Lab-scale assessment and adaptation of wastewater for cultivation of microalgal biomass for biodiesel production

Submitted in fulfilment of the requirements of the degree of Master of Applied Science in Biotechnology in the Faculty of Applied Sciences at the Durban University of Technology

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March

2015

Supervisor: Prof Faizal Bux

Declaration

Lab-scale assessment and adaptation of wastewater for cultivation of microalgal biomass for biodiesel production

Luveshan Ramanna

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

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2015

Reference Declaration in Respect of a Master's Dissertation

I, Luveshan Ramanna and Prof. Faizal Bux do hereby declare that in respect of the following dissertation:

Lab-scale assessment and adaptation of wastewater for cultivation of microalgal biomass for biodiesel production.

As far as we know and can ascertain: no other similar dissertation exists: all references as detailed in the dissertation are complete in terms of all personal communications engaged in and published works consulted.

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Approval

I hereby approve the final submission of the following dissertation.

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Prof. Faizal Bux

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Doctoral Degree in Technology: Biotechnology

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This 14th day of April 2015, at the Durban University of Technology.

Abstract

In light of the world's declining fossil fuel reserves, the use of microalgal biodiesel has come to the forefront as a potentially viable alternative liquid fuel. The depleting freshwater reserves make the feasibility of this concept questionable. The use of wastewater reduces the requirement for depleting freshwater supplies. This project aimed to determine the viability of municipal domestic wastewater effluent as a substrate for microalgal growth, in order to generate an economical and environmentally friendly source of biofuel. Wastewater effluents from three domestic wastewater treatment plants were characterized in terms of known microalgal nutrients viz., ammonia, phosphate and nitrates. Phosphate concentrations varied throughout the year and were found to be low (< 3 mgL^{-1}) whilst ammonia and nitrate concentrations ranged from 0 to 10 mgL^{-1} throughout the experimental period. These wastewaters were found to be suitable for cultivating microalgae. The study explored the cultivation of *Chlorella sorokiniana* on pre- and post-chlorinated domestic wastewater effluent to assess their potential as a medium for high microalgal culture density and lipid production. Post-chlorinated wastewater effluent was found to be superior to pre-chlorinated wastewater effluent, as evident by the higher biomass concentration. This wastewater stream did not contain high concentrations of bacteria when compared to pre-chlorinated wastewater effluent. Nitrogen is an essential nutrient required for regulating the growth and lipid accumulation in microalgae. Cultures growing in post-chlorinated effluent had a lifespan of 18 d. Residual nitrogen in wastewater effluent supported microalgal growth for limited periods. Supplementation using cheap, readily available nitrogen sources was required for optimal biomass and lipid production. Urea, potassium nitrate, sodium nitrate and ammonium nitrate were evaluated in terms of biomass and lipid production of *C. sorokiniana*. Urea showed the highest biomass yield of 0.216 gL^{-1} and was selected for

further experimentation. Urea concentrations (0–10 gL^{-1}) were assessed for their effect on growth and microalgal physiology using pulse amplitude modulated fluorometry. A concentration of 1.5 gL^{-1} urea produced 0.218 gL^{-1} biomass and 61.52 % lipid by relative fluorescence. Physiological stress was evident by the decrease in relative Electron Transport Rate from 10.45 to 6.77 and quantum efficiency of photosystem II charge separation from 0.665 to 0.131. Gas chromatography analysis revealed that C16:0, C18:0, C18:1, C18:2 and C18:3 were the major fatty acids produced by *C. sorokiniana*. Wastewater effluent has been considered an important resource for economical and sustainable microalgal biomass/lipid production. The study showed that *C. sorokiniana* was sufficiently robust to be cultivated on wastewater effluent supplemented with urea. The results indicate that supplemented wastewater effluent was an acceptable alternative to conventional media. Using a relatively cheap nitrogen source like urea can certainly improve the techno-economics of large scale biodiesel production.

Dedication

This work is dedicated to the betterment of science and can be regarded as yet another stepping stone in science's inexorable quest for renewable, sustainable energies such as biodiesel generation. May future generations be inspired and strive to fulfil this endeavour.

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Preface

The outputs of the Masters qualification include two Journal articles and a conference presentation:

Ramanna, L., Guldhe, A., Rawat, I. and Bux, F. **2014.** The optimization of biomass and lipid yields of *Chlorella sorokiniana* when using wastewater supplemented with different nitrogen sources. Bioresource Technology, **168:** 127–135.

Ramanna, L., Bhola,V.K., Rawat, I., Mutanda, T. and Bux, F. **2013.**The effect of nitrogen supplementation on domestic wastewater for cultivation of microalgae for biodiesel production. Oral presentation. International Conference on Applied Energy ICAE 2013, July 1-4, 2013, Pretoria, South Africa Paper ID: ICAE **2013-340**.

Govender, T., **Ramanna, L.,** Rawat, I. and Bux, F. **2012.** BODIPY staining, an alternative to the Nile Red fluorescence method for the evaluation of intracellular lipids in microalgae. Bioresource Technology, **114:** 507-511.

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List of Abbreviations

Acyl Carrier Protein- ACP

Adenosine Triphosphate- ATP

Ammonia- NH_3

Ammonium- NH_4

Ammonium Nitrate- NH_4NO_3

Analysis of Variance- ANOVA

β-Ketoacyl-ACP-Synthase III- KASIII

Blue Green Medium- BG11

Cadmium- Cd

Calcium- Ca

Carbon- C

Carbon Monoxide- CO

Carbon Dioxide- CO_2

Cetane Number- CN

Chlorine- Cl

Chromium- Cr

Cobalt- Co

Cold Filter Plugging Point- CFPP

Colony Forming Units- CFUs

Copper- Cu

Dry Cell Weight- DCW

Dissolved Oxygen- DO

Ethylene-Di-Amine-Tetra-Acetic Acid- EDTA

Electron Transport Rate- ETR

Fatty Acid- FA

Fatty Acid Methyl Esters- FAME

Free Fatty Acid- FFA

Fatty Acid Synthase Enzyme- FAS

Gas Chromatography- GC

GC- Mass Spectroscopy- GC-MS

GC-Flame Ionization- GC- FID

Greenhouse Gases- GHG's

High Performance Liquid Chromatography- HPLC

Hydrochloric Acid- HCl

Hydrogen- H

Iron- Fe

Lead- Pb

Manganese- Mn

Magnesium- Mg

Mercury- Hg

Modulated Light- ML

Monounsaturated Fatty Acid- MUFA

Negative Control- NC

Nickel- Ni

Nitrate- NO_3

Nitrite- NO_2

Nitrogen- N

Nitrous Oxide- NO_x

Orthophosphate- PO_4

Oxygen- O_2

Optical Density- OD

Photobioreactors- PBRs

Phosphate- P

Photosynthetically Active Photon Flux Density- PPFD

Photosynthetically Active Radiation- PAR

Photosystem II- PS II

Polyunsaturated Fatty Acid- PUFA

Potassium- K

Potassium Nitrate- KNO_3

Plastoquinones- QA

Pulse Amplitude Modulated Fluorometry- PAM

Relative Electron Transport Rate- rETR

Relative Fluorescence per cell- rf/cell

Republic of South Africa- RSA

Saturated Fatty Acid- SFA

Saturation Pulse- SP

Silver- Ag

Sodium Nitrate- $NaNO_3$

Sub Saharan Africa- SSA

Sulphur- S

Sulphuric Acid- H_2SO_4

Sulphur Oxides- SO_x

Too Numerous To Count- TNTC

Total Suspended Solids- TSS

Thin Layer Chromatography- TLC

Triacylglyceride- TAG

United States of America- USA

Unsaturated fatty acid- UFA

United Kingdom- UK

Wastewater- WW

Wastewater Treatment Plant- WWTP

Water footprint- WF

Zinc- Zn

List of Mathematical Symbols

Alpha value (significance)- α

Autoclave pressure- psi

Biomass at time zero- N_0

Biomass at time of the exponential phase- N

Dark fluorescence yield- F'

Days- d

Degrees celcius- ^{o}C

Fluorescent units- a.u

Grams per litre- gL^{-1}

Hours- h

Intrinsic fluorescence- F_o

Kilogram- kg

Litres- L

Maximum efficiency of PS II- F_v/F_m

Maximum fluorescence in a light adapted sample- F'_m

Maximal fluorescence yield- F_m

Meters- m

Meters per second- $m^{-2}s^{-1}$

Micromole- µmol

Micro litre- µL

Minutes- min

Nano meter- nm

Natural log- ln

Number of samples- n

Percent- %

Per day- d^{-1}

Per millilitre- mL^{-1}

Per litre- L^{-1}

PS II operating efficiency- F'_q/F'_m

Regression constant- R^2

Rotations per minute- rpm

Seconds- s

Specific growth rate- µ

Standard error of the mean- SEM

Time- t

Time zero- t_0

Variable fluorescence- F_v

Weight volume per volume- *v*/*v*

Milligrams- mg

Milligrams per litre- mgL^{-1}

Millilitre- mL

Chapter 1 : Introduction

1.1 General Introduction

The world′s projected energy requirement for 2030 will be 50 % more than it was in 2014. The transport sector alone accounts for 30 % of the global energy consumption. Over the last 30 years we have witnessed a sudden increase in the number of transport vehicles globally, most of which are privately owned cars (Singh *et al.*, 2014). With this in mind, the demand for fossil fuels is ever-increasing and the cost of these fuels is escalating (Chisti, 2008). The world's energy demand in the transport sector is predicted to grow by 2 % per year. This would inevitably lead to 80 % higher emissions in terms of greenhouse gasses (GHG's) (Singh *et al.*, 2014). For this reason, and many more, the International Energy Agency has deemed the use of fossil fuels as unsustainable due to their non-renewability and rapid depletion (Khan *et al.*, 2009). Their combustion adds to pollutants in the air and contributes a net increase to the percentage of global warming (Singh *et al.*, 2011). It has, therefore, become important to develop and promote renewable energy sources capable of sequestering atmospheric carbon dioxide (CO_2), minimizing dependency on fossil fuels, and maintaining environmental and economic sustainability (Singh *et al.*, 2011, Brennan and Owende, 2010).

Renewable biofuels such as microalgal biodiesel, biogas and bio-hydrogen etc., are prospective fuels to sustain future generations (Brennan and Owende, 2010, Chisti, 2013, Singh *et al.*, 2014). The use of biofuels reduces carbon monoxide (CO), hydrocarbons and sulphur oxide (SO_x) emissions, thus helping in reducing the greenhouse effect and global

warming (Mutanda *et al.*, 2011b). The by-products of these biofuels are biodegradable, hence contributing to the sustainability as well as reliability of renewable resources (Brennan and Owende, 2010). Thus, renewable biofuels are eco-friendly, nontoxic and can replace the conventional carbonaceous fuels such as petrol and diesel.

The term biofuel refers to a gas or liquid fuel that is derived from organic biomass. Biodiesel, which is produced by a transesterification reaction, consists of fatty acid methyl esters (FAME), and may promise an alternative to the conventional diesel fuel. Presently, biodiesel is produced from the oil of plants, such as sunflower, rapeseed, soybean, non edible crops as well as used vegetable oil and animal fats (Khan *et al.*, 2009). The major concerns with regard to the use of crop-based oils as sources of alternate fuels are: crops have short life spans, they require extensive land for cultivation, and also using plant oils for biofuel production would affect the global food demand (Mata *et al.*, 2010). It is therefore essential to find non-food based alternate feed stocks for biodiesel production. Among the various biodiesel feed-stocks available, microalgae present a source of opportunity as they contain the necessary fatty acids (FA) used for biodiesel production (Mutanda *et al.*, 2011b).

Nitrogen (N), phosphate (P) and carbon (C) are the main nutrients required for microalgal production. Organic N is used to make up biological molecules including proteins, chlorophylls, peptides, enzymes, energy transfer molecules (such as ADP, ATP), and genetic materials (RNA, DNA), (Cai *et al.*, 2013a). Nitrogen limitation may be used as a means of inducing neutral lipid accumulation within microalgal cells, whilst P is used for energy production (Bhatnagar *et al.*, 2010, Chisti, 2007). These two nutrients contribute 10-20 % of the total biodiesel production cost (Bhatnagar *et al.*, 2010, Chisti, 2013). Water and inorganic nutrients are recognized as important limiting resources when considering large scale

biodiesel production. The extensive utilization of water and nutrients for microalgal propagation can be offset by combining microalgal cultivation with wastewater (WW) treatment (Wu *et al.*, 2014).

Utilizing WW for microalgal propagation could reduce 90 % of the water demand. Also, depending on the WW source (i.e. domestic, agricultural or industrial), it may reduce the requirement for additional fertilizers (Wu *et al.*, 2014, Chen *et al.*, 2015). Microalgae can integrate biofuel production with CO_2 sequestration. Flue gas originating from power plants or other emission sources can be sequestered offering an efficient C capture and recycle opportunity, whilst generating carbon credits (Zhou *et al.*, 2014).

Moreover the organic constituents found in WW can also be used for mixotrophic growth of microalgae. Microalgae cultivated under mixotrophic growth conditions produce higher biomass and lipid when compared to photoautotrophic growth conditions. Wastewater would provide the essential resource for large-scale microalgal propagation in terms of water resources (Chen *et al.*, 2015). Microalgae in turn would clean up the WW, by reducing any N and P in the WW aiding wastewater treatment plants (WWTPs) to meet environmental standards (Zhou *et al.*, 2011). Growing microalgae in WW will also diminish the need for chemical fertilizers. Through the employment of WW, the zero-waste concept will be further implemented. This will encourage a more sustainable industry for microalgal biofuels. Integrated phyco-remediation and biofuel technologies have emerged as the only source of sustainable production of biofuels (Chen *et al.*, 2015). Due to the fact that microalgal cultivation using WW as a resource holds promising sustainability, a lot of research has been focused on this topic (Bhatnagar *et al.*, 2010, Wu *et al.*, 2014).

1.2 Aim and Objectives

The aim of this study was to evaluate the viability of domestic wastewater as a growth medium for microalgal cultivation and lipid production for biodiesel.

To achieve the aims the objectives were:

i. Physico-chemical and biological characterization of domestic wastewater over a one year period
ii. Assessment of the potential of pre- and post-chlorinated domestic wastewater for microalgae cultivation
iii. Determination of growth kinetics using different nitrogen sources supplemented in wastewater
iv. Optimisation of biomass and lipid yields of *Chlorella sorokiniana*
v. Elucidation of lipid profiles from biomass grown on wastewater and BG11 to assess its suitability for biodiesel production.

Chapter 2 : Literature Review

2.1 Significance of Biofuels

Energy is an essential requirement for human expansion as well as the maintenance of current and future living standards. Currently, fossil fuels such as coal, petroleum and natural gas are the main global energy sources (Kirrolia *et al.*, 2013). These fuel reserves are now becoming exhausted (Wu *et al.*, 2014), whilst their combustion has increased the accumulation of GHG's in the atmosphere, resulting in global warming (Arias-Penaranda *et al.*, 2013). Biofuels originating from food/agricultural crop sources show potential as alternative transport fuels (Chisti, 2013). However, food crop sources cannot be utilized due to global food demands and the ever increasing population, which are expected to double in the next 50 years. Therefore, the need to develop renewable and sustainable energy and fuel sources is paramount.

Biofuels production could also broaden opportunities like diversifying income and fuel supply sources, promoting employment in rural areas and increasing the security of energy supply among others (Mata *et al.*, 2010). The biofuels most commonly produced are biodiesel and bioethanol (Chisti, 2008). Biodiesel can be produced from plant and animal fats (Mata *et al.*, 2010). The United States presently produces biodiesel from soybeans (Rawat *et al.*, 2011, Fon Sing *et al.*, 2011). The use of plant oils for biodiesel production is extremely controversial. Plants require arable land for cultivation and their slow growth rates make them unsuitable for large scale biodiesel production (Makareviciene *et al.*, 2013). Currently there is a rapid global increase in the utilization of biodiesel in the transport sector. Land dedicated to the cultivation of plants for biodiesel production will also increase. This development endangers the natural environment by destroying natural habitats and

ecosystems as well as contaminating the underground and surface waters with fertilizers used to increase crop yield (Makareviciene *et al.*, 2013). Biodiesel is more eco-friendly compared to petro-diesel. This fuel contributes zero net CO_2 or sulphur (S) and overall less GHG's to the atmosphere when compared to petro-diesel. With increasing environmental concern and legislation, these factors benefit the acceptability of biodiesel (Chisti and Yan, 2011, Wang *et al.*, 2009a, Rawat *et al.*, 2013a). The immense need for renewable green fuel sources (Borowitzka and Moheimani, 2013), has brought about research focussing on alternate sources for biodiesel production. Environmentally friendly biodiesel from microalgae is a promising petro-diesel alternative (Rawat *et al.*, 2013a, Cai *et al.*, 2013a).

