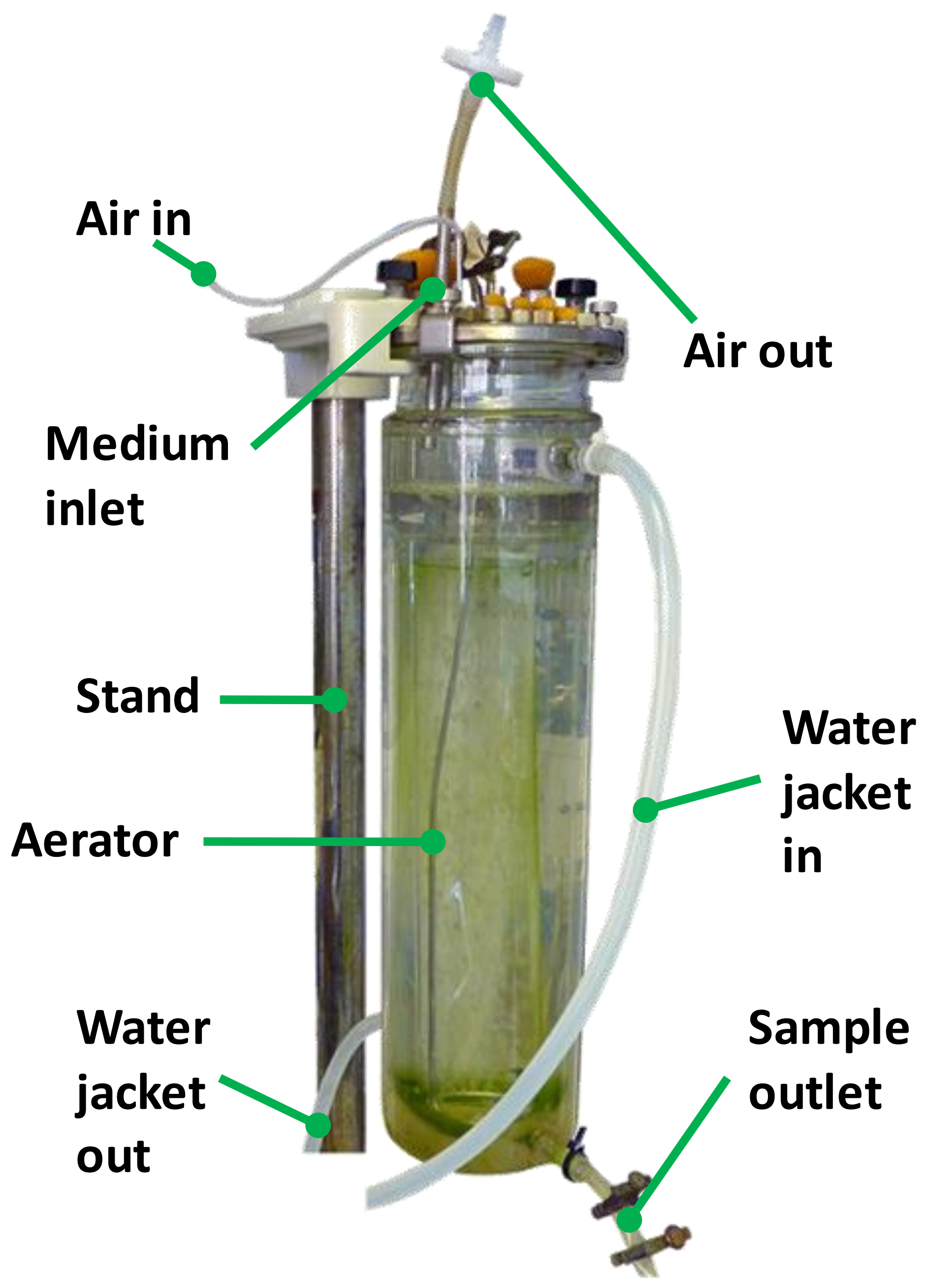


Figure 5.4. Empty fermenter vessel after harvesting algae showing the key characteristics of air inlet and oulet, water jacket and sample outlet. Picture taken by Krys Bangert.

