

The use of PAM Fluorometry to Assess Microalgal Physiological Stress for the Production of Biodiesel

Sarah Anne White

Supervisor: Prof F Bux (Durban University of Technology)

Co-Supervisors: Dr A Anandraj (Mangosuthu University of Technology)
 Dr FM Swalaha (Durban University of Technology)

**A dissertation submitted in compliance with the requirements for the Masters
Degree in Technology in the Department of Biotechnology, Durban University
of Technology**

March 2011

The use of PAM Fluorometry to Assess Microalgal Physiological Stress for the Production of Biodiesel

Sarah Anne White

I hereby declare that the dissertation represents my own work. It has not been submitted before for any diploma/degree or examination at any University.

Sarah Anne White

I hereby approve the final submission of the following dissertation.

Prof Faizal Bux
Supervisor
Institute for Water and
Wastewater Technology

Dr Akash Anandraj
Co-Supervisor
Centre for Algal Biotechnology

This 30 day of MARCH, 2011, at the Durban University of
Technology.

ABSTRACT

Under environmental stress, most microalgae produce intracellular neutral lipids as a storage mechanism. In the biotechnology industry, these lipids are extracted and converted to microalgal biodiesel; however the extent of the stress is not measured for optimum lipid accumulation. In the series of studies undertaken, Pulse Amplitude Modulated (PAM) fluorometry was used to measure three types of physiological stress on *Chlorella* sp., a freshwater microalgal species. Biomass and lipid yields were also used as indicators of the induced stress conditions. Firstly, nutrient induced physiological stress and the subsequent synthesis of cellular neutral lipids was investigated. Optimization of neutral lipids occurred by initially maximizing the microalgal biomass and subsequently subjecting the harvested biomass to complete nutrient stress, using dH₂O. Complete nutrient stress induced the highest yield of intracellular neutral lipids compared to the absence of selected nutrients. Secondly, the acclimation of the freshwater *Chlorella* sp. to a range of salinity levels was investigated. The process of acclimation was monitored using PAM fluorometry. The culture showed the ability to acclimatize at a 40 ‰ growth medium; however physiological functioning was reduced, thereby reducing overall biomass production. At 20 ‰, the culture showed no significant difference in growth rate and physiological functioning after 12 days, compared to the control. This acclimation study showed the influence of stress placed on the microalgal cells when cultured at varying salinities. Thirdly, the influence of temperature on biomass production and lipid synthesis was investigated and the biodiesel produced was characterized according to carbon chain length, density and melting point. The Fatty Acid Methyl Esters (FAME) composition was found to be significantly altered when cultures were incubated under various pre-determined stress conditions. Dual stress (temperature and nutrients) was found to be more effective than single stress. This study has significant application at commercial scale biodiesel production and the technologies

developed would improve the economic feasibility of biodiesel production from microalgae.

PREFACE

Aspects of the work covered in this dissertation have and will be published and presented elsewhere:

Publications:

White, S., Anandraj, A., Bux, F. 2010. PAM fluorometry as a tool to assess microalgal nutrient stress and monitor cellular neutral lipids. *Bioresource Technology*, 102, 1675-1682 (See Appendix 1).

Viswanath, B., Mutanda, T., White, S., Bux, F. 2010. The Microalgae – A Future Source of Biodiesel. *Dynamic Biochemistry, Process Biotechnology and Molecular Biology*, 4(1), 37 - 47 (See Appendix 2).

Conference Proceedings:

White, S., Anandraj, A., Bux, F. 2010. PAM fluorometry as a tool to assess microalgal nutrient stress and monitor cellular neutral lipids. *Bioresource Technology*, 102, 1675-1682. 26TH congress of the Phycological Society of Southern Africa, 25-28 January 2011, Broederstroom, Magaliesberg

ACKNOWLEDGEMENTS

I would like to thank the following people and institutions:

- Prof Faizal Bux for the opportunity to study for my Masters degree
- Dr Akash Anandraj for his mentorship and support.
- The Centre for Algal Biotechnology at Mangosuthu University of Technology (MUT) for the use of specialised instrumentation and laboratories.
- National Research Foundation (NRF) and Durban University of Technology (DUT) for financial assistance.

TABLE OF CONTENTS

Abstract.....	ii
Preface.....	iv
Acknowledgements	v
List of Figures.....	ix
List of tables	xii
List of Abbreviations	xiii
Copyright.....	xiv
 Chapter One	 1
1 General Introduction and Literature Review	1
1.1 The Energy Crisis.....	1
1.2 Microalgal Biodiesel	4
1.3 Microalgal Cultivation	7
1.4 Physiological Stress	7
1.4.1 Nutrient Stress.....	8
1.4.2 Osmotic Stress	9
1.4.3 Temperature Stress.....	10
1.5 Cellular Neutral Lipid Synthesis	10
1.6 Pulse Amplitude Modulated (PAM) Fluorometry	12
1.7 Aims and Objectives of the Study	16
 Chapter Two	 17
2 Effect of nutrient stress on microalgal physiology and lipid yields	17
2.1 Introduction	17

2.2	Materials and Methods.....	21
2.2.1	Culture conditions.....	21
2.2.2	Nutrient stress	21
2.2.3	Hyper-Nutrient Stress	22
2.2.4	PAM Fluorometry.....	23
2.2.5	Chlorophyll a determination.....	23
2.2.6	Nile Red Staining.....	24
2.2.7	Lipid Extraction	21
2.2.8	Statistics	24
2.3	Results	26
2.4	Discussion.....	36
 Chapter Three.....		40
3	Acclimation of the freshwater <i>Chlorella</i> sp. to a marine environment	40
3.1	Introduction	40
3.2	Materials and Methods.....	43
3.2.1	Culture conditions.....	43
3.2.2	Salinity stress	43
3.2.3	PAM fluorometry.....	43
3.2.4	Chlorophyll a determination.....	45
3.2.5	Statistics	45
3.3	Results	46
3.4	Discussion.....	61

Chapter Four	66
4 Effect of temperature and nutrient stress on microalgal lipid yields	66
4.1 Introduction	66
4.2 Material and Methods	69
4.2.1 Culture conditions	69
4.2.2 Temperature stress	69
4.2.3 Dual stress	69
4.2.4 PAM fluorometry	70
4.2.5 Nile Red staining	71
4.2.6 Temperature stress for Lipid extraction	72
4.2.7 Lipid extraction	72
4.2.8 Transesterification	72
4.2.9 Gas Chromatography Analysis	73
4.2.10 Statistics	73
4.3 Results	74
4.4 Discussion	89
Chapter Five	95
5 Conclusions and Recommendations	95
5.1 Recommendations	97
6 References:	98
7 Appendices	1
7.1 Appendix 1: Technical Article	1
7.2 Appendix 2: Review Article	2

LIST OF FIGURES

Figure 1: Catalyzed reaction of triglycerides converted into biodiesel (Mutanda et al., 2011).	5
Figure 2: Biodiesel production process (modified from Demirbas, 2009a). ..	6
Figure 3: The Reactions of the Fatty Acid Synthesis Pathway (modified from (Ohlrogge and Browse, 1995)	11
Figure 4: A schematic diagram representing the triacylglyceride biosynthesis pathway (Hu et al., 2008)	12
Figure 5: The Electron Transport Chain of the Photosynthetic apparatus crucial for PAM Fluorometry (http://ideonex.com/2009/01/).....	13
Figure 6: Culture vessels containing the <i>Chlorella</i> sp. cells showing selective stress cultured in BG-11-N (A), BG-11-P (B) and BG-11-Fe (C). 27	
Figure 7: Growth Curves of <i>Chlorella</i> sp. cultured in BG-11 medium, selectively stressed media (BG-11-N, BG-11-P and BG-11-Fe) and complete stressed medium (distilled water).....	28
Figure 8: The physiological parameters (black bars, mean \pm SD), ETR (A); NPQ (B); F_v/F_m (C) and E_k (D), recorded under complete and selective stress conditions and the corresponding cellular lipid yields.	30
Figure 9: Composite images of <i>Chlorella</i> sp. cultures stained using Nile Red showing cellular neutral lipids in BG-11 medium (A), phosphate deficient medium (BG-11-P) (B), distilled water (C), nitrate deficient medium (BG-11-N) (D) and iron deficient medium (BG-11-Fe) (E).	31
Figure 10: The physiological parameters ETR (A); NPQ (B); F_v/F_m (C) and E_k (D) recorded under hyper-nutrient stress showing initial values (black bars, mean \pm SD) and values after the addition of the limited nutrients (grey bars, mean \pm SD).	34
Figure 11: Rapid Light Curves for the nutrient stressed cultures (black lines, mean \pm SD) and the hyper nutrient stressed cultures (grey lines, mean \pm SD) after the addition of 70 μ M of the specific depleted	

nutrient in BG-11-P medium (A), BG-11-N medium (B), BG-11 compared to distilled water (C) and BG-11-Fe medium (D).....	35
Figure 12: Chlorophyll a concentrations of <i>Chlorella</i> sp. cultured in BG-11 media at varying salinities	47
Figure 13: Growth Rates plotted against salinity for the <i>Chlorella</i> sp.	48
Figure 14: Culture vessels containing the <i>Chlorella</i> sp. cells cultured in BG-11 and varying salinities ranging from 5 ‰ to 40 ‰ at day 15. ...	48
Figure 15: Graphs depicting the Realtive Electron Transport Rate ($rETR_{max}$) of <i>Chlorella</i> cells cultured in BG-11 growth media at varying salinities over a 3 week period.	53
Figure 16: Graphs depicting the α values of <i>Chlorella</i> cells cultured in BG-11 growth media at varying salinities over a 3 week period	55
Figure 17: Graphs depicting the light saturating point (E_k) of <i>Chlorella</i> cells cultured in BG-11 growth media at varying salinities over a 3 week period.	58
Figure 18: Graphs depicting the Non Photochemical Quenching (NPQ) of <i>Chlorella</i> sp. cells cultured in BG-11 growth media at varying salinities over a 3 week period.	60
Figure 19: Temperature response curves depicting the relationship between the maximum electron transport rate (ETR_{max}) and the respective incubation temperatures (10°C to 45°C).....	76
Figure 20: Non photochemical quenching (NPQ) values of <i>Chlorella</i> samples incubated at temperatures ranging from 10°C to 45°C.	77
Figure 21: The E_k value or light saturation point obtained from the ETR_{max} value on the rapid light curve of cultures incubated at temperature ranging from 10 °C to 45 °C.	78
Figure 22: The quantum effeciency value (F_v/F_m) of the cultures incubated at temperatures ranging from 10 °C to 45 °C.	79
Figure 23: The α values depicting the photosynthtic efficiency of the cultures incubated at temperatures ranging from 10 °C to 45 °C.....	80
Figure 24: Composite images of cultures stained with Nile Red showing cellular neutral lipids of the <i>Chlorella</i> cells cultured in BG-11 at	

10°C (A), dH₂O at 10°C (B), BG-11 at 25°C (C), dH₂O at 25°C (D),
BG-11 at 40°C (E) and dH₂O at 40°C (F). 82

Figure 25: Transesterified microalgal biodiesel showing FAMES in the upper
yellow section and glycerol in the lower darker section. 83

Figure 26: A sample chromatogram showing the peaks and peak areas of
the FAMES present in the microalgal biodiesel..... 84

LIST OF TABLES

Table 1: A comparison of crop dependent and microalgal biodiesel production efficiencies (Chisti, 2007, Schenk et al., 2008)	3
Table 2: Summary of the physiological parameters and lipid yields measured by the PAM fluorometer of <i>Chlorella</i> sp. grown on complete and selective deficient media.	26
Table 3: Acclimation parameters for the samples incubated from 5 ‰ to 40 ‰.....	50
Table 4: PAM parameters for the <i>Chlorella</i> incubated at 40 ‰.....	51
Table 5: Summary of the $rETR_{max}$ values recorded from Day 1 to Day 19 from cells incubated at salinities ranging from 5 ‰ to 40 ‰ with BG-11 and Sea Water serving as controls.....	52
Table 6: Summary of the α values recorded at the various time frames in the <i>Chlorella</i> cells incubated at salinities ranging from 5 ‰ to 40 ‰ with controls.....	54
Table 7: Summary of the E_k values recorded at the various time frames in <i>Chlorella</i> incubated at salinities ranging from 5 ‰ to 40 ‰ with controls.....	57
Table 8: Summary of the NPQ values recorded at the various time frames in <i>Chlorella</i> incubated at salinities ranging from 5 ‰ to 40 ‰ with controls.....	59
Table 9: The PAM results induced by dual stress, expressed as a percentage change of the BG-11 control (day of acclimation).....	74
Table 10: Total lipid compositions and the composition of the fatty acid methyl esters in the single stress (temperature) and dual stress (nutrient and temperature).....	85
Table 11: Characteristics of the fatty acid methyl esters identified in the stressed microalgal samples	86

LIST OF ABBREVIATIONS

α	alpha
Chl <i>a</i>	Chlorophyll <i>a</i> (mg chl <i>a</i>)
E_k	Light Saturation.
ETR	Electron Transport Rate
rETR	Relative Electron Transport Rate
rETR _{max}	Maximum Relative Electron Transport Rate
FAME	Fatty Acid Methyl Esters
F	Dark fluorescence yield
F_o	Minimum fluorescence emitted in a dark adapted sample
F_m	Maximum fluorescence emitted in a dark adapted sample
F_o'	Minimum fluorescence emitted in a light adapted sample
F_m'	Maximum fluorescence emitted in a light adapted sample
F_v/F_m	Maximum Quantum Efficiency of PSII (dimensionless)
NPQ	Non Photochemical Quenching (dimensionless)
PAM	Pulse-Amplitude Modulation
PAR	Photosynthetically Active Radiation
PPFD	Photosynthetic flux density ($\mu\text{mol quanta m}^{-2}\text{s}^{-1}$).
PSI	Photosystem One
PSII	Photosystem Two
RLC	Rapid Light Curve
TAG	Triacylglycerides

COPYRIGHT

Information contained in this dissertation is protected by the South African Copyright Act (amended no 9 of 2002).

CHAPTER ONE

1 GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 THE ENERGY CRISIS

Currently there are two major energy crises faced by the world: one is the manner in which to substitute the crude oil supply with renewable energy and the other is to reduce greenhouse gas emissions which cause global warming (Chynoweth et al., 2001, Forsberg, 2009, Hansen et al., 2000). World energy demand is on the rise due to increased urbanization and industrialization (Dresselhaus and Thomas, 2001). The demand is predicted to increase by 1.8 percent per annum; however, crude oil reserves are rapidly declining. Global petroleum reserves are estimated to last until 2035 (Demirbas, 2008), emphasising the need for an alternative renewable fuel source (Demirbas, 2009b).

Global warming is caused by the release of greenhouse gases (CO_2 , CH_4 and N_2O) from the combustion of fossil fuels. These air pollutants have an effect on the geochemistry and the thermal characteristics of the biosphere (Crookes, 2006, Forsberg, 2009) causing an increase in the overall land and air temperatures. A possible solution to the current energy crisis is the use of alternative fuel sources produced from renewable sources. The use of renewable fuels is 'greener' with respect to most atmospheric pollutants as it is able to aid in closing the carbon cycle thereby reducing CO_2 emission into the atmosphere (Chynoweth et al., 2001).

In 2003, the White Paper on Energy was approved in South Africa by the Department of Minerals and Energy (DME). This document has a goal of developing 10 000 GWh of renewable energy capacity by the year 2013. This means that South Africa requires intensive research into renewable energy to achieve this goal. Current renewable energy sources that have been

investigated and are being implemented according to the DME White Paper include solar, hydropower, nuclear, wind, geothermal energy and biofuels.

Biofuels are liquid fuels formed from the conversion of biomass or their products. These include bio-ethanol, biogas, biodiesel, bio-hydrogen and bio-oil. Biodiesel is renewable, biodegradable, non-toxic and contains significantly reduced amounts of sulphur and polycyclic aromatic compounds (Demirbas, 2009b). It consists of mono alkyl esters that are derived from organic oils obtained from either plants or animals, through the process of transesterification with short chain alcohols (Demirbas, 2009b). Transesterification reaction consists of transforming triacylglycerides (TAGs) into fatty acid methyl esters (FAMEs), in the presence of an alcohol (Hoydoncx et al., 2004). Biodiesel has a higher oxygen percentage and a higher cetane number than conventional diesel which provides for a more complete combustion giving a cleaner burn and reducing emissions (Song et al., 2008). Biodiesel is thus a competitive, cleaner and renewable fuel source when compared to conventional petroleum diesel fuel (Demirbas, 2009b). The current commercially produced biodiesel utilises seed and food crops (Table 1), which minimises the land available for food crops contributing to a global food security crisis (Chisti, 2008, Vasudevan and Briggs, 2008). For this reason alternative sources or feedstocks need to be sought and microalgae have proven to be one such source.

There are numerous reasons that microalgae are favoured as a biodiesel source over plant crops (Rodolfi et al., 2009b). Microalgae require less water than terrestrial crops and can be grown in saline and brackish environments, they also require less arable land (Table 1) therefore reducing food security issues. Microalgae have a lipid yield of up to 80 percent dry cell weight whereas vegetable crops can accumulate on average between 15 to 40 percent total lipids (Singh and Singh, 2010). Microalgae have a generation time of between 4 and 24 hours and can be cultured continuously resulting in constant oil supply (Chisti, 2007). Maturation ponds at waste water treatment

plants provide a rich supply of nutrients for microalgae (Clavier et al., 2005), reducing the demand for freshwater and nutrients used for plant crops.

Table 1: A comparison of crop dependent and microalgal biodiesel production efficiencies (Chisti, 2007, Schenk et al., 2008)

Oil Source	Biodiesel (L/ha/year)	Area of global oil demand (ha x 10 ⁶)	Area required of global land mass (%)	Area of global arable land (%)
Cotton	325	15 002	100.7	756.9
Soybean	446	10 932	73.4	551.6
Mustard Seed	572	8 524	57.2	430.1
Sunflower	952	5 121	34.4	258.4
Rapeseed/Canola	1 190	4 097	27.5	206.7
Jatropha	1 892	2 577	17.3	130
Oil palm	5 950	819	5.5	41.3
Microalgae (30% TAG)	12 000	406	2.7	20.5
Microalgae (50% TAG)	98 500	49	0.3	2.5

Microalgae are unicellular microscopic, polyphyletic, generally photoautotrophic organisms that convert solar energy, carbon dioxide and water to algal biomass. These microorganisms are therefore cultivated as an energy crop (Greenwell et al., 2009). Microalgae have been commercially grown to produce metabolites such as lipids, carbohydrates, proteins numerous vitamins, antioxidants (Tokusoglu and Unal, 2003) and plant hormones (Yokoya et al., 2010). Some microalgae can produce more than 60 percent of their dry cell weight in the form of lipids under specific conditions (Chisti, 2007, Demirbas, 2009a, Rodolfi et al., 2009b, Ohlrogge and Browse, 1995). The economic feasibility of microalgal biodiesel production is

dependent on maximized growth and lipid yields. *Chlorella* (Chlorophyta or green algae) has been used as a potential feedstock for biodiesel due to its ability to synthesise TAGs with a carbon chain length (C_{16} and C_{18}) suitable for biodiesel (Meng et al., 2009, Rasoul-Amini et al., 2009).

1.2 MICROALGAL BIODIESEL

Biodiesel comprises of the lower alkyl fatty acids with esters of short chain alcohols which are primarily methanol or ethanol. They have a carbon chain length ranging between C_{14} – C_{22} however C_{16} and C_{18} are predominant for biodiesel production (Demirbas, 2009b). Microalgae can produce lipids with carbon chains ranging from C_3 – C_{38} . Some species such as *Dunaliella salina* produce longer hydrocarbon chains (C_{19} - C_{38}) (Rasoul-Amini et al., 2009).

Microalgal lipids can be converted to biodiesel using microemulsions, pyrolysis and transesterification (Demirbas, 2009a, Miao and Wu, 2006). The lipids (TAGs) extracted from microalgae are composed of three fatty acid molecules esterified to a glycerol molecule. During the transesterification process, methanol reacts with triglycerides to obtain biodiesel as an engine fuel. The reaction cleaves the fatty acids from the glycerol and binds them to a methyl group from the excess methanol, producing fatty acid methyl esters (FAMES) or biodiesel (Fig. 1). Glycerol results as a by-product (Meher et al., 2006). The reaction follows a stepwise process where the triglycerides are first converted to diglycerides and then to monoglycerides and finally to glycerol. This process occurs in three separate reactions (Chisti, 2007). Three moles of alcohol are required per mole of triglyceride to produce one mole of glycerol and three moles of biodiesel, because the reaction can be driven in either direction or maintained in equilibrium, methanol is usually added in excess to drive the forward reaction (Fukuda et al., 2001). Research has shown that for maximum conversion to the ester a ratio of 6:1 has proven appropriate (He et al., 2007, Meher et al., 2006).

The time taken for the reaction to occur is dependent on the temperature. A higher temperature can be used in conjunction with a higher pressure but this reduces the feasibility of the process as it will become energy intensive (Chisti, 2007). It has been found that the conversion rate increases with the reaction time. Research has shown that the most feasible temperature and time combination is to run the reactions, which are catalyzed by alkali substances, at a temperature of 60°C under atmospheric conditions. Reactions at 60°C usually take 90 minutes to complete (Chisti, 2007).

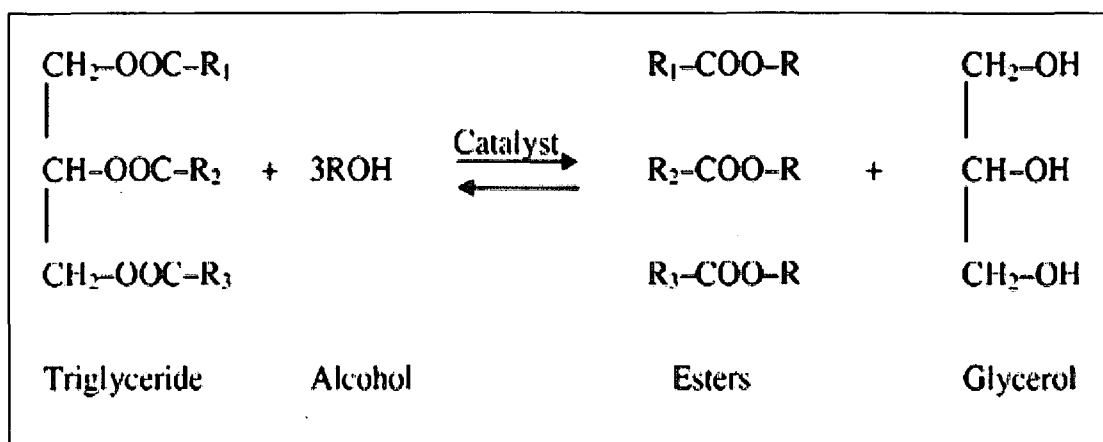


Figure 1: Catalyzed reaction of triglycerides converted into biodiesel (Mutanda et al., 2011).

The use of a catalyst such as sulphuric acid (H₂SO₄) or alkaline sodium hydroxide (NaOH) accelerates the transesterification process. Reactions that are catalyzed by alkalis are about 4000 times faster than acid catalyzed reactions and are therefore preferred. Consequently sodium hydroxide and potassium hydroxide (KOH) are commonly used as commercial alkali catalysts. After the transesterification process, methanol and fatty acid methyl esters separate into two liquid phases (Fig 2). The methanol is removed and recovered and can be reused for subsequent transesterification. Biodiesel is then repeatedly washed with water to remove traces of glycerol and methanol which ensures a high quality (Chisti, 2007) (Fig. 2).

Biodiesel can be used as a direct replacement or as a blending agent for diesel fuel for the running of diesel engines (Demirbas, 2009b). One hundred percent pure biodiesel called B100 fuel can be used in engines but it is most commonly blended with petroleum diesel in different proportions. The most common proportions are 20%, 5% and 2% termed B20, B5 and B2 blends respectively.

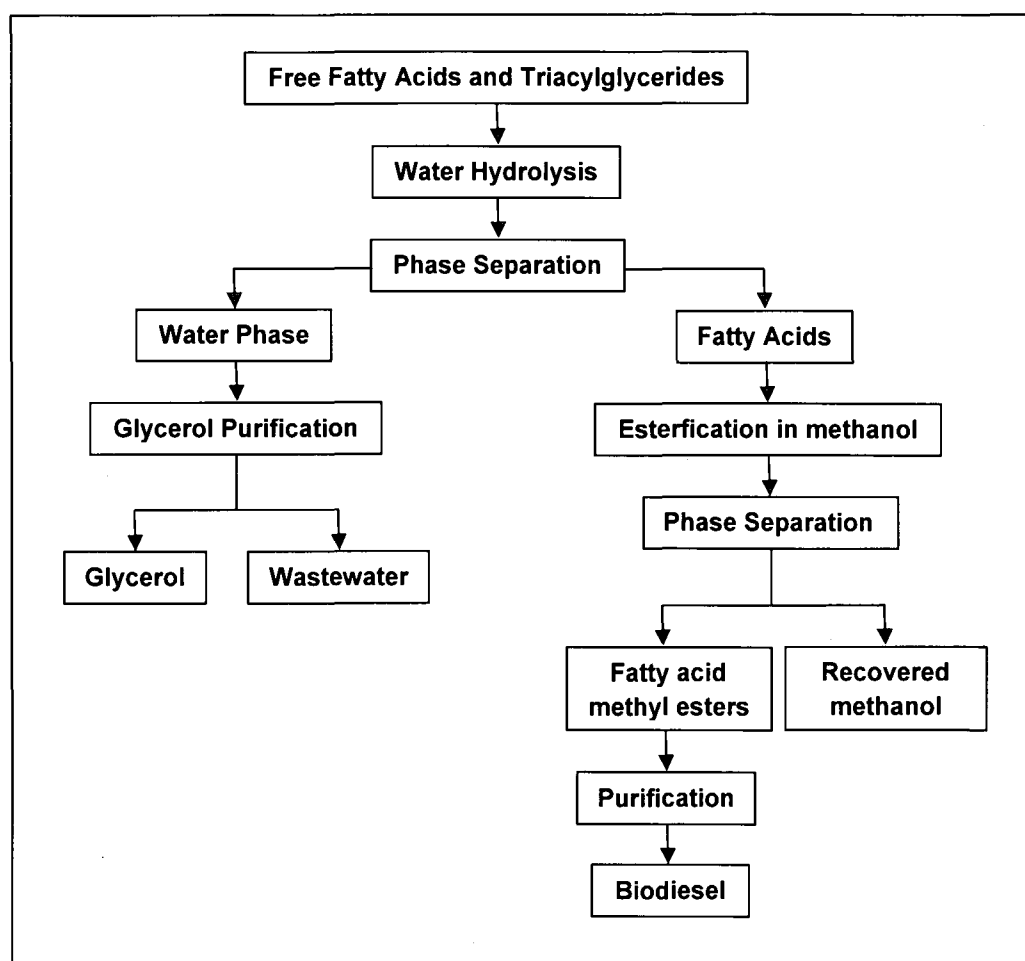


Figure 2: Biodiesel production process (modified from Demirbas, 2009a).

1.3 MICROALGAL CULTIVATION

The mass cultivation of microalgae occurs either by open raceway ponds or closed photobioreactors. Open raceway ponds consist of closed loop, oval shaped recirculation channels with paddle-wheels ranging from laboratory scale (3 m²) to commercial scale (440 000 m²) (Brennan and Owende, 2010, Spolaore et al., 2006). Raceway ponds have been in operation since the 1950's. Raceway ponds are open systems that are susceptible to the elements and are therefore very difficult to control. Photobioreactors are closed systems usually constructed from glass/perspex tubes that allow for a controlled environment and maximum light absorption (Chisti, 2007). The main disadvantage of using photobioreactors is the initial cost of the infrastructure and maintenance thereafter (Brennan and Owende, 2010, Chisti, 2007, Vasudevan and Briggs, 2008). Raceway ponds are therefore the preferred manner to mass produce microalgae, because raceway ponds are uncontrolled environments, physiological stress conditions may result when conditions become unfavourable.

1.4 PHYSIOLOGICAL STRESS

Physiological stress in organisms results from a deviation from normal growth conditions. Once stressors have acted on an environment or ecosystem, a stress-response relationship may develop. The ability of the ecosystem to adjust to the new state or to enter the recovery phase where the nominal steady state is re-established via homeostasis is crucial for survival of the components of the ecosystem (Power, 1999). Environmental stresses include nutrients, salinity, temperature, light and pH. Stress conditions have been found to induce intracellular neutral lipid yields in microalgae. Many microalgae have the ability to produce up to almost 80 percent dry cell weight of triacylglycerides (TAGs) as a storage lipid when under environmental stress (Chisti, 2007, Spolaore et al., 2006).

1.4.1 Nutrient Stress

Microalgae cultured in nutrient depleted conditions are more likely to suffer damage from incident light, reducing the physiological and metabolic functioning. This is due to a lack of nutrients for the construction of photoprotective compounds and for repair mechanisms (Dean et al., 2010, Herzig and Falkowski, 1989). Nutrient deprived algal cells have shown a decline in photosynthetic efficiency of photosystem Two (PS II) (Lippemeier et al., 1999, Petrou et al., 2008). Nutrient limitation will initially be reflected by a lower photosynthetic functioning and subsequently by cellular biochemical processes, eventually affecting growth and biomass production. Ecophysiological studies have shown that phytoplankton generally require an average ratio C:N:P of 106:16:1 (Redfield et al., 1963). Previous studies have monitored nutrient stress by a reduction in growth rates (Courchesne et al., 2009, Converti et al., 2009, Flynn, 1990) and nutrient uptake rates (Flynn, 1990). However, PAM Fluorometry has recently been used as a non invasive tool to monitor immediate changes in photosynthetic functioning (Beardall et al., 2001, Petrou et al., 2008). The F_v/F_m parameter for example, is generally used as an indicator of nutrient stress (Beardall et al., 2001, Timmermans et al., 2005). According to Dean et al., (2010), microalgae cultures deprived of nutrients show greater photo damage (Herzig and Falkowski, 1989).