2.2 Microalgae

Microalgae are microscopic organisms having a simple cellular structure with the capability to uptake large amounts of different nutrients (Kirrolia *et al.*, 2013). Some microalgae grow in aqueous suspension. Microalgae use the same photosynthetic process adopted by higher plants for producing food. Autotrophically grown microalgae use natural and synthetic light as an energy source and atmospheric C as a carbon source. This can be CO_2 originating from exhaust gases. Microalgae are able to grow heterotrophically as well. Heterotrophic growth means growth without light, and therefore requires substituting the inorganic C source with an organic C source dissolved in the nutrient medium (Hidalgo *et al.*, 2013, Makareviciene *et al.*, 2013). Microalgae can exist as single cells or as chains of cells, however, they do not form differentiated, multi-cellular complexes like macroalgae. Microalgae have a rapid growth rate (some species can have a doubling time of 24 hours), (Chisti, 2007). Comparatively, land plants are less efficient in the utilization of solar energy.

Land plants convert less than 0.5 % of the solar energy received into plant biomass, whereas microalgae can theoretically capture more than 10.0 % of solar energy (Chisti, 2013).

2.3 Lipid Production by Microalgae

Microalgae are able to produce lipids particularly triacylglycerides (TAG's), which, after extraction, are the most suitable for biodiesel production. Many microalgal species accumulate large quantities of lipids (between 15 and 75 % in dry cell matter depending on species and culture conditions) (Arias-Penaranda *et al.*, 2013, Hidalgo *et al.*, 2013). Table 2.1 shows the oil content of various microalgae.

Table 2.1: Percentage oil yields produced from some microalgae, extracted from (Hidalgo *et al.*, 2013).

Algal group	Microalgae	Oil content (% dry wt.)
Green algae	*Botryococcus braunii*	25-75
	Dunaliella primolecta	23
	Tetraselmis sueica	15-23
	Chlorella sp.	28-32
Diatoms	*Phaeodactylum tricomutum*	20-30
	Cylindrotheca sp.	16-37
	Nannochloris sp.	20-35
Eustigmatophytes	*Nannochloropsis* sp.	31-68
	Isochrysis sp.	25-33
Red algae	*Porphyridium cruentum*	9.5

The lipids found in microalgae may include polar lipids, neutral lipids, wax esters, sterols and hydrocarbons. Under favourable conditions, microalgae synthesize FAs which include medium-chain (C10–C14), long-chain (C16–18), very-long-chain (C20) and FA derivatives. The major membrane lipids are glycosylglycerides (e.g. monogalactosyldiacyl-glycerol, digalactosyldiacyl-glycerol and sulfoquinovosyldiacyl-glycerol), which are located in the chloroplast, along with high amounts of phosphoglycerides (e.g. phosphatidylethanolamine and phosphatidyl-glycerol), which can be located in the plasma membrane and many endoplasmic membrane systems (Chen *et al.*, 2008).

Under stressed growth conditions such as N deficiency, many microalgae amend their lipid biosynthetic pathways towards the formation and accumulation of neutral lipids, mostly in the form TAG. Unlike glycerolipids, TAGs don't play a structural role. They primarily serve as storage forms of C and energy.

2.4 Cultivation of Microalgae

Microalgae may be grown on land not suitable for farming or in deserts unsuitable for living. With fresh water becoming an increasingly scarce resource, microalgae may also be cultivated using saline, brackish, gray water or even WW (Hidalgo *et al.*, 2013, Han *et al.*, 2014). The growth of these organisms is hugely influenced by growing conditions, so manipulation of these parameters will result in a higher production of microalgal biomass (Makareviciene *et al.*, 2013).

2.4.1 Cultivation Options

Currently the only recommended methods for large scale microalgae production are open raceway ponds (ORPs) and closed photo bioreactors (PBRs) (Chisti, 2007, Rawat *et al.*, 2013a, Wang *et al.*, 2013b). Open raceway ponds are usually situated outdoors and rely on direct sunlight (Singh *et al.*, 2011, Resurreccion *et al.*, 2012), while PBRs can be situated indoors or outdoors to utilize natural light. Microalgae can be grown in either way, however, using open systems can present some challenges (Bahadar and Bilal Khan, 2013, Wang *et al.*, 2013b, Resurreccion *et al.*, 2012).

Open raceway ponds are operated at depths between 0.15 and 0.5 meters (m) (Chisti, 2007, Brennan and Owende, 2010, Fon Sing *et al.*, 2011). These systems are simple in structure, have moderately low equipment expenses, are easily maintained and have lower energy consumption rates when compared to PBRs (Wang *et al.*, 2013b, Rawat *et al.*, 2013b). However, these systems have a lot of disadvantages. Without adequate mixing/aeration, CO_2 may not be distributed to all microalgal cells; there is slow delivery of nutrients to microalgal cells, floatation and sedimentation of both viable and non-viable cells occurs and light penetration into deeper layers is limited. Contamination from environmental organisms, leaves, dust, etc., is rife within ORP systems. Difficulties associated with light penetration may be overcome by reduced the depth of the ORP which will increase productivity (Makareviciene *et al.*, 2013, Fon Sing *et al.*, 2011, Resurreccion *et al.*, 2012).

Closed PBRs have better control and regulation systems for temperature and pH. In this way they afford protection against contamination. They have reduced evaporation rates on account of being closed systems. They also show reduced CO_2 diffusion and O_2 build up because it can't escape into the air (Fon Sing *et al.*, 2011, Rawat *et al.*, 2013b, Resurreccion *et al.*, 2012). However, closed systems require higher initial investments than open systems. Closed systems are costly to construct and are associated with high maintenance (cooling expenses due to high daylight solar irradiance) and energy requirements owing to the circulation of the microalgal culture (Borowitzka and Moheimani, 2013, Makareviciene *et al.*, 2013, Rawat *et al.*, 2013b). Due to these reasons, ORPs seem to be the most suitable and sustainable culture system.

2.4.2 Land Usage

Large scale microalgal cultivation systems do not utilize expansive areas of arable land as plants do. Microalgae potentially have higher growth rates than land plants (Bahadar and Bilal Khan, 2013). Microalgae can potentially produce 30 times the quantity of lipid per unit area of land when compared to terrestrial crops (Costa and de Morais, 2011). The land selected for large-scale microalgal cultivation requires optimum climatic conditions for high productivity. This includes moderate to high yearly average sunlight, high temperatures and low rainfall (Chisti and Yan, 2011, Rawat *et al.*, 2013b). Furthermore, the land needs to be level for ease of construction of cultivation systems. The land chosen for construction needs to be fairly close to the major inputs like water and CO_2 to reduce transportation and supply costs (Borowitzka and Moheimani, 2013).

2.4.3 Water Usage

The production of large amounts microalgal biomass, irrespective of whether it is using ORPs or PBRs, requires substantial amounts of water (3700 kg water/kg biodiesel) (Rawat *et al.*, 2013b, Chisti, 2013). Since freshwater resources are limited, WW sources may be used to cultivate microalgae (Aresta *et al.*, 2005, Fon Sing *et al.*, 2011). When using ORPs in areas of high solar irradiation, evaporation rates can be in excess of 1.5 m per year. These evaporative losses can be replaced with WW rather than fresh water, owing to the very high volumes required (Borowitzka and Moheimani, 2013).

2.4.3.1 Wastewater

The demand for potable water is currently a universal priority. Wastewater treatment plants strive to produce water, free of nutrients, by employing treatments composed of numerous stages of physical, chemical and biological methods before discharge. Nitrogen is biologically removed from WW using two methods: (1) the uptake of nitrogenous compounds by bacteria, creating N rich biomass which is recycled back into the system as return activated sludge and, (2) by the oxidation of NH_4 forming NO_3, NO_2, and nitrous oxide (NO_x) which is evaporated to the atmosphere as gaseous N (Daims *et al.*, 2006). Phosphorus is the most difficult to remove from WW. Most treatment facilities remove P from WW effluents by chemical precipitation with Fe, alum, or lime. It can be removed by converting P ions in WW into a solid fraction. This fraction can be an insoluble salt precipitate (chemical precipitation with Fe, alum, or lime), which is removed via skimming. Phosphorus is also converted to a microbial mass as activated sludge via microbial activity (de-Bashan and Bashan, 2010). In this way it makes P not fully recyclable as it is removed with a range of waste products (de-Bashan and Bashan, 2010, Cho *et al.*, 2011, Pittman *et al.*, 2011). Precipitated P is either buried in landfills or further treated to generate sludge fertilizer (de-Bashan and Bashan, 2010, Pittman *et al.*, 2011).

Tertiary methods do not remove all of the N and P and residual nutrients can still be found in some effluents (Lizzul *et al.*, 2014). Optimization of the combination of WW treatment and biodiesel production from microalgae presents a way forward (Wang *et al.*, 2009a, Cabanelas *et al.*, 2013a). Growth of microalgae can effectively remove nutrients, heavy metals, organic contaminants, and pathogens from domestic WW (Muñoz and Guieysse, 2006, Pittman *et al.*, 2011, Han *et al.*, 2014). Microalgal cultivation in WW

effluent is highly desirable as it could serve a dual role of bioremediation of WW and generating biomass for biodiesel production (Rawat *et al.*, 2011). An advantage of using WW is that it can be fed directly into ORPs (Rawat *et al.*, 2011). Also, sustainable biomass yields can be achieved without the addition of expensive fertilizers (Kirrolia *et al.*, 2013, Wang *et al.*, 2013b, Wu *et al.*, 2014). Studies have demonstrated that the use of WW in ORPs results in moderate to high lipid production (Wang *et al.*, 2013b, Rawat *et al.*, 2013b). Growing algae on WW has the potential to lower the cost of biofuel production (Pittman *et al.*, 2011, Wang *et al.*, 2013a).

2.4.3.2 Microalgae and waste resources

Large-scale propagation of microalgae can be more costly than traditional crop production (Rawat *et al.*, 2013a, Chen *et al.*, 2015). Microalgae require an abundance of water and nutrients (Borowitzka and Moheimani 2013, Rawat *et al.*, 2013b). These expenses make up the bulk cost of microalgal production (Chisti 2013, Han *et al.*, 2014). In 2009, the global annual freshwater consumption was estimated at 3,908.3 billion m^3, most of the consumed water was turned into WW (Chen *et al.*, 2015). Moreover, total N and P concentrations in WW can be as high as 1000 mgL^{-1} in agricultural effluent (Wu *et al.*, 2014, Zhou *et al.*, 2014, Chen *et al.*, 2015). Due to its abundance of nutrients, WW can be used as a low cost nutrient source for microalgae cultivation (Olguin 2012, Wu *et al.*, 2014). Microalgal cultivation in WW effluent is highly desirable as it serves a dual role of bioremediation of WW and generating biomass for biodiesel production (Rawat *et al.*, 2013b). Microalgal treatment of WW offers a cheaper and more efficient means of removing nutrients and metals when compared to conventional tertiary WW treatment (Kirrolia *et al.*, 2013, Wang *et al.*, 2013a, Zhou *et al.*, 2014). For example, commercial approaches of P

removal do not recycle it as a fully sustainable product. It is retrieved along with various other waste products (Zhou *et al.,* 2014). The application of WW for microalgal biomass production would cut the water footprint (WF) (Chisti, 2013). Freshwater resources will be insufficient to support any large scale microalgal biomass or biofuel production. Without water recycle, the WF value can reach 3700 kg/kg biodiesel, which could be reduced by as much as 90 % with WW. The WF values for biodiesel produced from Jatropha and soybean has been estimated at 5787 kg/kg (Chen *et al.,* 2015) and 13,676 kg/kg (Chisti, 2013), respectively. Thus, the WF of WW based microalgal biodiesel is significantly lower than that for biodiesel from other sources. With WW, the WF mainly originates from evaporative loss during cultivation in open PBRs (Chen *et al.,* 2015). Furthermore, WW composition varies with sources (Olguin, 2012). As a result, the ability of microalgae to grow in different waste streams varies for each strain and each waste stream (Rawat *et al.,* 2013b). With this in mind, agricultural, industrial and municipal WW streams will be briefly discussed.

2.4.3.3 Agricultural wastewater

The use of animal WW for growing microalgae serves the dual purpose of providing a source of water and nutrients whilst simultaneously treating this type of abundant WW stream (Olguin, 2012, Hernandez *et al.,* 2013). Agricultural WW has a considerably higher concentration of nutrients when compared to municipal WW (Sahu *et al.,* 2013). Organic matters, high in N and P, are excreted as a high percentage of the animal's weight. Pig and poultry WW can be the most polluting wastes due to their higher organic matter contents in terms of Biological Oxygen Demand when compared to other animal WWs (Olguin, 2012). Studies show that microalgae are able to grow and efficiently remove nutrients from agricultural WW (Abou-Shanab *et al.,* 2013, Ji *et al.,* 2013). *Neochloris oleoabundans* was

cultivated on anaerobically digested dairy manure under batch conditions. The microalga was capable of assimilating 90–95 % of the initial NO_3 and NH_4. After day 6 of cultivation the microalga contained 10–30 % FAMEs (by dry weight) (Olguin, 2012).

2.4.3.4 Industrial wastewater

Most of the industrial WW produced globally pollutes the environment and creates health hazards. If 50 % of this WW (approximately 88 trillion gallons) is used for microalgal cultivation, it would yield approximately 247 million tons of microalgal biomass (0.7 gL^{-1} WW) and 37 million tons of microalgal lipid (roughly 0.42 kg or 0.13 gallons of algae oil per 1000 gallons of WW, with yield of 15 g algal oil per 100 g dry algae) (Zuka *et al.,* 2012). However, high heavy metal concentration, organic chemical toxins as well as relatively low N and P concentration in industrial WW have negative effects on microalgal cultivation. As a result, the application of industrial WW for microalgal based biofuel production is limited (Chen *et al.,* 2015). The majority of studies involving microalgal cultivation on industrial WW focus on WW from the palm oil mill industry, carpet mill WW and olive mill WW (Chinnasamy *et al.,* 2010, Di Iaconi *et al.,* 2010, Hernandez *et al.,* 2013, Zhou *et al.,* 2014, Chen *et al.,* 2015). The composition of industrial WW is complex. Carbon is often deficient whilst the N and P present are capable of supporting microalgal growth (Chinnasamy *et al.,* 2010). Wu *et al.,* (2012) found that *Chlamydomonas* sp. TAI-2 grown on industrial WW was capable of removing 100 % NH_4^+-N (38.4 mgL^{-1}) and NO_3^--N (3.1 mgL^{-1}) and 33 % PO_4^{3-}-P (44.7 mgL^{-1}) whilst accumulating 18.4 % lipid. Over 90 % of total FAs found were C14:0, C16:0, C16:1, C18:1, and C18:3 FAs, which could be utilized for biodiesel production. Sun *et al.,* (2013) focussed on the acclimation of *Chlorella pyrenoidosa* in diluted industrial WW for biomass production and the removal of chemical oxygen demand, NH_3-N and P. The

removal efficiency of chemical oxygen demand reached 89.2 %, while the total N and P decreased by 64.52 % and 82.20 %, respectively. Biomass concentrations reached 1.25 gL^{-1} and 38.27 % (dry basis, w %) of lipid content was found after microalgal cultivation.

2.4.3.5 Challenges when using agricultural and industrial wastewater for microalgal cultivation

Despite the positive outlook on the use of WW mediated biomass production (Rawat *et al.,* 2013b) some drawbacks include WW toxicity consideration for human handling and low toxicity levels for the microalgal growth (Zuka *et al.,* 2012). The occurrence of toxins and predators in these WWs may have negative impacts on microalgal growth. Additional organic carbon leads to contamination and competition between cultures (Zhou *et al.,* 2014). Bacterial contamination is expected to influence (inhibit or stimulate) microalgal biomass production, lipid content and nutrients removal. This results in the culture instability (Ma *et al.,* 2014, Wang *et al.,* 2013). Furthermore, compositions of industrial and agricultural WW streams may vary with sources. Microalgal cultures ability to valorise waste streams varies between strains and individual waste stream (Chen *et al*., 2015, Mohan *et al*., 2014). This issue cannot be controlled and close monitoring and adjustment of nutrient levels may be required. Utilization of these WWs will require recurrent cleaning of the culturing system (Rawat *et al.,* 2013b). The higher concentrations of nutrients present in agricultural WWs inhibit microalgal growth. A large portion of C is locked in large insoluble organic compounds which are unavailable for microalgal assimilation. Also, the high turbidity of agricultural WW reduces light penetration required for microalgal growth (Olguin, 2012, Sahu *et al.,* 2013, Zhou *et al.,* 2014, Chen *et al.,* 2015). As a result, agricultural WWs need to be diluted during storage and before use. Dilution of agricultural WW has an impact on biomass accumulation and nutrients removal (Arumugam *et al.,* 2013, Lizzul *et al.,* 2014,

Chen *et al*., 2015). Dilution requires large quantities of fresh water to dilute the concentrated animal WW (Sahu *et al.,* 2013; Zhou *et al.,* 2014; Chen *et al.,* 2015).