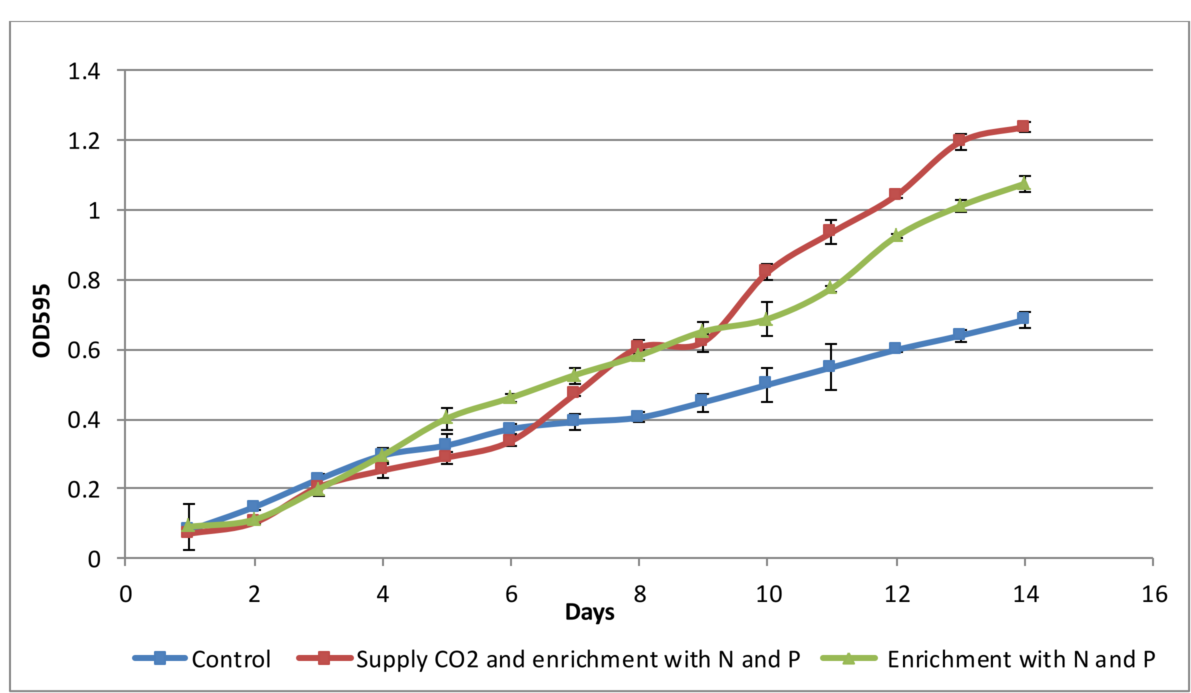


Figure 5.5. Growth of *T. suecica* using the 2 litre airlift photobioreactor under three different conditions in 0.4 M F/2 defined medium. Control (blue line) = normal medium bubbled with air; green line is defined F/2 medium enriched with N and P (19 ml of each defined F/2 medium nitrate and phosphate stock solution) bubbled with air; red line is enriched medium bubbled with 5% CO_2.

5.3 Discussion of the Algal Biorefinery Concept

The experimental work described in this chapter has laid down the groundwork for further exploration of the possibilities of using *T. suecica* as a feedstock for commercial production of algal biofuels. The scale-up experiment was successful and over a 14 day growth period no obvious problems were encountered with the cells being mixed by the airlift effect (Zimmerman *et al.*, 2011). *Tetraselmis suecica* has a cell wall so it is not susceptible to cell damage in the same way that the wall-less *Dunaliella* cells suffer damage (Bangert, 2013).

It is also clear that sufficient levels of N and P are essential to achieve the high biomass required for biodiesel production and the ability to source this N and P in a sustainable way will be crucial to the success of algal biodiesel production. As mentioned in section 5.1, wastewater is a good source of N and P and cleaning up of polluted water can potentially be combined with growth of algae for biofuels. In this discussion section the potential for setting up a biorefinery using *T. suecica* as the feedstock will be explored.

There are a number of different ways that an algal biorefinery can be set up and a fairly simple set-up is shown in Figure 5.6, which is taken from the paper of Razzak *et al.* (2013). The origins of the inputs to the algal culture are crucial and should include wastewater or seawater as sources of nutrients and industrial flue gas as the source of CO_2. Solar energy must be used, but this does not automatically mean an open pond, it could involve a closed photobioreactor situated outdoors. As much as possible of the medium should be recycled and any waste CO_2 from downstream processing should also be recycled back to the microalgal culture for re-use. The range of potential products is shown in Figure 5.6 and includes a mixture of fuels, animal feed and human health foods (i.e. a mixture of low value

high volume products such as fuels versus high value low volume products such as health food supplements).

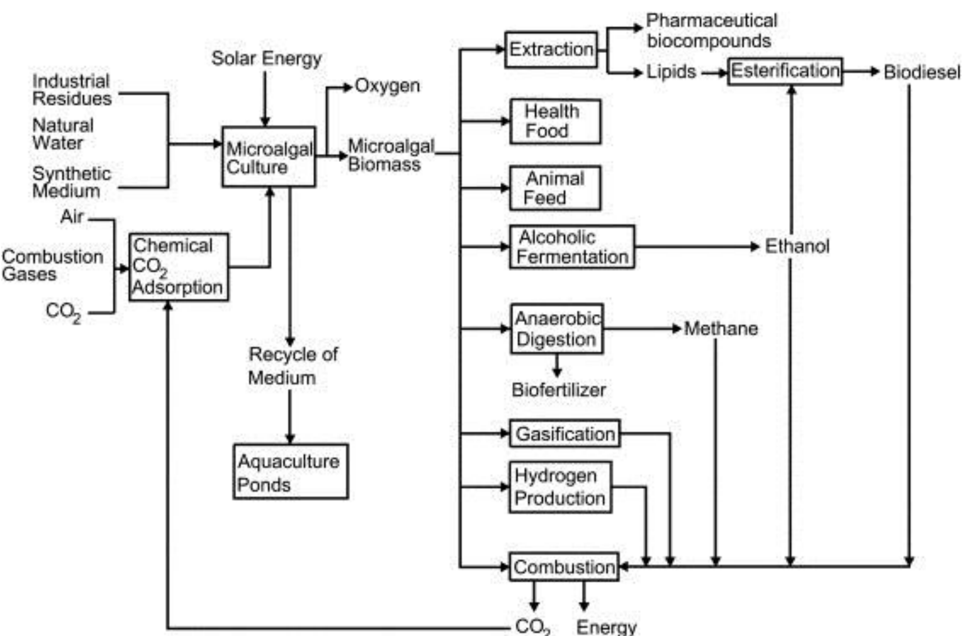


Figure 5.6. Outline of an algal biorefinery scheme. The microalgal culture requires the inputs shown in the top left of the diagram and the potential uses for the microalgal biomass are shown on the right hand side. Recycling of the medium and CO_2 are essential for the operation of the biorefinery. Modified from Razzak *et al.* (2013).