Nitrogen depletion has been found to decrease microalgal growth rates (Chen et al., 2010, Converti et al., 2009, Spolaore et al., 2006) and to increase the synthesis of intracellular neutral lipids (Illman et al., 2000, Hsieh and Wu, 2009, Pruvost et al., 2009, Rodolfi et al., 2009a). The depletion of phosphorus in the growth medium has been found to decrease growth and the total depletion of phosphorus (P) resulted in the death of the culture (Timmermans et al., 2005). Studies have found that available ATP in the cell decreased during P depletion (Beardall et al., 2001, Healey, 1982) thereby reducing photosynthetic functioning of the cell. Phosphorus deficiency has been shown to cause cessation of cellular growth and increase lipid

synthesis in some microalgae (Courchesne et al., 2009). The lack of iron (Fe) in growth media has been shown to reduce microalgal growth rates (Liu et al., 2007, Liu et al., 2008, Marchetti et al., 2010, Timmermans et al., 2005). However, by increasing the iron concentration, cellular neutral lipid synthesis was found to be induced (Liu et al., 2008, Marchetti et al., 2010). The addition of extra iron also had the ability to prolong the exponential growth phase thereby increasing the final cell density (Liu et al., 2008).

1.4.2 Osmotic Stress

Osmotic stress results from the change in salinity in the growth medium. Osmotic stress has opposing effects on freshwater and marine microalgae species. Acclimation to variable salinity usually occurs in estuarine species of microalgae. The ability of the cells to maintain homeostasis via changes in physiological function will determine the acclimation process (Parida and Das, 2005). *Chlorella* sp. has been found to tolerate salinities around 0.5 M NaCl (Alyabyev et al., 2007) whereas the marine or halotolerant microalga *Dunaliella* is well suited to grow at salinities above 1.5 M NaCl and would show stress responses in a freshwater medium (Alyabyev et al., 2007). The effect of osmotic stress has been commercially exploited to obtain specific value-added products from numerous microalgal species. *Haematococcus* is induced to produce astaxanthin (Boussiba, 2000, Kobayashi et al., 1992) and *Dunaliella* sp. has been shown to produce phytosterols (Francavilla et al., 2010). *Chlorococcum* spp. have been shown to increase the carotenoid accumulation when exposed to osmotic stress. The use of PAM fluorometry has shown to produce a greater amount of information regarding the changes in microalgal physiology when cells are exposed to osmotic stress conditions (Masojidek et al., 2000).

1.4.3 Temperature Stress

Temperature is a factor to which microalgal cells are continually responding. Growth rate has been shown to be significantly affected by temperature (Nedeva and Pouneva, 2009). In a higher temperature environment, the photosynthetic activity of the chloroplasts is the most heat sensitive process (Cherry and Nielson, 2004). Short exposure to high temperatures could irreversibly inactivate the oxygen evolving complex (Kreslavski et al., 2007). In addition PAM Fluorometry allows the rapid identification of potential injuries to microalgae where there is an absence of other visible symptoms (Pang et al., 2007, Lamote et al., 2007, Collen and Davison, 2001). It had been previously speculated that the ability of algae to alter the physical properties and thermal responses of their membrane lipids represents a strategy for enhancing physiological acclimation over a range of temperatures (Somerville, 1995, Hu et al., 2008). An increase in temperature has been shown to increase saturation of fatty acids in many algae species (Guschina and Harwood, 2006, Renaud et al., 2002). Temperature has also been shown to affect the total lipid content of the cells (Converti et al., 2009, Hu et al., 2008). High temperatures have also been shown to increase the total protein concentrations (Nedeva and Pouneva, 2009). This shows a temporary ability for the cells to withstand higher temperatures.

1.5 CELLULAR NEUTRAL LIPID SYNTHESIS

The synthesis of fatty acid is regarded as the most important pathway with regard to lipid production for biodiesel (Ohlrogge and Browse, 1995). The fatty acid synthesis occurs mainly in the chloroplast stroma resulting in a C₁₆ or C₁₈ carbon chain fatty acid from Acetyl-CoA and malonyl-CoA where at least thirty enzymes are required for this process (Ohlrogge and Browse, 1995) (Fig. 3).

The synthesis of fatty acids and glycerol into triacylglycerides (TAGs) occurs in the plastids (Hu et al., 2008). The occurrence and extent to which algae produce TAGs depends entirely on the species or strain of microalgae as well as the genetic makeup of the individual organism (Courchesne et al., 2009). The TAG synthesis pathway yields neutral lipids which are an effective manner in which to store fatty acids and indirectly energy. The sequence of the TAG synthesis can be seen in Fig. 4. The DAG and PA compounds are used in the production of polar lipids.

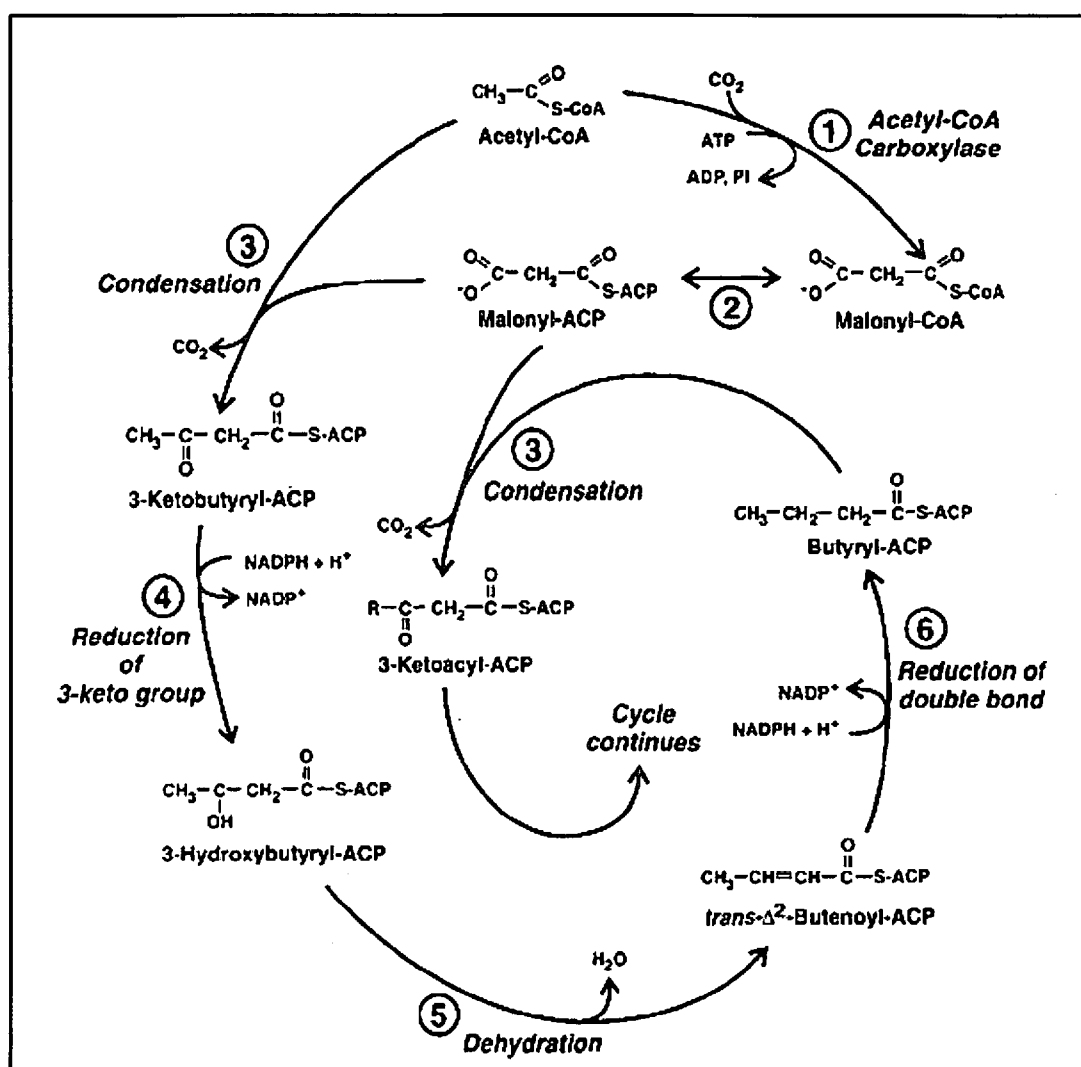


Figure 3: The Reactions of the Fatty Acid Biosynthesis Pathway (Ohlrogge and Browse, 1995)

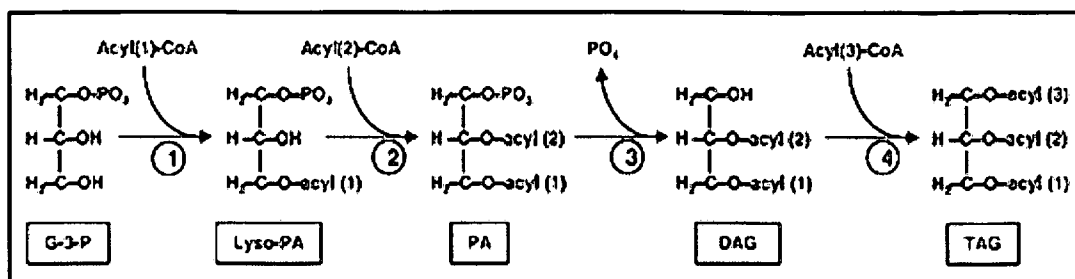


Figure 4: A schematic diagram representing the triacylglyceride biosynthesis pathway (Hu et al., 2008)

The TAG synthesis pathway is usually co-ordinated with the production of secondary carotenoids in microalgae (Zhekisheva et al., 2002, Rabbani et al., 1998). Under normal physiological conditions, microalgal cells contain primary carotenoids normally contained in the chloroplast. Green algae exhibit rapid light-dependant, reversible interconversion of the primary carotenoids to secondary carotenoids via the xanthophyll cycle (Demmig-Adams, 1990). The xanthophyll cycle has been shown to be related the non photochemical quenching (NPQ), a parameter obtained from the PAM Fluorometer. The synthesis of triacylglycerides into cytosolic neutral lipid bodies may, with a few exceptions, be the default pathway in algae under environmental stress conditions (Hu et al., 2008). These specific environmental stress factors inducing lipid synthesis are termed inductive conditions (Boussiba, 2000). The physiological role of TAGs is to act as a carbon and energy store, particularly in aged cells or cells cultured under inductive growth conditions. Lipid inductive conditions will yield changes in the physiology of the microalgae which can be monitored by PAM Fluorometry.

1.6 PULSE AMPLITUDE MODULATED (PAM) FLUOROMETRY

Pulse Amplitude Modulated (PAM) Fluorometry has become one of the most common, non-invasive and rapid techniques to measure the variability of

chlorophyll fluorescence and photosynthetic performance both in terrestrial plants (Juneau et al., 2005) and microalgae (Baker, 2008, Schreiber et al., 2002, Hartig et al., 1998, Oxborough et al., 2000). In recent years, the technique of chlorophyll fluorescence has become ubiquitous in plant ecophysiology (Juneau et al., 2002, Juneau et al., 2005). One of the main attractions of chlorophyll fluorescence is its ability to give a relative measure of photosynthesis (Maxwell and Johnson, 2000). The electron transport rate forms the basis for photosynthesis to release ATP for metabolic processes within the cell (Fig. 5).

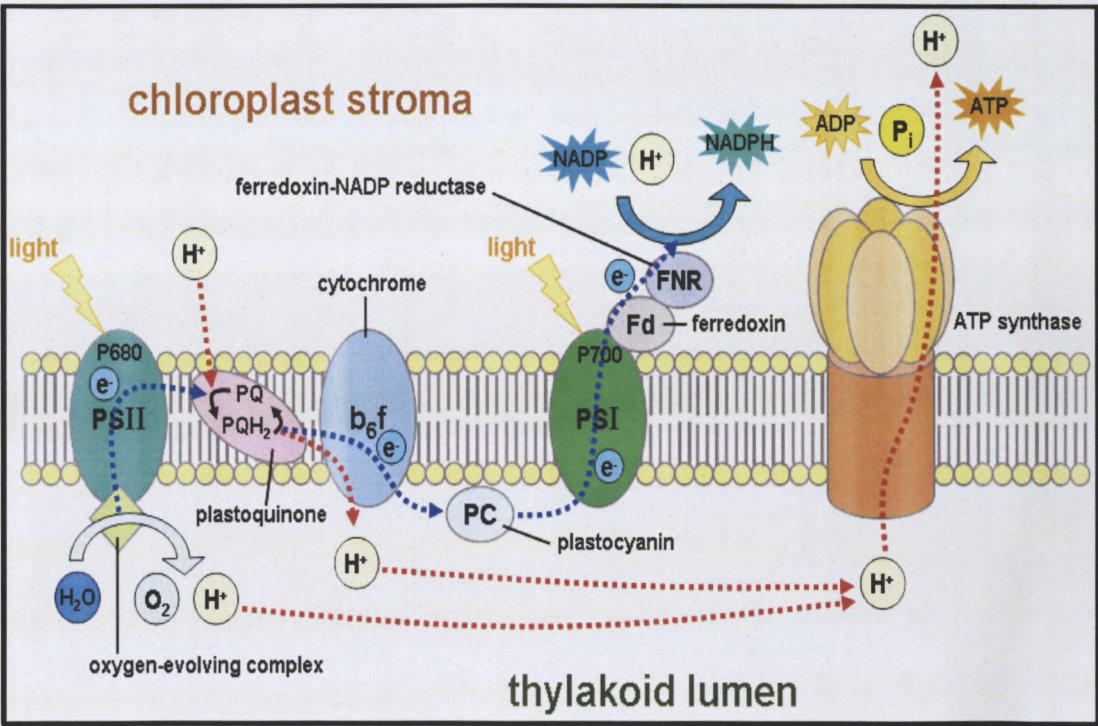


Figure 5: The Electron Transport Chain of the Photosynthetic apparatus crucial for PAM Fluorometry (<http://ideonexus.com/2009/01/>).

Tolerance to environmental stresses can be monitored using PAM Fluorometry (Maxwell and Johnson, 2000). Measurements made over a time period can yield information pertaining to non photochemical quenching, electron transport rates, quantum efficiency (F_v/F_m), the extent of induced

photoinhibition in response to light and temperature, and even to combined environmental stress factors (Bilger et al., 1995). The PAM Fluorometer uses a modulated measuring system. In this system the light is switched on and off at high frequency (i.e. modulated). The detector is tuned to detect only fluorescence excited by the measuring light. This allows the relative yield of fluorescence to be measured in the presence of background illumination and sunlight, making it advantageous over a non modulated system (Schreiber et al., 1986). The design of the Dual-PAM-100 is the latest fluorometer available allowing the state of photosystem I (PS I) and photosystem II (PS II) to be assessed simultaneously. In Chlorophytes, PS II is the dominant PS which will be the focus of this research (Baker, 2008).

PAM Fluorometry works on a model developed for PS II photochemistry in which the photochemistry competes with processes of fluorescence and heat loss for excitation energy in the pigment antennae of PS II (Butler, 1978, Baker, 2008). This model proposed that electron transfer from the reaction centre of PS II to the primary quinone acceptor of PS II quenches fluorescence. Increases in the heat loss result in non-photochemical quenching of fluorescence. This model therefore predicts that PS II fluorescence emission could be used to monitor changes in photochemistry.

Physiological stress is generally induced to stimulate the synthesis of neutral lipids. The economic feasibility of biodiesel production is dependent on maximizing the biomass yields and simulating optimum conditions for photosynthesis (Borowitzka, 1999). In this series of studies, the use of PAM Fluorometry was investigated as a potential tool to monitor physiological stress of the freshwater *Chlorella* species under specific environmental conditions.

In chapter two of this dissertation, the effect of complete and selective nutrient stress on biomass and lipid yields was investigated. An indigenous microalga was isolated and identified for potential application for microalgal

biodiesel production. The potential of the isolate to produce lipids was determined by stress induced by nutrient deprivation. Complete stress was induced where all nutrients were depleted and selective stress was induced where N, P and Fe were selectively depleted. These nutrients have shown to be crucial for microalgal growth as well as play a pivotal role in lipid synthesis (Redfield et al., 1963). The parameters of the PAM Fluorometer were used to assess the physiological stress of cultures under these conditions. A decrease in photosynthetic functioning indicated physiological stress, suggesting the onset of lipid synthesis. Lipid yields could therefore be maximized by monitoring nutrient stress with a PAM Fluorometer. Growth rate can also be increased by determining the optimum environmental conditions by PAM Fluorometry.

The commercial production of the hyper lipid producing isolate in raceway ponds has numerous challenges due to the copious amounts of freshwater required. Due to the geographical location of KwaZulu-Natal and the available sea water, the culturing of the *Chlorella* sp. in a marine environment would improve the overall economic feasibility and reduce the usage of freshwater. Chapter three therefore investigates the acclimation of the high lipid yielding *Chlorella* sp. to a marine medium using PAM Fluorometry. The acclimation of microalgae to a marine environment will have a dual benefit where it decreases planktonic contamination from fresh water species and the demand for freshwater.

Raceway ponds are preferred for use in the mass cultivation of microalgae for biodiesel production. These ponds are open systems and are susceptible to daily and seasonal environmental variability (Harun et al., 2010). The temperature of the pond water fluctuates according to a diurnal cycle as well as seasonally (Grobelaar, 2009a), influencing the growth and productivity. Chapter four of this thesis investigated the effects of temperature fluctuations on growth, lipid yields and characteristics of this microalgal isolate. The effect of dual stress versus single stress was also investigated as a means to

optimize FAME production. The biodiesel was characterized to determine optimum growth and stress conditions for the production of commercial grade biodiesel.

1.7 AIMS AND OBJECTIVES OF THE STUDY

The aim of this study was to monitor physiological stress using PAM fluorometry to optimize biomass and lipid production to increase feasibility of biodiesel production in the Kwa Zulu Natal area.

The objectives of this study were as follows:

1. To use PAM fluorometry to monitor the effect of selective and complete nutrient stress as well as hyper nutrient stress on growth and intracellular neutral lipid synthesis.
2. To monitor the acclimatisation of a freshwater *Chlorella* sp. to variable salinity levels and to determine the optimum salinity for growth.
3. To determine the effect of temperature on lipid, biomass and biodiesel production.
4. To determine the effect of dual stress compared to single stress for maximum cellular neutral lipid synthesis.

CHAPTER TWO

2 EFFECT OF NUTRIENT STRESS ON MICROALGAL PHYSIOLOGY AND LIPID YIELDS

2.1 INTRODUCTION

The majority of microalgal species have the capacity to produce and accumulate substantial amounts (20-60 percent) of triacylglycerols (TAGs) under stressful conditions (Chisti, 2007). Stress is generally defined as 'a deviation from nominal conditions as long as homeostasis permits' (Power, 1999). Previous studies have found these stressful conditions to include nutrient deprivation, particularly nitrogen, high light intensity, low temperature and high salinity concentrations (Li et al., 2008). The effect of these environmental conditions, whether they are adverse or favourable, can heavily affect the physiology of microalgae and hence the biochemical functioning (Rosenberg et al., 2008). This change in biochemical functioning is evident by the use of stress to induce the accumulation of specific value added products in numerous microalgal species, e.g. *Dunaliella salina* can be induced to accumulate β -carotene when cultured under irradiance stress and *Haematococcus pluvialis* can accumulate astaxanthin when cultured under osmotic stress (Masojidek et al., 2000, Boussiba, 2000). In the review, Boussiba (2000) discusses cellular physiology and stress response in further detail in the green algae *Haematococcus pluvialis*. Furthermore, nutrient stress has been shown to increase the accumulation of neutral intracellular lipids in many Chlorophytes (Chisti, 2007, Li et al., 2008) .

Maximum biomass and lipid yields are essential for an economically feasible production of microalgal biodiesel (Chisti, 2007). The growth conditions of microalgae as well as the preferred nutrient sources and concentrations need to be optimized to produce maximum biomass. In the same manner, maximum lipid yields are increased by employing pre-determined stress conditions (Rosenberg et al., 2008). Growth conditions and factors inducing stress can be monitored by the use of variable chlorophyll a fluorescence

(Schreiber et al., 2002). Fluorescence provides an extremely sensitive tool for examining energy metabolism in photosynthetic cells and the interactions between carbon and nutrient assimilation (Oxborough et al., 2000). Light energy absorbed by chlorophyll in the light-harvesting antennae of the chloroplast thylakoid membranes can either be absorbed to do photochemical work or re-emitted as heat, or fluorescence. These pathways compete for the light energy absorbed. Thus the proportion of energy used to do photochemical work is inversely related to the amount of fluorescence emission from chlorophyll *a* (Schreiber et al., 2002). The capacity for photochemical work is influenced by the stress of cells and any damage to the photochemical apparatus caused by photoinhibition (Eggert et al., 2007).

Pulse Amplitude Modulated (PAM) Fluorometry has become one of the most common, non-invasive and rapid techniques to measure the variability of chlorophyll fluorescence and photosynthetic performance both in terrestrial plants (Juneau et al., 2005) and microalgae (Baker, 2008, Hartig et al., 1988, Oxborough et al., 2000, Schreiber et al., 2002). The new dual-channel PAM chlorophyll fluorometer detects variability in photosynthetic activity in both photosystems I and II (Schreiber et al., 2002). The following PAM parameters are generally used to measure the physiological state of the organism: Relative Electron Transport Rate (rETR), Non Photochemical Quenching (NPQ), Maximum Quantum Efficiency (F_v/F_m) and Light Saturation (E_k). rETR is a measure of the rate of linear electron transport through photosystem II, which correlates to the overall photosynthetic performance of the microalgae (Juneau et al., 2005). In microalgae, exposure to photosynthetically supersaturating light, triggers the operation of energy dissipating processes that lower the yield of chlorophyll *a* fluorescence and are thus generally termed as NPQ processes (Masojidek et al., 2000). A high NPQ value has been found to be correlated with carotenoid interconversion into photo-protective pigments under light stress (Boussiba, 2000).

The E_k value defines the onset of light saturation as is obtained from curve fitting model by Platt et al (1980). It results from the ratio between the $rETR_{max}$ and the initial slope of the curve termed α . However, the E_k values may vary significantly from a rapid light curve and a conventional steady-state light curve (Serodio et al., 2005). E_k has also been found to be related to NPQ where NPQ dominates above the E_k saturation value (Henley, 1993). Photoinhibition is detected by the RLCs providing the threshold of irradiance a culture can tolerate, and showing at which light intensities photo damage will occur (Schreiber et al., 2002). The maximum quantum efficiency F_v/F_m is used to estimate nutrient limitation. Non-limited phytoplankton has F_v/F_m values from 0.6 to 0.7 (Kromkamp and Peene, 1999). The same study revealed that the F_v/F_m value has been found to be relatively constant in non-stressed cultures, and to decrease in nutrient stressed cultures.

The use of the PAM fluorometry has been widely used by ecologists to determine phytoplankton photosynthetic efficiency (Gustavs et al., 2009, Hartig et al., 1998, Oxborough et al., 2000, Petrou et al., 2008) and physiological stress which includes temperature (Eggert et al., 2007), salinity (Boussiba, 2000, Masojidek et al., 2000), nutrient (Li et al., 2008, Chisti, 2007) and irradiance (Eggert et al., 2007). However, the application of PAM fluorometry to monitor stress for the production of lipids and other value added products has not been well established in the algal biotechnology industry.

The synthesis and sequestration of triacylglycerols (TAGs) into the cytosolic lipid bodies can be detected by the dye Nile Red (9-diethylamino-5H-benzo(α)phenoxine-5-one) (Greenspan et al., 1985). The synthesis of neutral lipids has been found to be a protective mechanism for cells against stressful conditions (Courchesne et al., 2009). PAM fluorometry can be used to increase the yields of lipids by identifying the extent of stress induced by environmental factors. Previous studies have shown that the feasibility of

microalgal biodiesel production is dependent on maximum lipid yields as well as biomass production (Chisti, 2007).

This chapter therefore investigates the use of PAM Fluorometry to determine the effect of complete and selective nutrient stress on cellular neutral lipid synthesis.

2.2 MATERIALS AND METHODS

2.2.1 Culture conditions

The freshwater unicellular chlorophyte was sampled and isolated from a fresh water pond at the Durban Central within the KwaZulu-Natal region. The unicellular chlorophyte was purified until an axenic state, whereby sterile conditions were maintained to prevent contamination. The culture was monitored microscopically on a weekly basis to verify the lack of contamination. The chlorophyte was identified taxonomically and was cultured in aerated liquid BG-11 medium until early logarithmic phase was reached (Allen, 1973). Samples were cultured for 28 days at an irradiance of $28 \mu\text{mol m}^{-2}\text{s}^{-1}$ with a 14:10 hour light:dark cycle at a constant temperature of 25°C.

2.2.2 Nutrient Stress

Biomass was harvested at mid log phase (\pm day 3 obtained from growth curve, Figure 7) by centrifugation at 3000 rpm for 15 minutes (Hermle Z400). Short term selective nutrient stress was induced by re-suspending equal amounts of the harvested biomass pellet ($1000 \mu\text{g/L chl } a$) into three separate media (100ml), consisting of, BG-11 with no nitrates (BG-11 – N), BG-11 lacking phosphates (BG-11 – P), and BG-11 without iron (BG-11 – Fe). The BG-11 medium inoculated with $1000 \mu\text{g/L chl } a$, acted as the positive control. Complete nutrient stress was induced by inoculating $1000 \mu\text{g/L chl } a$ of the biomass pellet into distilled water (dH_2O). Growth of cultures and experimental samples was undertaken in triplicate. Sub-samples were taken at days 10 and 28 for the nutrient stress experiments.

2.2.3 Hyper-Nutrient Stress

Based on a previous study, hyper-nutrient stress was determined by inoculating $10 \mu\text{M}$ (Liu et al., 2008) of the limiting nutrient into a 10 and 28 day selectively nutrient starved sub-sample (2 mL), every hour for 8 hours. Sodium nitrate was added to the BG-11-N culture, ferric chloride was added

to the BG-11-Fe culture and dipotassium hydrogen phosphate was added to the BG-11-P culture. Growth of cultures and experimental samples was undertaken in triplicate. Rapid Light Curves were generated before the inoculation of the selective nutrients and also 8 hours later after final concentration of 70 μM had been reached.

2.2.4 PAM Fluorometry

Non invasive fluorescence measurements were obtained using a DUAL-PAM 100 Chlorophyll Fluorometer (Heinz Walz GmbH, Effeltrich, Germany). Light ($28 \mu\text{mol m}^{-2} \text{s}^{-1}$) and dark adapted samples were incubated for 15 minutes, at 25 °C. Aliquots of the culture (3 ml) were dispensed into a 10 mm quartz glass cuvette (10 X 10 x 40) containing a micro magnetic stirrer. Each cuvette was then inserted into the PAM fluorometer and Rapid light curves (RLCs) were generated. Rapid light curves provided detailed information on the saturation characteristics of the electron transport chain as well as the overall photosynthetic performance exhibited by the microalgae (Ralph and Gademann, 2005). The RLCs were generated by applying a sequence of increasing actinic irradiance in 15 preset discrete increments ranging from 8 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ to 710 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Each actinic light incubation lasted for 10 s before a saturation pulse of blue light (0.6 s at 10 000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) was applied to determine the rETR at each irradiance level. A Dual PAM software (v 1.9) was used to record all data and to generate the RLCs (Baker, 2008). The Dual PAM software calculated rETR as:

$$\text{rETR} = F'q/F'm \times \text{PPFD} \quad (1)$$

Where $F'q = (F'm - F')$,

$F'q/F'm$ is termed the PS II operating efficiency and estimates the efficiency at which light absorbed by PS II is used. At a given photosynthetically active photon flux density (PPFD) this parameter provides an estimate of the quantum yield of linear electron flux through PS II (Baker, 2008).

PSII quantum yield is also known as maximum quantum efficiency of photosystem II according to the formula:

$$F_v/F_m = (F_m - F_o)/F_m \quad (2)$$

where F_m is maximum fluorescence and F_o is minimum fluorescence resulting in the variable fluorescence F_v .

Non photochemical quenching (NPQ) is calculated and quantified by measuring the change in F_m to the final value F_m' (ΔF percent). It is calculated from the maximum fluorescence measured in the dark adapted sample and is the maximum fluorescence measured in the saturating light pulse (F_m').

$$NPQ = (F_m - F_m') / F_m' \quad (3)$$

The maximum photosynthetic efficiency can be determined from the Alpha (α) value which is the initial slope of the rapid light curve (RLC) (Ritchie, 2008). Alpha (α) was determined from the model by Platt et al (1980).

The saturation values (E_k) are obtained from the curve fitting model of Platt et al., (1980), a feature of the dual-PAM software. The value is determined from the interception point of the α value with the maximum photosynthetic rate which follows the equation:

$$E_k = rETR_{max} / \alpha \quad (4)$$

2.2.5 Chlorophyll a determination

Microalgal biomass was quantified by chlorophyll a (chl a) fluorescence, using a Turner Designs Trilogy Fluorometer, after extraction in 90 percent acetone at 4 °C for 24 hours (Parsons et al., 1984). Chlorophyll a standard (Sigma) was used to calibrate Trilogy fluorometer.

2.2.6 Nile Red staining

Cells were incubated in a Nile Red solution (0.25 mg/mL in acetone) for 5 minutes (Greenspan et al., 1985) and viewed under a fluorescent microscope (Zeiss Axioscope, Zeiss). A 450–490 nm excitation filter, a 510 nm diachronic mirror and a 515 nm barrier filter with 100 x objective lens were used to visualise the fluorescent yellow-gold lipid vacuoles in the microalgal cells. Blue light was used as the excitation light. Laser Scanning Confocal

Microscopy (LSCM) was used to determine the spatial positioning of the lipids from the 3D cell image. Cells incubated in Nile Red were visualized by a Zeiss 710 LSM with EC Plan-Neofluar 40 x/1.30 Oil DIC (Carl Zeiss) objective and an Argon-Ion 514.5 nm laser with a 591 nm- 721 nm filter and a dichroic beam splitter. Lipid to cell ratio was calculated using the Cell Profiler software (Lamprecht et al., 2007). This was achieved by converting all images to greyscale for pixel analysis. A threshold value was set manually to distinguish between the bright intensity of the lipid pixels and the remaining cell pixels. Pixel numbers were recorded and the ratio of 'lipid pixels' to 'cell pixels' was determined and converted to a percentage.

2.2.7 Lipid Extraction

Lipid extraction of the stressed cultures showing neutral lipid storage followed a modified method (Bligh and Dyer, 1959). This method has been used frequently and with great success since its development in 1959 and was chosen for these reasons. Oven dried biomass was weighed (1 g) into 100 ml Schott bottles. A total of 80 ml solvent was added in the order chloroform, methanol, distilled water in the ratio 1:2:0.8 (v/v/v). The samples were allowed to incubate for 18 hours after which they were placed in separating funnels and solvents were added to make the ratio 1:1:0.9 chloroform: methanol: distilled water (v/v/v). The lower chloroform phase was collected and dried where the lipids were measured per dry cell weight.