Low microalgal biomass productivity is a major obstacle when cultivating microalgae in industrial and agricultural WW (Zhou *et al.,* 2014). Low biomass productivity rates increase the consumptions on resources (such as increased the land occupation for cultivation) and energy involved in processing. Large scale microalgal propagation requires suitable sites for cultivation. These sites need to be situated close to WW sources. Additional collection and transportation of the WW would adversely affect the final profits of microalgal production (Chen *et al.,* 2015). The challenges for WW based microalgal cultivation also include the lack of high system efficiency and lack of low cost downstream processing. These problems must be overcome through scientific breakthroughs and innovation (Zhou *et al.,* 2014).

2.4.3.6 Using municipal domestic wastewater for microalgal cultivation

High concentrations of N and P removed from municipal WW can be used for growing microalgae (Rawat *et al.,* 2013a, Chen *et al.,* 2015). During municipal sewage treatment, different types of WW streams are generated at different stages in the process. These include raw WW before primary settling, raw WW after primary settling, WW after the activated sludge tank (effluent), primary clarifier effluent, secondarily treated effluent and a fraction derived from WW generated during return activated sludge (centrate) (Olguin 2012, Zhou *et al.,* 2014). Representative concentrations of influent NH_3-N and P in municipal WWs may fall into ranges of 20–40 mgL^{-1} and 1–10 mgL^{-1}, respectively (Kong *et al.,* 2010). These concentrations are adequate to support high productivities from most microalgal strains (Olguin 2012, Cabanelas *et al.,* 2013b). Centrate is generated from the dewatering of sludge

after primary and secondary settling. This WW stream is rich in phosphorus (ranging from 9.4 to 200 mgL^{-1}), N (ranging from 53 to 157 mgL^{-1}), and a variety of minerals such as Ca, Mg, K, Fe, Cu and Mn. Chemical Oxygen Demands can range from 1000 mgL^{-1} to around 2800 mgL^{-1} (Zhou *et al.*, 2012b). Centrate, however, contains numerous species of bacteria that would affect biomass accumulation, lipid content and nutrients removal (Ma *et al.*, 2014). The study conducted be Ebrahimian *et al.*, (2014) found NH_4 and P concentrations in secondary effluents to be 0.63 mgL^{-1} and 0.53 mgL^{-1}. Kong *et al.*, (2010) found NH_3 and P concentrations in secondary effluents to be 8.78 mgL^{-1} and 1.25 mgL^{-1} respectively. Cultivating microalgae in nutrient-rich raw municipal WW is an alternative that enhances microalgal biomass productivity (Rawat *et al.*, 2011, Olguin 2012, Shao *et al.*, 2014). This approach also serves a dual role of nutrient reduction in the WW and cost-effective biofuel feedstock production (Zhou *et al.*, 2014). However, these are laboratory-scale experimental results require confirmation in long term large scale production (Chen *et al*., 2015).

2.4.4 Nutrient Usage

Microalgae require N, P and C as macronutrients and other trace nutrients as micronutrients for cultivation. The composition of freshwater microalgae (in terms of N, P and C) is constant, although the N content will fluctuate according to environmental conditions and nutrient uptake of the specific strain of microalgae (Chisti, 2013). To achieve maximum microalgal growth rates, these nutrients need to be supplied in adequate quantities, so as not to limit growth. When using groundwater as culturing medium nutrients like potassium (K), in addition to N, P and C, may be needed to supplement microalgae growth (Han *et al.*, 2014). Cultivation of microalgae requires large quantities of nutrients (Borowitzka and Moheimani, 2013).

2.4.4.1 Nitrogen

Nitrogen is indispensable for the growth of microalgae. Nitrogen is available in multiple forms like ammonia (NH_3), nitrate (NO_3) or urea [$CO(NH_2)_2$]. However the form of N supplied to microalgae may affect the organisms functioning by changing its metabolic pathway and altering its FA composition (Borowitzka and Moheimani, 2013, Kamyab *et al.*, 2014). All microalgae assimilate inorganic N in the form of NO_3, nitrite (NO_2), and NH_4 (Cai *et al.*, 2013a). Nitrogen fertilizer requires energy to be produced which makes it far too expensive and energy intensive to use for production of biofuel (Chisti, 2013). Nearly 1.2 % of global energy utilization may be attributed to producing N fertilizers. Ammonium nitrate (NH_4NO_3) may be used for microalgal growth. Its production has an embodied energy value of 67 MJ kg^{-1} N. Also, fertilizer production will release CO_2. One of the benefits of using biodiesel is to decrease C emissions. Therefore, producing fertilizer through conventional technologies for the use in biodiesel production would be counterproductive (Chisti, 2013). A possible alternative may be to cultivate mixed cultures including N-fixing microalgae (heterocystous cyanobacteria) such as *Anabaena* (Chisti, 2013). These microalgae may release NH_3 and NO_2 into the medium making it available for other microalgal cultures (Borowitzka and Moheimani, 2013). A typical domestic WW stream consists of between 85 mgL^{-1} and 131.5 mgL^{-1} total N (Pittman *et al.*, 2011, Rawat *et al.*, 2013a).

2.4.4.2 Phosphorus

Microalgae require P at defined concentrations for cell growth and energy metabolism (Chisti, 2013). Phosphorus can be found in lipids, proteins, nucleic acids, as well as the intermediates of carbohydrate metabolism (Cai *et al.*, 2013b, Chu *et al.*, 2013). Throughout

microalgal metabolism, P in the forms of dihydrogen P and mono hydrogen P is integrated into organic compounds via phosphorylation, which generate ATP from ADP (Cai *et al.*, 2013b, Chu *et al.*, 2013). Phosphorus concentrations in WW streams can be as high as 10–100 mgL^{-1} (Wu *et al.*, 2014). Phosphorus from WW effluents are ideal for microalgae culturing, however, steadfast growth rates may be hard to achieve. This is due to variability in the composition of the WW. A lot of attention is being paid to P recovery methods from WW streams, however, feasibility on a large scale is questionable (Borowitzka and Moheimani, 2013). Like crude oil reserves, P is a non-renewable resource which will be depleted in the next 50–100 years (Chu *et al.*, 2013). Biodiesel production industries will compete directly with the food production industries for P fertilizers which are critical for food crop production (Chisti, 2013).

2.4.4.3 Carbon

Carbon is essential for cultivating microalgae for biodiesel production. Production of each ton of microalgae requires at least 1.83 tons of CO_2 (Chisti, 2013). Carbon, as CO_2, can be fixed from the atmosphere through the process of photosynthesis. Several strains of microalgae exhibit heterotrophic patterns using organic C forms, whilst other strains possess both autotrophic and heterotrophic behavioural patterns simultaneously (mixotrophy) (Campbell, 2008). Carbon is used as soluble carbonates required for cell growth by direct uptake or by the conversion of carbonate to free CO_2 through the activity of the enzyme carbonic anhydrase (Cai *et al.*, 2013a). The use of purchased CO_2 can contribute about 50 % of the total cost for biomass production (Chisti, 2007). Microalgal growth for biodiesel production will not be feasible unless the CO_2 required is available for free (Chisti, 2007). Finding cheap, available sources of C for microalgal cultivation is imperative to minimize the

cost impact of the process. Crude glycerol, which is a by-product of biodiesel production, has recently been considered as an alternative C source for heterotrophic microalgal cultivation (Bahadar and Bilal Khan, 2013, Cabanelas *et al.*, 2013b). On-site utilization of this glycerol will reduce the costs associated with biofuels production (Rawat *et al.,* 2013b). Presently, glycerol is cheaply available in large quantities (Cabanelas *et al.*, 2013b).

2.4.4.4 Other Nutrients

Micronutrients are also needed for microalgal cultivation as they are essential for microalgal growth (Raja *et al.*, 2014, Soares *et al.*, 2012). These nutrients make up the cell as well as part of the enzyme active centre in microalgal cells (Dou *et al.*, 2013). Iron (Fe) is the most essential nutrient required for microalgal cultivation. Iron is involved in enzymatic processes like oxygen metabolism, electron transfer, N assimilation, and DNA, RNA and chlorophyll synthesis. In domestic WWs, Fe is often bio-unavailable. Consequently, Fe is often supplied as chelated complexes thus rendering it bio-available (Markou *et al.,* 2014). Iron is favourable as it redirects the metabolic pathway from producing starch to producing lipid thereby increasing the lipid content of microalgae (Dou *et al.,* 2013). Magnesium (Mg) is required for microalgal biomass production. Microalgae have internal Mg concentrations ranging from 0.35 to 0.7 %. Magnesium is essential as it participates in fundamental cell processes like ATP reactions for C fixation. It is also a major enzymes activator. Magnesium constitutes part of the photosynthetic apparatus, in particular chlorophylls. It is provided to microalgae largely as $MgSO_4$. Domestic WWs are often deficient in Mg. Salts such as $MgNO_3$, magnesium chloride or dimagnesium phosphate can be used to supplement WW (Markou *et al.,* 2014). Manganese (Mn) is utilized during the growth of microalgae by being a catalyst for photosynthesis and an enzyme activator. The addition of ethylene-di-amine-

tetra-acetic acid (EDTA) into a culture medium would form a complex with the metal ions in solution which would promote their absorption and thereby the growth of the microalgae (Dou *et al.*, 2013). However, when supplied at too high a concentration, micronutrients can be toxic to most microalgae species (Dou *et al.*, 2013, Cai *et al.*, 2013a, Soares *et al.*, 2012). Some elements such as Mn promote oxidative degradation thus affecting the quality and preservation of oil and fats (Soares *et al.*, 2012).

2.5 Harvesting Strategies

The propagation of the microalgal biomass is the first part of the biodiesel production process. The second part deals with biomass harvesting and dewatering, lipid extraction and transesterification to biodiesel (Borowitzka and Moheimani, 2013, Kim *et al.*, 2013). Efficient recovery methods are essential to the economics of biodiesel production (Pragya *et al.*, 2013). Microalgal harvesting is expensive and accounts for 20–30 % of the total biodiesel production cost (Borowitzka and Moheimani, 2013). Harvesting methods depend on the uniqueness of the microalgal strain (size of the microalgal cells, densities of the culture etc., (Rawat *et al.*, 2013b, Pragya *et al.*, 2013). Diluted tiny-celled biomass slurries sometimes need a combination of recovery separation steps (Rawat *et al.*, 2013b, Bahadar and Bilal Khan, 2013), whilst larger microalgal cells can be centrifuged or filtered. Flocculation, gravity sedimentation, and flotation may also be used to harvest microalgae (Pragya *et al.*, 2013, Rawat *et al.*, 2013b, Kim *et al.*, 2013).

2.6 Lipid Extraction and Identification Methods

Once the biomass is concentrated and thickened, other processes are used to extract the lipids for biodiesel production (Halim *et al.*, 2012). An extensive variety of lipid extraction methods are available. Pre-treatment of certain microalgal biomass may be required before lipid extraction can be attempted (Rawat *et al.*, 2011, Pragya *et al.*, 2013, Rawat *et al.*, 2013b). Cellular disruption may be facilitated mechanically or chemically (Rawat *et al.*, 2011, Rawat *et al.*, 2013b). Various cell disruption methods exist which include microwave disruption, sonication, bead milling (Kim *et al.*, 2013), autoclaving (Rawat *et al.*, 2011), grinding, osmotic shock, homogenization (Rawat *et al.*, 2013b, Bahadar and Bilal Khan, 2013), freeze drying and change in osmotic potential (Rawat *et al.*, 2011). Lipid extraction may be performed using physical or chemical methods (solvent extractions), or a combination of the two (Rawat *et al.*, 2011). The selection of a method should be based on accuracy, efficiency, cost effectiveness, high throughput capability, robustness, precision, reproducibility (Rawat *et al.*, 2013b) and it should not damage the extracted lipids (Pragya *et al.*, 2013). A few conventional methods for lipid extraction include: Folch method, gravimetric method, the Soxhlet method (n-hexane) (Bahadar and Bilal Khan, 2013), and Bligh and Dyer method (mixture of chloroform and methanol) (Pragya *et al.*, 2013). Analytical methods used to identify and quantify microalgal lipids include Gas Chromatography (GC), Gas Chromatography Mass Spectroscopy (GC-MS), High Performance Liquid Chromatography (HPLC) and Thin Layer Chromatography (TLC) (Halim *et al.*, 2012). The method of lipid analysis should allow for the recovery of lipid extracts and at the same time avoid lipid decomposition (Rawat *et al.*, 2013b, Halim *et al.*, 2012). Extracted lipids need to be then transesterified into biodiesel (Bahadar and Bilal Khan, 2013).

2.7 Production of Biodiesel (Transesterification)

Transesterification transforms raw microalgal lipids into renewable, biodegradable and non-toxic biodiesel for direct utilization in unmodified diesel vehicles.

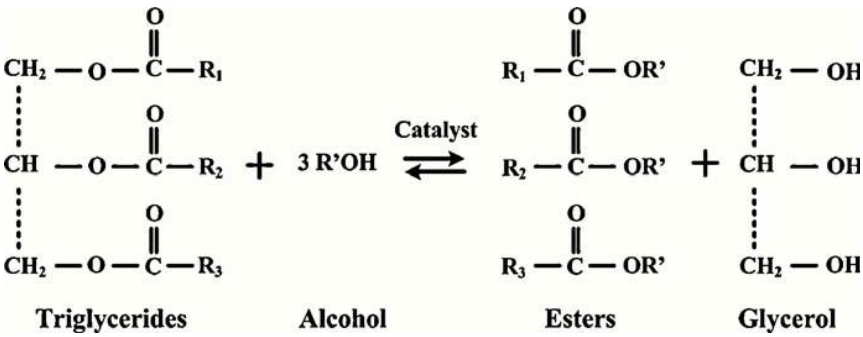


Figure 2.1: Transesterification of triglycerides (overall reaction) (Mata *et al.*, 2010).

Microalgal lipids are particular viscous and require conversion to lower molecular weight. The reaction proceeds as an equilibrium reaction which requires a catalyst to sustain the equilibrium shift as well as to improve the reaction rate (Rawat *et al.*, 2013b, Rawat *et al.*, 2011). The transesterification reaction involves TAGs (parent oil) and a short chain alcohol. It is completed in three steps where the TAG is successively converted to diglycerides and monoglycerides by displacement of the alcohol which forms glycerol (by-product), producing three molecules of FAME (biodiesel) (Velasquez-Orta *et al.*, 2013). This can be seen in Figure 2.1. The alcohols frequently utilized are methanol, ethanol, propanol, butanol, and amyl alcohol. Methanol is preferred due to its low cost as well as its physical and chemical advantages (Rawat *et al.*, 2013b, Rawat *et al.*, 2011).

The transesterification reaction uses homogeneous or heterogeneous catalysts (Mata *et al.*, 2010, Meher *et al.*, 2006). Homogeneous catalysts are able to attain high conversion rates while heterogeneous catalysts provide moderate conversions. Homogenous or heterogeneous catalysts may be acid or alkaline based. Homogenous catalysts include sulphuric acid (H_2SO_4) or hydrochloric acid (HCl) and sodium or potassium hydroxide/methoxide as alkaline catalysts (VanGerpen *et al.*, 2004, Velasquez-Orta *et al.*, 2013). Examples of heterogeneous catalysts are sulphated zirconia (acid based) and zinc aluminates or oxides (alkaline based) (Velasquez-Orta *et al.*, 2013). Alkali catalysts are more suitable for high level conversion of triglycerides to their corresponding esters, however, if the oil has high free fatty acid (FFA) content and water, an acid catalyst would be feasible (Meher *et al.*, 2006).

Microalgal biodiesel has the most potential to replace petroleum derived transport fuels, but it has its limitations. It is one of the most environmentally friendly fuels, but whether it can be produced inexpensively in sufficient quantities to significantly replace fossil fuels, needs to be proven (VanGerpen *et al.*, 2004, Makareviciene *et al.*, 2013, Chisti., 2013). Microalgal biodiesel has analogous combustion properties to petroleum diesel (Viswanath *et al.*, 2010). The CO_2 produced from the utilization of the biodiesel will be sequestered from the atmosphere by the subsequent culture of microalgae used to produce the next batch of biodiesel (Bowman *et al.*, 2006, Makareviciene *et al.*, 2013). Biodiesel is almost twice as thick as petroleum diesel, therefore, it has better lubricating properties (Bowman *et al.*, 2006), and leads to a better running and long lasting engine (Campbell, 2008, Chisti, 2007).