5.3.1 Inputs to the Microalgal Biorefinery

To grow algal biomass, all inorganic nutrients must be supplied in addition to a source of carbon (normally CO_2) and energy (normally solar energy). This may be stating the obvious, but when contemplating algal growth on a large scale, even minor nutrients are required in reasonably large amounts. The F/2 defined medium described in section 3.2.3 provides a good breakdown of all the nutrients required to grow a marine alga such as *Tetraselmis.* Sodium chloride, magnesium chloride, magnesium sulphate, calcium chloride and potassium chloride are (in decreasing order of weight required) the major salts required. Micronutrients

such as strontium chloride and potassium bromide may also be required depending on the algal species. A source of nitrogen (often sodium nitrate) and a source of phosphate are essential and other trace metals and vitamins may be required again depending on the species (see Table 2.4). The most obvious source of most of the salts is natural seawater, but supplementation with N and P will almost certainly be required to promote growth to a satisfactory level. A sustainable source of N and P is wastewater.

The N to P ratio in algal biomass varies from N:P 4:1 (under N limitation) to values reaching almost N:P 40:1 (Craggs *et al.*, 2013). The latter ratio corresponds to 10% N and 1% P on a dry weight basis. For scientists interested in the removal of N and P from wastewater to avoid the eutrophication of lakes and rivers, these ratios indicate that N is the key element and that if all N is removed then all P will also be sequestered into the algal biomass (Craggs *et al.*, 2013). In domestic wastewater, N concentrations can be found in the range of 15 to 90 mg l^{-1} and P concentrations range from 4 to 20 mg l^{-1} (Beuckels *et al.*, 2015). From the defined F/2 medium recipe (see section 3.2.3), the amount of N present is 20.3 mg l^{-1} and the amount of P present is 1.1 mg l^{-1}. Therefore based on the results in this chapter, the N and P concentrations in domestic wastewater would allow growth of *T. suecica* for at least 10 days and probably for up to 2 to 3 weeks, since the amounts of P and N in wastewater are normally in excess of the amounts of P and N in F/2 medium. It is reasonable to conclude that natural seawater supplemented with domestic wastewater would meet the requirements for growing *T. suecica* to satisfactory cell densities.

The other major input is CO_2, which can be found in high quantities in industrial flue gases. Zimmerman *et al.* (2011) successfully grew *Dunaliella* cells on industrial flue gas containing up to 23% CO_2. In the current work, we have demonstrated good growth of *T. suecica* up to

5% CO_2 (Figure 5.5). In a recent paper, Ferriols *et al.* (2013) demonstrated optimum growth of *Tetraselmis* at 10% CO_2, giving good grounds to suspect that *T. suecica* could grow well at the elevated CO_2 levels found in flue gases.

5.3.2 Recycling Medium in a Microalgal Biorefinery

The water requirement for growing microalgae to produce biodiesel on a large scale is potentially very large and in the previous section, it was suggested that the use of seawater supplemented with wastewater was a way to avoid this problem. However, use of seawater means that the algal biorefineries must be located near the coast close to a sewage treatment plant and near an industrial source of CO_2. Matching these criteria can quite quickly and dramatically reduce the availability of suitable sites (Farooq *et al.*, 2015). Various studies have tried to determine the water requirement for microalgal cultivation for biodiesel production and this includes water used indownstream processes to extract and purify the TAGs. This information is summarized in the paper by Farooq *et al.* (2015) and figures up to 3360 litres of water per litre of biodiesel produced are quoted. Re-cycling the medium can significantly decrease the water requirement as demonstrated for *Chlorella vulgaris* by Stephenson *et al.* (2010) with a decrease from 1611 litres of water required per litre of biodiesel to only 3.7 litres of water required per litre of biodiesel.

The main problems with the recycling of growth medium is that growth inhibiting chemicals may accumulate in the medium and broken cell debris may induce the growth of bacteria. There also tends to be an increase in salt concentrations as the medium is re-used (Ben-Amotz, 1995). In the present chapter, results were presented (Figures 5.1 and 5.2) that showed that no autoinhibitory chemicals were accumulated in *T. suecica* cultures over an

initial 10 day growth period. After supplementation with N and P, *T. suecica* cells grew well in the re-cycled medium.

This agrees well with a pilot scale culture of *Tetraselmis* MUR233 using recycled medium carried out by Fon Sing *et al.* (2014) in outdoor ponds in Western Australia. Electro-flocculation was used to remove the *Tetraselmis* cells and the recycled medium went through a settling tank before being returned to the culture pond. It was found that over a period of 5 months with recycling of medium at least twice weekly that the cells continued to grow well and coped with an increase in salinity from 5.5% w/v NaCl (approximately 1 M NaCl) to 12% w/v NaCl (approximately 2 M NaCl). The electro-flocculation method also sterilised the recycled medium and is an important part of this novel process (Fon Sing *et al.*, 2014).