2.2.8 Statistics

Statistical comparisons were performed using SPSS version 11.5.1 for Windows. All values were logarithmically (\log_{10}) normalised to meet the requirements for parametric statistical tests. One way analyses of variance (ANOVA) were used to test for differences in data ($p < 0.05$) between the nutrient stressed cultures and after the introduction of the depleted nutrient. Tukey's post hoc tests were used to locate the differences in the significant results. ANOVA was also used to determine significance in varying lipid

yields in the nutrient depleted growth media. Pearson's bivariate correlation was used to determine the relationship between photosynthetic efficiency (α) and cellular lipid yields.

2.3 RESULTS

Under environmental stress conditions, many microalgae alter their lipid biosynthesis to produce neutral lipids, mainly in the form of triacylglycerol (TAG). In this study on the identified *Chlorella* sp., the parameters of the PAM Fluorometer (rETR, NPQ, E_k and F_v/F_m) recorded significant physiological stress induced by nutrient limitation (Fig. 8, Table 2) which was later compared to lipid accumulation. A significant inverse correlation was shown between photosynthetic efficiency and cellular neutral lipid yields ($r = -0.8$, $p < 0.05$).

Table 2: Summary of the physiological parameters and lipid yields measured by the PAM fluorometer of *Chlorella* sp. grown on complete and selective deficient media at day 10.

	BG-11	dH ₂ O	BG-11-N	BG-11-Fe	Bg-11-P
rETR _{max} (rel. Units)	45±1.2	15±0.4	11±0.2	20±0.9	17±0.2
E_k (μmol m ⁻² s ⁻¹)	528±25	210±14	116±13	264±15	228±16
α	0.34±0.01	0.28±0.01	0.3±0.02	0.32±0.01	0.31±0.1
NPQ (rel. Units)	0.36±0.01	0.27±0	2.14±0.18	0.27±0.02	0.83±0.01
F_v/F_m (rel. Units)	0.84±0.02	0.67±0.01	0.54±0.01	0.65±0.02	0.60±0
Growth Rate (μg chl a L ⁻¹ d ⁻¹)	1.90±0.4	0.61±0.2	-1.02±0.1	0.76±0.3	1.12±0.3
Lipids (Cell Profiler percent)	14.92±1.3	48.71±3.2	30.15±3.9	3.81±2.8	4.39±1.1
Lipids (dry cell weight percent)	11±0.3	26±1.2	13±0.9	N	N

N – Negligible neutral lipids

The effect of nutrients on biomass and growth rates was significantly different in the various selectively stressed media ($p < 0.05$). This is evident by the selectively nutrient stressed samples in Fig. 6 depicting various intensities of green as well as the growth curves in Fig. 7.

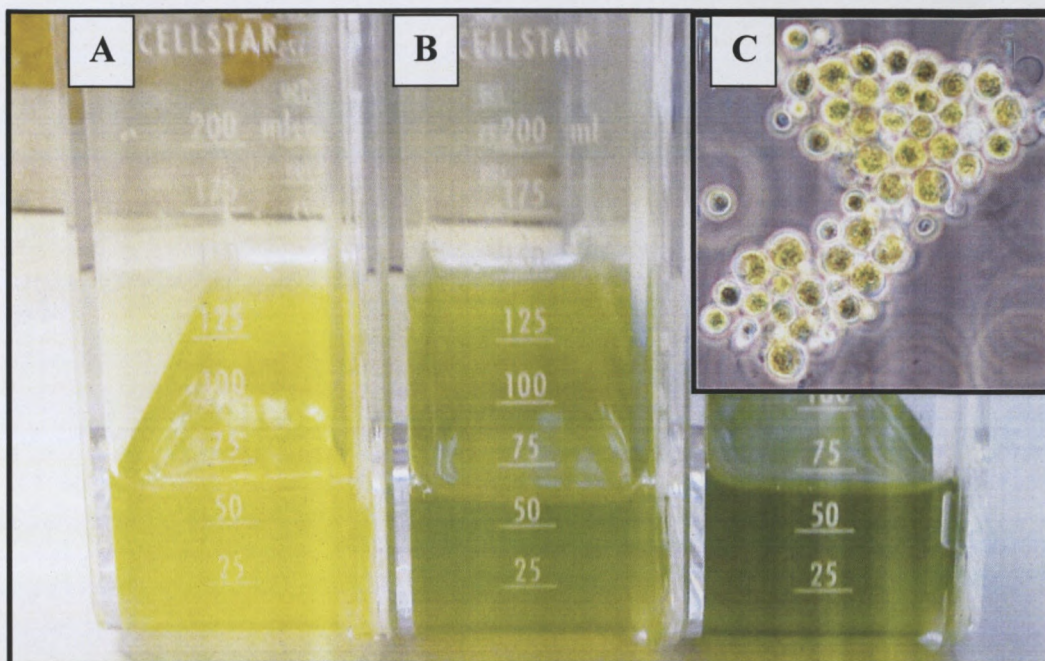


Figure 6: Culture vessels containing the *Chlorella* sp. cells showing selective stress cultured in BG-11-N (A), BG-11-P (B) and BG-11-Fe (C). A micrograph of the *Chlorella* sp. can be seen in the Insert.

The growth rates in Table 2 and Fig. 7 show the effect of selective stress versus complete stress compared to the BG-11 control. The highest growth rate can be seen in the BG-11 control sample. It can be seen that both complete and selective stress decrease the growth rate as well as the biomass production. The greatest decrease was evident in the samples incubated under complete stress (dH_2O) and under selective stress with nitrogen as the depleted nutrient (BG-11-N). This decrease in growth rate can be used as an indication of nutrient stress.

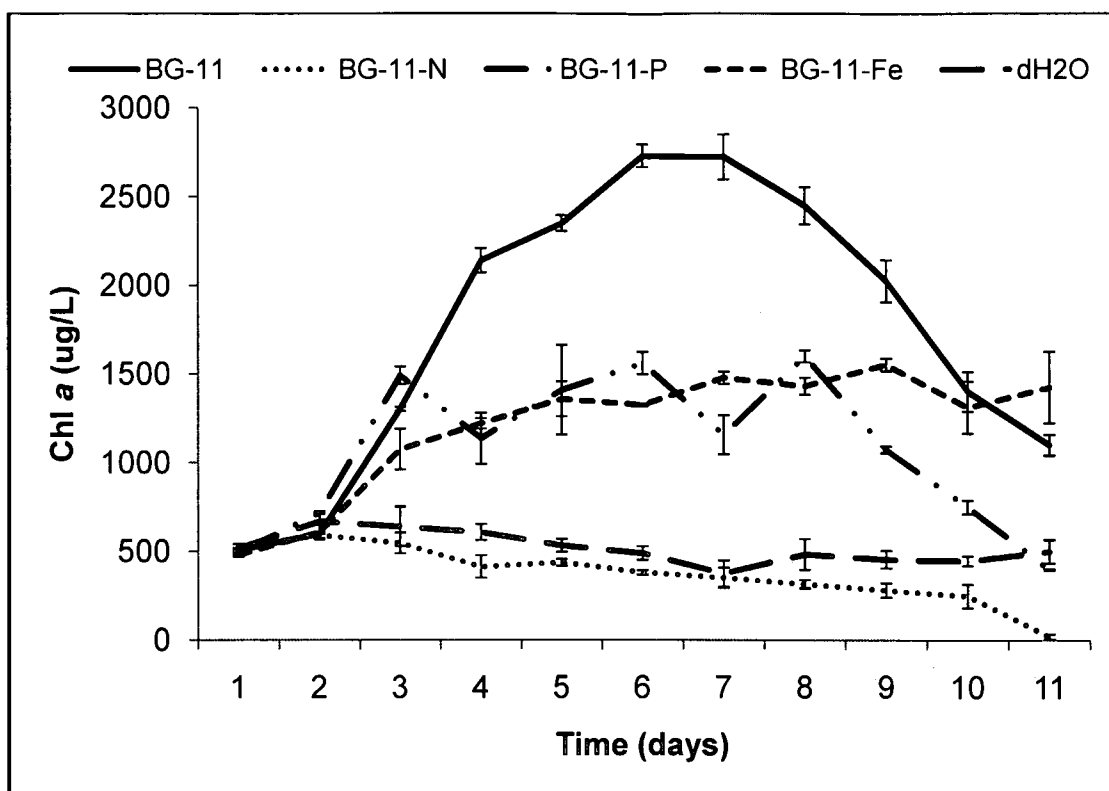


Figure 7: Growth Curves of *Chlorella* sp. cultured in BG-11 medium, selectively stressed media (BG-11-N, BG-11-P and BG-11-Fe) and complete stressed medium (distilled water).

Because nitrate is quantitatively the most important element contributing to the dry weight of algal cells, this depletion showed significant changes in the physiological parameters measured. Selected nutrient stress induced through a nitrate deficient medium (BG -11 – N) showed a 75 percent decrease in the maximum rETR to 11 (Fig. 8), compared to the control ($rETR_{max} = 45$). Further physiological stress was shown by the significant increase in the NPQ value (83 percent; $p < 0.05$). The NPQ value is generally interpreted with caution as the increase in thermal energy release may be caused by the operation of the xanthophyll cycle or slow reversible damages to the photosynthetic apparatus. This dissipation of thermal energy significantly increased only in the nitrate depleted culture ($p < 0.05$), a consequence of a reduction in photosynthetic efficiency (Fig 8).

In this study, cultures in the BG-11–N media showed no growth over the 12 day period ($-1.02 \mu\text{g chl } a \text{ L}^{-1}\text{d}^{-1}$) (Fig. 7), but cellular neutral lipid yields of 30 percent were recorded (Fig. 9) (Table 2). Nitrate depleted cultures in this study, showed a reduced threshold of photoinhibition. The E_k decreased by 78 percent, from $528 \mu\text{mol m}^{-2}\text{s}^{-1}$ in the BG-11 control culture to $116 \mu\text{mol m}^{-2}\text{s}^{-1}$ (Fig 8). The decrease of 12 percent in the α value, showed the reduction in photosynthetic efficiency under depleted nitrate concentrations (Table 2).

Nutrient stress in microalgae is generally detected by a decrease in maximum quantum efficiency (F_v/F_m) values (Beardall et al., 2001) which in this study decreased from 0.8 to 0.5 (Fig 8). Selective nutrient stress induced in this study showed a significant reduction in physiological functioning and stimulated the synthesis of neutral lipids over a 10 day period. Stressed conditions were identified by a combination of reduced physiological functioning which is necessary for the production of neutral lipids (Fig. 9).

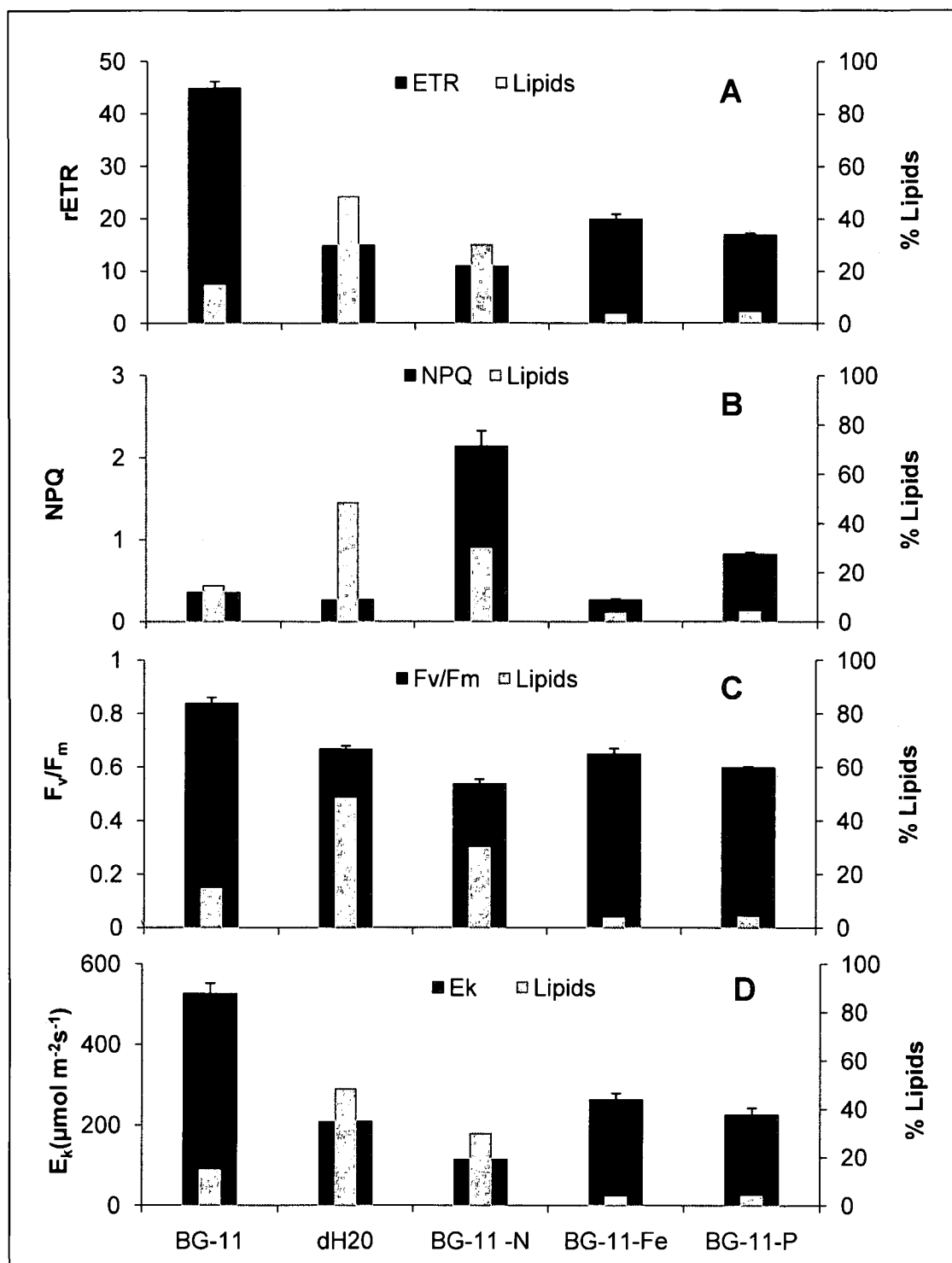


Figure 8: The physiological parameters (black bars, mean \pm SD), ETR (A); NPQ (B); F_v/F_m (C) and E_k (D), recorded under complete and selective stress conditions and the corresponding cellular lipid yields.

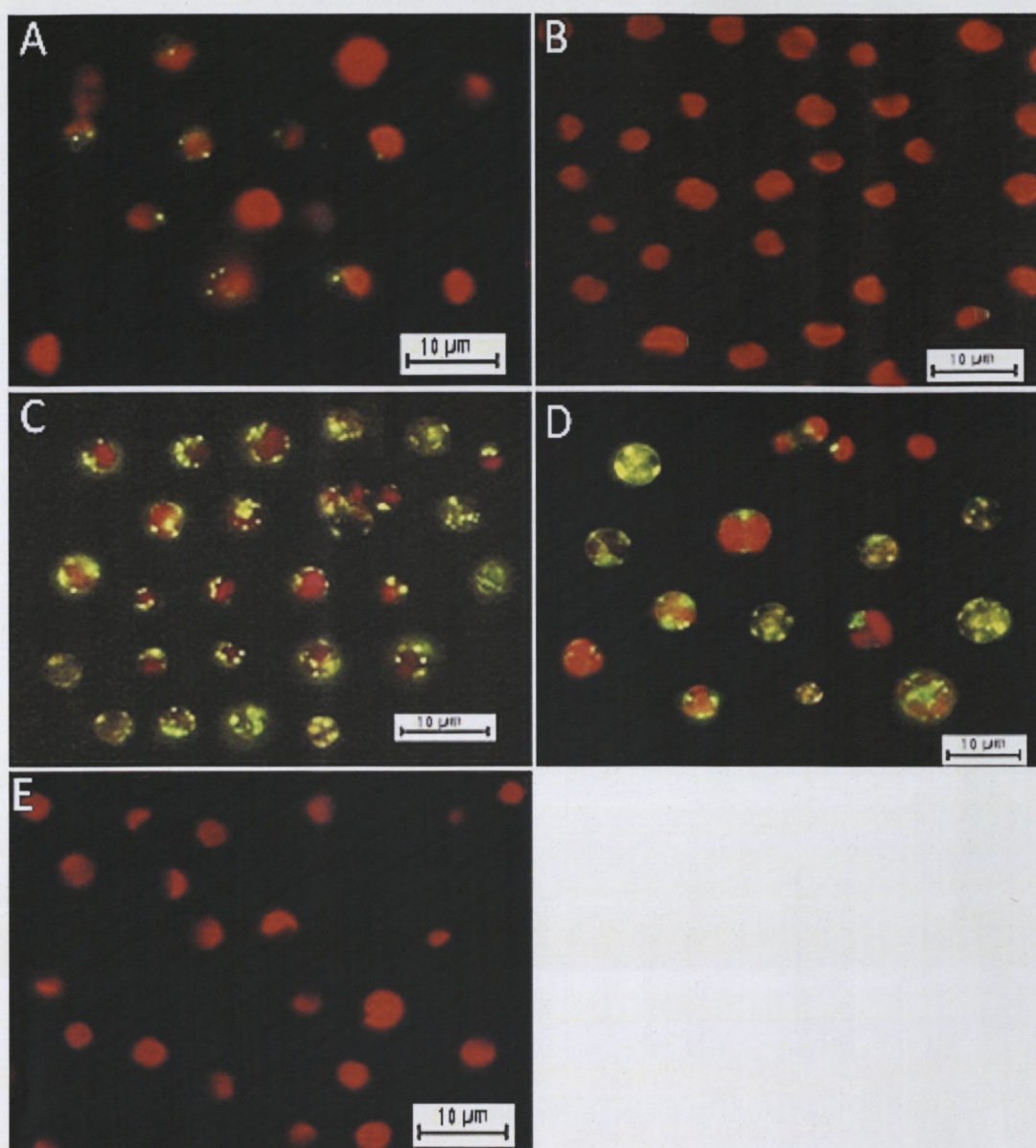


Figure 9: Composite images of *Chlorella* sp. cultures stained using Nile Red showing cellular neutral lipids in BG-11 medium (A), phosphate deficient medium (BG-11-P) (B), distilled water (C), nitrate deficient medium (BG-11-N) (D) and iron deficient medium (BG-11-Fe) (E).

Complete nutrient stress induced by incubating the culture in distilled water resulted in a greater neutral lipid yield (49 percent) compared to the nitrate deficient cultures (30 percent) and the BG-11 control (15 percent) (Fig 9) (Table 2). Not all cells in the culture contained maximum lipid, which showed a poor correlation to the dry cell weight lipid percentage (Table 2). Under complete nutrient stress, the physiological parameters, ETR, NPQ, E_k and F_v/F_m decreased by 67 percent, 29 percent, 60 percent and 20 percent respectively (Fig. 8, Table 2) compared to the BG-11 control. Photosynthetic efficiency showed the greatest decrease when incubated in distilled water having an α value of 0.28 compared to 0.34 in the control. In this study, complete nutrient stress induced greater synthesis of neutral lipids compared to stress by selective nutrients ($p < 0.05$) with a greater amount of lipid available for biodiesel production (49 percent).

Additional selective nutrient stress was induced in this study, using BG-11 media depleted of iron (BG-11-Fe). In this media, the culture recorded the least change in rETR (56 percent), compared to the BG-11 control (Fig 8). The NPQ value showed a reduction of 25 percent from the BG-11 control culture which is expected when ETR_{max} is reduced. The E_k value of the BG-11-Fe ($264 \mu\text{mol m}^{-2}\text{s}^{-1}$) was greater than that recorded for the culture under complete nutrient stress, showing a lower degree of stress (Fig 8). The minimal reduction in the photosynthetic efficiency supports the lower stress levels recorded in this sample. The maximum quantum efficiency value in the iron depleted media showed a reduction of 23 percent compared to the BG-11 control media. This culture recorded a lipid yield of 4 percent, which shows that iron depletion induces minimal stress. This minimal stress was also evident from the insignificant reductions in the physiological parameters recorded from PAM fluorometry.

Selective stress was induced on the *Chlorella* sp. by the incubation in phosphate depleted media (BG-11-P). The photosynthetic efficiency was shown to be reduced, evident by the 62 percent decrease in rETR and an

increase from 0.36 to 0.83 in the NPQ value (Fig. 8). The lipid yields in this medium were reduced to 4 percent compared to the control (15 percent) (Fig 9). Further physiological stress was evident by the reduced E_k value (57 percent), showing photo-sensitivity at a lower PAR. The decline in photosynthetic efficiency was also evident by a decrease in the F_v/F_m value (28 percent).

Hyper nutrified conditions were introduced by the addition of the selected limiting nutrient using the 10 and 28 day cultures. The addition of nitrate and phosphate to the limiting cultures showed significant physiological stress ($p < 0.05$). The addition of nitrate resulted in a significant decrease of both E_k (11 percent) and ETR (36 percent) ($p < 0.05$) (Figs. 10A, D and 11). The addition of excess phosphate into the limiting medium (BG-11 - P) resulted in a probable nutrient shock, i.e. no photosynthetic activity was recorded.

The re-supply of iron to the BG-11-Fe medium had a recovery effect whereby the rETR increased by 43 percent from 20 to 35 (Figs. 10A and 11). The E_k value increased by 28 percent from $264 \mu\text{mol m}^{-2}\text{s}^{-1}$ to $366 \mu\text{mol m}^{-2}\text{s}^{-1}$ showing a recovery effect (Fig. 10D) The F_v/F_m value increased from 0.65 to 0.9 showing a 28 percent increase (Fig. 10C). The absence of iron in the BG-11 - Fe showed significant stress, however photosynthetic recovery was evident upon the re-addition of the limited nutrient

Minimal changes in photosynthetic functioning were evident on day 28, probably a consequence of reduced nutrient uptake in the aging cells (data not shown).

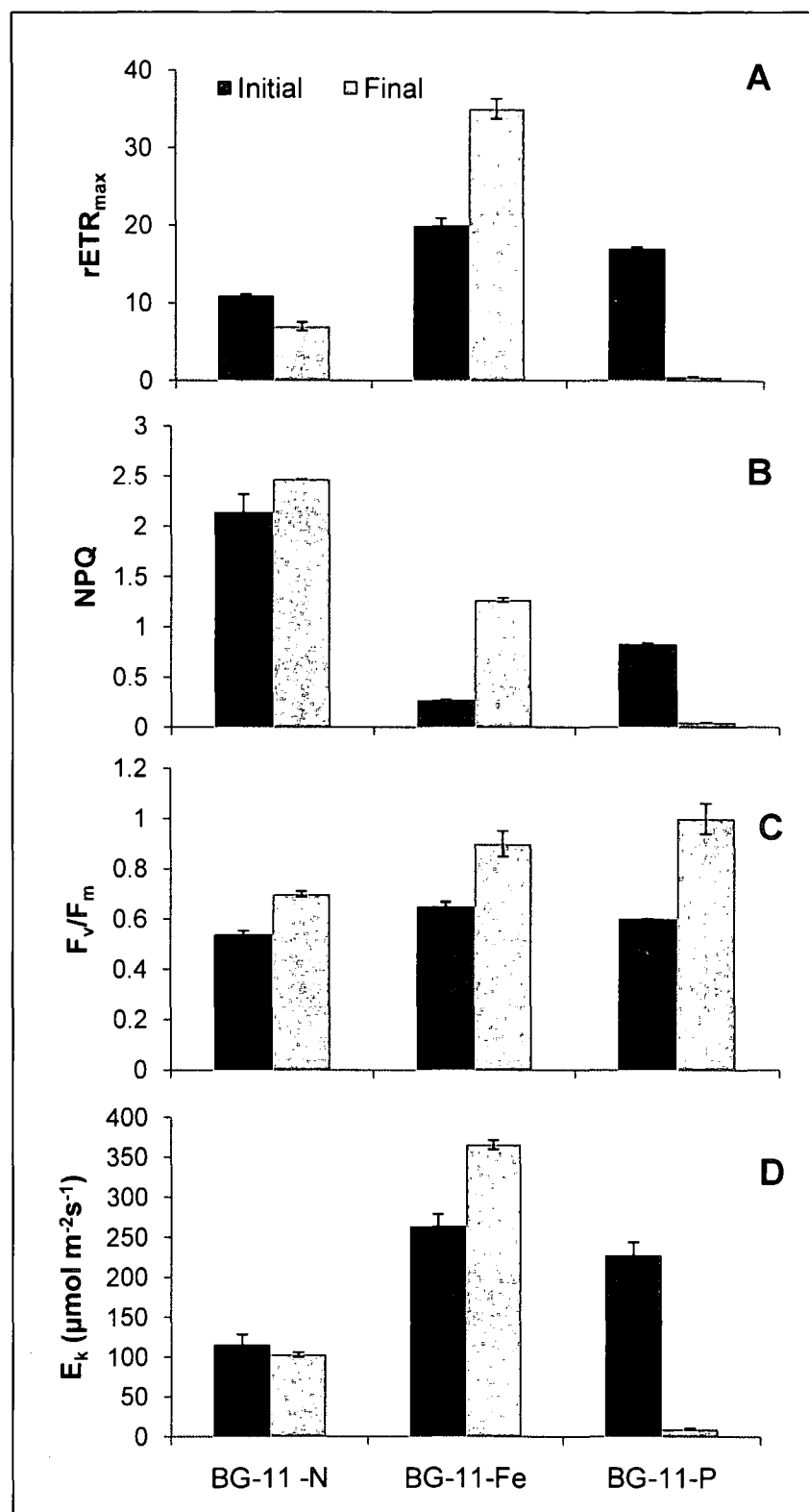


Figure 10: The physiological parameters ETR (A); NPQ (B); F_v/F_m (C) and E_k (D) recorded under hyper-nutrient stress showing initial values (black bars, mean \pm SD) and values after the addition of the limited nutrients (grey bars, mean \pm SD).

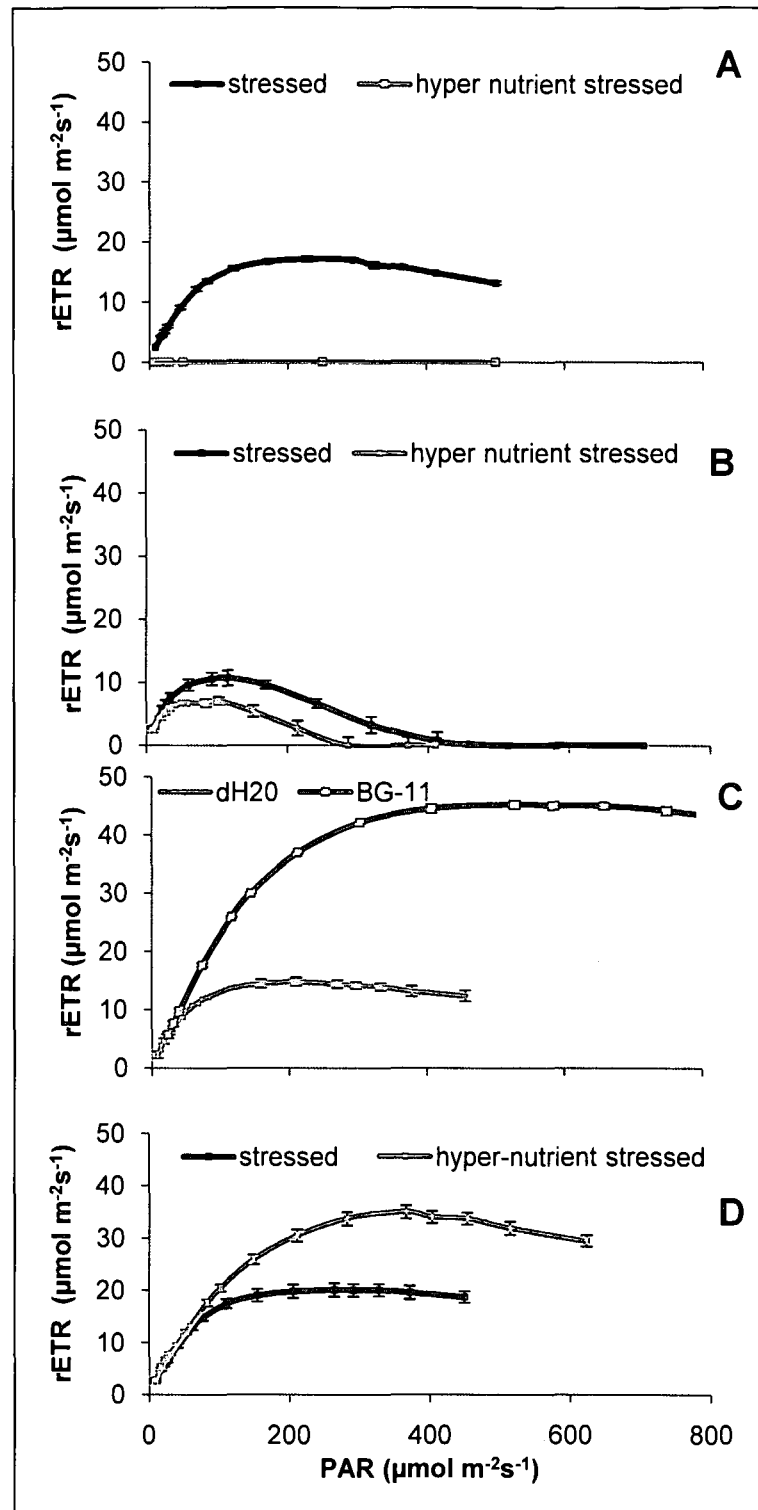


Figure 11: Rapid Light Curves for the nutrient stressed cultures (black lines, mean \pm SD) and the hyper nutrient stressed cultures (grey lines, mean \pm SD) after the addition of 70 μ M of the specific depleted nutrient in BG-11-P medium (A), BG-11-N medium (B), BG-11 compared to distilled water (C) and BG-11-Fe medium (D).

2.4 DISCUSSION

When microalgae are cultured under nitrogen deprivation, preferential degradation of the nitrogen containing macromolecules occurs, resulting in a decrease of total nitrogen content as well as the accumulation of excess carbon in the form of lipids (Dawes, 1976). Previous studies have reported lipid yields between 14 - 20 percent for *Chlorella vulgaris* under normal growth conditions (Spolaore et al., 2006) and 28 - 32 percent for *Chlorella* sp. (Harun et al., 2010) in the absence of nitrates. In this study, when the lipids were induced by nitrate depletion, the yields were found to be in the range according to literature stated above (30 percent) (Fig. 9D).