2.8 End products and applications of biomass usage

The high costs associated with biodiesel production can be considerably reduced by employing the biorefinery concept. This concept consumes all the components used in the biodiesel production process to produce useful products (Rawat *et al.*, 2011). Microalgae have high physiological diversity. A typical microalgal biorefinery process can simultaneously use the extracted lipids from microalgal cells to produce biodiesel, while the carbohydrates and cellulose can be used to produce bioethanol through fermentation (Singh *et al.,* 2014). Additionally, the carbohydrates, proteins and fats can be converted into methane and bio-hydrogen via anaerobic digestion. Residual biomass (high in protein and carbohydrate) may be utilized by the agricultural or aquaculture industry as a feed supplement (Zhu, 2015, Singh *et al.,* 2014). Thus, microalgal biofuel production can be integrated with the co-production of high value added products which will offer a promising opportunity to commercialize microalgal biofuels (Zhu, 2015).

2.9 Hypotheses

- If the nutrient concentrations in municipal domestic WW are high enough, then this WW stream would be acceptable to support the growth of *C. sorokiniana* for limited periods of time.
- If the FAs extracted from *C. sorokiniana* grown on domestic WW are between the C12 to C22 range, then this FA composition would be suitable for conversion to biodiesel.

2.10 Significance and Motivation of Research

With the ever increasing demand on the planet's finite fossil fuel reserves, the urgency for scientific endeavour in the quest for renewable and sustainable energy cannot be over emphasized. However, depleting freshwater reserves question the feasibility of this concept. Presently farmers use 2700 km^3 of water, projected figures estimate that farmers would need approximately 4000 km^3 of water in 2050, excluding drinking water. Biodiesel production from crops in 2030 would require 180 km^3 of water. Microalgae present a sustainable alternative to biofuel crops; however they require 11–13 million litres of water per hectare per year for cultivation in open ponds. People produce 3 billion tones of domestic WW annually. The use of WW reduces the requirement for depleting freshwater supplies as well as additional nutrient supplementation.

This study investigated the potential of producing microalgal biodiesel using domestic WW. It aimed to determine the viability of municipal domestic WW effluent as a substrate for microalgal growth and lipid production. The physico-chemical and biological characterization of WW effluents from three WWTPs was tested. The efficacy of pre- and post-chlorinated effluents as growth mediums for *Chlorella sorokiniana* was investigated. Four N sources were evaluated as supplements for domestic WW. Experimentation was carried out on lab-scale which took two years to reach fruition.

Chapter 3 : Wastewater characterization

3.1 Introduction

Large quantities of nitrogenous matter as well as carbohydrates, sugars, lipids as well as minute quantities of several synthetic organic chemicals make up the majority of WW (Ahmad *et al.*, 2012). Phosphorus and N are the major nutrients necessary for microalgal cultivation (Rawat *et al.*, 2011). Residential, agricultural and industrial release of P and N into water bodies like lakes and reservoirs causes eutrophication (Ahmad *et al.*, 2012, Cai *et al.*, 2013b). Wastes released into sewers are made up of P as orthophosphate (PO_4) (Hussain *et al.*, 2011, Rawat *et al.*, 2013a) and N in the form of NH_3 and NH_4. These waste products combine with water to form domestic WW (Rawat *et al.*, 2011). Organic matter found in WW varies from simple compounds to complex polymers (Huang *et al.*, 2010, Fernandes *et al.*, 2013). Organic matter comprises of C, H and O as well as elements such as S, P, Fe and NH_3 (Rawat *et al.*, 2011, Cho *et al.*, 2013). Wastewater also contains human pathogens like bacteria, viruses, and parasitic worms (Ahmad *et al.*, 2012). Domestic WW has less N and P than agricultural WW, however, toxic heavy metals such as Fe, zinc (Zn), copper (Cu), silver (Ag), nickel (Ni), mercury (Hg), cobalt (Co), cadmium (Cd), lead (Pb) and chromium (Cr) are frequently found in domestic WW at varying concentrations (Singh *et al.*, 2012). One of the main requirements in traditional WW treatment is the removal and reduction of nutrients and heavy metals to acceptable limits before its discharge into water bodies and reuse (Ding *et al.*, 2013). However, final effluent discharged from the WW treatment facilities may still have nutrients at lower concentrations (Lizzul *et al.*, 2014).

Microalgae have been utilised for the tertiary treatment of municipal WW for more than half a century. The nutrients required for microalgal growth (N, P, vitamins and trace metals) can be sourced directly from tertiary WW effluent. The composition of the WW has a critical impact on microalgal cultivation (Lizzul *et al.*, 2014). This objective aimed to characterize domestic WW effluent and compare the growth characteristics of *C. sorokiniana* on both domestic WW effluent and commercial medium. It also aimed to determine the viability of domestic WW as a medium for microalgal cultivation.

3.2 Methodology

3.2.1 Physical, chemical and biological analyses of wastewater

The WWs used in the study were collected from Kingsburgh Wastewater Treatment Plant (WWTP), Howick WWTP and Craigieburn WWTP. These plants were selected on the basis that they treat domesticated WW. Post-chlorinated WW effluents were sampled from the chlorine (Cl) contact tank. Wastewater was characterized weekly; however in the case that there was no significant variation in the data; samples were taken bi-weekly. Wastewater effluents were collected from the chosen sampling locations between 9 and 10 a.m. The final effluent was characterized for N, P and Cl. Nitrogen (NH_3 and NO_3) and P concentrations were determined by standard methods (APHA, AWWA, WEF, 1998), using a gallery discreet water analyzer (Thermo Scientific, USA) (APPENDIX A-D). Ammonia and P were determined via the automated spectrophotometric and automated colorimetric methods respectively. Chlorine levels were determined using a Cl pocket colorimeter (HACH, USA) as per Mutanda *et al.*, 2011a. A 10 mL WW effluent sample was filtered through a 0.45µ whatman filter paper. The samples were then added to the colorimeter cuvette. This was then

used to blank the machine. An N, N-diethyl-p-phenylenediamine-free Cl reagent sachet (0.1 g) was added to the cuvette and shaken for 21 seconds (s), then read.

The WW was also characterized with respect to temperature and pH. This was measured with a multi parameter meter (Yellow Springs Instruments, USA). Total suspended solids (TSS) were also measured. A 100 mL WW sample was poured into a pre-weighed beaker and then dried in an oven at 105 ^{o}C till constant weight (APHA, AWWA, WEF, 1998). Post-chlorinated final effluent was also biologically characterized. This was done to establish the total numbers of bacteria in the effluent; the standard plate count method was used (APHA, AWWA, WEF, 1998). Nutrient agar plates were used for this experiment. Bacterial numbers are indicated as colony forming units (CFUs) per mL. 1 mL of WW was swabbed onto nutrient agar plates, between 30 and 300 colonies were counted after cultivation in an incubator at 25 ^{o}C for 24 hours (h) as per standard methods (APHA, AWWA, WEF, 1998).

3.2.2 Microalga and Culture conditions

Chlorella sorokiniana, (GenBank accession number: AB731602.1), was isolated from the maturation pond at Kingsburgh WWTP, RSA. The strain was grown and maintained in complete BG11 medium (APPENDIX E) according to Watanabe *et al.* (2000). The microalga was exposed to GROlux irradiance of 120 µmol photons $m^{-2}s^{-1}$, a light: dark cycle of 16: 8 h and a temperature of 25 ±1 °C on an orbital shaker (OrbiShake shaker, Labotec, RSA) at 80 rpm. Sterilization of commercial media and glassware was done at 121 °C and 15 psi for 15 minutes (min) in an autoclave. Cultures were kept free from contamination by keeping the flasks cotton plugged. Culture purity was maintained by regular sub-culturing and

observation using the Nikon eclipse 80*i* microscope under 1000X magnification. All reagents (analytical grade used for media preparations) and consumables were purchased from either Merck (RSA), Polychem supplies (RSA) or Lasec (RSA). pH was monitored using a multi parameter meter.

3.2.3 Cultivation of microalgae in pre- and post chlorinated wastewater

A 100 mL microalgal sample was collected and centrifuged at 2096 x g for 15 min at 4 °C using a centrifuge (Heraeus multifuge centrifuge 4KR, USA). Cells were first washed with a diluted HCl solution, to remove any salts and remaining nutrient components, and washed thrice thereafter with distilled water before being inoculated in a 250 mL Erlenmeyer flask containing 100 mL of BG11 [positive control (PC)], pre-chlorinated effluent (from the clarifier) and post-chlorinated effluent (from the Cl contact tank). Wastewater samples were collected from the Kingsburgh WWTP in early April. The pH of the cultures was measured using the multi parameter meter. From the results obtained all other experiments were carried out using post-chlorinated WW from Kingsburgh WWTP.

3.2.4 Growth Kinetics

3.2.4.1 Microalgal biomass concentration

Microalgal growth was determined daily by optical density (OD_{680}) measurement (Wang *et al.*, 2009a), using a spectrophotometer (SpectroquantR Pharo 300, Merck, Germany). BG11 and WW was used to blank the instrument where required. Samples were vortexed for homogeneity before being read. Spectrophotometric determinations of biomass

correlated to dry cell weight [DCW (gL^{-1})] measurements by a standard curve (APPENDIX F).

$y = 0.1101x - 0.0009$

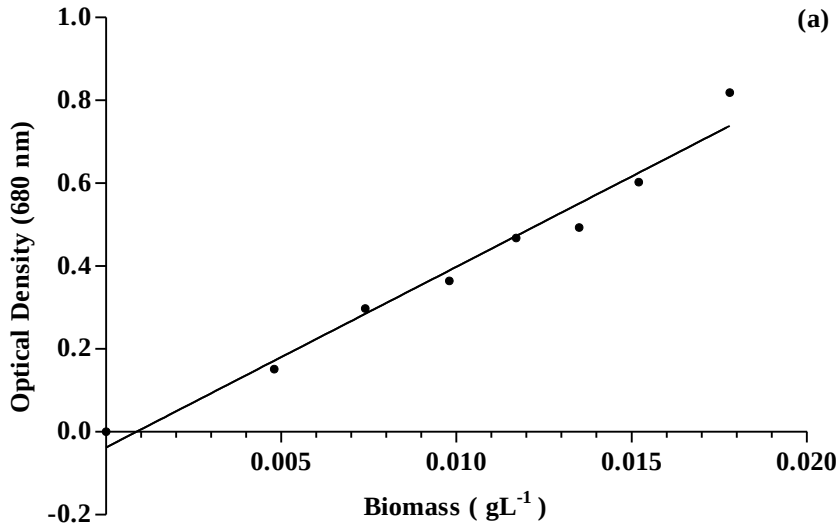
$R^2 = 0.9820$

Equation 3.1: Standard Curve equation for wastewater.

$y = 0.1403x + 0.0764$

$R^2 = 0.9916$

Equation 3.2: Standard Curve equation for BG11.



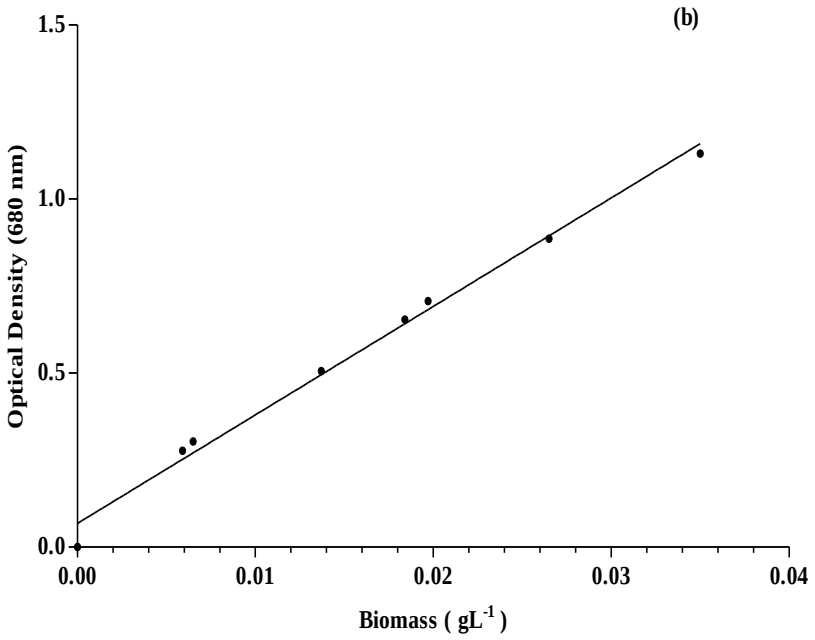


Figure 3.1: Standard curves of (a) wastewater, (b) BG11.

3.2.4.2 Average Growth Rate

The average growth rate of each microalgal strain was calculated from the natural log cell density from start to end of experimentation, as specified by (Lizzul *et al.*, 2014, Song *et al.*, 2013) i.e.

$$\mu = \frac{(ln_N - ln_{N0})}{(t - t_0)}$$

Equation 3.3: Average Growth Rate (d^{-1}) for microalga.

Where μ is the average specific growth rate, N_0 is biomass (gL^{-1}) at the beginning of experimentation (t_0) and N represents the biomass at the end time (t).

3.2.5 Statistical analysis

All experimentation was carried out in triplicate. The results were subjected to one way Analysis of Variance (ANOVA) at a 95 % confidence limit ($\alpha = 0.05$). Tukey's post hoc tests were used to locate the differences in the significant results. All statistical tests were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

3.3 Results

3.3.1 Physical analyses of wastewater

3.3.1.1 Temperature

The average monthly temperatures for the three WWTP effluents can be seen in Figure 3.2. The mean temperatures for all three plants followed a similar pattern throughout the year (± 5 ^{o}C between plants). From January to April there was a steady temperature between all three WWTPs. Temperatures declined from May to June and increased from August to December. July showed the lowest temperature in all three plants. The highest temperature can be noted for Howick WWTP in December which was 31.67 ^{o}C. α value above 0.05 verifies no significant differences in temperature throughout the year between each plant.

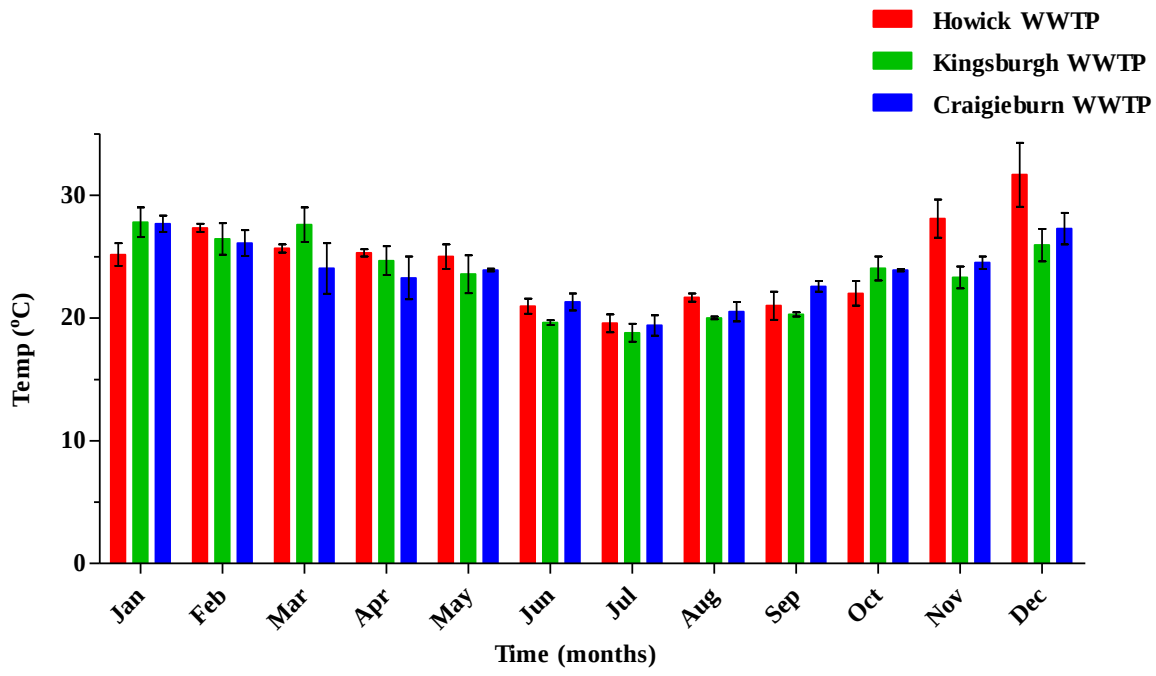


Figure 3.2: Mean temperatures of the wastewater samples of the three plants tested. Data are expressed as a mean ± SEM (n = 3).