5.3.3 Harvesting Cells and Extracting Products

One of the major constraints preventing the mass commercial exploitation of microalgae is harvesting. When algal cells are grown photoautotrophically (i.e. CO_2 as sole source of carbon), 1 to 2 g l^{-1} dry biomass is the maximum achievable because of the self shading in dense algal cultures (Gilmour and Zimmerman, 2012). Most industrial processes based on microbial biomass would normally expect to attain 100 g l^{-1} dry biomass before harvesting. This illustrates the challenge – to economically harvest very small microalgal cells from a dilute suspension of only 1 to 2 g l^{-1}. In many pilot studies for setting up algal biorefineries, centrifugation is used to harvest the biomass (e.g. Nurra *et al.*, 2014), but the cost in energy

and money rules out centrifugation as a way of harvesting mass cultures of algae (Gutierrez *et al.,* 2015).

The most commonly used harvesting methods utilize the fact that algal cells are negatively charged and can thus be caused to produce flocs of cells that can be precipitated out of suspension by adding positively charged (cationic) flocculants such as aluminium or iron sulphate (Razzak *et al*., 2013). Polyferric sulphate is more efficient with multiple positive charges to bind the cells together and biodegradable flocculants such as chitosan can also be used (Molina Grima *et al*., 2013). The latter is particularly useful for food products, but the efficiency of chitosan flocculation is decreased with increasing salinity, which is a drawback for marine algal species like *Tetraselmis* (Molina Grima *et al.,* 2013). Novel techniques, such as foam fractionation, which uses surfactants such as CTAB (cetyl trimethylammonium bromide) to break open the algal cells and produce a foam that is harvested from the top of a funnel attached to the fermenter (Coward *et al*., 2013). Also, microwave assisted hydrothermal pyrolysis has been shown to be effective using *Chlorella* biomass and a range of products (lipids, sugars, proteins) were produced (Budarin *et al*., 2012). This technique is particularly relevant to the biorefinery concept because it allows the simultaneous extraction of a number of products.

Filtration is also an option, but relatively cheap cellulose filters do not work with many small algal cells. Membrane filters and the use of a vacuum to drive the filtration can be used, but blocking of filters is a difficult problem to overcome (Molina Grima *et al*., 2003). No work has been done on harvesting during the current project, but there is no reason to think that *T. suecica* will not be amenable to the usual harvesting techniques.

The extraction of neutral lipids (TAGs) and other products from the harvested algal biomass can be achieved by a range of techniques including mechanical extraction (e.g. use of a bead mill), ultrasound (which produces cavitation bubbles) and chemical/solvent extractions (Bligh and Dyer solvent extraction or supercritical CO_2 extraction) (Razzak *et al.*, 2013). The procedures used in Chapter 4 to extract and transesterify neutral lipids from *T. suecica* showed that extraction of lipids should be straight forward.

5.3.4 Commercial Viability of a Microalgal Biorefinery

The commercial viability of microalgal biorefineries is essential to the establishment of viable industries based on microalgal biomass. The most obvious place to start the development of an algal biorefinery is to build on the existing high rate algal ponds (HRAP) which are used to clean up wastewater (Craggs *et al.*, 2013). The investment in harvesting technology would be repaid by the production of biodiesel to use elsewhere in the sewage works. To date, water companies have not seen the need to invest in this area, but hopefully this will change in the near future.

Another approach is to site an algal biorefinery (often called an algal farm in this context) on suitable marginal land and set up the infrastructure required for the refinery/farm from scratch (Abodeely *et al.*, 2014; Subhadra and Grinson-George, 2010). A fully integrated renewable energy park would include solar energy, wind energy, geothermal energy and algal biorefinery all on the same site with the production of biofuel and co-products, but with zero net carbon emissions (Subhadra, 2010). This is a theoretical concept and would require a very large initial investment to get underway. However, the idea of using valuable co-products to offset the cost of biofuel production is a good one and remains at the heart of the algal biorefinery concept.

5.4 Conclusions

The work described in this chapter shows that *T. suecica* can be grown successfully in a 2 litre photobioreactor under elevated (5% CO_2) conditions. It was possible to re-cycle the F/2 growth medium without problems from autoinhibitory chemicals, but it was noted that N and P became limiting within 10 days of culture in the novel defined F/2 medium. The analysis of the requirements for an algal biorefinery did not show up any obvious reasons not to use *T. suecica* as the algal feedstock, but more work is required to compare *T. suecica* to a range of algae to see which species are most suitable for the development of an algal biorefinery.

Chapter Six

General Discussion

6.1 General Discussion

Tetraselmis suecica has been identified as a good candidate for the production of biodiesel and other fine chemicals (Chisti, 2007) on the basis of high lipid content and the fact that *T. suecica* is tolerant to a range of environmental extremes including high salinity and pH (Chapter 3). As a marine alga, *T. suecica* is normally grown on seawater based media such as F/2, but all such media are complex media with unknown concentrations of salts. In the present work, a fully defined medium based on F/2 was developed (section 3.2.3) and shown to support good growth of *T. suecica* (Figure 3.7). This means that in future work, nutrient levels can be varied in a fully quantitative way and this will lead to further insights into the best growth conditions for *T. suecica.*

As part of the initial characterisation of *T. suecica*, NMR analysis was used to identify the compatible solute produced by the cells to balance the external osmotic pressure at high salinities. The data shown in Figure 3.11 indicate that mannitol acts as the compatible solute since its intracellular concentration increases with increasing salinity. This finding confirms the original work of Craigie *et al.* (1967) using a modern technique. The type of compatible solute affects the maximum salinity that can be tolerated by the organism and a six carbon polyol would be expected to allow growth up to about 1 M NaCl (Empadinhas and da Costa, 2008), which agrees well with the growth data shown in Figure 3.8.