Under nitrogen-limited conditions the efficiency of PSII decreases, primarily as a consequence of the reduced photosynthetic pigments, decreasing the rate of photosynthesis (Kana et al., 1997). The trend for lowered photosynthetic rates and increased lipid accumulation was shown in this study in the nitrogen depleted cells and in the cells under complete nutrient stress (distilled water). However, complete nutrient stress showed the greatest accumulation of lipids (48 percent total) and showed an increase of 53 percent compared to the maximum reported for *Chlorella* species. Neutral lipids are a result of carbon storage for cells under stressful conditions (Courchesne et al., 2009). Therefore more carbon is channelled into lipid synthesis pathways than carbohydrate synthesis pathways making complete nutrient stress an improved manner in which to stress the algae.

Iron is generally termed a micronutrient compared to the macronutrient status of nitrogen and phosphate and therefore resulted in minimal physiological interference in this study (Fig. 10). Iron is required for the assimilation of nitrate as well as having a functional role in photosynthesis via the electron carrier ferredoxin and iron sulphur proteins present on the acceptor side of photosystem I (Lax et al., 2007). The increased NPQ value recorded in the iron depleted cultures (Fig. 9B) may be due to the functioning of the xanthophyll cycle which is induced under stress conditions to protect the light

harvesting complexes from increased light intensity (Baker, 2008). This revealed that light energy was not being dissipated as thermal energy, thereby establishing that the culture was not under immense stress as the photo adaptive functions were still functioning. This can be compared to the selective nitrate stressed cultures and the complete nutrient stressed cultures which showed minimal photo adaptive functioning. In the absence of iron, carbon tends to be accumulated as intracellular glucose (Kaplan et al., 1986), and not necessarily as neutral lipids. As the F_v/F_m is generally used as an indicator for nutrient stress the reduced value was expected. Previous studies have also reported a decrease in F_v/F_m values under osmotic, light and nutrient stress (Lu et al., 1999, Beardall et al., 2001).

Phosphorus is one of the major nutrients required for normal algal growth with a particular role in energy (ATP) and electron transfer processes (Beardall et al., 2001). Under phosphate limiting stress, microalgae tend to decrease the synthesis of proteins, chlorophyll and DNA, while increasing the production of carbohydrates rather than lipids (Healey, 1982). In this study the low photosynthetic efficiencies experienced (low E_k and $rETR_{max}$) are indicators of this change in metabolic functioning. In the absence of nitrates and phosphates, the microalgal culture under study had a N:P ratio less than 16:1, which is generally reported as stressful for phytoplankton communities (Redfield et al., 1963). This stress will reduce the normal cellular functions such as photosynthesis (ETR), respiration and some enzyme activities (Liu et al., 2007). In the case of phosphorus, the effect of limitation is indirect and less immediate in reducing the rate of synthesis of proteins in the photosynthetic apparatus.

The control of the regulators that can direct carbon into the lipid synthesis pathway has been shown to be controlled by numerous factors such as temperature, salinity and nutrient concentrations. This is known as biochemical or metabolic engineering. The metabolic engineering via the process of selective nutrient stress had shown to be favourable when

selecting for carbohydrate or lipid synthesis pathways. This approach has shown to be very useful for enhancing lipid production by numerous studies reported in the review by (Courchesne et al., 2009). This strategy can be employed for other microalgal strains to accumulate other metabolites such as beta carotene and astaxanthin (Leon-Banares et al., 2004).

Hyper nutrified conditions were shown to introduce stressful effects after the addition of excess nitrates and phosphates (Fig. 10). Previous studies (Petrou et al., 2008) have shown that nutrient starved algae redirect energy from photosynthetic processes towards maximising nutrient uptake upon nutrient re-addition. This redirection of energy leads to a net decrease in the capacity of cells to dissipate energy photochemically. This results in an increased NPQ value as a means of photoprotection. The possible nutrient shock recorded after the addition of phosphate resulted in an insignificant change in the physiological parameters recorded. This may be due to insufficient enzymes being available for nutrient uptake (Beardall et al., 2001).

The *Chlorella* cells that were cultured for 28 days showed minimal change in the physiological parameters. A possible cause for this is the age of the cells. The peak of the growth curve was shown to be achieved near day 7 therefore the cells are in stationary phase nearing the death phase. This has been shown in previous studies where the increase in nutrient concentrations has little to no effect on the growth or biomass in cultures at stationary phase (Fabregas et al., 1984).

The resupply of iron showed a recovery effect in contrast to hyper nutrient stress displayed after the addition of excess nitrates and phosphates. (Fig. 10) This shows that the addition of iron is able to delay the onset of photoinhibition and has an effect on the photoprotection of the microalgae. This is due to the increase in iron which forms part of the flavoprotein ferredoxin-NADP⁺ reductase which transports electrons between PSI and NADP⁺. This photoprotective recovery has been seen in natural

phytoplankton of the southern ocean and equatorial pacific regions where the resupply of iron has shown a significant increase in quantum efficiency values (Beardall et al., 2001).

Although the extent of nutrients on lipid production and biomass production is crucial for feasible biodiesel production, numerous other factors play a role in the feasibility of large scale microalgal biodiesel. The cost of freshwater compared to sea water has a financial implication when cultured at large scale. The salinity will have an influence on the biomass and lipid production and therefore the extent of this stress should be determined.

CHAPTER THREE

3 ACCLIMATION OF THE FRESHWATER *CHLORELLA* SP. TO A MARINE ENVIRONMENT

3.1 INTRODUCTION

Growing commercial microalgal cultures in saline open raceway ponds has the advantage of reducing the level of contamination from freshwater organisms. Contamination of raceway ponds can have huge financial implications while reducing the quality of the value added product. Furthermore, fresh water is a limiting resource in South Africa and is essential for the production of food crops. Growing microalgae at a large commercial scale for the sustainable production of biodiesel would directly compete with essential food crops for freshwater. Saline water is freely available for raceway ponds constructed on the coast, saving on the cost and utilization of a scarce resource. Cultivating microalgae in saline water is a physiologically energy intensive and may compromise the overall productivity.

Osmotic stress in microalgae has been found to: i) increase the rates of biopolymers, ii) lipid catabolism, iii) changes in the rates of energy metabolism, iv) changes in the membrane permeability with the interruption of ion homeostasis (Alyabyev et al., 2007). These osmoregulatory responses to salt stress are the key factors to cell survival. It is important to understand the combined effect of these factors to determine the tolerance and/or adaptive mechanism of microalgae strains to marine environments. Osmoregulation in microalgae has been exploited commercially to induce the synthesis of certain value added products such as β -carotene from *Dunaliella* sp. and astaxanthin from *Haematococcus* sp. (Francavilla et al., 2010).

Osmoregulation in microalgae is the active regulation of the osmotic pressure to maintain the homeostasis of water content; that is it keeps the cellular

cytoplasmic fluids from becoming too diluted or too concentrated. The adjustment to water stress or osmotic stress results from increased salinity levels in the growth medium. The movement of water from high water potential to low water potential will result in cell dehydration and place the microalgal cells under osmotic stress (Brodribb and Hill, 2000). The cells therefore have to counterbalance the rise in extracellular solutes by numerous species specific processes. By understanding the effect of osmotic stress, freshwater microalgae can be grown in saline media through the process of acclimation. Acclimation in this study was regarded as the temporary change in physio-chemical functioning of the microalgae to the external conditions. The photo-physiological parameters can be monitored during the acclimatization process by the variable chlorophyll *a* levels using a Pulse Amplitude Modulated (PAM) Fluorometry.

Pulse Amplitude Modulated (PAM) Fluorometry has become one of the most common, non-invasive and rapid techniques to measure the variability of chlorophyll fluorescence and photosynthetic performance both in terrestrial plants (Juneau et al., 2005) and microalgae (Baker, 2008, Schreiber et al., 2002, Hartig et al., 1998, Oxborough et al., 2000). The new dual-channel PAM chlorophyll fluorometer detects variability in photosynthetic activity in both photosystems I and II (Schreiber et al., 2002). The following PAM parameters are generally used to measure the physiological state of the organism: Relative Electron Transport Rate (rETR), Non Photochemical Quenching (NPQ), Photosynthetic efficiency (α) and Light Saturation (E_k). rETR is a measure of the rate of linear electron transport through photosystem II, which is correlated with the overall photosynthetic performance of the microalgae (Juneau et al., 2005). In microalgae, exposure to photosynthetically supersaturating light triggers the operation of energy dissipating processes that lowers the yield of chlorophyll *a* fluorescence and are thus generally termed as Non Photochemical Quenching (NPQ) processes (Masojidek et al., 2000). A high NPQ value has been found to be correlated with carotenoid interconversion into photo-protective pigments

under light stress (Kuster et al., 2004). The E_k value defines the onset of light saturation as is obtained from curve fitting models (Platt et al., 1980). It results from the ratio between the $rETR_{max}$ and the initial slope of the curve termed α . The E_k values may vary significantly from a rapid light curve and a conventional steady-state light curve (Serodio et al., 2005). E_k has also been found to be related to NPQ where NPQ dominates above the E_k saturation value (Henley, 1993). Photoinhibition is detected by the RLCs providing the threshold of irradiance a culture can tolerate and showing at which light intensities photo damage will occur (Schreiber et al., 2002).

The use of the PAM fluorometry has been widely used by ecologists to determine phytoplankton photosynthetic efficiency (Hartig et al., 1998, Gustavs et al., 2009, Petrou et al., 2008, Oxborough et al., 2000) and stressors which include temperature, irradiance (Eggert et al., 2007), salinity (Boussiba, 2000, Masojidek et al., 2000) and nutrient (Li et al., 2008, Chisti, 2007). However, the application of PAM fluorometry to monitor the process of acclimation has not been well established in the algal biotechnology industry. Microalgal strains can be genetically engineered to grow under specific conditions and produce maximum yields of the value added product. Alternatively, the microalgal strain can be physiologically acclimatized to specific conditions of growth. A previous study has shown that the *Chlorella* strain under study exhibited high cellular lipid yields (49 percent) under complete nutrient stress (White et al., 2011) and would be best suited for large scale production of biodiesel. The economic feasibility is however threatened by the cost of freshwater and possible contamination of an open raceway pond. The use of freely available marine water would reduce the overall production cost and the possibility of contamination of freshwater microalgae.

This study therefore investigates the use of PAM Fluorometry to monitor the acclimatization of a freshwater *Chlorella* sp. to variable salinity levels and to determine the optimum salinity for growth.

3.2 MATERIALS AND METHODS

3.2.1 Culture Conditions

The freshwater chlorophyte *Chlorella* sp. was cultured in aerated BG-11 growth media until mid-logarithmic growth phase was reached (\pm day 4) (Allen, 1973). Samples were incubated at an irradiance of $35 \mu\text{mol m}^{-2}\text{s}^{-1}$ with a 14:10 hour light: dark cycle and at a constant temperature of 25 °C.

3.2.2 Salinity Stress

Samples (100 ml) were cultured in salinities ranging from 5 ‰ to 40 ‰ salinity. BG-11 medium and sea water (no nutrients added) were used as positive and negative controls respectively. Sea water was filtered using a $0.22 \mu\text{m}$ filter (Whatman G/FF) and autoclaved (121 °C, 15 min) and was used as the base of the BG-11 media. Distilled water was added to the sea water to reduce the salinity (35 ‰) and natural sea salt was added to increase the salinity. The salinity was measured using a refractometer. Salinities were re-evaluated after the addition of the nutrient stock solutions and salt was added to obtain the required salinity. Samples were then cultured for 19 days at $35 \mu\text{mol m}^{-2}\text{s}^{-1}$ with a 14:10 light: dark cycle at a constant temperature of 25 °C. Physiological measurements (PAM Fluorometry) were recorded twice a week in triplicate.

3.2.3 PAM Fluorometry

Non invasive fluorescence measurements were obtained using a DUAL-PAM 100 Chlorophyll Fluorometer (Heinz Walz GmbH, Effeltrich, Germany). Light ($28 \mu\text{mol m}^{-2} \text{s}^{-1}$) and dark adapted samples were incubated for 15 minutes, at 25 °C. Aliquots of the culture (3 ml) was dispensed into a 10 mm quartz glass cuvette (10 X 10 x 40) containing a micro magnetic stirrer. The cuvette was then inserted into the PAM fluorometer and Rapid light curves (RLCs) were generated. RLCs provided detailed information on the saturation

characteristics of the electron transport chain as well as the overall photosynthetic performance exhibited by the microalgae (Ralph and Gademann, 2005). The RLCs were generated by applying a sequence of increasing actinic irradiance in 15 preset discrete increments ranging from 8 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ to 710 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Each actinic light incubation lasted for 10 s before a saturation pulse of blue light (0.6 s at 10 000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) was applied to determine the rETR at each irradiance level. A Dual PAM software (v 1.9) was used to record all data and to generate the RLCs (Baker, 2008). The Dual PAM software calculated rETR as:

$$\text{rETR} = F'q/F'm \times \text{PPFD} \quad (1)$$

Where $F'q = (F'm - F')$,

$F'q/F'm$ is termed the PS II operating efficiency and estimates the efficiency at which light absorbed by PS II is used. At a given photosynthetically active photon flux density (PPFD) this parameter provides an estimate of the quantum yield of linear electron flux through PS II (Baker, 2008).

NPQ is calculated and quantified by measuring the change in F_m to the final value F_m (ΔF percent). NPQ is calculated from the maximum fluorescence measured in the dark adapted sample and is the maximum fluorescence measured in the saturating light pulse (F_m').

$$\text{NPQ} = (F_m - F_m')/F_m' \quad (2)$$

The maximum photosynthetic efficiency can be determined from the Alpha (α) value which is the initial slope of the rapid light curve (RLC). Alpha (α) was determined from the model by Platt et al (1980).

The light saturation values (E_k) are obtained from the curve fitting model of Platt et al., 1980 a feature of the dual-PAM software. The value is determined from the interception point of the α value with the maximum photosynthetic rate which follows the equation:

$$E_k = \text{rETR}_{\text{max}}/\alpha \quad (3)$$

3.2.4 Chlorophyll a determination

Microalgal biomass was quantified by chlorophyll a (chl a) fluorescence, using a Turner Designs Trilogy Fluorometer. Chl a was extracted in 90 percent acetone at 4 °C for 24 hours (Parsons et al., 1984). A chl a standard (*Anacystis nidulans* - Sigma) was used to calibrate the fluorometer.

3.2.5 Statistics

Statistics were calculated by using SPSS version 11.5.1 for Windows. All values were logarithmically (\log_{10}) normalised to meet the requirements for parametric statistical tests. One way analyses of variance (ANOVA) were used to test for differences in data ($p < 0.05$) between the nutrient stressed cultures and after the introduction of the depleted nutrient. Tukey's post hoc tests were used to locate the differences in the significant results. Correlations were determined using Pearson's Correlation co-efficient.

3.3 RESULTS

Optimum cellular acclimatization of the freshwater *Chlorella* sp. occurred at 20 ‰, although physiological functioning was still evident up to 40 ‰. The acclimatization period varied between 8 to 12 days for the lower range of salinities. Acclimation was identified by a sustained reduction in the ETR_{max} and α values.

Chlorophyll *a* concentrations (Fig. 12) and growth rates (Fig. 13) showed the ability of the microalgal culture incubated at salinities under 25 ‰ to tolerate the effects of osmotic stress and continue biomass production. Microalgal cells cultured in the BG-11 medium (control) showed a growth rate of $1.8 \mu\text{g chl } a \text{ L}^{-1}\text{d}^{-1}$ and depicted a typical growth curve consisting of the lag phase, exponential phase and the stationary phase. The growth rates of cultures in salinities of 5 ‰ to 20 ‰ ($1.5 \mu\text{g chl } a \text{ L}^{-1}\text{d}^{-1}$ to $1.6 \mu\text{g chl } a \text{ L}^{-1}\text{d}^{-1}$) were not significantly different from those of the control ($p > 0.05$). The lower salinities showed greater chlorophyll *a* yields and the corresponding growth was also evident by the darker green colour change (Fig. 14). Cells incubated at 35 ‰ and 40 ‰ showed negative growth rates of $-0.5 \mu\text{g chl } a \text{ L}^{-1}\text{d}^{-1}$ and $-0.8 \mu\text{g chl } a \text{ L}^{-1}\text{d}^{-1}$, respectively. Cells cultured in pure sea water hindered growth over the 15 day period and the chlorophyll *a* levels declined at a rate of $0.4 \mu\text{g chl } a \text{ L}^{-1}\text{d}^{-1}$. It can also be noted that the cells incubated in sea water and the cells incubated at 35 ‰ follow the same trend. This is because sea water has an average salinity of 35 ‰; therefore this proves the authenticity of the method.

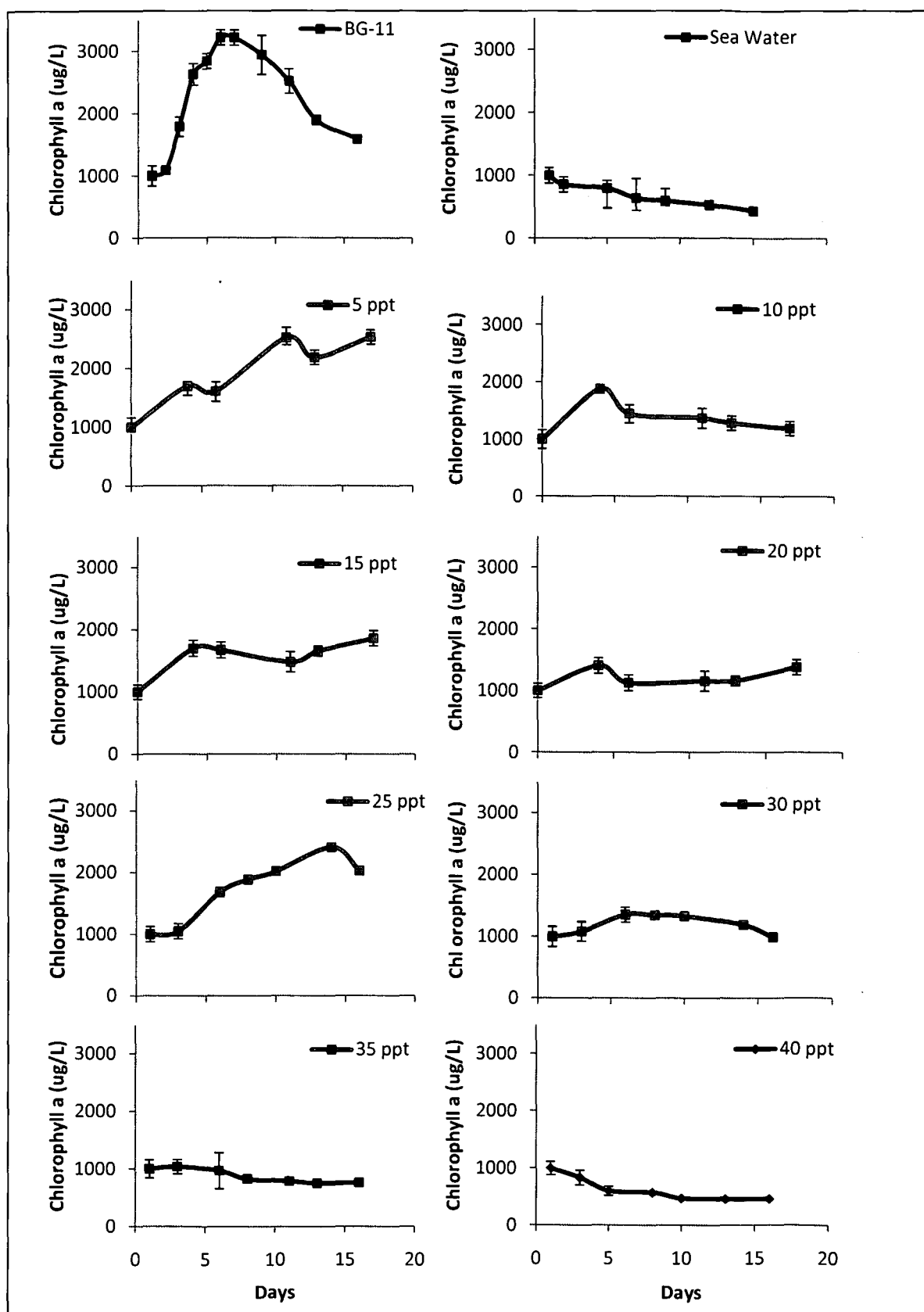


Figure 12: Chlorophyll a concentrations of *Chlorella* sp. cultured in BG-11 media at varying salinities

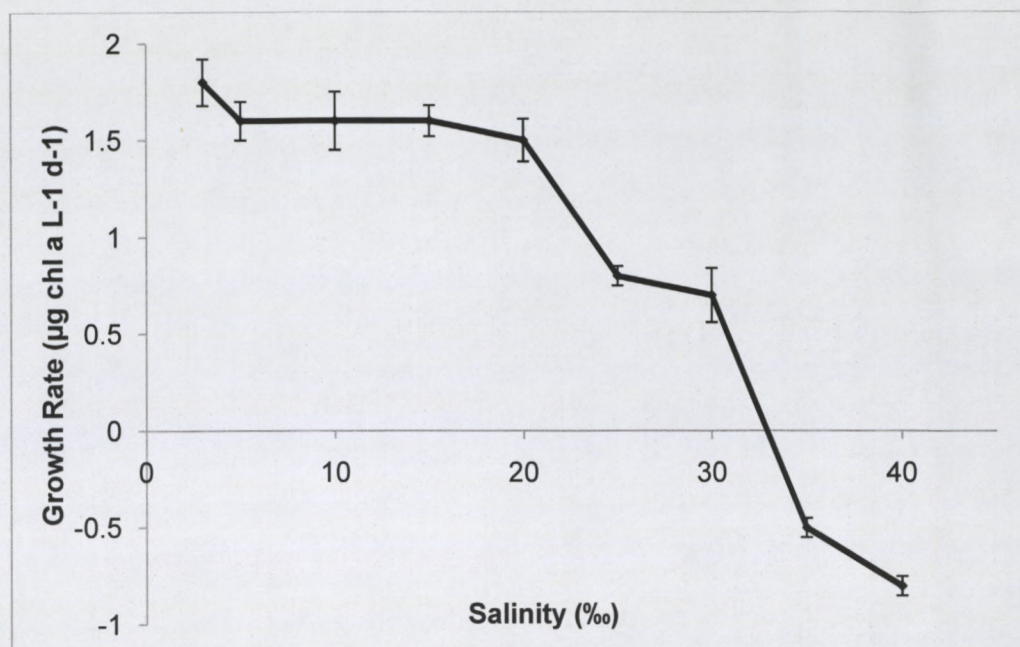


Figure 13: Growth Rates plotted against salinity for the *Chlorella* sp.

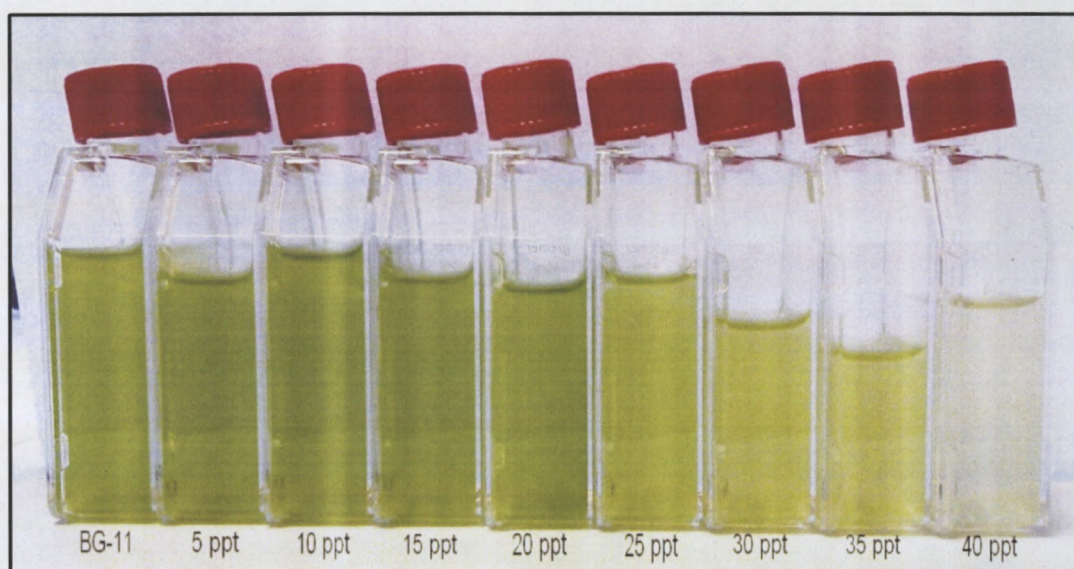


Figure 14: Culture vessels containing the *Chlorella* sp. cells cultured in BG-11 and varying salinities ranging from 5 ‰ to 40 ‰ at day 15.

Salinity was also found to have a significant impact on the physiology of *Chlorella* sp. during the acclimatization process as recorded by the physiological PAM parameters (rETR, E_k , NPQ and α).

A general trend emerged that rETR max values increased with increasing salinities. From 5 to 20 ‰, the rETR_{max} ranged from 14 to 24 during the acclimation period (days 1 – 8) and was significantly lower ($p < 0.05$) (6 to 12) following the acclimation (days 12 – 15). At salinities ranging from 25 to 35, rETR_{max} varied between 5 and 38, prior to acclimation (Table 3). Post acclimation, there was a marginal difference in rETR_{max} values (15 - 28). At 40 ‰ the highest value during the acclimation period (days 1 – 22) was 118 (Fig 15, Table 4). Subsequently, the highest from day 26 – 36 was 29 showing the reduced energy requirement needed to cope with the increased osmotic stress due to the acclimation. During days 1 to 8, no photosynthetic responses were detected in the 40 ‰ media, delaying the onset of acclimation by 11 days. The average rETR values (100) for cultures in 40 ‰ were significantly higher ($p < 0.05$) during the acclimation period compared to the lower salinity cultures (Fig. 15). The rETR, recorded in cultures incubated in natural sea water ranged from 12 to 20, generally showing a steady decrease over time (Table 5).

The photosynthetic efficiency of the culture (α) (Table 6, Fig. 16) was significantly correlated with salinities from 5 ‰ to 35 ‰. From 5 to 20 ‰, there were no significant differences in α compared to the control. The photosynthetic efficiency also showed no significant difference ($p > 0.05$) during and post acclimation. α significantly increased ($p < 0.05$) at salinities between 25 ‰ and 35 ‰ compared to the control and showed no significant difference ($p > 0.05$) during and post acclimation. At 40 ‰, α remained consistent (0.42) throughout the acclimation period and significantly decreased to 0.34 post acclimation ($p < 0.05$). Compared to the control culture, α was significantly higher in the 40‰ ($p < 0.05$). Sea water was constant.

Table 3: Acclimation parameters for the samples incubated from 5 ‰ to 40 ‰

Salinity (‰)	Acclimation parameters				
	Period (days)	rETRmax	α	$E_k (\mu\text{mol m}^{-2}\text{s}^{-1})$	NPQ
5	12	12 ± 2.5	0.28 ± 0.07	145 ± 16	1.83 ± 0.10
10	12	8 ± 1.4	0.32 ± 0.05	88 ± 8	1.46 ± 0.09
15	12	9 ± 1.2	0.30 ± 0.0	143 ± 14	1.60 ± 0.10
20	12	9 ± 1.4	0.30 ± 0.03	100 ± 8	1.52 ± 0.12
25	12	15 ± 2.9	0.32 ± 0.04	398 ± 12	0.62 ± 0.09
30	12	23 ± 2.8	0.34 ± 0.06	508 ± 25	0.94 ± 0.10
35	15	28 ± 3.1	0.37 ± 0.05	369 ± 38	4.10 ± 0.10
40	26	28 ± 2.1	0.38 ± 0.0	205 ± 24	1.69 ± 0.11

Table 4: PAM parameters for the *Chlorella* incubated at 40 ‰.

Day	rETR _{max} ($\mu\text{mol m}^{-2}\text{s}^{-1}$)	E _k ($\mu\text{mol m}^{-2}\text{s}^{-1}$)	α	NPQ
1	0	0	0	0
5	0	0	0	0
8	0	0	0	0
12	71±2.9	751±16	0.42±0.00	10±0.23
15	118±3.9	282±19	0.42±0.08	10<
19	93±2.7	223±15	0.42±0.02	10<
22	116±3.8	293±13	0.42±0.03	9.60±0.17
26	28±1.3	205±17	0.38±0.06	2.60±0.14
29	28±1.2	499±18	0.34±0.02	2.76±0.25
33	29±0.9	463±3	0.34±0.02	3.85±0.12
36	27±0.8	296±10	0.34±0.01	1.66±0.09

Table 5: Summary of the $rETR_{max}$ values recorded from Day 1 to Day 19 from cells incubated at salinities ranging from 5 ‰ to 40 ‰ with BG-11 and Sea Water serving as controls.