3.3.1.2 pH

The pH values of two plants 'effluents were relatively stable throughout the year (± pH between plants), as seen in Figure 3.3. Howick WWTP and Kingsburgh WWTP had a steady pH between 6 and 8, however, Craigieburn WWTP had an erratic pH pattern. There was an increase in pH in January where it was stable till March. It dropped in April and increased in May. From June to September it continued to range between 7 and 8. Craigieburn WWTP showed the lowest recorded pH of 5.23 in January whilst the highest pH of 8.3 in October.

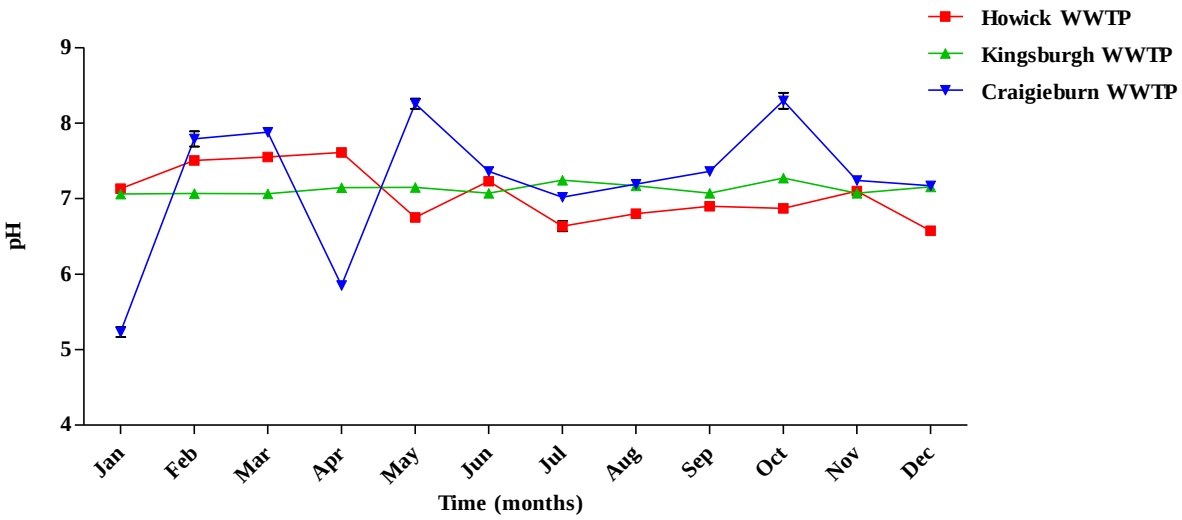


Figure 3.3: Mean pH of the wastewater samples of the three plants tested. Data are expressed as a mean ± SEM (n = 3).

3.3.1.3 Total suspended solids

From Figure 3.4, all three WW plants' effluents TSS fluctuated within the year but within a fairly narrow range. The lowest TSS was demonstrated by Howick WWTP in

February which was 3.73 mgL^{-1}, whilst the highest was demonstrated by Craigieburn WWTP in October which was 8.57 mgL^{-1}. Kingsburgh WWTP ranged from 4 mgL^{-1} to 6 mgL^{-1} throughout the year.

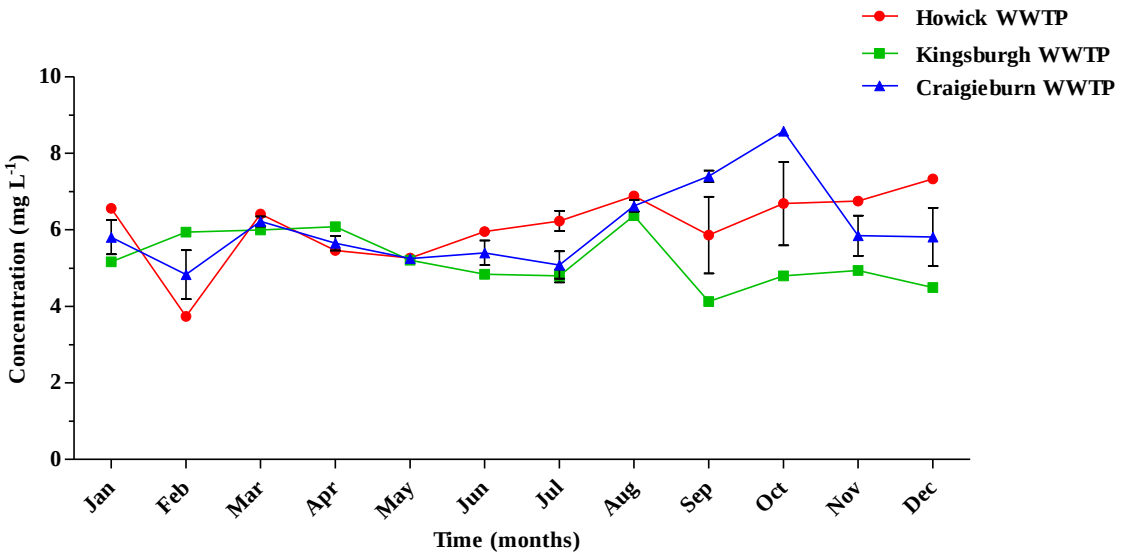


Figure 3.4: Total suspended solids in the wastewater samples of the three plants tested. Data are expressed as a mean ± SEM (n = 3).

3.3.2 Chemical analyses of wastewater

3.3.2.1 Ammonia

From the data obtained (Figure 3.5) the NH_3 concentrations varied between each plant tested. In January, Howick WWTP had an effluent NH_3 concentration of 8.43 mgL^{-1} which was the highest concentration recorded for this plant. This was considerably higher than Kingsburgh WWTP and Craigieburn WWTP as their concentrations were 2.14 mgL^{-1} and 1.48 mgL^{-1} respectively. All three plants showed an NH_3 concentration of below 5 mgL^{-1} from February to May. From Figure 3.5 it was observed that Kingsburgh WWTP and Craigieburn WWTP showed an increase in June. The WW effluent from Kingsburgh WWTP

showed a significant increase in NH_3 concentration ($\alpha < 0.05$) from May to August. August had the highest concentration of NH_3 (11.8 mgL^{-1}) amongst all three plants. Craigieburn WWTP showed a consistent NH_3 concentration below 5 mgL^{-1} throughout the year except for June which was 5.43 mgL^{-1}. This was in contrast to Kingsburgh WWTP. Their NH_3 concentrations follow a variable pattern.

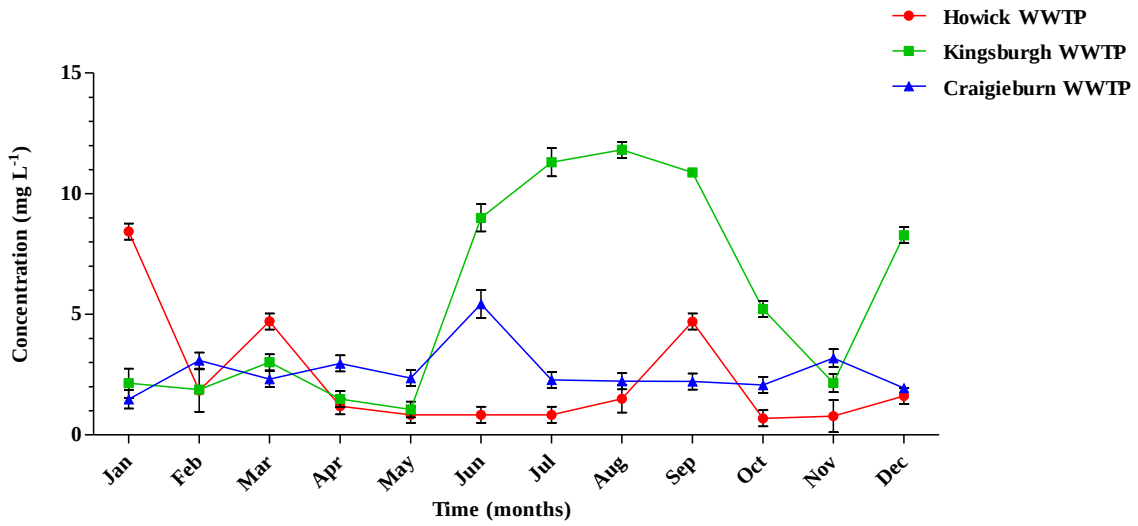


Figure 3.5: Ammonia concentrations in the wastewater samples of the three plants tested. Data are expressed as a mean ± SEM (n = 3).

3.3.2.2 Nitrate

Yearly average NO_3 concentration in WW of all the treatment plants can be seen in Figure 3.6. The average NO_3 concentrations for Kingsburgh WWTP effluent were comparatively higher in the first six months (January- July) than Howick WWTP and Craigieburn WWTP. The highest NO_3 concentration at Kingsburgh WWTP was 7.52 mgL^{-1} in February. After June the NO_3 concentrations at Kingsburgh WWTP dropped considerably to below 2 mgL^{-1}. Both Howick WWTP and Craigieburn WWTP effluents had low NO_3 concentrations of below 2 mgL^{-1} in the first six months of the year. Howick WWTP showed

NO_3 concentrations over 2 mgL^{-1} only in July and August which were 2.16 mgL^{-1}, 2.99 mgL^{-1} respectively. Compared to the other plants, Craigieburn WWTP had high NO_3 concentrations only at the end of the year (October- December) which were 2.08 mgL^{-1}, 2.09 mgL^{-1} and 2.30 mgL^{-1} respectively.

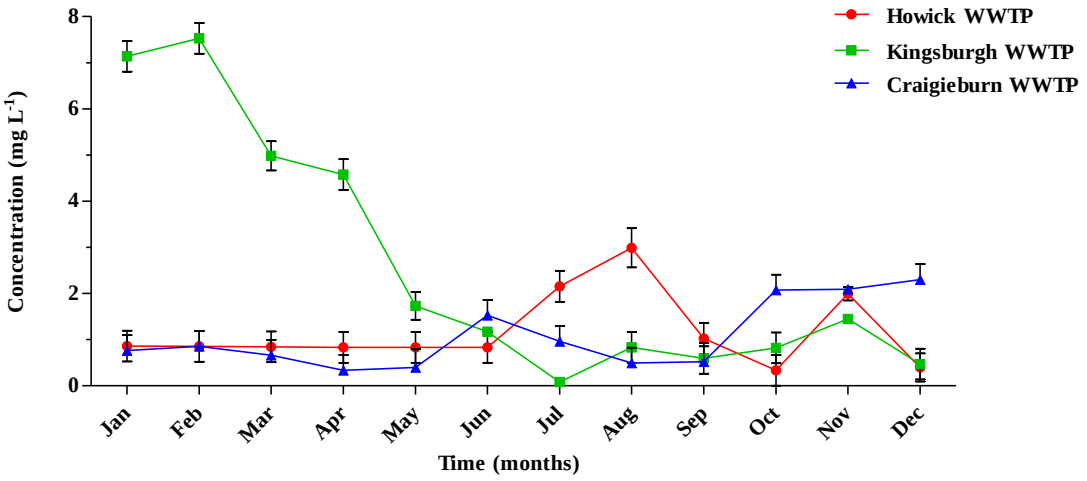


Figure 3.6: Nitrate concentrations in the wastewater samples of the three plants tested. Data are expressed as a mean ± SEM (n = 3).

3.3.2.3 Phosphorus

Focussing on the results obtained for P concentrations in all three plant effluents (Figure 3.7), it was observed that Kingsburgh WWTP had concentrations of below 0.5 mgL^{-1} throughout the year. From March to July and November there was no P in the effluent. The highest P concentration of 2.42 mgL^{-1} recorded was in January for Howick WWTP. This concentration then decreased to 0 mgL^{-1} but increased again in April to 0.09 mgL^{-1}. It decreased to 0 mgL^{-1} in August and September and showed a P concentration of 1.86 mgL^{-1} in October. Craigieburn WWTP showed concentrations of below 1 mgL^{-1} from January to April and then increased in May to 1.24 mgL^{-1}, thereafter decreasing till December.

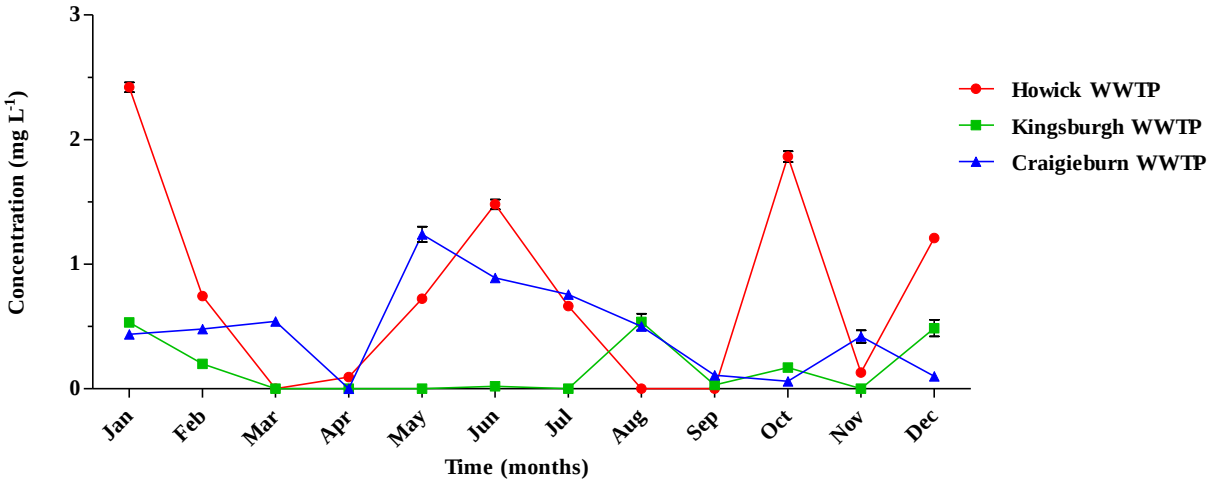


Figure 3.7: Phosphorus concentrations in the wastewater samples of the three plants tested. Data are expressed as a mean ± SEM (n = 3).

3.3.2.4 Chlorine

All three WWTP effluents showed Cl levels below 0.2 mgL^{-1} throughout the study period (Figure 3.8). The Cl results expressed below exhibit fairly high standard error around the means. Kingsburgh WWTP showed Cl levels ranging between 0.16 mgL^{-1} in February and 0.06 mgL^{-1} in October; this being the highest of all three plant effluents. Howick WWTP demonstrated Cl levels below 0.1 mgL^{-1} throughout the year. The highest Cl concentration was seen in July whilst the lowest was seen in December which was 0.09 mgL^{-1} and 0.02 mgL^{-1}, respectively. Craigieburn WWTP showed levels below 0.05 mgL^{-1} during the study period except for January, February and August which were 0.07 mgL^{-1}, 0.05 $mgL^{-1}$and 0.05 mgL^{-1}, respectively. High standard error of the mean (SEM) in April (Howick), June (Howick), July (Kingsburgh) and November (Kingsburgh) was due to variation of Cl concentrations in the samples. Low SEM was due to Cl concentrations being kept constant at the WWTPs. Samples was taken at the same time throughout the experimentation period. The

sample tank may have not had proper mixing, therefore, Cl gas being fed to the tank may have had an impact on the variable concentrations. Chlorine flow rates were not taken into consideration, this may have caused the inconsistent SEM values.

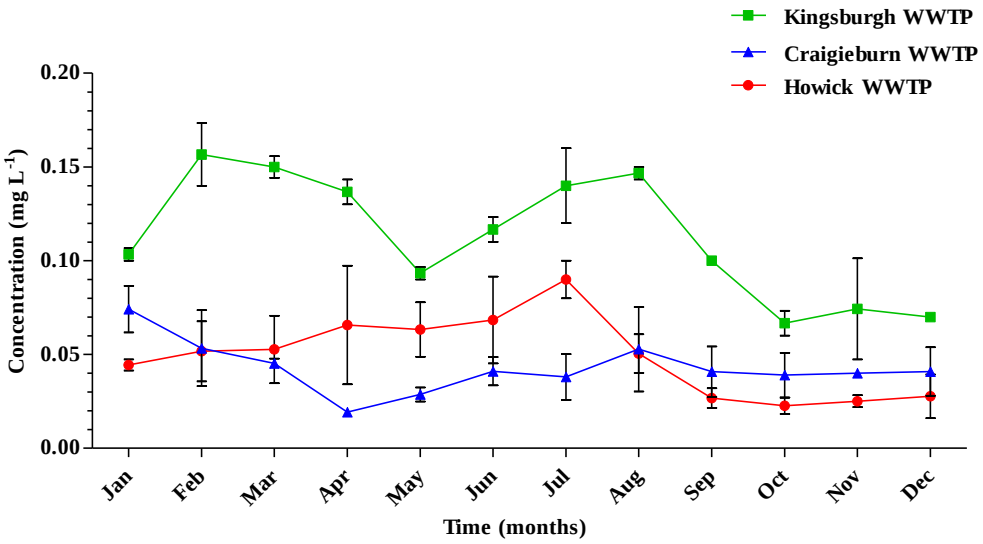


Figure 3.8: Chlorine concentrations in the wastewater samples of the three plants tested. Data are expressed as a mean ± SEM (n = 3).