The work described in Chapter 4 aimed to identify the best conditions for neutral lipid accumulation in *T. suecica*. The results shown in Figure 4.5 indicate that high salinities (0.6 M NaCl and above) do induce more TAG synthesis after 4 weeks incubation. The effect was not particularly strong and reflects the fact that *T. suecica* grows well up to 1 M NaCl.

Increasing the salt stress above 1 M NaCl may induce more TAG accumulation, but it will also likely reduce the growth rate. In a parallel experiment to determine the effect of pH on TAG synthesis, growing cells at pH 9 (0.4 M NaCl) for two weeks led to the highest level of TAG accumulation (Figure 4.6). Putting these two results together suggests that 1 M NaCl and pH 9 would be the best growth conditions for TAG synthesis.

The fatty acid profiles were also determined using GC-MS and increasing salinity induced the synthesis of fatty acids with one double bond (monounsaturated), which are ideal for biodiesel production (Knothe, 2005; Stansell *et al.*, 2012). It appears likely that growing *T. suecica* at high salinity will result in a favourable mix of fatty acids for biodiesel (Chapter 4).

In the final results chapter (Chapter 5), preliminary experiments on scaling up the culture of *T. suecica* and re-using medium were successfully carried out showing that there were no obvious drawbacks to growing *T. suecica* on a large scale. The analysis of algal biorefinery requirements described in section 5.3 showed that *T. suecica* meets the major criteria for a feedstock into an algal biorefinery. The key points are that *T. suecica* can grow well at high salinity, high pH and high CO_2. Co-products such as carotenoids and polyunsaturated fatty acids can be produced from *Tetraselmis* and it has been used successfully as a feed for shellfish and fish farming (Fabregas *et al.*, 1984).

6.2 Future Work

The work on environmental stress effects on TAG production by *T. suecica* should be continued to confirm the optimum conditions for TAG production, which appear to be 1 M NaCl and pH 9. Also, increasing the salinity stress beyond 1 M NaCl should be attempted to see if the increased production of TAG will outweigh the likely decrease in growth rate. A full quantification of TAG will be carried out using the triolein concentration curve which was produced as part of the current work. This will enable a % TAG to be calculated on a dry weight basis.

Once the optimum growth conditions for TAG synthesis are fully established, the GC-MS work will be repeated to examine the fatty acid profile with respect to biodiesel production.

Supplementing the medium with wastewater from a sewage works will determine how well *T. suecica* can grow on wastewater and what level of wastewater addition to F/2 medium leads to the best growth and TAG synthesis. The re-cycling of medium will be further tested by increasing the number of times the medium is re-used until it will no longer support growth. At this point, the spent medium will be tested for toxin build-up to understand the limitations to re-cycling of the medium.

Further scaling up of the cultures of *T. suecica* will be carried out using larger fermenters up to 200 litres. This will allow a full investigation of the properties of *T. suecica* when grown in mass culture. If the 200 litre culture proves successful, the University of Swansea has larger fermenters which can be used to test biofuel production in *T. suecica* in a several thousand litre capacity outdoor fermenter.

Chapter Seven

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Chapter 8

Appendices

Appendix A. Relationship between OD_{595} and dry weight for *T. suecica* cells grown at different salinities

To allow dry weight to be calculated from OD measurements, concentration curves were produced as shown in Figures A.1 and A.2. The data for 0.4 and 0.8 M NaCl grown *T. suecica* cells were similar and are plotted as one graph (Figure A.1). However, the relationship between OD_{595} and dry weight was significantly different for the cells grown in 1 M NaCl and thus a separate concentration curve was produced (Figure A.2).

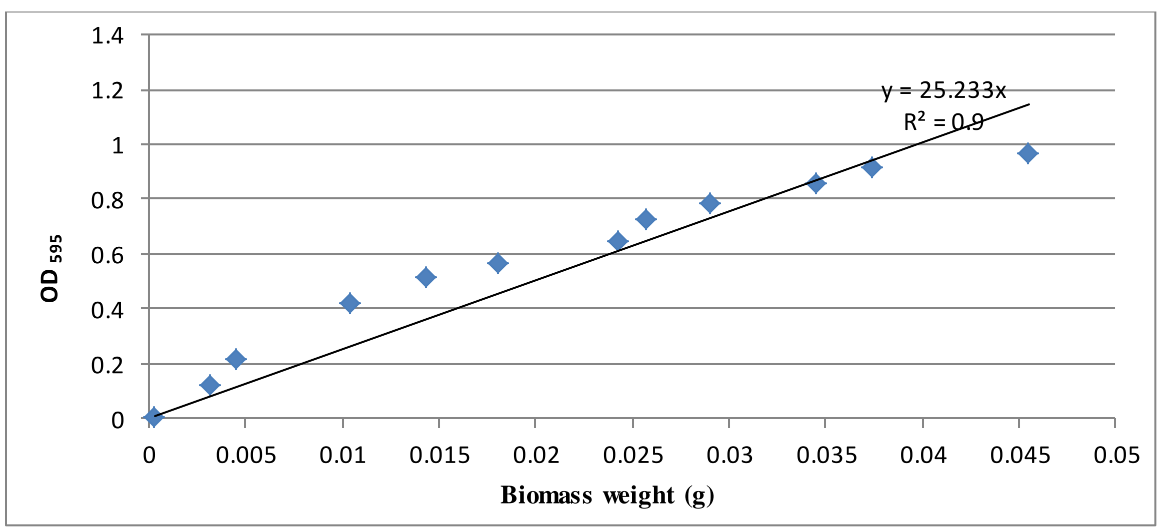

Figure A.1. Average of dry weight versus OD concentration curves for 0.4 M NaCl grown and 0.8 M NaCl grown *T. suecica* cells.