	$rETR_{max}$ values (relative units)									
	BG-11	5 ‰	10 ‰	15 ‰	20 ‰	25 ‰	30 ‰	35 ‰	40 ‰	Sea Water
Day 1	5 ± 1.2	21 ± 2.3	15 ± 2.6	8 ± 1.4	22 ± 2.7	17 ± 1.5	14 ± 2.9	5 ± 0.9	0	20 ± 2.8
Day 5	17 ± 2.3	20 ± 2.9	18 ± 3.1	14 ± 1.8	15 ± 2.1	22 ± 3.2	38 ± 4.8	30 ± 4.2	0	15 ± 2.6
Day 8	16 ± 2.6	24 ± 4.8	22 ± 3.7	20 ± 2.8	19 ± 2.7	34 ± 5.8	38 ± 5.3	24 ± 2.7	0	13 ± 2.9
Day 12	8 ± 1.7	12 ± 2.5	8 ± 1.4	9 ± 1.2	9 ± 1.4	20 ± 2.5	23 ± 3.2	29 ± 3.9	71 ± 2.9	13 ± 1.9
Day 15	7 ± 1.4	6 ± 1.9	6 ± 1.8	6 ± 1.5	7 ± 2.2	15 ± 2.9	23 ± 2.8	28 ± 3.1	118 ± 3.9	12 ± 2.4

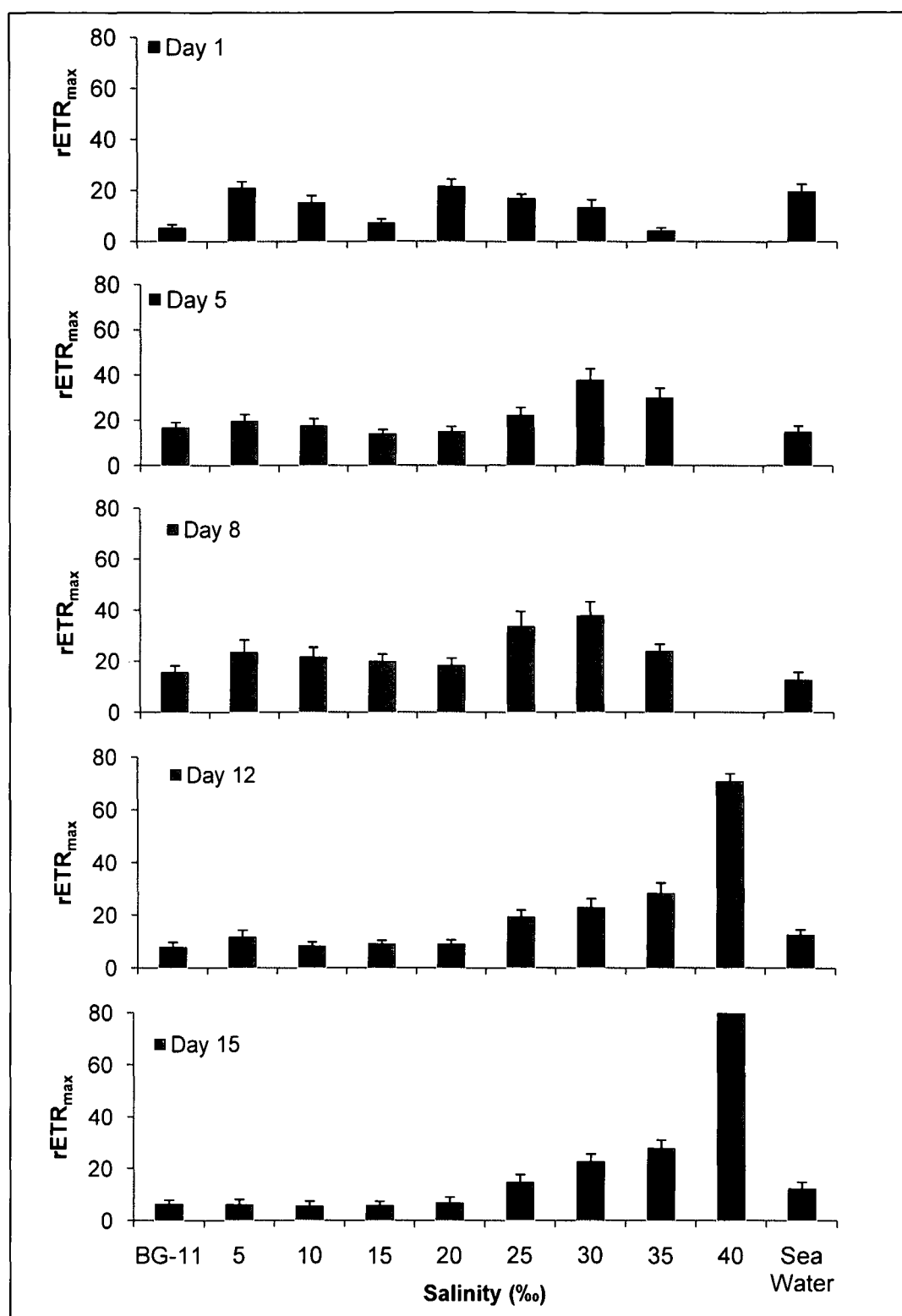


Figure 15: Graphs depicting the Relative Electron Transport Rate ($rETR_{max}$) of *Chlorella* cells cultured in BG-11 growth media at varying salinities over a 3 week period.

Table 6: Summary of the α values recorded at the various time frames in the *Chlorella* cells incubated at salinities ranging from 5 ‰ to 40 ‰ with controls.

	α values (relative units)									Sea Water
	BG-11	5 ‰	10 ‰	15 ‰	20 ‰	25 ‰	30 ‰	35 ‰	40 ‰	
Day 1	0.20±0.04	0.32±0.05	0.25±0.03	0.16±0.03	0.30±0.01	0.33±0.02	0.29±0.04	0.18±0.04	0	0.31±0.05
Day 5	0.30±0.02	0.27±0.01	0.31±0.05	0.27±0.03	0.31±0.02	0.34±0.04	0.33±0.01	0.33±0.01	0	0.31±0.03
Day 8	0.23±0.06	0.29±0.04	0.29±0.05	0.28±0.09	0.31±0.07	0.34±0.06	0.33±0.08	0.33±0.05	0	0.32±0.07
Day 12	0.23±0.06	0.28±0.07	0.32±0.05	0.30±0.00	0.30±0.03	0.32±0.04	0.34±0.06	0.35±0.01	0.42±0.00	0.32±0.07
Day 15	0.27±0.06	0.24±0.04	0.28±0.05	0.27±0.09	0.28±0.07	0.32±0.06	0.34±0.08	0.37±0.05	0.42±0.08	0.31±0.07

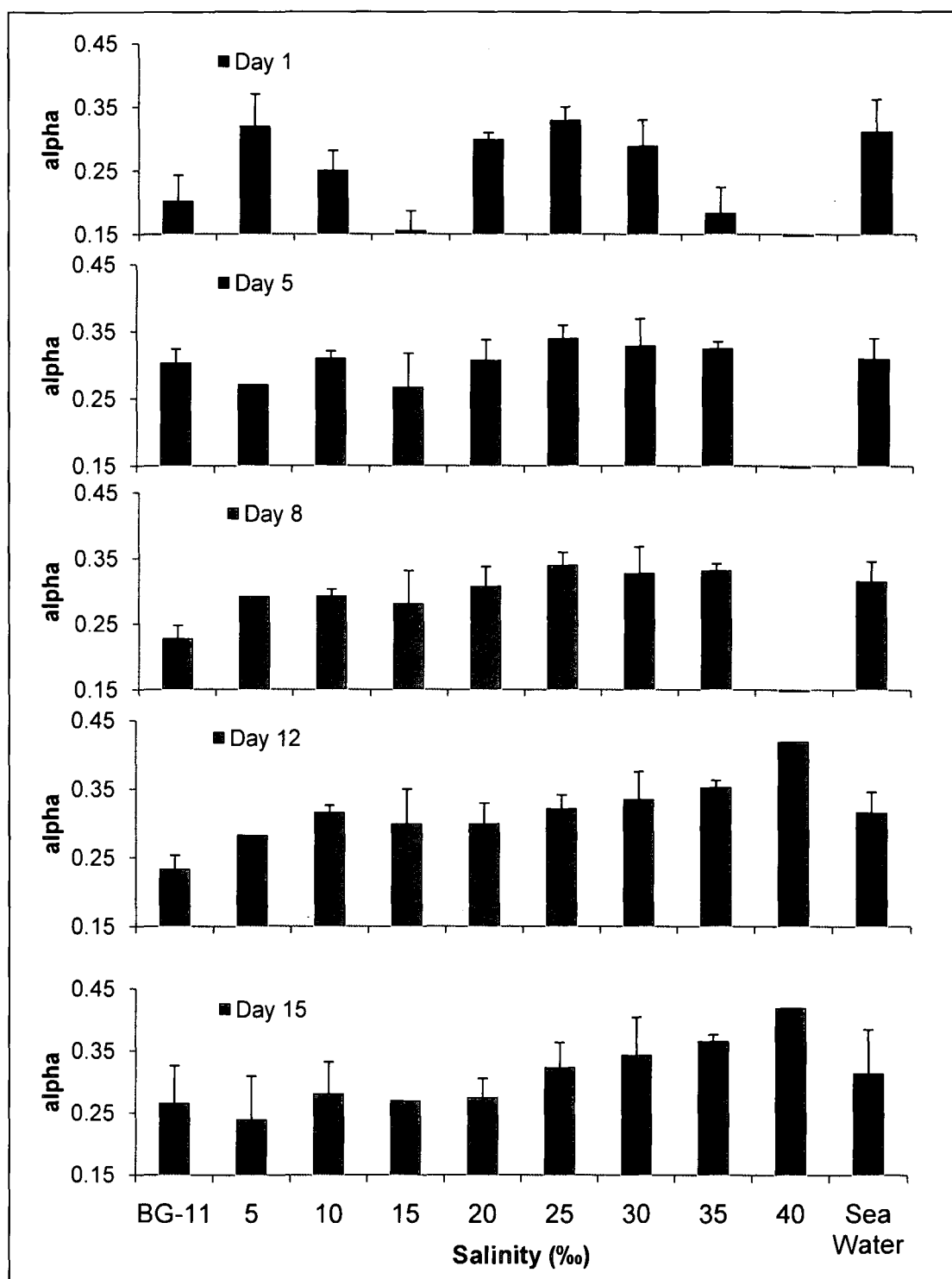


Figure 16: Graphs depicting the α values of *Chlorella* cells cultured in BG-11 growth media at varying salinities over a 3 week period

The light saturating point of photosynthesis, evident by the E_k value was directly correlated with the salinity ($r = 0.9$, $p < 0.05$). Samples incubated at salinities between 5 ‰ and 20 ‰ showed a significant increase in the E_k values on day 1 compared to the BG-11 control (Table 7, Fig. 17). No other significant differences were recorded until post acclimation where the E_k values decreased significantly ($p < 0.05$). In contrast to the lower salinities, the 25 ‰ to 35 ‰ samples recorded decreased initial E_k values. Thereafter the E_k values increased until the acclimation point. A significant reduction in the E_k value was recorded post acclimation ($p < 0.05$). Samples incubated at 40 ‰ recorded a significant decrease in the E_k value on day 12. The value then remained constant until the acclimation point at day 22 where it increased to an average of $450 \mu\text{mol m}^{-2}\text{s}^{-1}$. The samples incubated in natural sea water showed a steady reduction from $269 \mu\text{mol m}^{-2}\text{s}^{-1}$ to $170 \mu\text{mol m}^{-2}\text{s}^{-1}$ over the 15 day period.

The dissipation of excess energy, recorded by the NPQ value, showed a general trend of increased NPQ value with increased salinities (Table 8, Fig. 18). However, the opposite was evident on day 1 where NPQ was indirectly correlated to salinity ($r = -0.9$, $p < 0.05$). Salinities ranging from 5 ‰ to 20 ‰ showed increasing NPQ values during acclimation, however no significant differences ($p > 0.05$) were evident compared to the BG-11 control culture. After the acclimation process, a significant reduction was recorded in the NPQ to values between 0.8 and 1.0 ($p < 0.05$). Samples incubated at 25 ‰ and 35 ‰ showed significant increases in NPQ during acclimation with values ranging from 1.5 to 8.5. Post acclimation showed no significant change in the NPQ values ($p > 0.05$). Samples incubated at 40 ‰ showed maximum NPQ values of 10 from day 12 to day 19. This value decreased significantly ($p < 0.05$) after acclimation showing a decrease of 73 percent on day 26. Samples incubated in natural sea water showed a direct correlation with time ($r = 0.9$, $p < 0.05$).

Table 7: Summary of the E_k values recorded at the various time frames in *Chlorella* incubated at salinities ranging from 5 ‰ to 40 ‰ with controls.

	E_k ($\mu\text{mol m}^{-2}\text{s}^{-1}$)									
	BG-11	5 ‰	10 ‰	15 ‰	20 ‰	25 ‰	30 ‰	35 ‰	40 ‰	Sea Water
Day 1	123±23	368±32	293±21	272±27	513±10	271±13	120±17	61±9	0	269±24
Day 5	354±34	410±25	320±29	356±26	250±27	393±29	528±45	243±24	0	247±17
Day 8	268±27	309±28	269±26	308±38	237±25	568±49	575±46	229±26	0	173±20
Day 12	78±7	145±16	88±8	143±14	100±8	398±13	508±25	303±29	751±16	171±14
Day 15	76±6	67±8	66±9	66±5	96±8	172±10	400±28	369±38	282±19	170±18

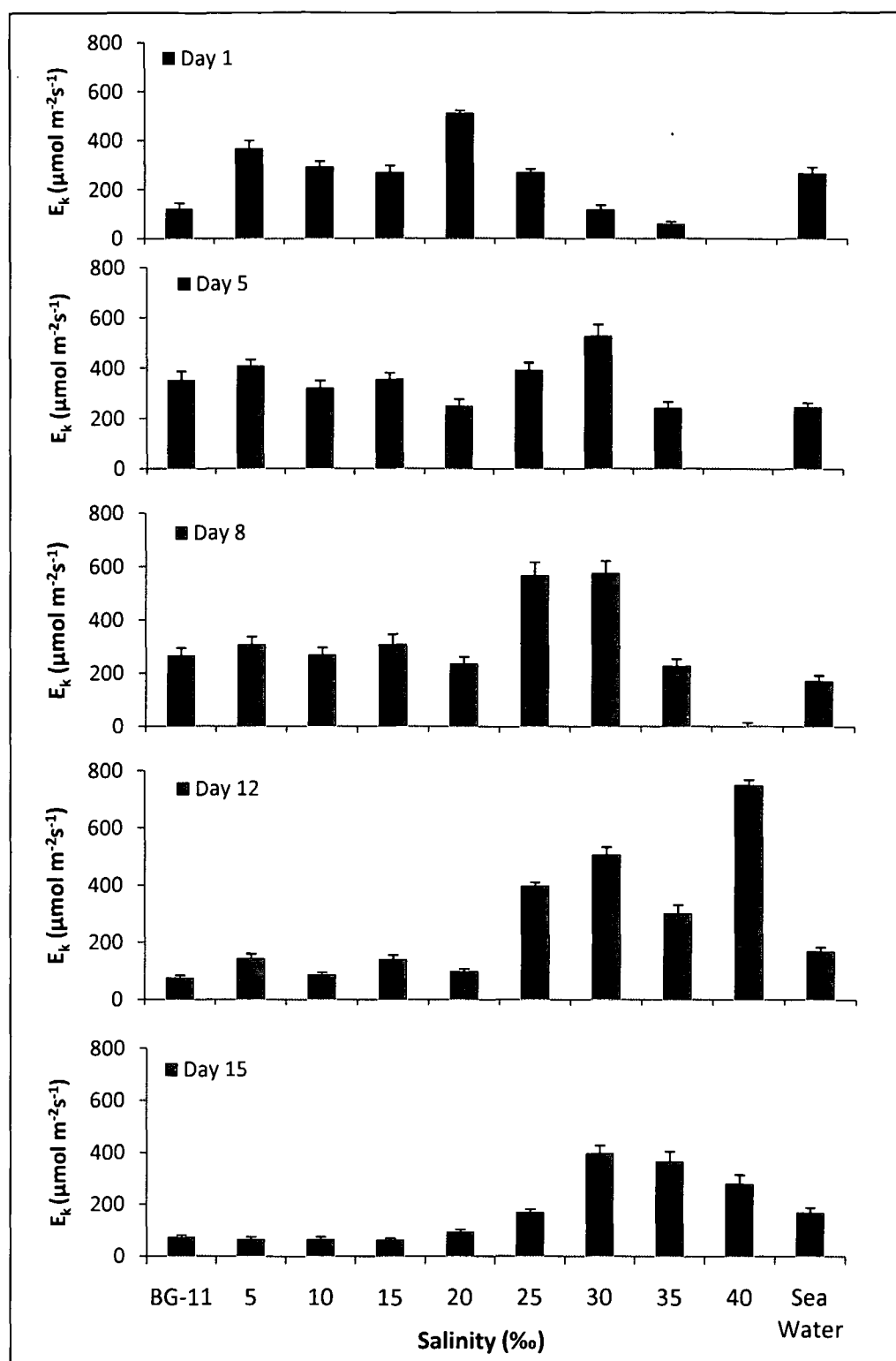


Figure 17: Graphs depicting the light saturating point (E_k) of *Chlorella* cells cultured in BG-11 growth media at varying salinities over a 3 week period.

Table 8: Summary of the NPQ values recorded at the various time frames in *Chlorella* incubated at salinities ranging from 5 ‰ to 40 ‰ with controls.

	NPQ (relative units)									Sea Water
	BG-11	5 ‰	10 ‰	15 ‰	20 ‰	25 ‰	30 ‰	35 ‰	40 ‰	
Day 1	1.40±0.7	1.80±0.03	1.94±0.04	0.90±0.01	1.77±0.19	1.12±0.03	0.92±0	0.20±0.01	0	1.08±0.01
Day 5	1.30±0.02	0.65±0.01	0.90±0.04	0.60±0.01	0.41±0.2	2.20±0.66	8.90±0.8	3.50±0.4	0	1.18±0.1
Day 8	1.90±0.09	2.27±0.16	2.05±0.11	1.40±0.09	1.65±0.09	2.05±0.1	5.10±0.2	3.20±0.09	0	1.57±0.07
Day 12	1.00±0.01	1.83±0.1	1.46±0.09	1.60±0.1	1.52±0.12	0.62±0.09	0.94±0.1	1.50±0.2	10±0.23	1.90±0.1
Day 15	0.80±0.06	0.80±0.08	0.90±0.09	0.80±0.1	1.00±0.9	2.60±0.06	5.10±0.1	4.10±0.1	10<	1.75±0.2

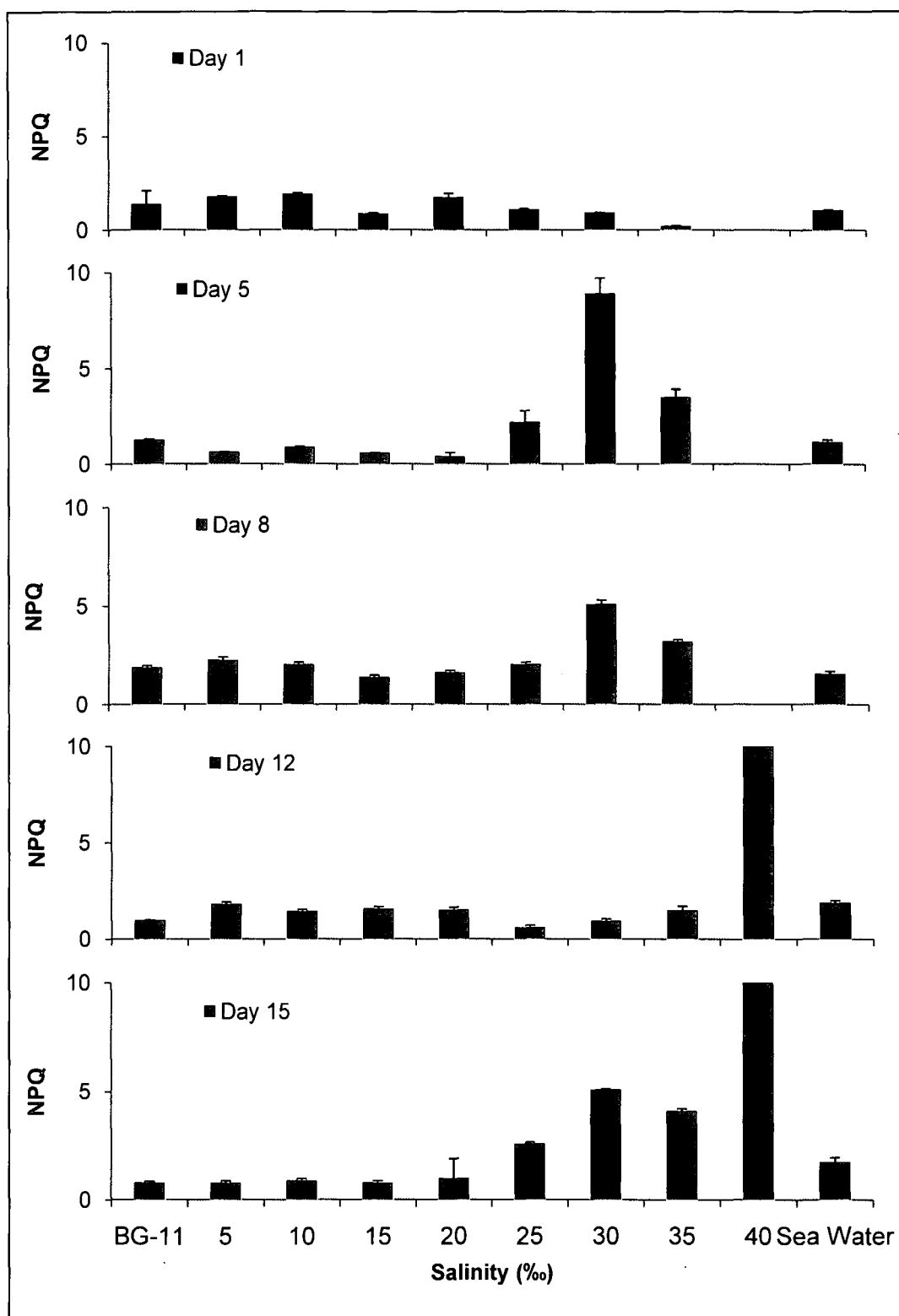


Figure 18: Graphs depicting the Non Photochemical Quenching (NPQ) of *Chlorella sp.* cells cultured in BG-11 growth media at varying salinities over a 3 week period.

3.4 DISCUSSION

Cellular acclimation was easily monitored by the variability of $rETR_{max}$, E_k , NPQ and the α values. In this study, the freshwater *Chlorella* sp. acclimatized to lower salinities (5 ‰ to 20 ‰) over a period of 12 days. However, the culture had taken longer (22 to 26 days) to acclimatize at higher salinities (25 ‰ to 40‰). The osmotic stress induced by the transition of the culture from a freshwater to a marine medium was recorded by the change in photosynthetic performance. Acclimation to osmotic stress was said to occur once a sustained reduction in the rETR was recorded after gradually increasing over 8 days (Table 4). The acclimatized culture showed similar rates of growth and photosynthetic performance to the control culture. From days 12 to 19 there was no significant difference in rETR ($p>0.05$) compared to the control, which suggested that the culture had acclimated to the lower saline media (Table 3).

The rETR may be best suited to monitor microalgal acclimation, as it is also the initial process in ATP synthesis. At lower salinities (5 ‰ to 20 ‰), $rETR_{max}$ increased proportionally during acclimation ($r = 0.9$, $p < 0.05$) (Table 3, Fig. 15). With increasing salinity, the energy demand also increases as more ATP is required in the energy intensive osmoregulation process (Dluzniewska et al., 2007, Jampeetong and Brix, 2009). At higher salinities rETR (25 ‰ to 35 ‰), the culture showed no sustained reduction in rETR in the given time period, showing the need for extra ATP yields. At 40 ‰, rETR showed a sustained reduction however, the overall $rETR_{max}$ value was significantly higher than the lower salinity samples (Table 4). A rise in the electron transport rate which increases the energetic rate has been previously found in *Chlorella* (Alyabyev et al., 2007). This ensures a quick and effective acclimation to salinity stress which may be caused by evaporation in raceway ponds.

An increase in the photosynthetic efficiency (α) was found to be directly correlated with increasing salinity ($r = 0.8$, $p < 0.05$) (Table 6). This shows that the overall photosynthetic functioning was increased for acclimation to occur. Although α shows an increase in photosynthesis, evidence of the increased energy requirement, it does not depict the time frame in which acclimation occurred. At lower salinities (5 ‰ to 20 ‰), increased α values were recorded showing an increase in the functioning of the photosynthetic apparatus. A further increase was evident in the samples at higher salinities (25 ‰ to 35‰) reiterating the increased need for extra ATP to counterbalance the osmotic stress. Salt tolerance seems to rely upon the inducible expression of an Na^+/H^+ antiporter (Tartari and Forlani, 2008). This allows for the compartmentalization or removal of excess Na^+ ions thereby reducing the osmotic pressure (Apse et al., 1999). Studies have found that maintaining the gene expression and regulation of this antiporter pump is crucial for the acclimation of plant cells especially microalgal cells to salinity changes (Dietz et al., 2001). This *Chlorella* strain shows the ability to acclimatize to increased salinities by the possible functioning of the antiporter.

At the lower salinities the overall light saturation value was not significantly increased compared to the BG-11 control value. At salinities between 25 ‰ and 40 ‰ there was a significant increase in the light saturating value ($p < 0.05$) (Fig. 17). This proportional increase in the E_k values with salinity indicates a greater ability to harvest incoming light energy from the light harvesting antennae (LHA). Previous research has found that salinity stress induces an increased cross sectional area of the PSII LHA (Masojidek et al., 2000). It can therefore be hypothesized that this greater area collects more electrons as well as the inactive PSII centres collecting extra electrons. This phenomenon has also been reported elsewhere in other microalgae species (*Chlorococcum* and *Scenedesmus*) under high irradiance (Masojidek et al., 1999). Another mechanism for the increased ability of the cells to withstand greater light intensities is the production of secondary pigments which aid in

photo protection of the photosynthetic apparatus. Photosynthetic organisms have evolved to develop photoadaptive and photoprotective mechanisms (Bjorkman and Demmig-Adams, 1994) which synthesise carotenoids in the xanthophyll cycle (Boussiba, 2000, Hagen et al., 1994). This biochemical change is thought to be the conversion of carotenoid pigments to various secondary forms to protect the microalgae (Brunet and Lavaud, 2010, Horton and Ruban, 2005). The common commercial producer of primary and secondary carotenoids is *Haematococcus* sp. however, *Chlorella* has also been found to produce these carotenoids (Rise et al., 1994). The transformation of these protective pigments will allow for greater incoming solar radiation which will increase the absorbed light thereby increasing the light saturating ability and hence, E_k value. Although the E_k value may allude to changes in the biochemical functioning of the xanthophyll cycle of the cell, it cannot pinpoint the exact acclimation period. ETR is still however, favoured as the dominant PAM parameter to distinguish between the acclimation and post acclimation phases (Table 4).

Irradiance stress increases energy yields which in turn increases the heat in the cell. This excess heat is dissipated and this process is reflected and recorded by the NPQ values. There was an indirect correlation between NPQ and salinity ($r = -0.9$, $p < 0.05$) (Fig. 18) showing that less energy was dissipated in the pre acclimation period. This implies that majority of the energy was needed for increased ATP synthesis for the pre acclimation phase. During the acclimation process NPQ values were significantly higher than the BG-11 control. Non photochemical quenching of chlorophyll fluorescence allows for the dissipation of excessively absorbed light energy. Several mechanisms may be involved such as the energy dissipation by the carotenoids, state-transitions, spill over of electron energy by the LHA and photo-inhibition-associated quenching (Krause and Weis, 1991, Horton et al., 2005). The increased requirement for extra energy to account for the osmotic imbalance may result in the activation of these processes during the

acclimation period. The loss of energy as heat, as recorded by the NPQ value, may give some insight into the acclimation period due to the sudden reduction in NPQ after acclimation. The cells incubated at 40 ‰ demonstrate this clearly by the significant reduction in NPQ from 9.6 to 2.6 after acclimation ($p < 0.05$). The samples at the lower salinities (5 ‰ to 25 ‰) also showed significant reductions in the NPQ after acclimation ($p < 0.05$). NPQ in conjunction with rETR has the potential to monitor of the acclimation process as well as the acclimation period of the microalgae cells.

Open ponds are limited by light intensity, salinity and contamination which influence the feasibility of the commercial microalgal production (Masojidek and Torzillo, 2007). Cultivation of this acclimated *Chlorella* sp. at 20 ‰ possesses numerous beneficial attributes to the commercial production of microalgae, particularly in raceway ponds. The increase in the E_k value of the acclimated *Chlorella* culture shows that more photosynthetically active radiation (PAR) will be able to be harvested in the cells. Increased salinity will limit the growth of protozoa contaminants. This will help in maintaining high productivity by ensuring a dominant microalgal species and limit the loss of nutrients to foreign organisms (Brennan and Owende, 2010). A high growth rate will also reduce the contamination risk by out-growing slower growers in the culture system (Griffiths and Harrison, 2009). A raceway pond with a salinity of 20 ‰ will save 57 percent of freshwater by substituting sea water. Sea water also contains numerous trace metals which will add extra nutrients to the raceway ponds increasing the productivity. The largest raceway pond-based microalgal production facility occupies an area of 440 000 m² with a depth of roughly 30 centimetres (Spolaore et al., 2006). This equates to the need of 132 000 m³ of water for the raceway pond. In a country experiencing water shortages, this would not be advantageous to the economy.

Generally α implies high photosynthetic function which is normally correlated with increased biomass productivity. Under salinity stress growth is compromised for the survival and acclimation to new environmental

conditions. This concept has limited numerous studies where growth rates were used to measure acclimation (Eggert et al., 2007). This method does not monitor the physiological changes during the acclimation process. Physiological changes are the first to occur, followed by biochemical changes and lastly changes in growth. Biochemical changes have also been used to determine acclimation (Eggert et al., 2007a, Eggert et al., 2007b) however, the initial physiological changes are still not monitored. The use of PAM fluorometry can reveal the initial changes thereby giving an advantage in determining the absolute duration and intensity of the acclimation process.

Chlorella sp. and other Chlorophytes have been shown to achieve stable photosynthetic efficiencies and productivities in raceway pond systems (Hase et al., 2000). By culturing this *Chlorella* strain in a raceway pond with a salinity of 20 ‰, the overall functioning of the pond will be enhanced, contamination will be reduced, photoinhibition will be reduced and productivity will be maintained if not increased. The aforementioned improvements in cultivation increases the desired product, thus reducing the cost of downstream processing per unit produced (Borowitzka, 1992). The feasibility of commercial biomass production will be increased thereby reducing the overall cost of microalgal biodiesel production.

Although the use of sea water in commercial raceway ponds has an impact on the feasibility and optimization of biomass production, temperature fluctuations in the ponds have shown a positive impact on the growth and lipid production and require investigation.

CHAPTER FOUR

4 EFFECT OF TEMPERATURE AND NUTRIENT STRESS ON MICROALGAL LIPID YIELDS

4.1 INTRODUCTION

Lipid classes have been studied in microalgae for many reasons namely, bio-indicators of the algae origin (Gerin and Goutx, 1993), aquaculture for larval feeding (Parrish and Wangersky, 1990) and biodiesel production (Chisti, 2007). Despite these wide applications for lipid studies, few reports are related to lipid classes in response to environmental factors (Tang et al., 2007). Environmental conditions such as light, nutrients, photoperiod and, in particular, temperature, induce greater variability in lipid composition (Gordillo et al., 1998). On average, temperature requirements of microalgae vary from cryophilic (0 - 15°C), to mesophilic (15 - 25°C), to thermophilic (30- 40°C). (Morgan-Kiss et al., 2002) Microalgal functioning responds instantaneously to changes in environmental temperatures.