3.3.3 Biological analysis of wastewater

Of the three plants examined for bacteria in the post-chlorinated effluents, Kingsburgh WWTP had values below 50 CFUs/mL throughout the year. Only in January and February Craigieburn WWTP showed countable pates. Howick WWTP showed bacterial values of over 300 CFUs/mL in March, July and October. These values were too numerous to count (TNTC) as per standard methods.

Table 3.1: **Bacterial standard plate counts per mL of the three WWTPs tested. Data are expressed as a mean ± SD (n = 3). Values expressed as colony forming units (CFUs).**

	Howick	Kingsburgh	Craigieburn
Jan	256 ± 129	36 ± 4	208 ± 21
Feb	247 ± 75	44 ± 23	288 ± 51
Mar	TNTC	48 ± 24	TNTC
Apr	271 ± 152	45 ± 30	TNTC
May	271 ±96	36 ± 6	TNTC
Jun	258 ± 87	36 ± 8	TNTC
Jul	TNTC	32 ± 6	TNTC
Aug	297 ± 125	35 ± 7	TNTC
Sep	282 ± 132	40 ± 12	TNTC
Oct	TNTC	43 ± 14	TNTC
Nov	267 ± 155	38 ± 23	TNTC
Dec	278 ± 160	39 ± 25	TNTC

3.3.4 Efficacy of pre- and post chlorinated wastewater for microalgal cultivation

On day 1 of the time course study the *C. sorokiniana* concentration in all three media were between 0.014 gL^{-1} and 0.018 gL^{-1} (Figure 3.9). The culture growing in pre-chlorinated WW showed the highest biomass concentration of 0.056 gL^{-1} on day 10. The biomass concentration of post-chlorinated WW showed steady growth and the highest biomass concentration of 0.104 gL^{-1} was achieved on day 19. This was the highest biomass concentration of all three cultures, recorded for the 21 d experiment.

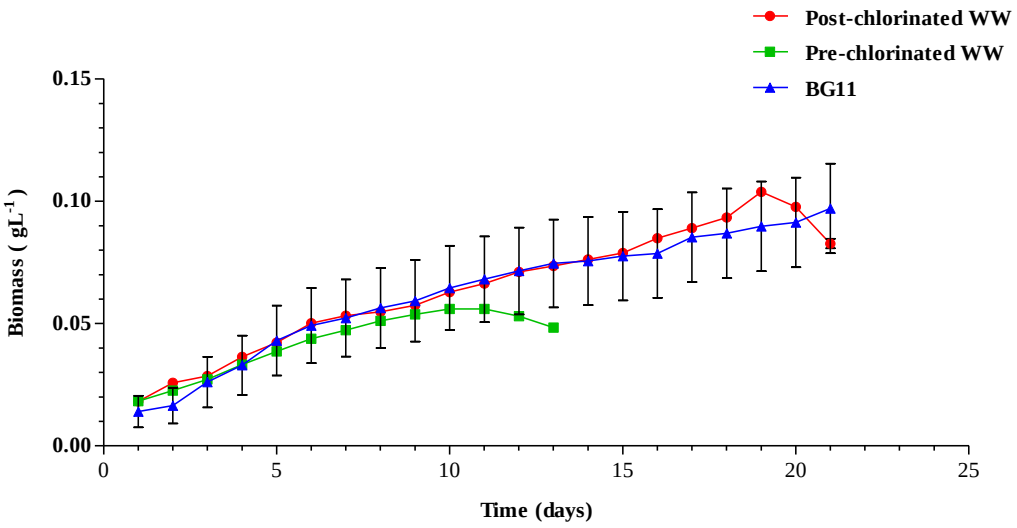


Figure 3.9: **Growth curves of *C. sorokiniana* in pre-chlorinated wastewater, post-chlorinated wastewater and BG11 media. Data are expressed as a mean ± SEM (n = 3).**

The culture growing in BG11 also showed a steady growth. Growth continued up till day 21 whilst the other cultures declined. The highest biomass concentration achieved by this culture was 0.097 gL^{-1}. The culture growing in pre-chlorinated WW showed the lowest average growth rate of 0.074 d^{-1}, which can be seen in table 3.2.

Table 3.2: **Average Growth Rates of *C. sorokiniana* cultivated in wastewater and BG11 media.**

Medium	**Growth Rate** (d^{-1})
Pre-chlorinated	0.074
Post-chlorinated	0.111
BG 11	0.097

3.4 Discussion

3.4.1 Physical analyses of wastewater

3.4.1.1 Temperature

The average monthly temperatures for the three WWTP effluents were similar throughout the year. All three plants are situated in the province of Kwa-Zulu Natal, RSA. This province has warm summers and relatively mild winters (MacKellar *et al.*, 2014). This is evident in Figure 3.2. The summers in RSA last from December to February. On the graph it was observed that the temperature did not drop below 25 ^{o}C. All three plant effluents showed temperature drops of below 25 ^{o}C from June to September as these months are winter months in RSA. Temperature is an important cultivation parameter for microalgal growth and should be around 20 ºC- 30 ºC (Chisti, 2007). The efficiency of microalgal activity normally decreases at low temperatures (Breuer *et al.*, 2013), whilst cell growth increases with temperature until the temperature optimum is reached. Further increases in temperature may reduce microalgal growth drastically (Lizzul *et al.*, 2014). There are many studies conducted on the effect of temperature on microalgal growth (Markou and Nerantzis, 2013, Breuer *et al.*, 2013, Li *et al.*, 2013). Temperature may also affect the cells sensitivity to high light intensity, causing the tolerance to high light intensities to increase, however, sub-optimal temperature induces photo-inhibition even at lower light intensities. The cultivation temperature also affects cell composition (Chisti, 2007). Lipid, along with carbohydrate synthesis, increases. Microalgae may accumulate up to 60–65 % lipid of DCW when cultivated under temperature stressed conditions (Li *et al.*, 2013). The effect of temperature is different from strain to strain.

In a study conducted by Van Wagenen *et al.* (2012), *Nannochloropsis salina* was subjected to light intensities ranging from 5–850 μmol $m^{-2}s^{-1}$ and temperatures between 13 and 40 ºC. Exponential growth rate was expressed for the maximum acclimated culture. The microalga had a growth rate of 1.3 d^{-1} at 23 °C and 250 μmol m^{-2} s^{-1}. The microalga used in their study was the thermo-tolerant, fast growing chlorophyte microalga *C. sorokiniana* which showed optimal growth between 35 and 40 ºC (Lizzul *et al.*, 2014, Li *et al.*, 2013). Li *et al.* (2013) analyzed the growth of *C. sorokiniana* whilst looking at the influence of environmental temperature and medium factors, such as C and N source and their initial concentrations. *C. sorokiniana* was found to be able to tolerate up to 42 ºC and showed the highest growth rate of 1.60 d^{-1} at 37 ºC. In a 5 L batch fermentation, the DCW increased dramatically from 0.9 gL^{-1} to 37.6 gL^{-1} in the first 72 h of cultivation, with the DCW productivity being 12.2 $gL^{-1}d^{-1}$.

The seasonal temperature variation at all three WWTP effluents were suitable for microalgal cultivation throughout the year and were found to be within the range of the average temperatures for the region (MacKellar *et al.*, 2014). Most microalgae will grow between these sub-tropical temperature ranges. Also, cultivation of a thermo-tolerant, microalga such as *C. sorokiniana* may be done at much higher temperature ranges.

3.4.1.2 pH

Most microalgae are not capable of functioning at low pH ranges as this will lead to a decrease in biomass productivity ultimately leading to culture death (Rawat *et al.*, 2013b). The pH of all three plant effluents was relatively stable throughout the year, as seen in Figure 3.3. All three plants showed a pH range of between 5 and 8. pH has a significant effect on

microalgal growth as it controls all metabolic and physiological functions of the cell (Rawat *et al.*, 2013b). The pH range for biological life is quite narrow. Extreme pH is known to damage biological processes in natural treatment units. Microalgae grown in ORPs are prone to contamination. pH levels above 8.3 are desirable because some microalgal consumers such as protozoans and rotifers are inhibited under these conditions and are therefore completely eliminated from open systems (Rawat *et al.*, 2011). The optimal pH range for microalgal growth varies between species. Freshwater microalgae have an optimal value close to 8. Higher or lower from this value leads to a reduction in biomass productivity. Microalgae like *Amphora* sp. and *Ankistrodesmus* sp. have grown uninhibited at a pH of 9 and 10. pH levels exceeding 11 may occur in high rate microalgal pond systems. This is caused by the consumption of CO_2 in the form of carbonic acid by the process of photosynthesis (Rawat *et al.*, 2013b). The pH values demonstrated for all three WWTPs were found to be suitable for the cultivation of microalgae, however, Craigieburn WWTP effluent was low in January and April. Kingsburgh WWTP effluent pH would be ideal for cultivating microalgae as the pH was close to neutral throughout the year. Microalgae have been found to change and maintain their pH values to around 10 (Rawat *et al.*, 2013b), this would negate the need for chemical buffered controlled pH regulators.

3.4.1.3 Total suspended solids

Total suspended solids are a measure of the combined content of all inorganic and organic substances contained in the aqueous suspension in molecular or micro-granular suspended form. Any particle or micro-organism smaller than 2 micron is considered a dissolved solid. Total suspended solids are a function of evaporation rates (Rawat *et al.*, 2011).

Total suspended solids are of particular importance when looking at WW clarity (Chen *et al.*, 2015). This measurement is associated with turbidity. Turbidity measurements are based on clarity, which is determined by the depth of sunlight penetration in WW (Chen *et al.*, 2015). The depth sunlight reaches may be referred to as the photic zone. The deeper the photic zone of WW effluent, the greater the potential for photosynthetic activity. High turbidity values inhibit photosynthesis in microalgae by reducing sunlight penetration into the culture medium. This decrease will lead to microalgal growth decline as well as a decreased DO output (Arumugam *et al.*, 2013, Lizzul *et al.*, 2014).

Microalgae, cultivated in the WW streams were able to use the organic and inorganic compounds for their primary production (Moheimani and Borowitzka, 2006, Chen *et al.*, 2015). The TSS of all three plant effluents ranged from 3 mgL^{-1} to around 9 mgL^{-1} throughout the year (Figure 3.4). Total suspended solid concentrations less than 20 mgL^{-1} are said to be clear. Water with TSS levels between 40 and 80 mgL^{-1} appear cloudy, while water with concentrations over 150 mgL^{-1} usually appears dirty (DWAF, 1999). The nature of the particles that comprise the suspended solids may cause these numbers to vary. High TSS values can be attributed to bacterial contamination as well as foreign particles (dust and clay) in the WW effluent. Dust and clay particles cannot be utilized by microalgal cells (Park *et al.*, 2011, Park *et al.*, 2012).

3.4.2 Chemical analyses of wastewater

3.4.2.1 Ammonia and Nitrate

Domestic effluents sometimes contain high concentrations of inorganic N that, when released, may cause eutrophication of receiving water bodies (Sahu *et al.*, 2013). Among

these contaminants, NH_3 is a severe problem. Although NH_3 is used as a source for N for microalgal growth, high concentrations of NH_3 (>100 mgL^{-1}) can inhibit growth of different strains of microalgae (Yuan *et al.*, 2011, Wu *et al.*, 2014). Ammonia concentration along with pH is contributing factors when considering microalgal growth (Kim *et al.*, 2013).

From the findings, it was seen that the effluent WW had a pH range of between 5 and 8 (Figure 3.3). Ammonia is toxic and toxicity is affected by pH. Ammonia-N (NH_3-N) has a more toxic form at high pH and a less toxic form at low pH, un-ionized NH_3 and ionized NH_4^+, respectively. At higher pH values, N in the form NH_3 is frequently found in WW. The presence of un-ionized NH_3, the toxic form, increases as pH rises and decreases as pH falls which causes NH_3 to become more ionized. Concentrations above 85 mgL^{-1} with a pH greater than 8 were toxic to *Dunaliella* species while *C. vulgaris* had a 50 % growth reduction when grown in a concentration of 330 mgL^{-1}. Another study showed that *C. vulgaris* did not grow at a pH 8-9 with a concentration of 700 mgL^{-1} (Kim *et al.*, 2013). From the results obtained, it was observed that all three WWTP effluents had NH_3 concentrations lower than 15 mgL^{-1} throughout the year (Figure 3.5). In Howick WWTP, the highest concentration was seen in January (8.43 mgL^{-1}). Kingsburgh WWTP showed the highest in August at 11.81 mgL^{-1} while Craigieburn WWTP showed NH_3 peak in June. All three plants showed NH_3 concentrations of below 5 mgL^{-1} from February to May. Kingsburgh WWTP and Craigieburn WWTP showed an increase in June. The WW effluent from Kingsburgh WWTP had a significant increase ($\alpha < 0.05$) from May to August. These were the maximum concentrations obtained. Craigieburn WWTP showed a constant NH_3 concentration all through the year except in June. This was different when compared to Howick WWTP and Kingsburgh WWTP which showed an inconsistent pattern. The levels of NH_3 found in the WWs tested were well below that found to reduce growth when compared to the study done by Kim *et al.*

(2013). They investigated the growth rate characteristics of N when using NH_3Cl as N sources for batch culturing of the heterotrophic microalga *C. sorokiniana* in proteose medium. The N concentrations examined were 10 mgL^{-1}, 20 mgL^{-1}, 40 mgL^{-1}, 80 mgL^{-1} and 160 mgL^{-1}. When using NH_3 as a N source, the maximum growth rate was 0.6 d^{-1}, with 160 mgL^{-1} NH_3. This study showed that *C. sorokiniana* was able to grow at high NH_3 concentrations.

He *et al.* (2013) studied the feasibility of cultivating *C. vulgaris* with WW containing high NH_3-N concentrations. *C. vulgaris* was maintained in BBM before being transferred into a PBR containing secondary effluent. Treatments were designated as N30 (17 mgL^{-1} NH_3), N55 (39 mgL^{-1} NH_3), N70 (52 mgL^{-1} NH_3), N85 (65 mgL^{-1} NH_3), N100 (77 mgL^{-1} NH_3), N175 (143 mgL^{-1} NH_3) and N245 (207 mgL^{-1} NH_3). Cell density was positively correlated with the NH_3 concentrations. It was found that the average specific growth rate of *C. vulgaris* was 0.92 d^{-1} at N30, but declined to 0.33 d^{-1} at NH_3 concentrations of N55– N175. The study also evaluated the microalgal biomass in terms of proteins, carbohydrates and lipids. As the NH_3 treatments increased from N30 to N245, it was found that the concentration of proteins increased gradually from 12 % to 42 %; carbohydrates increased initially, peaking at 45 % for the N175 medium and the lipid content reached a maximum of 32 % using N55 medium. Different concentrations of NH_3 produced and accumulated different compounds within the cell. With the N245 medium, the cell density dropped to 0.9 gL^{-1}. This was attributed to the inhibition of cellular growth which was also evident by a decline in productivity of both proteins and carbohydrates. This study demonstrated the use of high NH_3 concentrations for microalgal cultivation. In a N rich environment, microalgae will tend to utilize N, promoting growth as well as fixing CO_2, leading to a high productivity of proteins and carbohydrates. The mechanism of NH_3 utilization will be discussed in Chapter 4. Microalgae are able to

grow over a wide NH_3 range. Previous studies found that microalgae can withstand NH_3 concentrations from between 17 mgL^{-1} (He *et al.,* 2013) to 160 mgL^{-1} (Kim *et al.,* 2013) depending on the species of algae chosen.

When compared to Howick WWTP (0.03 mgL^{-1}) and Craigieburn WWTP (0.36 mgL^{-1}) the NO_3 concentrations at Kingsburgh WWTP were higher (5.41 mgL^{-1}) (Figure 3.6). Kingsburgh WWTP effluent had a NO_3 concentration of 7.52 mgL^{-1} in February. Following June the NO_3 concentrations at Kingsburgh WWTP declined considerably, concentrations were below 2 mgL^{-1}. From the results obtained in this study, it was seen that the NO_3 concentrations for Kingsburgh WWTP increased in May and December. This can be explained as a loss in the nitrification process. Many factors, including light, dissolved oxygen (DO), pH, temperature or organic matter may result in an accumulation of NO_3 in WWTPs due to imbalances in nitrification and denitrification processes (Mahapatra *et al.*, 2013, Zhang *et al.*, 2012, Karya *et al.*, 2013).