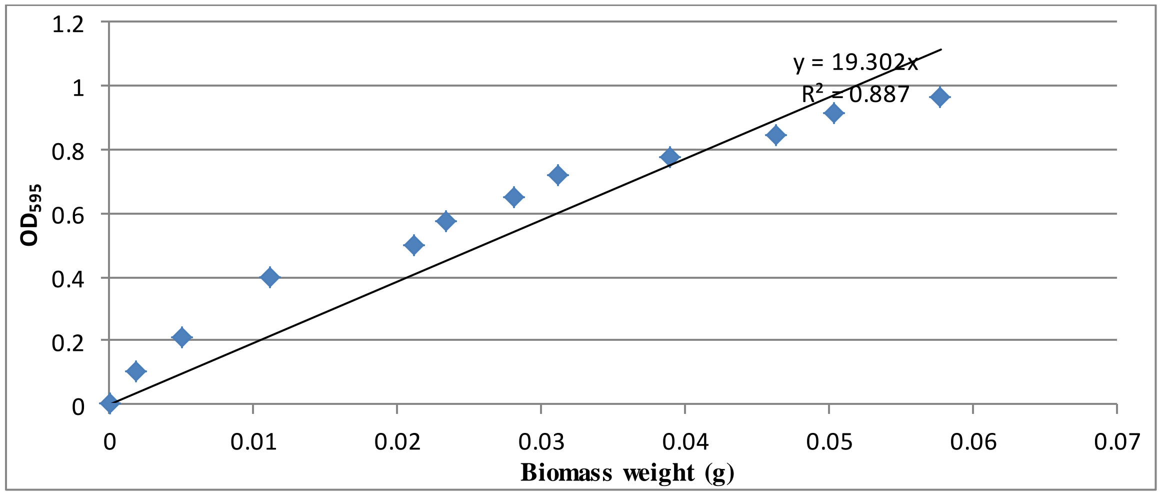

Figure A.2. Dry weight versus OD concentration curve for 1 M NaCl grown *T. suecica* cells.

Appendix B. Relationship between OD_{595} and cell count for *T. suecica* cells grown at different salinities

To allow cell number to be calculated from OD values, concentration curves were produced. The data for 0.4 and 0.8 M NaCl grown *T. suecica* cells were similar and are plotted as one graph (Figure B.1). However, the relationship between OD_{595} and cell number was significantly different for the cells grown in 1 M NaCl and thus a separate concentration curve was produced (Figure B.2).

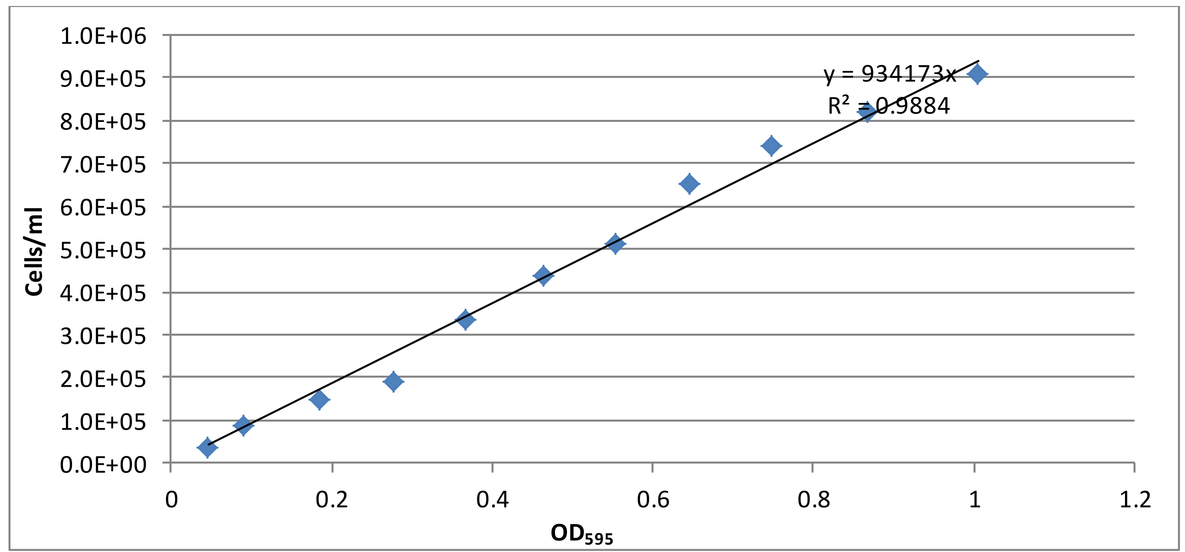

Figure B.1. Average cell count versus OD calibration curve for 0.4 M and 0.8 M NaCl grown *T. suecica* cells.