South Africa and in particular the KwaZulu-Natal region has shown to have an attractive climate for algal production due to the high average temperatures and solar radiation levels. These high temperatures range from 25°C to 40°C ambient temperature, causing water temperatures roughly similar in shallow raceway ponds (Grobbelaar, 2009b). During winter, these temperatures can cool to around 15°C to 20°C inducing sub-optimal conditions for microalgal growth. Temperatures on the water surface may increase at a rate of up to 4°C per hour throughout the solar irradiation period (Morris and Kromkamp, 2003). The efficient production of biomass and lipids in raceway ponds is dependent upon location, as atmospheric temperature is the primary factor influencing productivity (Mutanda et al., 2011, Borowitzka, 1999, Harun et al., 2010). These preferences make pond positioning and strain selection very important. On the other hand, bioreactors require cooling systems to maintain optimum growth temperature. Any temperature induced

alterations on the microalgal physiology will cause changes in the fluorescence of the chlorophyll molecules which can be monitored by the use of Pulse Amplitude Modulated (PAM) fluorometry.

PAM fluorometry is able to measure fluorescence parameters obtained during steady-state electron transport and is frequently used to evaluate the photosynthetic efficiency of plants (Juneau et al., 2005). The new dual-channel PAM chlorophyll fluorometer detects variability in photosynthetic activity in organisms in both photosystems I and II (Schreiber et al., 2002). The following PAM parameters measure physiological health of the organism: electron transport rate (ETR), non photochemical quenching (NPQ), maximum quantum efficiency (F_v/F_m) and light saturation (E_k). ETR is a measure of the rate of linear electron transport through photosystem II, which is correlated with the overall photosynthetic efficiency of the microalgae (Fracheboud and Leipner, 2003). In microalgae, exposure to photosynthetically supersaturating light, triggers the operation of energy dissipating processes that lowers the yield of chlorophyll a fluorescence and are thus generally termed as non-photochemical quenching (NPQ) processes (Muller et al., 2001). A high NPQ value has been found to be correlated with carotenoid interconversion into photo-protective pigments under light stress (Kuster et al., 2004). Photoinhibition is detected by the light saturation (E_k) value and is the threshold of irradiance a culture can tolerate. The maximum quantum efficiency F_v/F_m is used to estimate nutrient limitation. Non-limited phytoplankton has F_v/F_m values from 0.6 to 0.7 (Kromkamp and Peene, 1999). The F_v/F_m value has been found to be relatively constant in non-stressed cultures, and to decrease in nutrient stressed cultures (Rohacek, 2002). PAM fluorometry has been widely used by ecologists to determine microalgal photosynthetic efficiency, (Gustavs et al., 2009) and stress (Berden-Zrimec et al., 2008). However, its application to monitor stress for the production of lipids and other value added products in the algal biotechnology industry has been poorly reported.

Microalgae require a favourable environmental temperature to grow (Grobbelaar, 2009c), as these processes are enzyme dependent. However, unfavourable temperatures may induce physiological stress, leading to the synthesis of neutral triacylglycerols. The synthesis and sequestration of triacylglycerols (TAGs) into the cellular neutral lipids can be detected by the dye Nile Red (9-diethylamino-5H-benzo(a)phenoxine-5-one) (Greenspan et al., 1985). PAM fluorometry can be used to monitor increases in lipid yields by identifying and measuring the extent of stress induced by temperature.

The objectives of this aspect of the study are therefore: 1) to investigate the use of PAM Fluorometry to monitor the effect of temperature stress on cellular neutral lipids; 2) to obtain the optimum temperature for maximum biomass yields and; 3) to investigate the effect of dual environmental stresses on biomass and lipid yields.

4.2 MATERIAL AND METHODS

4.2.1 Culture conditions

The freshwater algal *Chlorella* sp. was cultured in aerated BG-11 growth medium until mid-logarithmic growth phase was reached (day 4) (Allen, 1973). Samples were incubated at an irradiance of $35 \mu\text{mol m}^{-2}\text{s}^{-1}$ with a 14:10 hour light: dark cycle and at a constant temperature of 25 °C.

4.2.2 Temperature stress

Samples (100 ml) were incubated in BG-11 growth medium at temperatures ranging from 10 °C to 45 °C (at 5 °C intervals) for 30 minutes to acclimatise to temperature change. The temperature range is representative of the annual water temperatures experienced in a sub-tropical climate on the coast of KwaZulu-Natal. A water bath was connected to the incubation chamber of the DUAL-PAM 100 Fluorometer to maintain the constant specific temperature. Measurements of physiological parameters were undertaken in triplicate.

4.2.3 Dual stress

Dual stress was induced by incubation in dH₂O (complete nutrient stress from chapter 2) and subsequent incubation at temperatures ranging from 10 °C to 45 °C (at 5°C intervals) for 30 minutes to acclimatise to temperature change. A water bath was connected to the temperature controlled incubation chamber of the DUAL-PAM 100 Fluorometer to maintain the constant specific temperature. Measurements of physiological parameters were undertaken in triplicate.

4.2.4 PAM fluorometry

Non invasive fluorescence measurements were obtained using a DUAL-PAM 100 Chlorophyll Fluorometer (Heinz Walz GmbH, Effeltrich, Germany). Light ($28 \mu\text{mol m}^{-2} \text{s}^{-1}$) and dark adapted samples were incubated for 15 minutes, at 25 °C. Aliquots of the culture (3 ml) were dispensed into a 10 mm quartz glass cuvette (10 X 10 x 40) containing a micro magnetic stirrer. The cuvette was then inserted into the PAM fluorometer and Rapid light curves (RLCs) were generated. RLCs provided detailed information on the saturation characteristics of the electron transport chain as well as the overall photosynthetic performance exhibited by the microalgae (Ralph and Gademann, 2005) . The RLCs were generated by applying a sequence of increasing actinic irradiance in 15 preset discrete increments ranging from $8 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ to $710 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. Each actinic light incubation lasted for 10 s before a saturation pulse of blue light (0.6 s at $10\,000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) was applied to determine the rETR at each irradiance level. A Dual PAM software (v 1.9) was used to record all data and to generate the RLCs (Baker, 2008). The Dual PAM software calculated rETR as:

$$\text{rETR} = F'q/F'm \times \text{PPFD} \quad (1)$$

Where $F'q = (F'm - F')$,

$F'q/F'm$ is termed the PS II operating efficiency and estimates the efficiency at which light absorbed by PS II is used. At a given photosynthetically active photon flux density (PPFD) this parameter provides an estimate of the quantum yield of linear electron flux through PS II (Baker, 2008).

PSII quantum yield also known as maximum quantum efficiency of photosystem II according to the formula:

$$F_v/F_m = (F_m - F_o)/F_m \quad (2)$$

where F_m is maximum fluorescence and F_o is minimum fluorescence resulting in the variable fluorescence F_v .

NPQ is calculated and quantified by measuring the change in F_m to the final value F_m (ΔF percent). NPQ is calculated from the maximum fluorescence measured in the dark adapted sample and is the maximum fluorescence measured in the saturating light pulse (F_m').

$$NPQ = (F_m - F_m') / F_m' \quad (3)$$

The slope of the RLC is termed the alpha (α) value, and can illustrate the maximum photosynthetic efficiency. Alpha (α) was determined from the model by Platt et al (1980).

The saturation values (E_k) are obtained from the curve fitting model of Platt et al., 1980 a feature of the dual-PAM software. The value is determined from the interception point of the maximum photosynthetic rate with the α value which follows the equation:

$$E_k = rETR_{max} / \alpha \quad (4)$$

4.2.5 Nile Red staining

Cells were incubated in a Nile Red solution (0.25 mg/mL in acetone) for 5 minutes (Greenspan et al., 1985) and viewed under a fluorescent microscope (Zeiss Axioscope, Zeiss). A 450–490 nm excitation filter, a 510 nm diachronic mirror and a 515 nm barrier filter with 100 x objective lens were used to visualise the fluorescent yellow-gold lipid vacuoles in the microalgal cells. Blue light was used as the excitation light. Lipid to cell ratio was calculated using the Cell Profiler software (Lamprecht et al., 2007). This was achieved by converting all images to greyscale for pixel analysis. A threshold value was set manually to distinguish between the bright intensity of the lipid pixels and the remaining cell pixels. Pixel numbers were recorded and the ratio of 'lipid pixels' to 'cell pixels' was determined and converted to a percentage.

4.2.6 Temperature Stress for Lipid extraction

A total of eighteen litres of microalgal biomass was harvested through centrifugation from a 20L culturing vessel (500ml containers - 3000 rpm for 15 minutes) (Hermle Z400). The pellets were removed and resuspended in triplicate in 400ml dH₂O and BG-11 (control) (chlorophyll *a* = 1000µg/L). Each inoculated media was incubated at 10°C, 25°C and 40°C for seven days in a water bath. Light intensity was maintained at 35 µmol m⁻²s⁻¹ over the incubation period. After culturing, the biomass from each incubation vessel was completely dried at 60°C for lipid analysis.

4.2.7 Lipid extraction

Lipid extraction of the stressed cultures followed a modified lipid extraction method (Bligh and Dyer, 1959). Oven dried biomass was weighed (1g) into 100ml Schott bottles. A total of 80 ml solvent was added in the order chloroform, methanol, distilled water in the ratio 1:2:0.8 (v/v/v). The samples were allowed to incubate for 18 hours after which they were placed in separating funnels and solvents were added to make the ratio 1:1:0.9 chloroform: methanol: distilled water (v/v/v). The lower chloroform phase was collected and was transesterified into FAMES.

4.2.8 Transesterification

Transesterification followed a modified protocol (Lewis et al., 2000). The chloroform was allowed to evaporate from the lipid samples leaving crude microalgal oil. Ten millilitres of a fresh solution of methanol: HCl: chloroform in the ratio 10:1:1 (v/v/v) was added at 90°C for 60 minutes. Transesterification tubes were removed from the heat and allowed to cool. Distilled water (1ml) was added to each tube. The FAMES were extracted by the addition of 4 ml hexane: chloroform 4:1 (v/v) solution (Lewis et al., 2000).

4.2.9 Gas Chromatography Analysis

The samples were run on a GC/MS (Perkin Elmer) system equipped with a mass spectrometer detector. Separation was performed on a PE-5 (30m x 0.25mm x 1.0 μ m) with helium as the carrier gas (flow rate 1ml/min) with a linear velocity of 44cm/sec. The temperature settings were as follows: Injector 250°C, oven temperature 280 °C with an initial temperature of 50 °C with a ramp temperature of 10 °C per min till 280 °C. The characteristics of the identified FAMES were obtained from the Lipid Bank (<http://www.lipidbank.jp>) and used for further comparison.

4.2.10 Statistics

Statistics were calculated by using SPSS version 11.5.1 for Windows. All values were logarithmically (log₁₀) normalised to meet the requirements for parametric statistical tests. One way analyses of variance (ANOVA) were used to test for differences in data ($p < 0.05$) between the temperature stressed cultures and the lipid yields. Tukey's post hoc tests were used to locate the differences in the significant results. ANOVA was also used to determine significance in varying lipid yields at varying temperatures. Correlations were determined using Pearson's Correlation co-efficient.

4.3 RESULTS

The optimum temperature for maximum biomass production from this *Chlorella* isolate was 35°C. Temperature was found to significantly influence the FAME composition. In this study the PAM Fluorometer parameters (ETR, NPQ, F_v/F_m , E_k and α) recorded significant physiological changes with varying incubation temperatures ($p < 0.05$). Dual stress (temperature and nutrient stress) was also found to be more effective in inducing greater physiological stress than single stress alone (Table 9).

Table 9: The PAM results induced by dual stress, expressed as a percentage change of the BG-11 control (day of acclimation).

	Temperature (°C)							
	10	15	20	25	30	35	40	45
ETR _{max}	71±2.1	62±3.2	57±1.6	28±1.9	22±0.5	53±1.4	52±1.8	80±3.7
E_k	15±0.5*	32±1.6	6±1.3*	53±2.9*	33±1.8*	26±2.7*	45±3.2*	31±1.2*
NPQ	32±1.1*	10±1.5*	77±4.2*	48±2.1*	18±0.6	3±0.2	1*±0.1	26±1.3
F_v/F_m	64±1.7	55±1.8	51±1.2	37±0.6	43±2.2	48±2.7	51±2.4	66±3.7
α	56±2.1	40±2.0	66±1.3	52±1.7	68±3.7	70±4.2	83±4.2	77±4.2

*denotes increase compared to the control

The temperature response curves in Fig. 19 describe the relationship between temperature and electron transport rate. This was characterized in the temperature induced stressed sample by the steady rise in ETR with temperatures until the optimum temperature (T_{opt}) was reached. In the BG-11 control sample, the ETR_{max} values ranged from 6 at 10°C to a maximum value of 34 at 35°C. The ETR_{max} significantly correlated with the growth temperature until the optimum of 35°C (T_{opt}) was reached ($r = 0.7$; $p < 0.01$). Temperatures higher than 35 °C were found to induce photosynthetic stress by the reduction in ETR_{max} . The ETR_{max} significantly decreased by 15 percent and 68 percent at 40 and 45 °C, respectively. Photosynthetic stress was also induced in the lower temperature ranges with a decrease of 82 percent in the ETR_{max} value when incubated at 10°C.

Under dual stress, samples incubated in distilled water showed the greatest amount of photosynthetic stress due to the greatest reduction in the ETR_{max} values (Table 9, Fig. 19). Cultures incubated at 40°C showed a 52 percent decrease in ETR_{max} value and a decrease of 71 percent was evident in at 10°C. The T_{opt} was reduced to 30 °C compared to the BG-11 control of 35°C showing a greater induced stress by the use of distilled water. The least difference in ETR_{max} values between the single stress and dual stress samples was evident between 25°C and 30°C. This shows that the effect of dual stress was greater at the temperature extremes (10°C and 45°C).

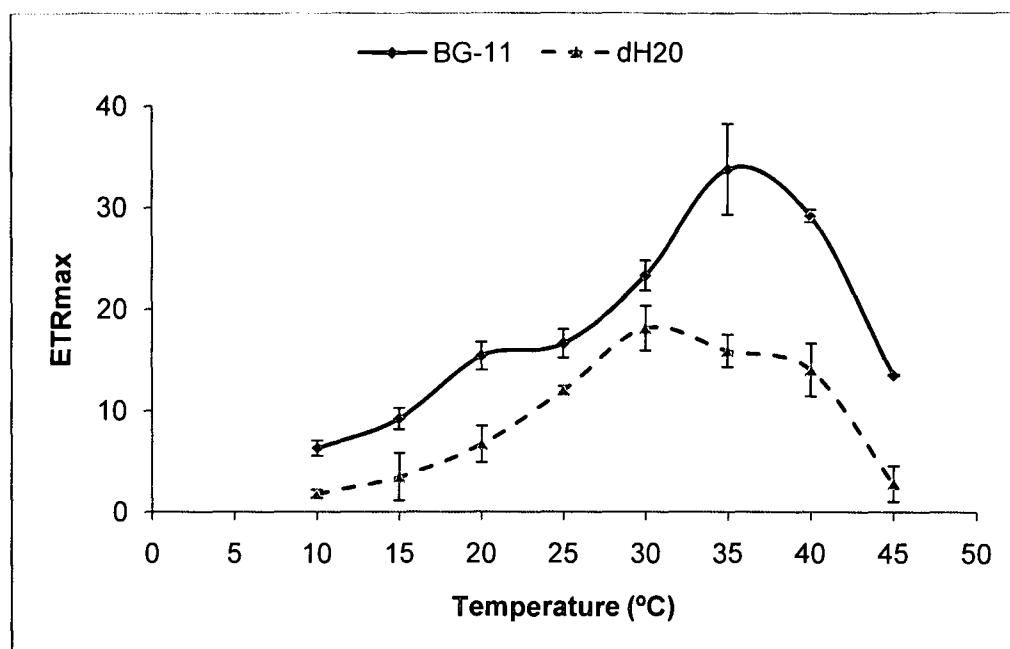


Figure 19: Temperature response curves depicting the relationship between the maximum electron transport rate (ETR_{max}) and the respective incubation temperatures (10°C to 45°C).

Photosynthetic stress was also evident by the non photochemical quenching (NPQ) values (Fig. 20). NPQ values show the dissipation of heat at varying temperatures. In the BG-11 control samples, NPQ values were found to be directly correlated with an increase in temperature ($r = 0.9$; $p < 0.01$). The highest NPQ value of 0.63 was measured in cultures incubated at 45°C, whereas the lowest value of 0.1 was present in cultures incubated at 10°C (Fig. 20). There was no significant change in recorded NPQ values between 10°C to 20°C.

Cultures subjected to dual stress illustrated a greater dissipation of heat in the NPQ values compared to the control. A significant increase in NPQ was evident between 20 °C and 25 °C compared to the BG-11 control ($p < 0.05$). At higher temperatures no significant changes were evident compared to the BG-11 control.

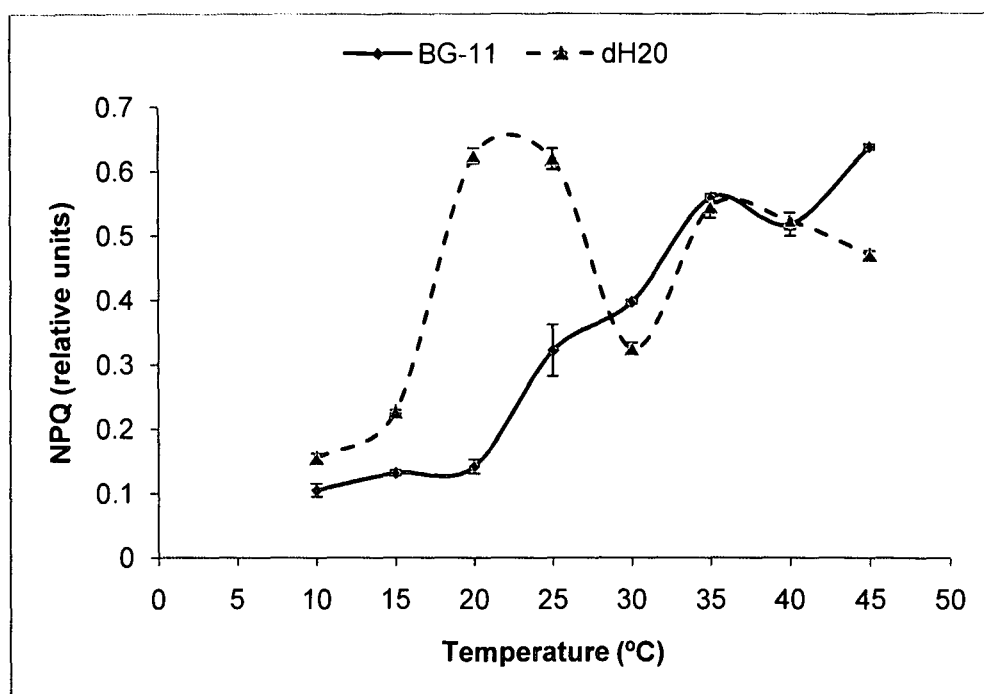


Figure 20: Non photochemical quenching (NPQ) values of *Chlorella* samples incubated at temperatures ranging from 10°C to 45°C.

In the control culture (BG-11), the highest E_k value of $422 \mu\text{mol m}^{-2}\text{s}^{-1}$ was identified in the sample incubated at 35 °C (Fig. 21). The E_k values were found to be directly correlated with temperature until the maximum E_k value at the T_{opt} ($r = 0.8$, $p < 0.05$). Possible photosynthetic stress was evident at the high temperatures by the decrease of 25 percent and 43 percent in the E_k value at 40°C and 45°C, respectively (Fig. 21). Colder incubation temperatures (10°C) also induced photosynthetic stress, evident by the decrease of 85 percent in the E_k value.

Dual stress affected the E_k values allowing for greater light saturation values. Samples incubated under complete nutrient stress (dH₂O) showed significant increases in the maximum E_k value recorded at $572 \mu\text{mol m}^{-2}\text{s}^{-1}$ between 35°C and 40°C ($p < 0.05$). Higher temperatures (45°C) decreased the light saturating ability of the samples however these value were still significantly

higher than the BG-11 control samples ($p < 0.05$). At cooler temperatures (10°C to 20°C) the E_k values were not significantly different from the control, however they did show a marked reduction of 33 percent.

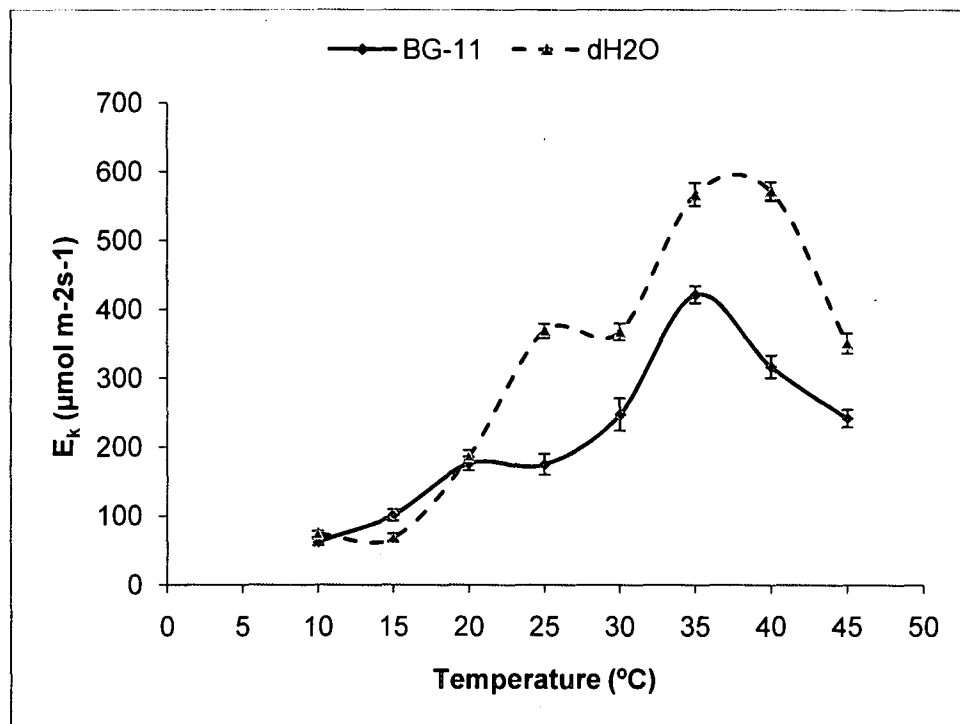


Figure 21: The E_k value or light saturation point obtained from the ETR_{max} value on the rapid light curve of cultures incubated at temperature ranging from 10 °C to 45 °C.

The quantum efficiency values (F_v/F_m) are generally used to indicate nutrient stress. In this study the F_v/F_m values were found to range from 0.64 to 0.74 (Fig. 22) in the BG-11 culture. The values were found to have no significant changes amongst the range of temperatures measured ($p > 0.05$).

Nutrient stress was evident in the dual stress samples by the decrease in the F_v/F_m value of 37 percent in the distilled water sample (Table 9). The effect of temperature and hence dual stress was found to enhance the total stress,

evident by the slight decrease in the F_v/F_m at the extreme temperature ranges (10°C and 45°C) (Fig. 22). The lack of significant change in the recorded values shows that temperature has no direct effect on the F_v/F_m values and cannot be used alone as an indicator of temperature stress. It does however show nutrient stress very effectively when complete stress is compared to the positive control.

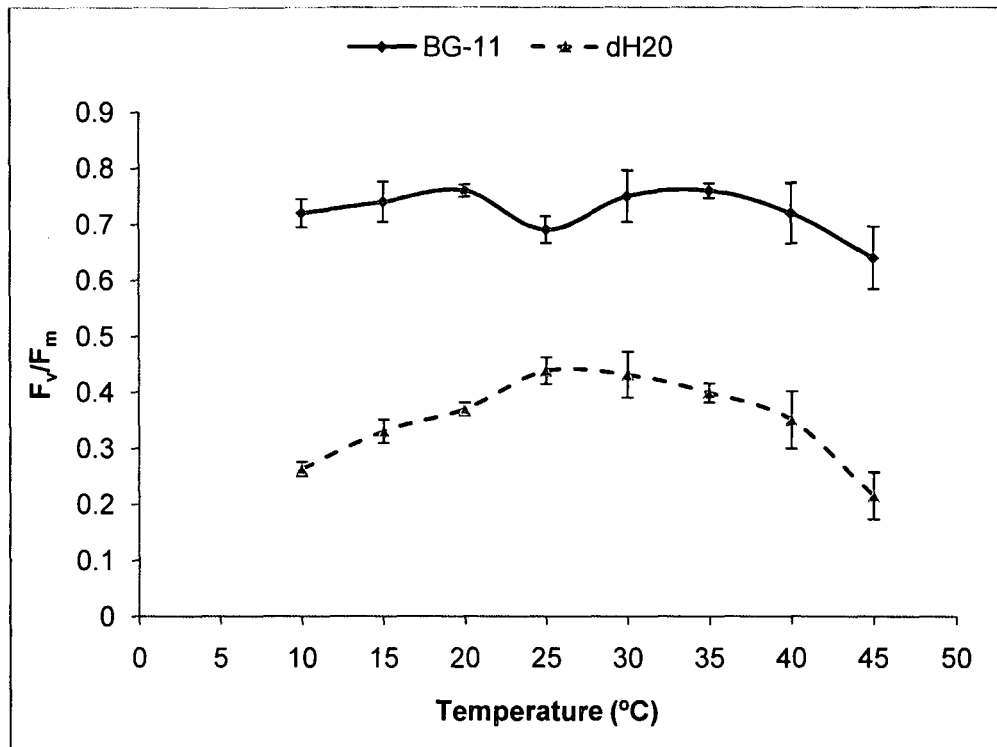


Figure 22: The quantum efficiency value (F_v/F_m) of the cultures incubated at temperatures ranging from 10 °C to 45 °C.

α values were found to have a significant increase in the samples incubated at temperatures ranging from 20°C to 40°C ($p < 0.05$). The highest α^{ETR} value (0.28) was recorded at samples incubated at 20°C (Fig. 23). A reduction in photosynthetic efficiency was evident in samples incubated at both cooler temperatures (10°C, 15°C) and warmer temperatures (45°C). This was evident by a decrease in the α value of 51 percent at 10°C and a 41percent

decrease at 45°C (Fig. 23). The greater α value denotes greater photosynthetic efficiency therefore higher temperatures (25°C – 35°C) are optimal for microalgal growth. The constant α value between 25°C and 40°C shows this is a optimal functioning temperautre and eludes to the acclimation of the isolate to warmer temperautre indicative of a sub tropical climate. The effect of complete nutrient stress in conjunction with temperature stress increased the T_{opt} where the maximum α value was recorded to be 25°C. Maximum stress was recorded at extreme temperatures (35°C - 45°C) with reductions of 70 to 83 percent compared to the BG-11 control.

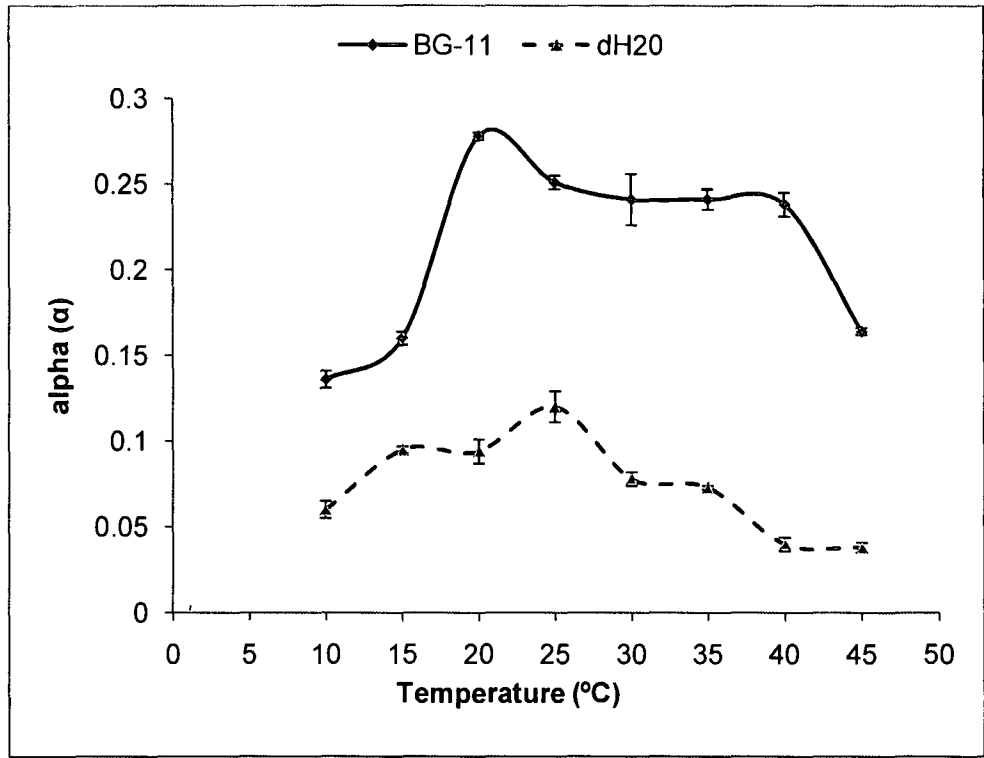


Figure 23: The α values depicting the photosynthtic efficiency of the cultures incubated at temperatures ranging from 10 °C to 45 °C.

Stressed cultures, evident from the physiological measurements recorded from the PAM fluorometry, were stained with Nile Red to determine the presence of neutral lipids. Stress was evident from low ETR values,

increased NPQ values and low α value recorded in the cultures. From the results, 10°C and 40°C showed the most stress and were used for further studies. Although 45°C showed greater stress, this temperature is too high for effective enzymatic functioning and was not used for this reason. Previous studies have shown that 10°C and 40°C should not denature the enzymes critical for neutral lipid synthesis due to the indigenous nature of the isolate (Nedeva and Pouneva, 2009).

The temperature stress induced the synthesis of neutral lipids which was visible using Nile Red staining (Fig. 24). *Chlorella* cells incubated in BG-11 at pre-determined temperatures of 10°C and 40°C showed significant difference in the amount of neutral lipids compared to the control temperature of 25°C ($p < 0.05$). This shows that temperature has the potential to cause stress that will affect cellular neutral lipid yields. There was a 32 percent increase in lipid accumulation in the BG-11 culture incubated at 40°C and a 25 percent increase in the lipid yields incubated at 10°C.