Howick WWTP and Craigieburn WWTP demonstrated low NO_3 concentrations in their effluents for the first six months of the year. Howick WWTP only had NO_3 levels of over 2 mgL^{-1} in July and August. Craigieburn WWTP only showed high NO_3 concentrations between October and December. From the data obtained, it can be seen that all three WWTPs showed variable NO_3 and NH_3 concentrations throughout the year. It should be noted that although limited, the NH_3 concentrations in the WW effluents can support microalgal growth.

3.4.2.2 Phosphorus

Phosphorus is one of the essential elements for microalgal growth. It can be stored in the cell as polymetaphosphate or used for other cell functions (Yuan *et al.*, 2012, Sahu *et al.*, 2013). The form of P which is mainly utilized by microalgae is PO_4. Organic P in WW's will be hydrolyzed to PO_4 by the extracellular enzyme alkaline phosphatase, its production is stimulated by a P deficiency (Yuan *et al.*, 2012). Microalgae have an internal inorganic P pool, in specific a polyphosphate pool, which is said to be full when the P is present in excess. These internal storage pools sustain microalgal growth by storing adequate levels of P to provide for as many as 20 cell divisions in the complete absence of external P sources. Under photosynthetic growth the uptake of P requires energy, therefore, uptake rates are slower in dark than in light environments (Chu *et al.*, 2013, Sahu *et al.*, 2013).

From the P results obtained in all three plants (Figure 3.7), Kingsburgh WWTP effluent had concentrations of below 0.5 mgL^{-1} throughout the year. From March to July and November there was no P in the effluent. The highest P concentration was 2.42 mgL^{-1} recorded was in January by Howick WWTP. This concentration then decreased but increased again in May and April. It decreased to 0 mgL^{-1} in August and September and showed a P concentration of 1.86 mgL^{-1} in October. Craigieburn WWTP showed effluent concentrations of less than 1 mgL^{-1} from January to April and then increased in May to 1.24 mgL^{-1}, thereafter decreasing till December. Phosphorus concentrations varied between the WWTPs, as well as from month to month. Phosphate is inherently dilute in domestic WW streams. Typical concentrations found in effluent are below 10 mgL^{-1} (Yuan *et al.*, 2012, Rawat *et al.*, 2013a). Tertiary WW effluents may contain P in adequate concentrations to support microalgal growth (Rawat *et al.*, 2013), however, the present study found there was no P in

WWs during a few months in the year. Therefore, supplementation with P is necessary for certain WW effluents to promote microalgal growth. Lizzul *et al.* (2014) grew *C. sorokiniana* on both WW influent and commercial medium (Bold's basal medium) whilst assessing the influence of exhaust gas on the process. The levels of P in the media fluctuated at around 10 mgL^{-1} and 15 mgL^{-1}. It is interesting that in all types of media there was little indication of P uptake. Microalgae do not require high concentrations of P for growth, however, P is necessary for growth. The WWs tested show no P concentrations for certain months in the year. Zhou *et al.,* (2013) cultivated *Chlorella* sp. NJ-18 in BG 11 medium with varying concentrations of P. Under P-deficient conditions, biomass concentrations reached 0.69 gL^{-1}. The lipid content was also much higher in P deficient conditions (37.20 %) than that with other concentrations. This study shows that even with no P, microalgae are still able to grow and produce lipid. The WWs tested would need to be supplemented with some form of P to sustain microalgal growth for longer periods of time.

When considering biodiesel production, high concentrations of P (> 10 mgL^{-1}) are not required for microalgal cultivation. Phosphorus is incorporated into polar lipids, especially phospholipids present in the cell wall (Rawat *et al.*, 2013b). These lipids are not desirable for biodiesel production as they play a negative role in transesterification (Sahu *et al.*, 2013). However, a P limitation may affect N uptake resulting in lowered growth rates (Han *et al.*, 2014). In addition, the uptake of P is influenced by pH, uptake rates decrease in acidic and relatively alkaline environments. From Figure 3.3 it was observed that all three plant effluents had neutral to alkaline pH values. Using these WW streams for microalgal cultivation would be ideal. Lack of ions, such as K, Na and Mg, in certain mediums also decreases the P uptake rate. In WW, both microalgae and bacteria use PO_4 for nutrition; therefore they compete for the available PO_4 (Chu *et al.*, 2013).

3.4.2.3 Chlorine

In order to prevent pollution of receiving water bodies, WWTPs have to adhere to effluent discharge standards. Limits are set for a wide range of chemical compounds, toxicity, and bacterial discharge (i.e., *Escherichia coli*). In order to meet the requirements for the bacterial discharge limit, the WWTPs evaluated here disinfect the effluent using chlorination (Pignata *et al.,* 2012). All three WWTPs showed Cl levels below 0.2 mgL^{-1} throughout the experimental period (Figure 3.8). The Cl results obtained display fairly high standard error around the means. Kingsburgh WWTP showed the highest Cl levels ranging between 0.16 mgL^{-1} and 0.06 mgL^{-1}. These Cl levels were sufficient enough to keep effluent bacterial levels low (table 3.1). Howick WWTP demonstrated Cl levels below 0.1 mgL^{-1} throughout the year. The highest Cl concentration was seen in July and lowest in December which was 0.09 mgL^{-1} and 0.02 mgL^{-1}, respectively. These Cl results were also quite low, nevertheless this plant only reached TNTC values in March, July and October. The Cl levels revealed that this plant was able to effectively disinfectant the effluent. Craigieburn WWTP showed levels equal to or less than 0.05 mgL^{-1} during the experimental period except for January (0.07 mgL^{-1}). These Cl concentrations were unable to completely disinfect the WW effluent. From table 3.1 it was seen that the bacterial levels from February onwards were TNTC. This was due to low Cl content and short processing times (Wang *et al.,* 2013b). From the results obtained it can be seen that Cl levels greater than 0.1 mgL^{-1} are needed to effectively remove harmful bacteria in WWs.

Mutanda *et al.,* (2011a) grew *Chlorella* sp. in post-chlorinated WW with a 0.2 mgL^{-1} free chlorine concentration. Biomass yields reached 69.8 mgL^{-1} at 0.2 mgL^{-1} chlorine dosage. It was found that there was a gradual increase in biomass production with increase in Cl dosage. Further experimentation was conducted to validate their findings. Sodium

hypochlorite was supplemented to WW in concentrations varying from 0.2 to 1 mgL^{-1}. It was found that *Chlorella* sp. was able to tolerate Cl concentrations up to 0.4 mgL^{-1}. The highest Cl concentration found amongst the three WWTPs tested was 0.16 mgL^{-1}. This concentration, theoretically, is low enough to cultivate *Chlorella* sp.

3.4.3 Biological analysis of wastewater

Wastewaters with high concentrations of NH_3 are used for cultivation of microalgae. However, the bacteria present in these WW can inhibit the growth of microalgae and compete with microalgae for nutrients e.g. N and P (Wang *et al.*, 2013a). Kingsburgh WWTP showed bacteria values of less than 50 CFUs/mL throughout the year. These results demonstrate the effectiveness of the chlorination process to keep bacterial populations at acceptable levels. Wastewater effluents from Craigieburn WWTP were TNTC in January and February. Howick WWTP had values that were TNTC in March, July and October (table 3.1). When cultivating microalgae in WW, it may be desirable to closely monitor microbial levels in the WW. Pathogenic bacteria will impact negatively on microalgal growth either by direct attack depending on cell-to-cell contact or indirect attack mediated by extracellular compounds (Wang *et al.*, 2013b). They may out-compete the target microalgal strain for essential nutrients (Pittman *et al.*, 2011). In turn, microalgae may cause detrimental effects on bacterial growth and activity by increasing the pH, the DO concentration or by excreting inhibitory metabolites (Cho *et al.*, 2011). However, some studies demonstrate a beneficial interaction between microalgae and bacteria in WWTPs. Urease bacteria are able to produce NH_4 as a by product of $CO(NH_2)_2$ metabolism, which is utilized by the microalgae. Organic by-products released from microalgae serve as additional substrates for bacteria. The O_2 produced by microalgae during photosynthesis is released into the medium and can be used by aerobic

bacteria, reducing O_2 levels and aiding in the efficient oxidation of organic matter. Correspondingly, bacteria disintegrate and degrade organic matter providing the CO_2, minerals and nutrients which is used by the microalgae (Subashchandrabose *et al.*, 2011, Mahapatra *et al.*, 2013). Microalgal bacterial interactions are not limited to simply a CO_2/O_2 exchange.

3.4.4 Efficacy of pre- and post chlorinated wastewater for microalgal cultivation

Kingsburgh WWTP effluent was chosen for the rest of the experiments based on the WW characterization results. This plant demonstrated higher NH_3 and NO_3 concentrations (Figures 3.5 and 3.6) with bacterial concentrations low, so as not to inhibit microalgal growth. The results obtained show the microalga grew without observable lag phase. *C. sorokiniana* used in the present study was isolated from the Kingsburgh WWTP, which suggests the microalga may have been acclimatized to the WW conditions. Microalgae isolated directly from WW or from bodies of water or local environments adapt and produce better results under specific conditions (Han *et al.*, 2014, Wu *et al.*, 2014, Kamyab *et al.*, 2014).

The culture growing in pre-chlorinated WW showed the highest biomass concentration of 0.056 gL^{-1} on day 10 (Figure 3.9). This culture showed the slowest growth rate of 0.074 d^{-1} (table 3.2). Experiments with pre-chlorinated WW proved unsuccessful for supporting microalgal growth. This could be attributed to the presence of large concentrations bacteria, present in the clarifier, which outcompete microalgal growth (Mutanda *et al.*, 2011a). The effects of bacteria on microalgal growth have been previously discussed in Section 3.4.3. The biomass concentration of post-chlorinated WW showed steady growth. It

can be postulated that; (1) the Cl in the post-chlorinated WW aided microalgal growth (Mutanda *et al.*, 2011a), (2) the high concentrations of bacteria present in pre-chlorinated WW negatively impacted on microalgal growth, or (3) there were not enough nutrients present in this WW stream to support microalgal growth for a pro-longed period. The culture growing in BG11 grew for a longer period when compared to post-chlorinated WW. The nutritional components in post-chlorinated WW are far less than BG11 medium. The relatively high biomass in post-chlorinated WW (0.104 gL^{-1} on day 19) can be due to the small quantities of organic matter found in the WW (Han *et al.*, 2014). This is evident from the high TSS values obtained (Figure 3.4).

The growth kinetics of *C. sorokiniana* using pre-chlorinated, post-chlorinated WW, and BG11 media indicate there was a significant difference between growth rates ($\alpha > 0.05$). Post-chlorinated had a higher average growth rate (0.111 d^{-1}) when compared to pre-chlorinated and BG11 which were 0.074 d^{-1} and 0.097 d^{-1}, respectively. This indicated that WW effluent may be used as a growth substrate to cultivate microalgae. The residual nutrients in post-chlorinated WW were able to support microalgal growth for limited periods of time. The growth rate in BG11 medium was 0.097d^{-1} as compared to 0.111 d^{-1} in post-chlorinated WW. The post-chlorinated WW culture declined at day 19, the microalga must have consumed all residual nutrients left in the WW by day 19. Post-chlorinated WW can be utilized for large-scale microalgal cultivation, however, it is highly complex in terms of composition (Wu *et al.*, 2014). In a study conducted by Mutanda *et al.* (2011a) *Chlorella* spp. was grown on post-chlorinated WW with residual free Cl. The microalga showed no growth in pure de-ionized water (0 mgL^{-1} Cl) but the highest growth rate and biomass yield was achieved at 0.2 mgL^{-1} free Cl dosage in WW containing nutrients. Their study hypothesized that chloride ions act as microalgal micronutrients. The authors concluded that low dosages

of free Cl were found to enhance microalgal growth (Mutanda *et al.*, 2011a, Mutanda *et al.*, 2011b); however, additional experimentation needs to be conducted to investigate the effect of Cl on microalgal growth (algicidal or nutritional effects).

Chapter 4 : Determination of growth kinetics using different nitrogen sources supplemented in wastewater

4.1 Introduction

Biodiesel production from microalgae is dependent on the microalgal growth rate and lipid productivity (Chen *et al.*, 2011). Lipid accumulation in microalgae may be improved by altering cultivation conditions. Nitrogen is central to the cultivation of microalgae and lipid regulation. Ammonia, $CO(NH_2)_2$ and NO_3, owing to their availability and to their relatively low-cost, are often selected as N sources for mass cultivation of microalgae (Lin and Lin, 2011, Hsieh and Wu, 2009, Kim *et al.*, 2013). These N sources are frequently found in domestic WW. Ammonium and $CO(NH_2)_2$ account for between 40 and 60 % of the N found in untreated municipal domestic WW (Eustance *et al.*, 2013). Final effluent discharged from the WW treatment processes is another important source of nutrients, although at lower concentrations (Lizzul *et al.*, 2014). Supplementing WW with N fertilizer for optimal growth is expensive and proves to be unfeasible for biodiesel production (Eustance *et al.*, 2013). Thus, options for cheaper sources of N need to be investigated (Borowitzka and Moheimani, 2013).

The most appropriate N source and concentration must be identified for each microalgal species as this is a vital factor which affects growth and lipid regulation (Hsieh and Wu, 2009, Chia *et al.*, 2013). Nitrogen is available in several forms. The amount and form of N that is supplied to the microalgae may affect the cellular composition of the algae, altering lipid content and FA composition, as well as the growth rate and culture stability (Borowitzka and Moheimani, 2013). All microalgae assimilate inorganic N in the forms of

NO_3 and NH_4 (Cai *et al.*, 2013a). Nitrogen stressed (deprivation) conditions are effective means of inducing neutral lipid accumulation within microalgal cells (Singh *et al.*, 2014).

Nitrogen limitation results in cellular changes including: reduction of the cellular content of the thylakoid membrane, acyl hydrolase activation and induction of phospholipid hydrolysis. These changes result in an increase in intracellular FA acyl-CoA while N limitation activates diacylglycerolacyl transferase, which converts acyl-CoA to TAG. Consequently, N deficiency ultimately leads to an increase in lipid and TAG content in microalgal cells (Xin *et al.*, 2010a). This chapter focussed on identifying the most suitable N source for the cultivation of *C. sorokiniana* and to determine the optimal concentration of this N source for microalgal biomass production, lipid production and microalgal physiology in WW.

4.2 Methodology

4.2.1 Microalga and culture conditions

C. sorokiniana was maintained as per section 3.2.2. Microalgal growth was determined as per section 3.2.4.

4.2.2 Nitrogen source and concentration

Urea (1.5 gL^{-1}), sodium nitrate ($NaNO_3$) (1.5 gL^{-1}), NH_4NO_3 (1.5 gL^{-1}) and potassium nitrate (KNO_3) (1.5 gL^{-1}) were used to supplement post-chlorinated WW collected from Kingsburgh WWTP in April. Experimentation was carried out in triplicate over a period of

21 d. Post-chlorinated WW was used as a control. Nitrogen concentrations in WW were under 5 mgL^{-1} whilst there was no P present. The pH was of the culture were measured as per section 3.2.1. All experimentation was carried out in triplicate. All statistical analysis was carried out as per section 3.2.4.

4.2.3 Semi quantitative lipid determination

BODIPY 505/515 (4,4-difluro-1,3,5,7-tetramethyl-4-bora-3a, 4adiaza-s-indacene, Invitrogen Molecular Probes, Carlsbad, CA) was used to determine lipid content of microalgal cells via the relative fluorescence staining technique. Quantitative estimation of lipids was performed using BODIPY staining as per the protocol described by Govender *et al.* (2012) using a Carl Zeiss Axiolab HBO 50 epi-fluorescence microscope and Zeiss Axiovision 8 software (Carl Zeiss, Germany). The excitation wavelength was 488 nm, and the emission wavelength was 490–535 nm, respectively. Image analysis was performed using Olympus analysis version 5 software to quantify lipid percentage. Ten replicates for each culture were analysed to get an average cell lipid content. The total cell area was quantified. The microalgal cultures were put in dark bottles. A dye concentration of 0.067 μgmL^{-1} was added to 3 mL of microalgal culture, at cell densities between 1 X 10^{-5} and 1 X 10^{-7} $cellsmL^{-1}$. Cultures were stored away from light to protect the dye from photo bleaching. Cultures were left for 2 min to allow for sufficient dye penetration, and then viewed.