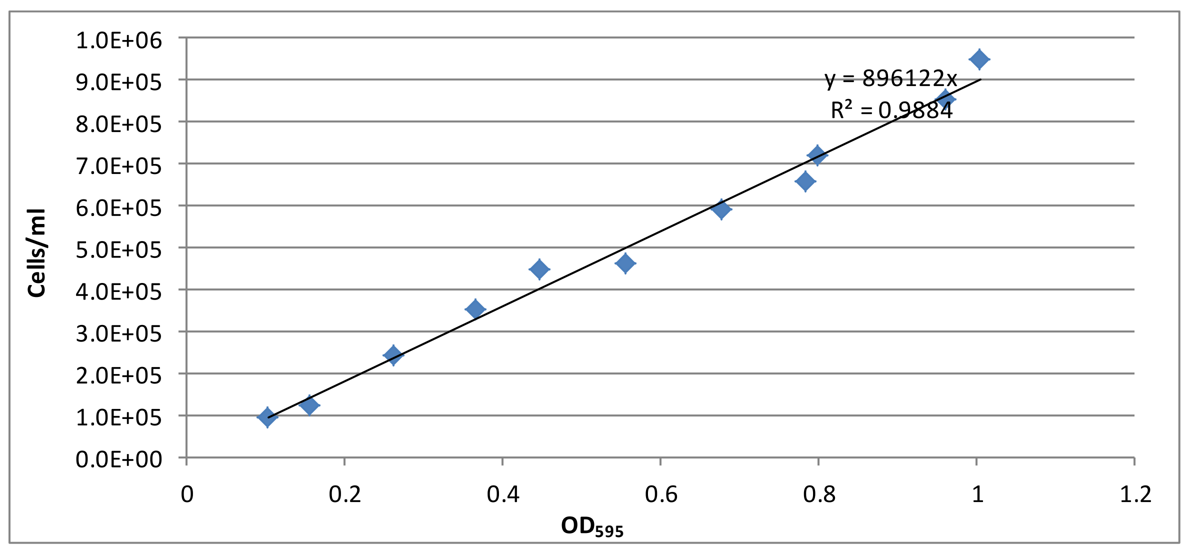

Figure B.2. Cell count versus OD calibration curve for 1M NaCl grown *T. suecica* cells.

Appendix C. Neutral Lipid Content of *T. suecica* Cells Grown at Different Salinities and pH Values - 10 mins reading

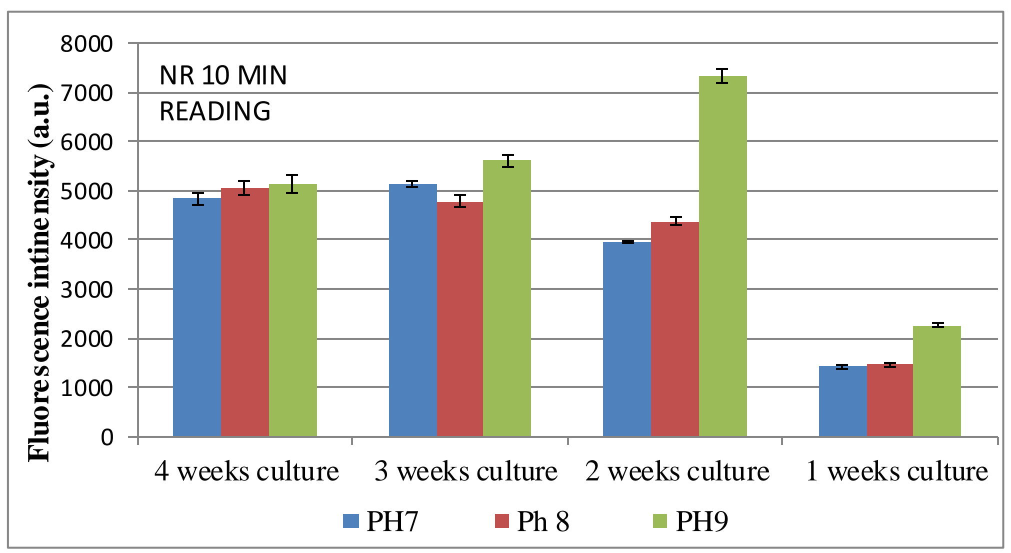

Figure C.1. Neutral lipid content measured by NR fluorescence of *T. suecica* in defined F/2 medium with different pH values. Each column represents the mean of four reading plus / minus standard deviation.