Single stress factors (nutrients and temperature independently) have been found to successfully increase the amount of neutral lipids present in *Chlorella sp.* cells. The effect of combining stress factors was employed to determine the effect of two different stresses namely nutrients and temperature. *Chlorella* cells incubated under complete nutrient stress (i.e. distilled water) showed a significant increase in neutral lipid yields to 55 percent when incubated at 40°C (Fig. 24). Cells incubated at 10°C showed a significant decrease in lipid yields to 12 percent as well as a decrease in cell viability.

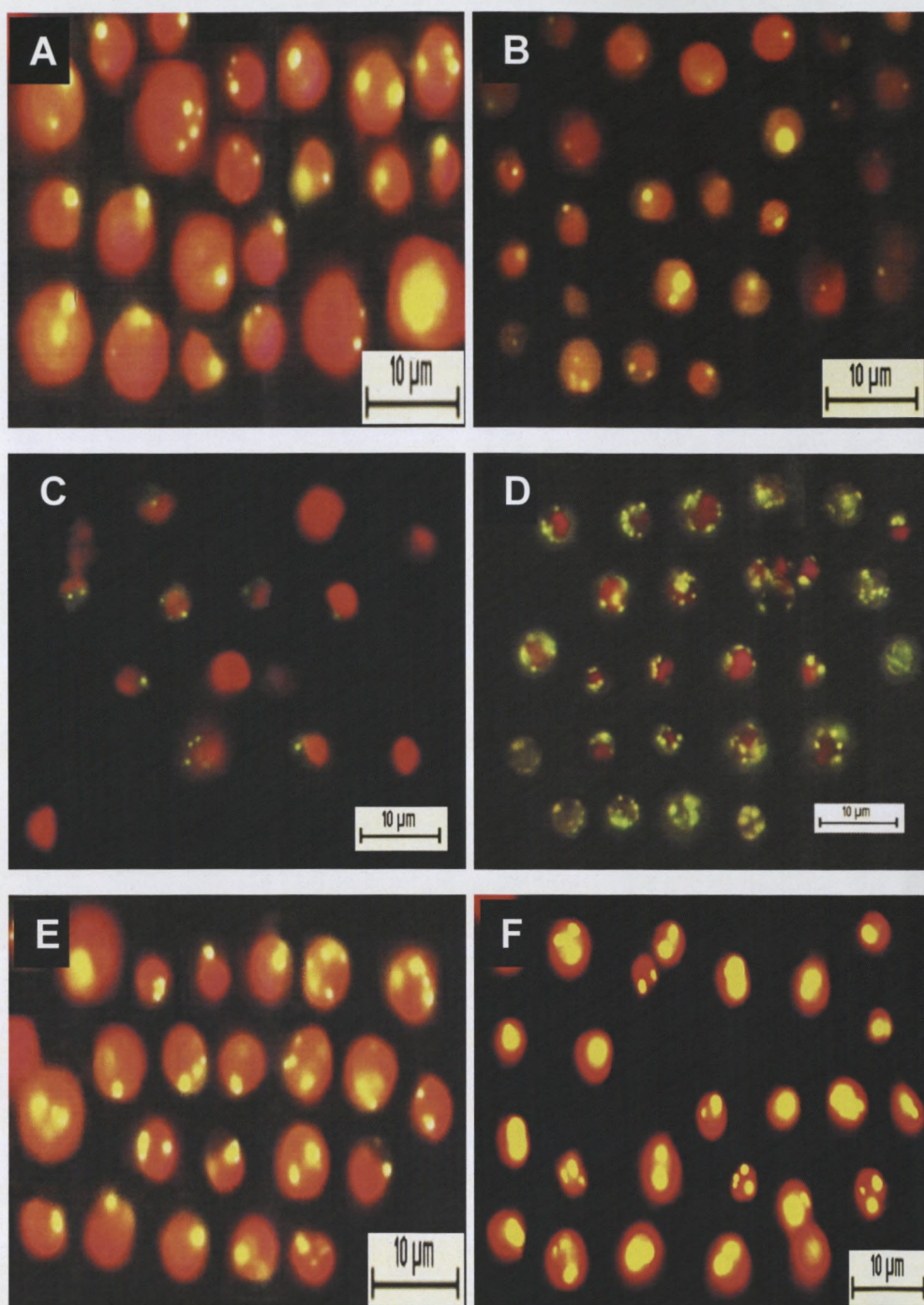


Figure 24: Composite images of cultures stained with Nile Red showing cellular neutral lipids of the *Chlorella* cells cultured in BG-11 at 10°C (A), dH₂O at 10°C (B), BG-11 at 25°C (C), dH₂O at 25°C (D), BG-11 at 40°C (E) and dH₂O at 40°C (F).

The extracted lipids were transesterified into FAMES or biodiesel resulting in the separation of fatty acids from the glycerol. The FAMES can be seen in Fig. 25 in the upper yellow phase layer and the glycerol in the brown/dark brown lower phase layer. The FAMES were then analysed by GC/MS to determine the composition.

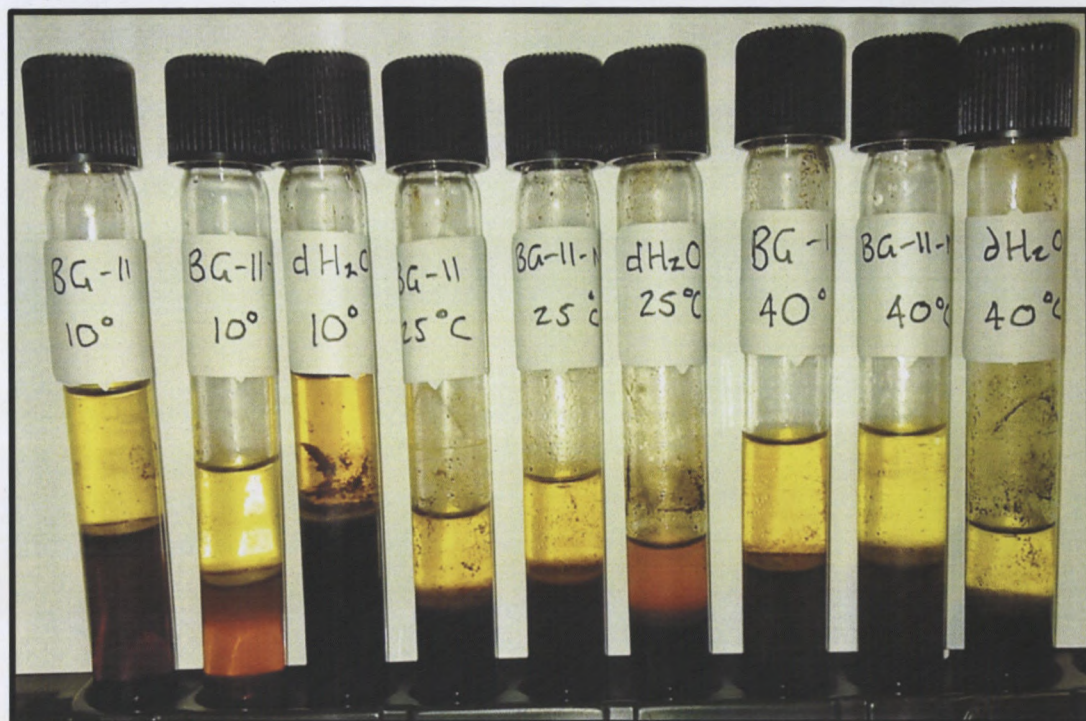


Figure 25: Transesterified microalgal biodiesel showing FAMES in the upper yellow section and glycerol in the lower darker section.

Each sample revealed a chromatogram showing the peaks of the specific FAMES. A chromatogram example can be seen in Fig. 26. Peak areas were calculated to determine percentage composition of the various FAMES present in each sample.

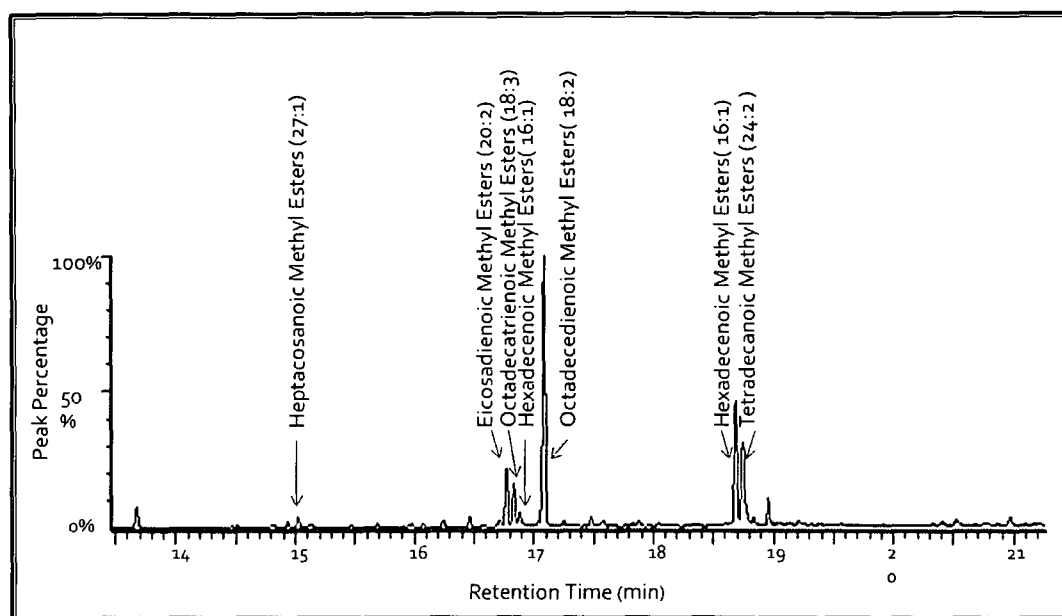


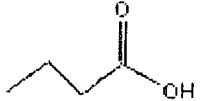
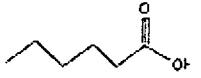
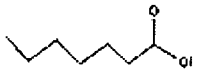
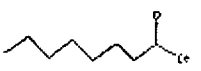
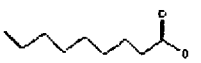


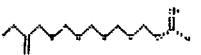
Figure 26: A sample chromatogram showing the peaks and peak areas of the FAMES present in the microalgal biodiesel


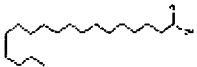
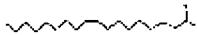
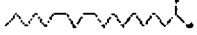

The samples cultured under single temperature stress showed a change in the number of FAMES present at the varying temperatures (Table 10). Thirteen FAMES were identified in the analysed chromatograms. Ten FAMES were present at both 10°C and 25 °C in the BG-11 control sample which was reduced to seven FAMES at 40°C. Temperature extremes induced the formation of Butanoic acid methyl esters. Most of the lower chain FAMES (6:0, 7:0, 8:0, 14:0 and 14:1) were absent at 40°C. The C₁₆ and C₁₈ FAMES which are used for viable biodiesel production were present at all of the incubated temperatures. When dual stress was employed the number of identified FAMES was decreased from nine to seven at both 10°C and 40°C. The 6:0 and 14:0 FAMES were found to be absent at 10°C. 4:0, 10:0 and 14:1 were totally absent from the complete nutrient stress sample under all temperatures monitored. Eicosanoic acid methyl esters were induced at 25°C which were not present in any other samples. The FAMES used primarily for biodiesel production were all present except for 18:0 at 40°C probably due to increased unsaturation with temperature decreases.

Table 10: Total lipid compositions and the composition of the fatty acid methyl esters in the single stress (temperature) and dual stress (nutrient and temperature)

Shorthand	Systematic Name	Trivial Name	10°C		25°C		40°C	
			BG-11	dH ₂ O	BG-11	dH ₂ O	BG-11	dH ₂ O
Total lipid composition (percent)			20	12	15	49	22	55
4:0	Butanoic acid methyl ester	Butyric	x				x	
6:0	Hexanoic acid methyl ester	Caproic	x		x	x		x
7:0	Heptanoic acid methyl ester	Heptylic	x	x	x	x		x
8:0	Octanoic acid methyl ester	Caprylic	x	x	x	x	x	x
9:0	Nonanoic acid methyl ester	Pelargonic	x	x	x			
10:0	Decanoic acid methyl ester	Capric			x		x	
14:0	Methyl tetradecanoate	Myristic	x			x		x
14:1	Methyl Z-11-tetradecanoate	Methylmeristic			x			
16:0	Hexadecanoic acid methyl ester	Palmitic	x	x	x	x	x	x
18:0	Octadecenoic acid methyl ester	Stearic	x	x	x	x	x	
18:1	9-Octandecenoic acid methyl ester	Oleic	x	x	x	x	x	x
18:2	9,12-Octadecadienoic ester	Linoleic	x	x	x	x	x	x
20:0	Eicosanoic acid methyl ester	Arachidic				x		

Table 11: Characteristics of the fatty acid methyl esters identified in the stressed microalgal samples

Trivial Name	Chemical Structure	Formula	Mol Wt	Melting point (°C)	Boiling point (°C)	Refractive Index (at 20°C)	Density (at 20°C)
Butyric		C ₄ H ₈ O ₂	88.105	-7.9	163.5	1.339	0.958
Caproic		C ₆ H ₁₂ O ₂	116.158	-3.4	205.8	1.416	0.929
Heptylic		C ₇ H ₁₄ O ₂	130.185	-10.5	223.0	1.423	0.922
Caprylic		C ₈ H ₁₆ O ₂	144.210	16.7	239.7	1.428	0.910
Pelargonic		C ₉ H ₁₈ O ₂	158.235	12.5	255.6	1.432	0.907
Capric		C ₁₀ H ₂₀ O ₂	172.265	31.6	270.0	1.428	0.885
Myristic		C ₁₄ H ₂₈ O ₂	228.371	53.9	250.0	1.427	0.862
Methylmyristic		C ₁₅ H ₃₀ O ₂	242.398	25.8	-	0.432	-

Palmitic		$C_{16}H_{32}O_2$	256.424	63.1	268.0	1.430	0.848
Stearic		$C_{18}H_{36}O_2$	284.447	69.6	213.0	1.433	0.839
Oleic		$C_{18}H_{34}O_2$	282.461	16	234.0	1.458	0.898
Linoleic		$C_{18}H_{32}O_2$	280.445	-5.0	229.0	1.471	0.903
Arachidic		$C_{20}H_{40}O_2$	312.520	77.0	204.0	1.425	0.824

The characteristics of the identified FAMES are present in Table 11. These show that the variation of FAMES and FAME quantities will affect the chemical quality of the microalgal biodiesel. It can be seen that the melting points and boiling points increase as the carbon length increases. A variation in this trend results when double bonds are present in the chain. The refractive indices increase as the molecular weight and carbon chain length increase. The density was found to decrease as the chain length increases. These values are important to determine the chemical characteristics of the biodiesel making it suitable to comply with American Society of Testing and Materials (ASTM) biodiesel standard requirements. It is therefore important to determine FAME compositions for biodiesel quality control.

4.4 DISCUSSION

In this study, temperature has been shown to induce physiological stress in microalgae and subsequently synthesize neutral triacylglycerols. Temperature was also shown to affect the FAMES composition when applied at extreme temperatures of 10°C and 40°C. Dual stress was found to have a greater increase on the stress recorded by PAM Fluorometry than temperature alone. Changes in the FAME compositions were also induced by the use of dual stress, however, a minimal effect on the FAME necessary for biodiesel production was found. This study also revealed that temperature was a very important abiotic factor for the optimization of biomass, important for feasible biodiesel production (Fig. 24).

The effect of short term temperature stress has been shown to affect the physiological parameters recorded by PAM Fluorometry. The correlation between ETR_{max} and temperature is critical for increasing biomass yields. Incubation at 35°C increased the ETR_{max} by 100 percent compared to control (25°C), and a 270 percent increase from the 15°C culture (Fig. 19). Previous studies (Blanchard et al., 1996) have modelled the relationship between primary production and ETR. It was found that an increase of only 10 degrees could potentially increase primary production by up to 40 percent. This shows that the optimization of temperature is crucial for biomass production. Another study (Zhang et al., 1999) found an increase of 50 percent in productivity when the temperature was increased from 20°C to 37°C.

Greater productivity has been reported for high temperature strains in comparison to lower temperature strains (Sheehan et al., 1998). This is because of the ability of the former to utilize higher temperatures and irradiance levels as indicated by their higher light saturating values for growth and photosynthesis. This was evident with the greater E_k (Fig. 21) and increased ETR_{max} recorded from cultures incubated at higher temperatures.

Greater light absorption results in more energy available for biomass production instead of mechanisms to protect from cooler temperatures. Increased temperatures would therefore be advantageous for raceway ponds.

Minimal changes were identified in the maximum photosynthetic efficiency range between 20°C and 40°C with significant reductions at the temperature extremes (Fig. 23). This shows minimal PS II efficiency changes in water temperatures expected in the KwaZulu-Natal region. Previous studies (Blanchard et al., 1996) have found that temperature stress is limited to the extremes however the extremes are specific for the microalgal species. This was evident in this study as samples incubated at 10°C and temperatures greater than 40°C revealed the greatest temperature induced stress evident by a decreased ETR and α value.

Electron transport rates and photosynthesis under heat stress have to cope with the generation of reactive oxygen species (ROS) (Liu and Pang, 2009). The generation in ROS disrupts cellular homeostasis leading to the damage of molecular molecules (DNA, RNA and particularly chlorophyll proteins) and membranes by inducing peroxidation (Halliwell, 2006). This will hinder enzymatic reactions and reduce photosynthetic activity and efficiency as oxygen evolution and variable fluorescence will be reduced. As a protective response to the effects of ROS, the microalgal defence system has non-enzymatic and enzymatic manners in which to cope with the resulting stress from the ROS. Previous studies (Pouneva and Nedeva, 2009) have found that an increase in temperature increased the amount of available polypeptides as well as inducing the synthesis of new polypeptides. These polypeptides may be involved in the protection of the ROS and would cause a deviation from the normal resulting in stress. The response to the decrease in photosynthesis at samples incubated at higher temperatures may also be attributed to the increase in photorespiration. This is also

amplified by temperature dependant changes in the solubility of CO₂ and O₂ (Raven and Geider, 1988).

The T_{opt} values found in this study (35°C) (Fig. 19) are higher than those found in previous studies (Blanchard et al., 1997) where the T_{opt} was found to 25°C. A possible explanation to this is that the algae strain is indigenous to a sub tropical climacteric region and was originally acclimatized to increased temperatures. The algae isolated in this study are also more tolerant to a wider range of temperatures compared to other studies (Morris and Kromkamp, 2003) making them ideal for large scale raceway ponds. In previous studies by Morris and Kromkamp (2003), incubation at 40°C showed the total elimination of fluorescence and no physiological response whereas this study showed a physiological response at 45°C. This strain can, however, tolerate higher temperatures until the T_{max} is reached (estimated at 50°C to 55°C). It is however important to compare T_{opt} and T_{max} from the microalgal isolated from the same of similar climacteric regions.

From the results of this study, it is evident that low temperatures (0°C to 5°C) induce 'dormancy' in the growth patterns of the microalgae. All the physiological parameters recorded at decreased temperatures (10°C and 15°C) showed significantly reduced recordings compared to the 25°C control. Enzymes are known to decrease activity at lower temperatures. This will slow down all metabolic reactions in the cell reducing the demand for energy. The microalgal cell will adjust photosynthetically to this change in energy demand by decreasing the electron transport rate and increasing photoinhibition (Huner et al., 1993). The total energy requirement for CO₂ fixation is reduced due to the speed at which the fixation is occurring, reducing the physiological functioning of the cell. Microalgae incubated at 10°C will not have correct metabolic functioning due to the slow electron transfer and synthesis of compounds and will reduce the cell viability at that particular temperature. This study showed that microalgal cells have the

capacity to adjust photosynthetically to the prevailing environmental conditions. This will be advantageous in the cooler winter months (15°C to 20°C) where microalgae can still function even if at a reduced efficiency.

The relative stability of the quantum efficiency as seen over the wide temperature range as found in this study, has been previously recorded (Morris and Kromkamp, 2003). The F_v/F_m value has been generally used to indicate nutrient stress not temperature stress. The dual stress samples showed significant reduction in the F_v/F_m values possibly due to the induced nutrient stress and not the temperature stress. This shows that quantum efficiency may not necessarily monitor temperature stress (Fig. 22).

The dissipation of extra energy (NPQ) was greatest when exposed to dual stress, at the lower temperatures (15°C and 25°C) (Fig. 20). This is due to the photosynthetic apparatus absorbing more light than can be readily dissipated through carbon fixation, resulting in excess heat. The photochemical reactions may have proceeded at rates independent of temperature while the enzyme dependant reactions are decreased by the lower temperatures (Raven and Geider, 1988). Excess energy results which are removed via heat (NPQ).

Temperature has been found to have a major effect on the fatty acid composition of microalgae (Hu et al., 2008). It has been previously speculated that algae has the ability to alter its physiological and thermal properties in response to changes in ambient temperatures, however the underlying regulatory mechanisms are still unknown (Somerville, 1995). This changing of physiological responses as well as lipid yields was evident in this study. The increase in temperature from 25°C to 40°C increased the lipid yields (Fig. 24). This was possibly due to the increased activity of the key regulatory enzymes involved in the synthesis of lipids (Sheehan et al., 1998). The main regulatory enzymes in the lipid synthesis pathway have been found to be Acetyl CoA carboxylase (ACCase) (Ohlrogge and Browse, 1995,

Sheehan et al., 1998). By 1988, the above researchers had shown the correlation of lipid accumulation with increased ACCase levels under selective nutrient depletion. It was also shown that increased lipid levels correlated with increased ACCase gene expression.

The reduction of neutral lipid yields at lower temperatures is probably due to the decrease in enzymatic functioning (Fig. 24). The activation energy required for the reaction will be greater than the decreased ambient temperature of the culture media and the cellular components. Less energy will be available in the cell as all photosynthetic functioning is reduced. Decreased enzymatic functioning and minimal energy result in sub-optimal conditions for enzyme dependant lipid biosynthesis.

The lipid content of microalgae was strongly influenced by the pre-determined culture parameters altering the FAMES produced. C₁₆ and C₁₈ FAMES were consistent throughout the single and dual stress samples present however the amount of each one was possibly varied. C₁₆ and C₁₈ FAMES have been found previously in *Chlorella* species (Nagashima et al., 1995, Rasoul-Amini et al., 2009) and the characteristics of these FAMES make them appropriate for biodiesel. By fully understanding the effects of varied growth conditions, the ability to engineer the algae to produce the desired FAMES appropriate for biodiesel. The absence of specific FAMES was found by incubation at higher temperatures (Table 10). This may be possibly due to the functioning of enzymes, in particular saturases and desaturases, or the production of short chain FAMES to protect the cell from the excess heat stress. In experiments using radioactive labelled fatty acids, it was shown that under normal temperatures labelled lipids remained relatively unchanged. At cooler temperatures, however, the labelled fatty acid region was transferred to polar lipids, showing the unsaturation and reduction in carbon chain length (Guschina and Harwood, 2006). This shows that lipids are used in membrane renewal and biogenesis due to damage from cold temperature making temperature a crucial factor for

optimizing lipid production. It has been reported that cooler temperatures promote the unsaturation of fatty acids possibly due to a protective mechanism or an acclimation mechanism (Nagashima et al., 1995, Wada and Murata, 1990). The lack of variation between the polyunsaturated fatty acid methyl esters (18:1 and 18:2) shows the need for unsaturation at all temperatures measured possibly to protect against temperature fluctuations.

The FAME variation will affect the microalgal biodiesel specifications due to the variation in melting point, densities and boiling points. The unsaturated FAMES do not follow the correlations of the carbon length to increase/decrease in temperature. This makes them very important for compliance to standards. It is important to understand the diesel characteristics crucial for quality and compliance to numerous standard bodies. These bodies include Europe (EN 14214:2003), Germany (DIN V 51606), South Africa (SANS 342 - 2006) and USA (ASTM D 6751-07b) with ASTM being the most favoured biodiesel standard (www.biofuelsystems.com/specification.htm). By understanding the characteristics of the various FAMES present in the sample, the compliance to national or international biodiesel standards can be determined.

Future studies are required to assess the influence of multiples synergistic stresses on the composition of FAMES required for quality microalgal biodiesel.

CHAPTER FIVE

5 CONCLUSIONS AND RECOMMENDATIONS

In this study, PAM Fluorometry was shown to be an effective tool to maximise biomass production and to identify stress conditions necessary for the synthesis of cellular neutral lipids. The conditions favourable to maximum biomass production were found to be the inverse of conditions favourable for the synthesis of cellular neutral lipids. Improving the productivity of microalgae biodiesel necessitates a two stage system. In the first stage, conditions for maximum growth are determined using the PAM Fluorometer. Once maximum biomass is recorded, the culture is harvested and prepared for stage two. During this stage the harvested biomass is incubated under predetermined nutrient depleted conditions to stimulate the maximum production of cellular neutral lipids. Complete nutrient stress was found to induce greater lipid yields than selective nutrient stress which has previously been found to be the preferred mechanism to induce lipids according to literature. Even though the strain under study produced high lipid and biomass yield, production at commercial scale would utilize large volumes of freshwater inflating production costs. Cultivating *Chlorella* species in a marine environment would reduce freshwater demand and maintenance of raceway ponds.

In Chapter 2 of the thesis, the isolated *Chlorella* species was shown to acclimatize to salinities of up to 40 ‰. The pre-acclimation period and the duration in which the acclimation occurred were directly proportional to the salinity. PAM Fluorometry was able to successfully monitor the physiological changes experienced due to the induced osmotic stress. The ETR parameter showed the potential to be used as an indicator of the acclimation period. Minimal stress was experienced in samples between 5 ‰ and 20 ‰ compared to the BG- 11 control showing that a salinity of 20 ‰ is favourable as a culture medium. Mass production of this *Chlorella* sp. at a salinity of 20‰ will reduce the amount of freshwater required and reduce the

freshwater algal contaminants as well as bacteria and protozoa that thrive in freshwater. The tolerance and acclimation of this strain to the effects of salinity will mitigate the effect of evaporation and not hinder the supply of freshwater for mass production of microalgae in raceway ponds.

Furthermore, productivity in raceway ponds can be reduced by the sub optimum temperatures recorded during the diurnal and seasonal periods. This may have a significant influence on biomass production and lipid yields. In chapter 3 of the thesis, the use of PAM Fluorometry was able to effectively detect and monitor the effect of temperature with regards to optimum growth and optimum stress conditions. The electron transport rate and photosynthetic efficiency values were found to be most adapted at monitoring this stress. Temperature fluctuations were found to have a significant effect on the physiology of microalgae thereby making it an important environmental factor necessary for optimizing biomass production for this isolate. This *Chlorella* sp. was able to be successfully cultured at a temperature range between 15°C and 40°C making it a suitable strain for large scale production in race way ponds in the KwaZulu-Natal region. The effect of dual stress was found to have the greatest increase in the lipid yield showing on average 55 percent lipid than single stress alone. Using these pre determined stress conditions the resulting FAMES were identified to have a carbon chain length of between C₄ to C₂₀. The identification of FAMES between C₁₆ and C₁₈ shows the potential for quality biodiesel due to the correct characteristics of these FAMES.

Microalgae show great potential as a feedstock for biodiesel production, however, the economic feasibility can only be determined at pilot scale. The series of studies undertaken in this thesis provide a comprehensive baseline dataset for implementation at a larger scale.

5.1 RECOMMENDATIONS

- The use of PAM Fluorometry to monitor physiological responses to growth and stress conditions can improve the understanding required to fully maximise growth and lipid yields and should be used in related studies.
- The *Chlorella* sp. shows the potential to be used in large-scale biodiesel production in race way ponds in the KwaZulu-Natal region, due to its ability to accumulate large quantities of cellular neutral lipids and tolerance to local temperatures.
- This *Chlorella* sp. strain has shown the ability to be cultured at a salinity of 20 ‰ thereby reducing the need for freshwater and the growth of foreign contaminants while increasing photosynthetic functioning.
- Temperature is a critical factor and should be considered for the large scale production of biodiesel as it influences the biomass production and the FAME characteristics.
- Microalgae as a feedstock are a cheap, renewable and sustainable manner in which to produce alternative fuels when petroleum reserves have become depleted.

6 REFERENCES:

- Allen, M.M. 1973. Methods for cyanophyceae. In: Stein JR (ed) Handbook of phycological methods: culture methods and growth measurements. Cambridge University Press, Cambridge, UK, pp 127–138.
- Alyabyev, A.J., Loseva, N.L., Gordon, L.K., Andreyeva, I.N., Rachimova, G.G., Tribunskih, V.I., Ponomareva, A.A., Kemp, R.B. 2007. The effect of changes in salinity on the energy yielding processes of *Chlorella vulgaris* and *Dunaliella maritima* cells. *Thermchimica Acta*, 458, 65-70.
- Apse, M.P., Aharon, G.S., Sneddon, W.A., Blumwald, E. 1999. Salt tolerance conferred by over expressions of a vacuolar Na⁺/H⁺ antiport in *Arabidopsis*. *Science*. 285, 1256-1258.
- Baker, N.R. 2008. Chlorophyll Fluorescence: A probe of photosynthesis In Vivo. *Annual Review of Plant Biology*, 59, 89-113
- Beardall, J., Young, E., Roberts, S. 2001. Approaches for determining phytoplankton nutrient limitation. *Aquatic Science*, 63, 44-69.
- Berden-Zrimec, M., Drinovec, L., Molinari, I., Zrimec, A., Umani, S.F., Monti, M. 2008. Delayed fluorescence as a measure of nutrient limitation in *Dunaliella tertiolecta*. *Journal of Photochemistry and Photobiology B: Biology*. 92, 13-18.
- Bilger, W., Schreiber, U., Bock, M. 1995. Determination of the quantum efficiency of photosystem II and of non-photochemical quenching of chlorophyll fluorescence in the field. *Oecologia*, 102, 425-432.
- Bjorkman, O., Demmig-Adams, B. 1994. Regulation of photosynthetic light energy capture, conversion and dissipation in leaves of higher plants.

(Schultz, E.D., and Caldwell, M. (Eds) *Ecological Studies*. New York, Springer Verlag.

Blanchard, G.F., Guarini, J.M., Gros, J.M., Richard, P. 1997. Seasonal effect on the relationship between the photosynthetic capacity of intertidal microphytobenthos and temperature. *Journal of Phycology*, 33, 723-728.