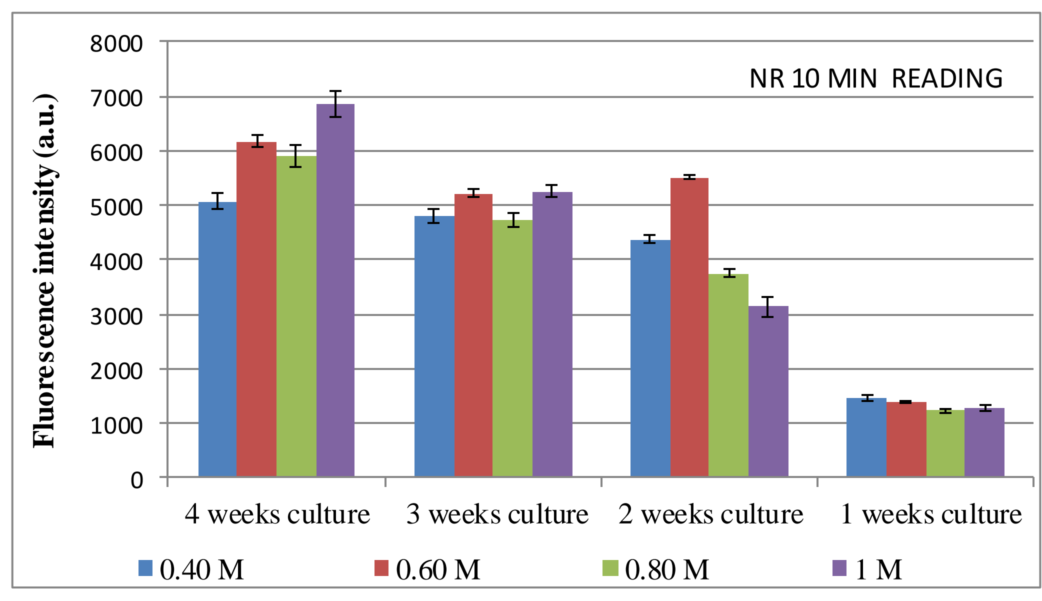

Figure C.2. Neutral lipid content measured by NR fluorescence of *T. suecica* in defined F/2 medium with different concentrations of NaCl. Each column represents the mean of four reading plus / minus standard deviation.

Appendix D. Neutral Lipid Content of *T. suecica* Cells Grown at Different Salinities and pH Values - 15 mins reading

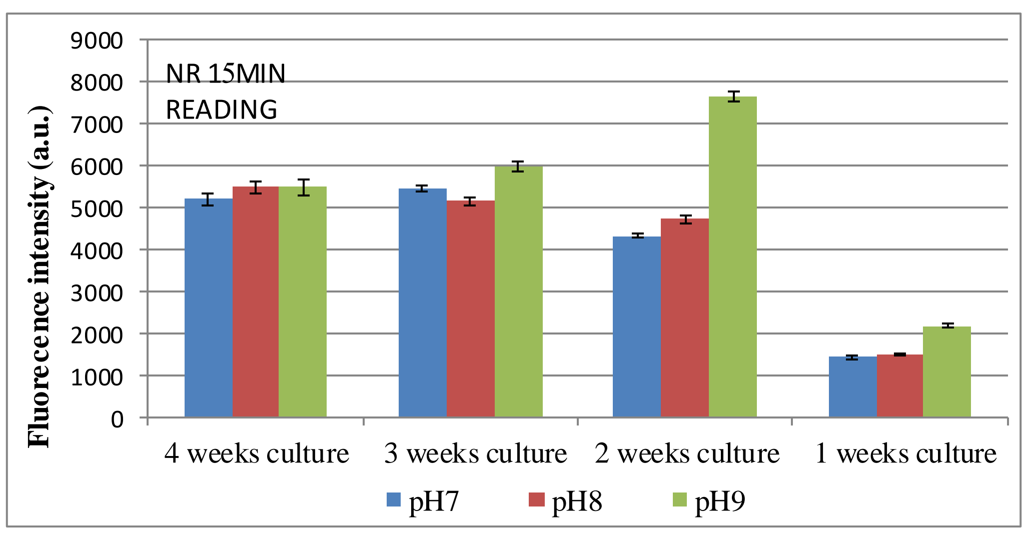

Figure D.1. Neutral lipid content measured by NR fluorescence of *T. suecica* in defined F/2 medium with different concentrations of NaCl. Each column represents the mean of four reading plus / minus standard deviation

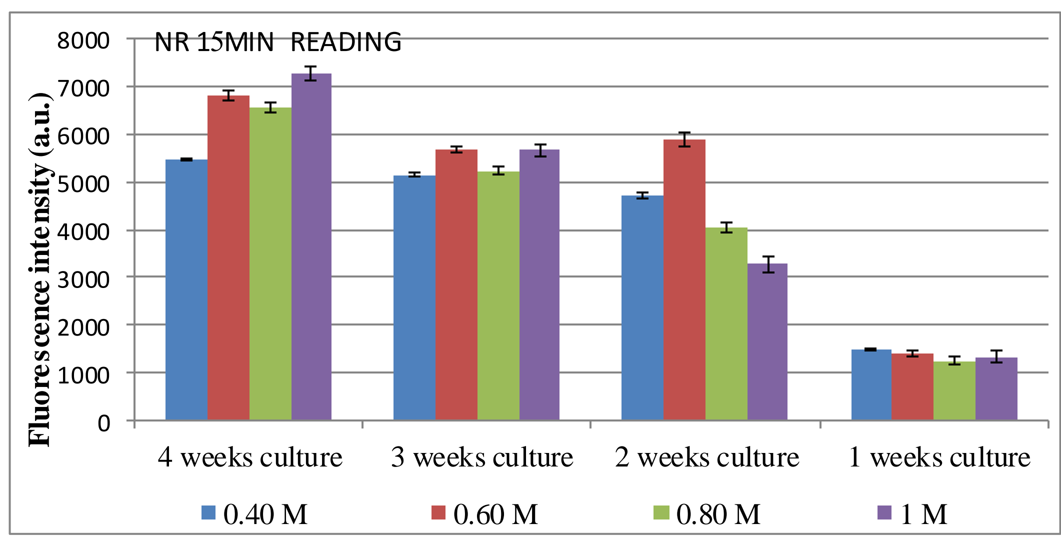

Figure D.2. Neutral lipid content measured by NR fluorescence of *T. suecica* in defined F/2 medium with different concentrations of NaCl. Each column represents the mean of four reading plus / minus standard deviation