Blanchard, G.F., Guarini, J.M., Richard, P., Gros, P., Mornet, F. 1996. Quantifying the short term temperature effect on light saturated photosynthesis on intertidal microphytobenthos. *Marine Ecology Progress Series*, 134, 309-313.

Bligh, E.G., Dyer, W.J., 1959. A rapid method for total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, 37, 911-917.

Borowitzka, M.A., 1992. Algal biotechnology products and processes-matching science and economics, *Journal of Applied Phycology*, 4, 267-279.

Borowitzka, M.A., 1999. Commercial production of microalgae: ponds, tanks, tubes and fermenters. *Journal of Biotechnology*, 70, 313-321.

Boussiba, S., 2000. Carotenogenesis in the green alga *Haematococcus pluvialis*: Cellular physiology and stress response. *Physiologia Plantarum*, 108, 111-117.

Brennan, L., Owende, P. 2010. Biofuels from microalgae-A review of technologies for production, processing and extractions of biofuels and co-products. *Renewable and Sustainable Energy Reviews*, 14, 557-577.

- Brodribb, T.J., Hill, R.S. 2000. Increases in water potential gradient reduce xylem conductivity in whole plants. Evidence from a low pressure conductivity method. *Plant Physiology*, 123, 1021-1028.
- Brunet, C., Lavaud, J. 2010. Can the xanthophyll cycle help extract the essence of the microalgal functional response to a variable light environment? *Journal of Plankton Research*. DOI: 10.1093/plankt/fbq10
- Butler, W.L. 1978. Energy distribution in the photochemical apparatus of photosynthesis. *Annual Review of Plant Physiology*, 29, 345-378.
- Chen, M., Tang, H., Ma, H., Holland, T.C., Simon, K.Y., Salley, S.O. 2010. Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*. *Bioresource Technology*. (DOI: 10.1016/j.biortech.2010.09.062)
- Cherry, J.H., Nielson, B.L. 2004. Metabolic engineering of chloroplasts for abiotic stress tolerance. Daniell, H., Chase, C.D., (Eds) *Molecular Biology and Biotechnology of Plant Organelles*. Dordrecht, Springer.
- Chisti, Y. 2007. Biodiesel from Microalgae. *Biotechnology Advances*, 25, 294-306.
- Chisti, Y. 2008. Biodiesel from microalgae beats bioethanol. *Trends in Biotechnology*, 26, 126-131.
- Chynoweth, D.P., Owens, J.M., Legrand, R. 2001. Renewable methane from anaerobic digestion of biomass. *Renewable Energy*, 22, 1-8.
- Clavier, J., Boucher, G., Chauvaud, L., Fichez, S. 2005. Benthic response to ammonium pulses in a tropical lagoon: implications for coastal

environmental processes. *Journal of Experimental Marine Biology and Ecology*, 316, 231-241.

Collen, J., Davison, I.R. 2001. Seasonality and thermal acclimation of reactive oxygen metabolism in *Fucus vesiculosus* (Phaeophyceae). *Journal of Phycology*, 37, 474-481.

Converti, A., Casazza, A.A., Ortiz, E.Y., Perego, P., Del Borghi, M. 2009. Effect of temperature and nitrogen concentration on the growth and lipid content on *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. *Chemical Engineering and Processing: Process Intensification*, 48, 1146-1151.

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

Crookes, R.J. 2006. Comparative Bio-Fuel performance in internal combustion engines. *Biomass and Bioenergy*, 30, 461-468.

Dawes, E.A. 1976. Endogenous metabolism and the survival of starved prokaryotes. *Symposium of the Society for General Microbiology*, 26.

Dean, A.P., Sigee, D.C., Estrada, B., Pittman, J.K. 2010. Using FTIR spectroscopy for rapid determination of lipid accumulation in response to nitrogen limitation in freshwater microalgae. *Bioresource Technology*, 101, 4499-4507.

Demirbas, A. 2008. Biofuels sources, biofuel policy, biofuel economy and global biofuel projections. *Energy Conversion and Management*, 49, 2106-2116.

- Demirbas, A. 2009a. Production of Biodiesel from Algae Oils. *Energy Sources, Part A*, 31, 163-168.
- Demirbas, A. 2009b. Progress and recent trends in biodiesel fuels. *Energy Conversion and Management*, 50, 14-34.
- Demming-Adams, B. 1990. Carotenoids and photoprotection in plants. A role for the xanthophyll zeaxanthin. *Biochemica et Biophysica Acta*, 1020, 1-24.
- Dietz, K.J., Tavakoli, N., Kluge, C., Mimura, T., Sharma, S.S., Harris, G.C., Chardonnens, A.N., Gollack, D. 2001. Significance of the V-type ATPase for the adaptation to stressful growth conditions and its regulation on the molecular and biochemical level. *Journal of Experimental Botany*, 52, 1969-1980.
- Dluzniewska, P., Gessler, A., Dietrich, H., Schnitzler, J.P., Teuber, M., Rennenberg, H. 2007. Nitrogen uptake and metabolism in *Populus canescans* as affected by salinity. *New Phytologist*, 173, 279-293.
- Dresselhaus, M.S., Thomas, I.L. 2001. Alternative energy technologies. *Nature*, 414, 332-337.
- Eggert, A., Nitschke, U., West, J., Michalik, D., Karsten, U. 2007a. Acclimation of the intertidal red alga *Bangiopsis subsimplex* (Stylonematophyceae) to salinity changes. *Journal of Experimental Marine Biology and Ecology*, 343, 176-186.
- Eggert, A., Raimund, S., Michalik, D., West, J., Karsten, U. 2007b. Ecophysiological performance of the primitive red alga *Dixoniella grisea* (Rhodellophyceae) to irradiance, temperature and salinity stress:

growth responses and the osmotic role of mannitol. *Phycologia*, 46, 22-28.

Fabregas, J., Abalde, J., Herrero, C., Cabezas, B., Veiga, M. 1984. Growth of the marine microalga *Tetraselmis suecica* in batch cultures with different salinities and nutrient concentrations *Aquaculture*, 42, 207-215.

Flynn, K. 1990. The determination of nitrogen status in microalgae. *Marine Ecology Press Series*, 61, 297-307.

Forsberg, C.W. 2009. Sustainability by combining nuclear, fossil and renewable energy sources. *Progress in Nuclear Energy*, 51, 192-200.

Fracheboud, Y., Leipner, J. 2003. The application of chlorophyll fluorescence to study light, temperature and drought stress. IN Deell, J. R., & Toivonen, P. M. A. (Eds.) *Practical Applications of Chlorophyll Fluorescence in Plant Biology*. Dordrecht, Kluwer Academic Publishers.

Francavilla, M., Trotta, P., Luque, R. 2010. Phytosterols from *Dunaliella tertiolecta* and *Dunaliella salina*: A potentially novel industrial application. *Bioresource Technology*. DOI: 10.1016/j.biortech.2009.12.139.

Fukuda, H., Kondo, A., Noda, H. 2001. Biodiesel fuel production by transesterification of oils. *Journal of Biosciences and Bioengineering*, 92, 405-416.

Gerin, C., Goutx, M. 1993. Separation and quantification of phospholipids from marine bacteria with the Latroscan Mark IV TLCFID. *Journal of Planar Chromatography*, 6, 307-312.

- Gordillo, F.J.L., Goutx, M., Figueora, F.L., Niell, F.X. 1998. Effects of light intensity, CO₂ and nitrogen supply on lipid class composition of *Dunaliella viridis*. *Journal of Applied Phycology*, 10, 135-144.
- Greenspan, P., Mayer, E.P., Fowler, S.D. 1985. Nile Red: a selective fluorescent stain for intracellular lipid droplets. *Journal of Cell Biology*, 100, 965-973.
- Greenwell, H.C., Laurens, M.L., Shields, R.J., Lovitt, R.W., Flynn, K.J. 2009. Placing microalgae on the biofuels priority list: a review of the technological challenges. . *Journal of Royal Society Interface*. DOI: 10.1098/rsif.2009.0322.
- Griffiths, M., Harrison, S.T.L., 2009. Lipid productivity as a key characteristic for choosing algal species for biodiesel production. *Journal of Applied Phycology*. DOI:10.1007/s10811-008-9392-7.
- Grobbelaar, J.U., 2009a. Factors governing algal growth in photobioreactors: the “open” versus “closed” debate. *Journal of Applied Phycology*, 21, 489-492.
- Grobbelaar, J.U., 2009b. From laboratory to commercial production: a case study of a *Spirulina* (*Arthrospira*) facility in Musina, South Africa. *Journal of Applied Phycology*, 21, 523-527.
- Grobbelaar, J.U. 2009c. Upper limits of photosynthetic productivity and problems of scaling. *Journal of Applied Phycology*, 21, 519-522.
- Guschina, I.A., Harwood, J.L. 2006. Lipids and lipid metabolism in eukaryotic algae. *Progress in Lipid Research*, 45, 160-186.

- Gustavs, L., Eggert, A., Michalik, D., Karsten, U. 2009. Physiological and biochemical responses of green microalgae from different habitats to osmotic and matric stress. *Protoplasma*. DOI:10.1007/s00709-009-0060-9.
- Hagen, C., Braune, W., Bjorn, L.O. 1994. Functional aspects of secondary carotenoids in *Haematococcus lacustris* (Girod) Rostafinski (Volvocales) III. Action of a 'sunshade'. *Journal of Phycology*, 30, 241-248.
- Halliwell, B. 2006. Reactive species and antioxidants: redox biology is a fundamental theme of aerobic life. *Plant Physiology*, 141, 312-322.
- Hansen, J., Sato, M., Ruedy, R., Lacis, A., Oinas, V. 2000. Global warming in the twenty-first century: An alternative scenario. *PNAS*, 97, 9875-9880.
- Hartig, P., Wolfstein, K., Lippemeier, S., Coljin, F. 1998. Photosynthetic activity of natural microphytobenthos populations measured by fluorescence (PAM) and ¹⁴C-tracer methods: a comparison. . *Marine Ecology Progress Series*, 166, 53-62.
- Harun, R., Singh, M., Forde, G.M., Danquah, M.K. 2010. Bioprocess engineering of microalgae to produce a variety of consumer products. *Renewable and Sustainable Energy Reviews*, 14, 1037-1047.
- Hase, R., Oikawa, H., Sasao, C., Morita, M., Watanabe, Y. 2000. Photosynthetic production of microalgal biomass in a raceway system under greenhouse conditions in Sendai City. *Journal of Bioscience and Bioengineering*, 89, 157-163.

- He, H., Wang, T., Zhu. 2007. Continuous production of biodiesel fuel from vegetable oil using supercritical methanol process. *Fuel*, 86, 442-447.
- Healey, F.P. 1982. Phosphate. IN Carr, N. G., & Whitton, B. A. (Eds.) *The Biology of Cyanobacteria*. Oxford, Blackwell Scientific.
- Henley, W.J. 1993. Measurement and interpretation of photosynthesis light-response curves in algae in the context of photoinhibition and diel changes. *Journal of Phycology*, 29, 729-739.
- Herzig, R., Falkowski, P.G. 1989. Nitrogen limitation in *Isochrysis galbana* (Haptophyceae) Photosynthetic energy conversion and growth efficiencies. *Journal of Phycology*, 25, 462-471.
- Horton, P., Ruban, A. 2005. Molecular design of the photosystem II light-harvesting antenna: photosynthesis and photoprotection. *Journal of Experimental Botany*, 56, 365-373.
- Horton, P., Wentworth, M., Ruban, A. 2005. Control of the light harvesting function of chloroplast membranes: the LHCII aggregation model for non-photochemical quenching. *FEBS letters*, 579, 4201-4206.
- Hoydoncx, H.E., De Vos, D.E., Chavan, S.A., Jacobs, P.A. 2004. Esterification and transesterification of renewable chemicals. *Topics in Catalysis*, 27, 83-96.
- Hsieh, C.H., Wu, W.T. 2009. Cultivation of microalgae for oil production with a cultivation strategy of urea limitation. *Bioresource Technology*, 100, 3921-3926.
- Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Siebert, M., Darzins, A. 2008. Microalgal triacylglycerols as feedstock's for biofuel

production: perspectives and advances. *The Plant Journal*, 54, 621-639.

Huner, N.P.A., Oquist, G., Hurry, V.M., Krol, M., Falk, S., Griffith, M. 1993. Photosynthesis, photoinhibition and low temperature acclimation in cold tolerant plants. *Photosynthesis Research*, 37, 19-39.

Illman, A.M., Scragg, A.H., Shales, S.W. 2000. Increase in *Chlorella* strains calorific values when grown in low nitrogen medium. *Enzyme and Microbial Technology*, 27, 631-635.

Jampeetong, A., Brix, H. 2009. Effects of NaCl salinity on growth, morphology, photosynthesis and proline accumulation of *Salvinia natans*. *Aquatic Botany*, 91, 181-186.

Juneau, P., Elberdey, A., Popovic, R. 2002. PAM Fluorometry in the Determination of the Sensitivity of *Chlorella vulgaris*, *Selenastrum capricornutum*, and *Chlamydomonas reinhardtii* to Copper. *Archives of Environmental Contamination and Toxicology*, 42, 155-164.

Juneau, P., Green, B.R., Harrison, P.J. 2005. Simulation of Pulse-Amplitude-Modulated (PAM) fluorescence: Limitations of some PAM-parameters in studying environmental stress effects *Photosynthetica*, 43, 75-83.

Kana, T., Geider, R.J., Critchley, C. 1997. Regulation of photosynthetic pigments in micro-algae by multiple environmental factors: a dynamic balance hypothesis. *New Phytologist*, 137, 629-638.

Kaplan, D., Richmond, A.E., Dubinsky, Z., Aaronson, S. 1986. Algal Nutrition. In Richmond, A. (Ed.) *CRC Handbook of Microalgal Mass Culture*. Israel, CRC Press.

- Kobayashi, M., Kakizono, T., Yamaguchi, K., Nisho, N., Nagai, S. 1992. Growth and astaxanthin formation of *Haematococcus pluvialis* in heterotrophic and mixotrophic conditions. *Journal of Fermentation and Bioengineering*, 74, 17-20.
- Krause, G.H., Weis, E. 1991. Chlorophyll fluorescence and photosynthesis: the basics. *Annual Review of Plant Physiology and Plant Molecular Biology*, 42, 313-349.
- Kreslavski, V.D., Carpentier, R., Klimov, V.V., Murata, N., Allakhverhiev, S.I. 2007. Molecular mechanisms of stress resistance of the photosynthetic apparatus. *Biochemistry (Moscow) Supplement Series A: Membrane and Cell Biology*, 1, 185-205.
- Kromkamp, J., Peene, J. 1999. Estimation of phytoplankton photosynthesis and nutrient limitation in the Eastern Scheldt estuary using variable fluorescence. *Aquatic Ecology*, 33, 101-104.
- Kuster, A., Schaible, R., Schobert, H. 2004. Light acclimation of photosynthesis in three charophyte species. *Aquatic Botany*, 79, 111-124.
- Lamote, M., Johnson, L.E., Lemoine, Y. 2007. Interspecific differences in the response of juvenile stages to physical stress: Fluorometric responses of fucoid embryos to variation in meteorological conditions. *Journal of Phycology*, 43, 1164-1176.
- Lamprecht, M.R., Sabitini, D.M., Carpenter, A.E. 2007. CellProfiler™: free, versatile software for automated biological image analysis. *Biotechniques*, 42, 71-75.

- Lax, J.E.M., Arteni, A.A., Boekema, E.J., Pistorius, E.K., Michel, K., Rogner, M. 2007. Structural response of Photosystem 2 to iron deficiency: Characterization of a new Photosystem 2–LhA complex from the cyanobacterium *Thermosynechococcus elongatus* BP-1. *Biochimica et Biophysica Acta*, 1767, 528-534.
- Leon-Banares, R., Gonzalez-Ballester, D., Galvan, A., Fernandez, E. 2004. Transgenic microalgae as green cell factories. *Trends in Biotechnology*, 22, 45-52.
- Lewis, T., Nichols, P.D., McMeekin, T.A. 2000. Evaluation of extraction methods for recovery of fatty acids from lipid-producing microheterotrophs. *Journal of Microbiological Methods*, 43, 107-116.
- Li, Y., Horsman, M., Wang, B., Wu, N., Lan, C.Q. 2008. Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans*. *Applied Microbiology and Biotechnology*, 81, 629-636.
- Lippemeier, S., Hartig, P., Colijn, F. 1999. Direct impacts of silicate on the photosynthetic performance of the diatom *Thalassiosira weissflogii* assessed by on- and off-line PAM fluorescence measurements. *Journal of Plankton Research*, 21, 269-283.
- Liu, F., Pang, S.J. 2009. Performances of growth, photochemical efficiency, and stress tolerance of young sporophytes from seven populations of *Saccharina japonica* (Phaeophyta) under short-term heat stress. *Journal of Applied Phycology*. DOI 10.1007/s10811-009-9445-6.
- Liu, W., Au, D.W.T., Anderson, D.M., Lam, P.K.S., Wu, R.S.S. 2007. Effects of nutrients, salinity, pH and light: dark cycle on the production of reactive oxygen species in the alga *Chattonella marina*. *Journal of Experimental Marine Biology and Ecology*, 346, 76-86.

- Liu, Z.Y., Wang, G.C., Zhou, B.C. 2008. Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*. *Bioresource Technology*, 99, 4717-4722.
- Lu, C., Guiseppe, T., Vonshak, A. 1999. Kinetic response of photosystem II photochemistry in cyanobacterium *Spirulina platensis* to high salinity is characterized by two distinct phases. *Australian Journal of Plant Physiology*, 26, 283-292.
- Marchetti, A., Varela, D.E., Lance, V.P., Johnson, Z., Palmucci, M., Giordino, M., Armbrust, E.V. 2010. Iron and silicic acid effects on phytoplankton productivity, diversity, and chemical composition in the central equatorial Pacific Ocean. *Limnology and Oceanography*, 55, 11-29.
- Masojidek, J., Torzillo, G. 2007. Mass cultivation of fresh water microalgae. *Encyclopedia of Ecology*, 2008, 2226-2235.
- Masojidek, J., Torzillo, G., Koblizek, M., Kopecky, J., Bernardini, P., Sacchi, A., Komenda, J. 1999. Photoadaptation of two members of the Chlorophyta (*Scenedesmus* and *Chlorella*) in laboratory and outdoor cultures: changes of chlorophyll fluorescence quenching and the xanthophyll cycle. *Planta*, 209, 126-135.
- Masojidek, J., Torzillo, G., Kopecky, J., Koblizek, M., Nidiaci, L., Komenda, J., Lukavska, A., Sacchi, A. 2000. Changes in chlorophyll fluorescence quenching and pigment composition in the green alga *Chlorococcum* sp. grown under nitrogen deficiency and salinity stress. *Journal of Applied Phycology*, 12, 417-426.
- Maxwell, K., Johnson, G.N. 2000. Chlorophyll fluorescence- a practical guide. *Journal of Experimental Botany*, 51, 659-668.

- Meher, L.C., Sagar, D.V., Naik, S.N. 2006. Technical aspects of biodiesel production by transesterification-a review. *Renewable and Sustainable Energy Reviews*, 10, 248-268.
- Meng, X., Yang, J., Xu, X., Zhang, L., Nie, Q., Xian, M. 2009. Biodiesel production from oleaginous microorganisms. *Renewable Energy*, 34, 1-5.
- Miao, X., Wu, Q. 2006. Biodiesel production from heterotrophic microalgal oil. *Bioresource Technology*, 97, 841-846.
- Morgan-Kiss, R., Ivanov, A.G., Williams, J., Khan, M., Huner, N.P.A. 2002. Differential thermal effects on the energy distribution between photosystem II and photosystem I in thylakoid membranes of a psychrophilic and a mesophilic alga. *Biochimica et Biophysica Acta*, 1561, 251-265.
- Morris, E.P., Kromkamp, J.C. 2003. Influence of temperature on the relationship between oxygen and fluorescence-based estimates of photosynthetic parameters in a marine benthic diatom (*Cylindrotheca closterium*). *European Journal of Phycology*, 38, 133-142.
- Muller, P., Li, X-P., Niyogi, K. 2001. Non-photochemical quenching. A response to excess light energy. *Plant Physiology*, 125, 1558-1566.
- Mutanda, T., Ramesh, D., Karthikeyan, S., Kumari, S., Anandraj, A., Bux, F. 2011. Bioprospecting for hyper-lipid producing microalgal strains for sustainable biofuel production. *Bioresource Technology*, 102, 57-70.
- Nagashima, H., Matsumoto, G.I., Ohtani, S., Momose., H. 1995. Temperature acclimation and the fatty acid composition of an Antarctic

green alga *Chlorella*. *Proceedings of the NIPR Symposium on Polar Biology*, 8, 194-199.

Nedeva, D., Pouneva, I. 2009. Changes in electrophoretic profiles of proteins and some antioxidant enzymes in Antarctic Alga *Choricystic minor* and *Chlorella* sp. as affected by temperature and oxidative stress. *Biotechnology and Biotechnology Equipment* 23, 233-236.

Ohlrogge, J., Browse, J. 1995. Lipid Biosynthesis. *The Plant Cell*, 7, 957-970.

Oxborough, K., Hanlon, A.R.M., Underwood, G.J.C., Baker, N.R. 2000. In vivo estimation of the photosystem II photochemical efficiency of individual microphytobenthos cells using high resolution imaging of chlorophyll a fluorescence. *Limnology and Oceanography*, 43, 1207-1221.

Pang, S.J., Jin, Z.H., Sun, J.Z., Gao, S.Q. 2007. Temperature tolerance of young sporophytes from two populations of *Laminaria japonica* revealed by chlorophyll fluorescence measurements and short term growth and survival performances in tank culture. *Aquaculture*, 262, 493-503.

Parida, A.K., Das, A.B. 2005. Salt tolerance and salinity effects on plants: a review. *Ecotoxicology and Environmental Safety*, 60, 324-349.

Parrish, C.C., Wangersky, P.J. 1990. Growth and lipid composition of the marine diatom *Chaetoceros gracilis*, in a laboratory and mass culture turbidostats. *Journal of Plankton Research*, 12, 1011-1021.

Parsons, T.R., Maita, Y., Lalli, C.M. 1984. *A manual of chemical and biological methods for seawater analysis*, Pergamon Press.

- Petrou, K., Doblin, M.A., Smith, R.A., Ralph, P.J., Shelly, K., Beardall, J. 2008. State transitions and non photochemical quenching during a nutrient induced fluorescence transient in phosphate starved *Dunaliella tertiolecta*. *Journal of Phycology*, 44, 1204 - 1211.
- Platt, T., Gallegos, C.L., Harrison, W.G. 1980. Photoinhibition of photosynthesis in natural assemblages of marine phytoplankton. *Journal of Marine Research*, 38, 687-701.
- Pouneva, I., Nedeva, D. 2009. Changes in electrophoretic profiles of proteins and some antioxidant enzymes in Antarctic alga *Choricystis minor* and *Chlorella sp.* as affected by temperature and oxidative stress. *Biotechnology and Biotechnology Equipment*, Special Edition, 233-236.
- Power, M. 1999. Recovery in Aquatic Ecosystems: Considerations for definition and measurement. *Journal of Aquatic Ecosystems Stress and Recovery*, 6, 179-180.
- Pruvost, J., Van Vooren, G., Cogne, G., Legrand, J. 2009. Investigation of biomass and lipids production with *Neochloris oleoabundans* in photobioreactor. *Bioresource Technology*, 100, 5988-5995.
- Rabbani, S., Beyer, P., Von Lintig, J., Hugueney, P., Kleinig, H. 1998. β -carotene synthesis driven by triacylglycerol deposition in the unicellular alga *Dunaliella bardawil*. *Plant Physiology*, 116, 1239-1248.
- Ralph, P.J., Gademann, R. 2005. Rapid light curves: A powerful tool to assess photosynthetic activity. *Aquatic Botany*, 82, 222-237.
- Rasoul-Amini, S., Ghasemi, Y., Morowvat, M.H., Mohaghehzadeh, A. 2009. PCR amplification of 18S rRNA, single cell protein production and

- fatty acid evaluation of some naturally isolated microalgae. *Food Chemistry*, 116, 129-136.
- Raven, J.A., Geider, R.J. 1988. Temperature and algal growth. *New Phytologist*, 110, 441-461.
- Redfield, A.C., Ketchum, B.H., Richards, F.A. 1963. The influence of organisms on the composition of sea water. In *Sea*. (Ed.) Hill M.N. New York, Interscience.
- Renaud, S.M., Thinh, L.V., Lambrinidis, G., Parry, D.L. 2002. Effect of temperature on growth, chemical composition and fatty acid composition of tropical Australian microalgae grown in batch cultures. *Aquaculture*, 211, 195-214.
- Rise, M., Cohen, E., Vishkautsan, M., Cojocaru, M., Gottlieb, H., Arad, S. 1994. Accumulation of secondary carotenoids in *Chlorella zofingiensis*. *Journal of Plant Physiology*, 44, 287-292.
- Ritchie, R.J. 2008. Fitting light saturation curves measured using modulated fluorometry. *Photosynthesis Research*, 96, 201-215.
- Rodolfi, L., Zittelli, G.C., Bassi, N., Padovani, G., Biondi, N., Bonini, G., Tredici, M.R. 2009. Microalgae for Oil: Strain Selection, Induction of Lipid Synthesis and Outdoor Mass Cultivation in a Low-Cost Photobioreactor. *Biotechnology and Bioengineering*, 102, 100-112.
- Rohacek, K. 2002. Chlorophyll fluorescence parameters: the definitions, photosynthetic meaning and mutual relationships. *Photosynthetica*, 40, 13-29.

- Rosenberg, J.N., Oyler, G.A., Wilkinson, L., Betenbaugh, M.J. 2008. A green light for engineered algae: redirecting metabolism to fuel a biotechnology revolution. *Current Opinion in Biotechnology*, 19, 430-436.
- Schenk, P.M., Thomas-Hall, S.K., Stephans, E., Marx, U.C., Mussgnug, J.H., Posten, C., Kruse, O., Hankamer, B. 2008. Second Generation Biofuels: High-Efficiency Microalgae for Biodiesel Production. *Bioenergy Research*, 1, 20-43.
- Schreiber, U., Muller, J.F., Haugg, A., Gademann, R. 2002. New type of dual-channel PAM chlorophyll fluorometer for highly sensitive water toxicity biotests. *Photosynthesis Research*, 74, 317-330.
- Schreiber, U., Schliwa, U., Bilger, W. 1986. Continuous recording of photochemical and non-photochemical chlorophyll fluorescence with a new type of modulation fluorometer. *Photosynthesis Research*, 10, 51-62.
- Serodio, J., Vieira, S., Cruz, S., Barroso, F. 2005. Short term variability in photosynthetic activity of microphytobenthos as detected by measuring rapid light curves using variable fluorescence. *Marine Biology*, 146, 903-914.
- Sheenan, J., Dunahey, T., Beneman, J., Roessler, P. 1998. A look back at the U.S Department of Energy's Aquatic Species Program: Biodiesel from Algae., National Renewable Energy Lab, Department of Energy, Golden, Colorado, U.S.A. Report NREL/TP-580-24190.
- Singh, S.P., Singh, D. (2010) Biodiesel production through the use of different sources and characterization of oils and their esters as the

substitute of diesel: A review. *Renewable and Sustainable Energy Reviews*, 14, 200-216.

Somerville, C. 1995. Direct tests of the role of membrane lipid composition in low-temperature induced photo-inhibition and chilling sensitivity in plants and cyanobacteria. *Proceedings of the National Academy of Science*, 92, 6215-6218.

Song, D., Fu, J., Shi, D. 2008. Exploitation of Oil-bearing Microalgae for Biodiesel. *Chinese Journal of Biotechnology*, 24, 341-348.

Spolaore, P., Joannis-Cassan, C., Duran, E., Isambert, A. 2006. Commercial applications of microalgae. *Journal of Biosciences and Bioengineering*, 101, 87-96.

Tang, D., Shi, S., Li, D., Hu, C., Liu, Y. 2007. Physiological and biochemical responses of *Scytonema javanicum* (cyanobacterium) to salt stress. *Journal of Arid Environments*, 71, 312-320.

Tartari, A., Forlani, G. 2008. Osmotic adjustments in a psychrophilic alga, *Xanthonema* sp. (Xanthophyceae). *Environmental and Experimental Botany* 63, 342-350.

Timmermans, K.R., Van der Wagt, B., Veldhuis, M.J.W., Maatman, A., De Baar, H.J.W. 2005. Physiological responses of three species of marine pico-phytoplankton to ammonium, phosphate, iron and light limitation. *Journal of Sea Research*, 53, 109-120.

Tokusoglu, O., Unal, M.K. 2003. Biomass Nutrient Profiles of three microalgae: *Spirulina platensis*, *Chlorella vulgaris* and *Isochrysis galbana*. *Journal of Food Science*, 68, 1144-1148.

- Vasudevan, P.T., Briggs, M. 2008. Biodiesel production - current state of the art and challenges. *Journal of Indian Microbiology and Biotechnology*, 35, 421-430.
- Wada, H., Murata, N. 1990. Temperature-induced changes in the fatty acid composition of cyanobacterium, *Synechocystis* PCC6803. *Plant Physiology*, 92, 1062-1069.
- White, S., Anandraj, A., Bux, F. 2011. PAM Fluorometry as a tool to assess microalgal nutrient stress and monitor cellular neutral lipids. *Bioresource Technology*. 102, 1675-1682.
- Yokoya, N.S., Stirk, W.A., Van Staden, J., Novak, O., Tureckova, V., Pencik, A., Strnad, M. 2010. Endogenous cytokinins, auxins and abscisic acid in red algae from Brazil. *Journal of Phycology*. DOI: 10.1111/j.1529-8817.2010.00898.x
- Zhang, X., Kurano, N., Miyachi, S. 1999. Outdoor culture of a cyanobacterium with a vertical flat-plate photobioreactor: effects on productivity of the reactor orientation, distance setting between the plates and culture temperature. *Applied Microbiology and Biotechnology*, 52, 781-786.
- Zhekisheva, M., Boussiba, S., Khozin-Goldberg, I., Zarka, A., Cohen, Z. 2002. Accumulation of oleic acid in *Haematococcus pluvialis* (Chlorophyceae) under nitrogen starvation or high light is correlated with that of astaxanthin esters. *Journal of Phycology*, 38, 325-331.
- <http://ideonexus.com/2009/01> (Accessed 14 September 2010)
- <http://www.biofuelsystems.com/specification.htm> (Accessed 1 October 2010)