4.3 Results

4.3.1 The effect of nitrogen source on biomass

Growth was observed for all N sources. All the N sources showed a steady increase in growth between days 1 to 9. Small error bars show good reproducibility of the results. It was observed (Figure 4.1) that $NaNO_3$ showed a steady increase in growth up to day 9, thereafter leading to the decline or death phase, as opposed to $CO(NH_2)_2$, KNO_3 and NH_4NO_3 that only displayed a considerable reduction in growth following day 16. The control culture grew till day 6 then started to decline. The WW supplemented with $CO(NH_2)_2$ produced the highest biomass concentration of 0.216 gL^{-1} on day 16, and thus proved to be the best N source for further investigation. Ammonium nitrate proved the least effective N source, producing only 0.106 gL^{-1} biomass on day 14. Due to the fact that $CO(NH_2)_2$ is the cheapest N source, it was further tested to determine the optimal concentration.

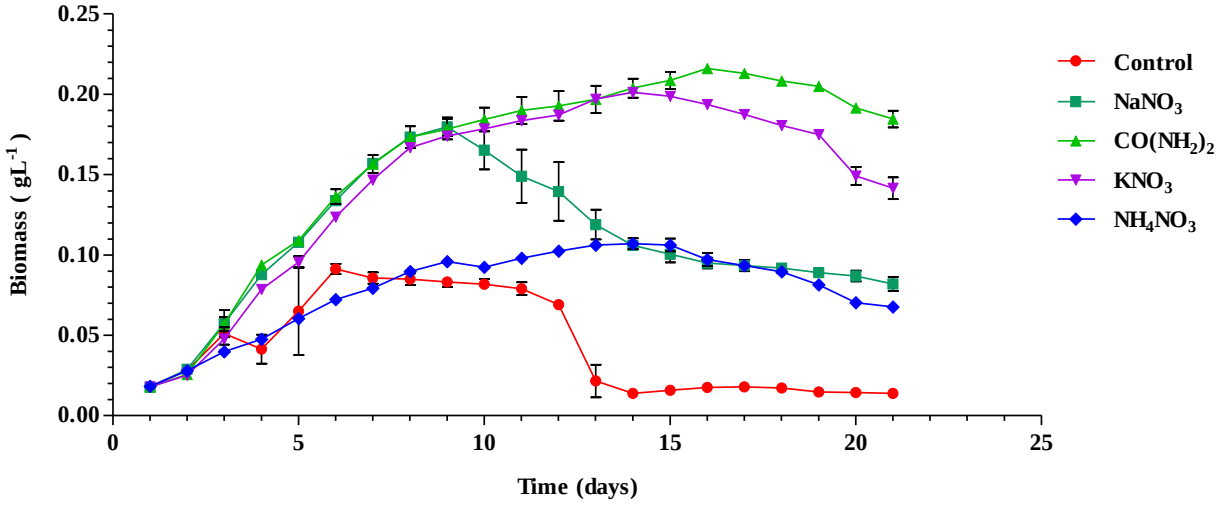


Figure 4.1: **Growth curves of *C. sorokiniana* cultivated in wastewater with different nitrogen sources. Data are expressed as a mean ± SEM (n = 3).**

From the table it can be seen that the culture inoculated with $CO(NH_2)_2$ had the fastest growth rate of 0.116 d^{-1}, followed by the KNO_3 supplemented culture which was 0.102 d^{-1}. Ammonium nitrate demonstrated the slowest growth rate of 0.065 d^{-1}. The control showed a negative growth rate. The initial cell density was higher than the final cell density.

Table 4.1: Average Growth rates of *C. sorokiniana* cultivated in wastewater with different nitrogen sources.

N source	**Growth Rate** (d^{-1})
Control	-0.014
$NaNO_3$	0.075
$CO(NH_2)_2$	0.116
KNO_3	0.102
NH_4NO_3	0.065

The daily pH variation of each supplemented media was determined and the results are shown in Figure 4.2. At the beginning of the experiment, all culture media showed an initial pH of around 8. The pH of all media ranged between 6 and 10 throughout the experiment. It was observed that the pH of all culture media, except NH_4NO_3, exhibited a trend. These supplemented cultures showed a slight increase and subsequent decrease after day 10. pH of the NH_4NO_3 supplemented culture decreased steadily after day 5. On day 21 the NH_4NO_3 supplemented culture medium pH was 5.5. This was much lower than that of the other culture media. The control showed the highest pH of 10.1 on day 11. Urea and KNO_3 had increased from around 7.7 to around 9.2 at day 21.

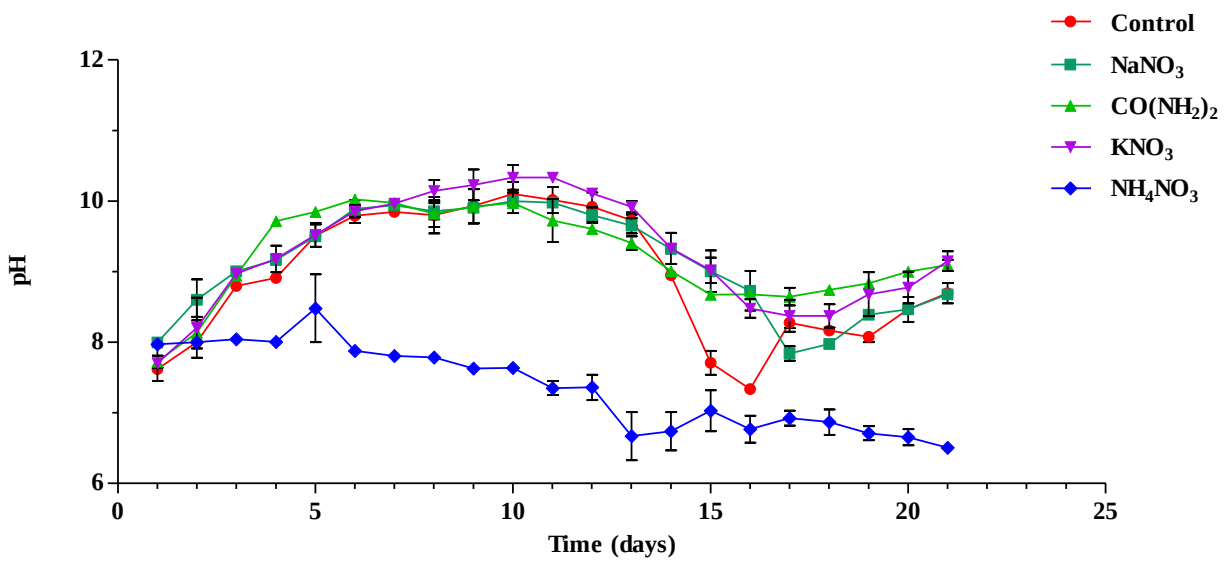


Figure 4.2: pH of *C. sorokiniana* cultivated in wastewater with different nitrogen sources. Data are expressed as a mean ± SEM (n = 3).

4.3.2 The effect of nitrogen source on lipid productivity

The results obtained show that all N sources induced lipid production by the microalgae. There was an initial lipid concentration of 10 % relative fluorescence per cell (rf/cell) lipid (Figure 4.4a). Urea produced the highest of 48.31 % rf/cell (Figures 4.3 and 4.4d). The control, KNO_3 and NH_4NO_3 had lipid concentrations below 35 % which can be seen in Figures 4.4b, e and f. Potassium nitrate showed the lowest lipid of 23.26 % rf/cell. A high concentration of NH_4NO_3 inhibited cell growth (Figure 4.1), and as cell growth declined, the lipids were used up as a form of energy.

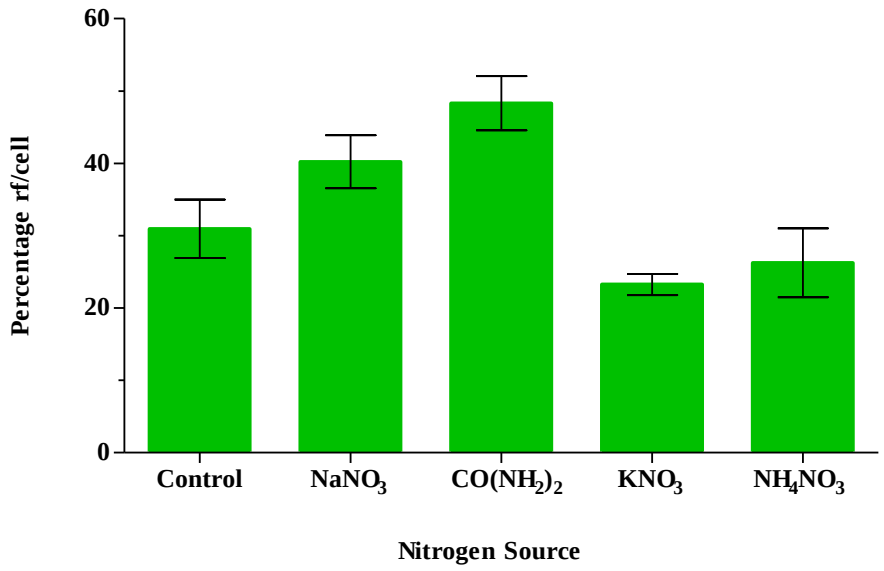


Figure 4.3: Lipid content of microalgae under different nitrogen sources as determined by relative fluorescence at day 20. Data are expressed as a mean ± SEM (n = 10).

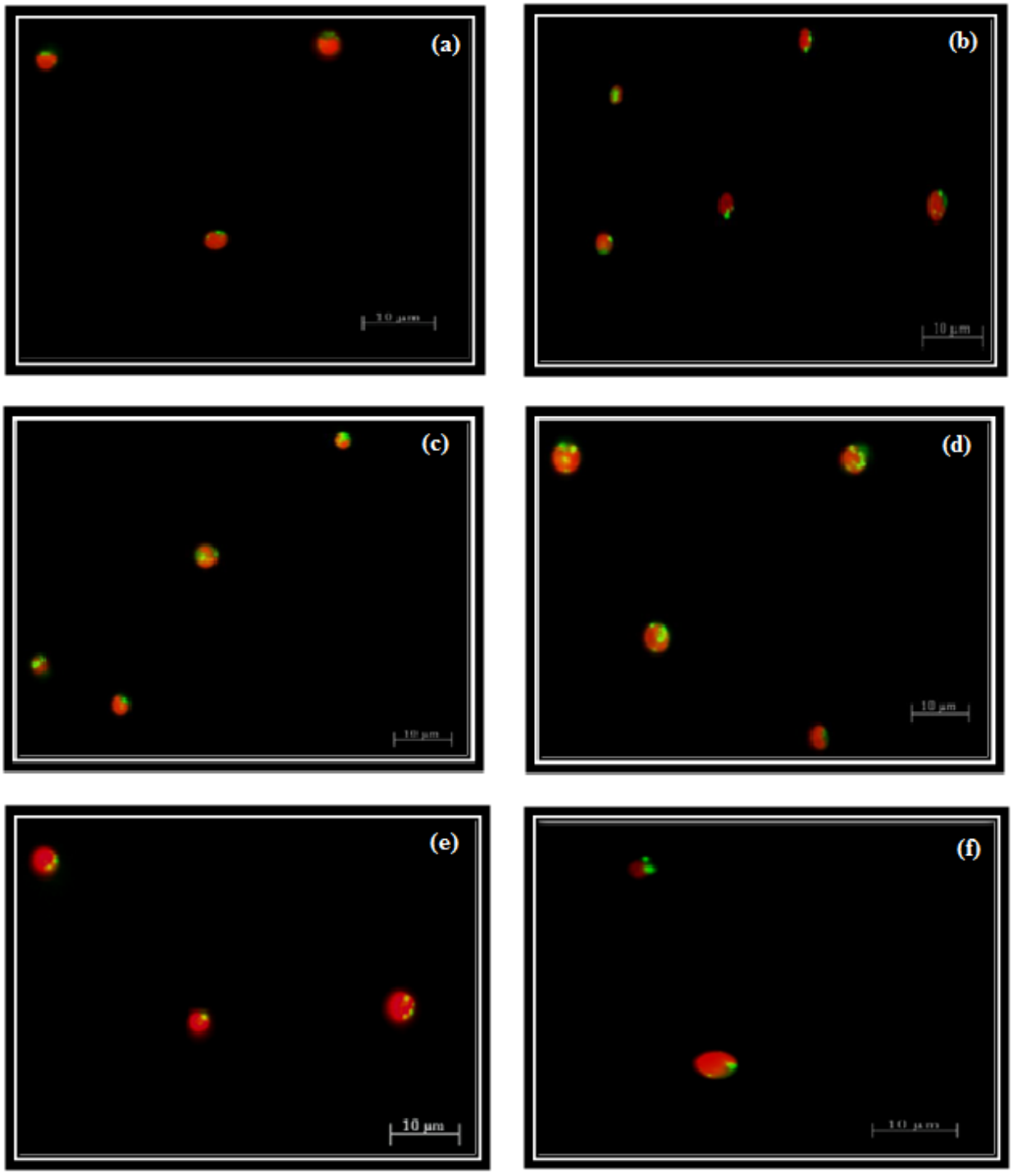


Figure 4.4: Micrographs showing Bodipy fluorescent staining of lipids in microalgae under different nitrogen sources at day 20. (a) Initial lipid concentration. (b) Control. (c) Sodium nitrate. (d) Urea. (e) Potassium nitrate. (f) Ammonium nitrate. Data are expressed as a mean ± SEM (n = 10).

4.4 Discussion

4.4.1 The effect of nitrogen source on biomass and lipid productivity

Research shows that a variety of N sources can be used for microalgal growth (Arumugam *et al.*, 2012, Lin and Lin, 2011, Hulatt *et al.*, 2012, Hsieh and Wu, 2009). High concentrations of NH_4NO_3 dissociates in solution to form NH_4 and NO_3. From the results it was observed that these products inhibited biomass growth (Figure 4.1). This was also evident from table 4.1 showing that *C. sorokiniana* had the slowest growth rate of 0.136 d^{-1} when supplemented with NH_4NO_3 as a supplement source. This could be due to the fact that microalgal cells are unable to control passive diffusion of NH_3, which is in equilibrium with NH_4 ions, across the plasma membrane. At high concentrations, this will deplete tricarboxylic acid cycle intermediates, thereby disrupting cellular respiration (Chen *et al.*, 2011). The cultures supplemented with NH_4NO_3 also showed a significant decrease in pH during the experiment. This caused growth inhibition due to the low buffering capacity of the medium. Therefore, when using NH_4NO_3 as a N source for microalgal cultivation it may be necessary to use biological buffers and pH controllers to prevent a decrease in pH. The use of buffers is only economical when conducting laboratory-scale experiments, when moving to large scale applications, chemical pH controllers may be utilized (Eustance *et al.*, 2013). Microalgae are able to grow over a wide pH range, however, suitable pH for growth can be largely species-dependent (Li *et al.*, 2013). Another reason for the decrease in the pH is the translocation of a proton out of the microalgal cell to maintain cell neutrality during uptake of cations. This acidification of the medium inhibits cell division (Eustance *et al.*, 2013). Ammonium as a N source is generally preferred by *Chlorella* sp., however, the study conducted by Li *et al.* (2013) showed that ammonium chloride and NH_4NO_3 hardly supported microalgal growth. They concluded that growth was related to the pH decrease (final pH 4.0) due to the

assimilation of ammonium ions. These findings correlate with our results. Ammonium nitrate supplemented in WW decreased the culture medium pH and was found to be the least affective N source tested.

Even though NH_4NO_3 and $CO(NH_2)_2$ are used for mass cultivation of microalgae, $CO(NH_2)_2$ and NO_3 are more suited for the growth and lipid accumulation in *Chlorella* sp. than NH_3 (Lin and Lin, 2011). Urea supplemented cultures showed a stable pH at first. This was due to the lack of ionic charge. A decrease in pH after day 10 can also be associated with acidification of the medium. This led to a decrease in cell viability ultimately causing chlorophyll degradation (Eustance *et al.,* 2013). Urea dissociates to form CO_2 and NH_4NO_3 in solution. This NH_4 is directly absorbed into the cell and accumulates to form amino acids, which are beneficial in the formation of chlorophylls that are essential in the photosynthetic process. (Wijanarko, 2011, Kim *et al.,* 2013). Ammonium is the basic unit of N required by microalgae to produce amino acids. Thus NH_4 is preferred over NO_3 (Eustance *et al.*, 2013). Urea supplemented cultures produced the highest biomass concentration of 0.216 gL^{-1}, and the fastest growth rate of 0.116 d^{-1}. Another benefit of utilizing $CO(NH_2)_2$ is that NH_4 is the most easily regenerated N source from microalgal biomass after lipid extraction. This is accomplished through the degradation of proteins at some stage in the secondary processing of biomass (Eustance *et al.,* 2013). The decline in growth in $CO(NH_2)_2$ supplemented cultures on day 16 can be associated with N limitation.

The traditional gravimetric method to extract and detect lipids in microalgae takes approximately 3–4 d, including drying time, and will need at least 100 mg wet biomass (Govender *et al.,* 2012). However, with fluorescent measurement by Bodipy 505/515, the process of lipid measurement was more simple, rapid, and sensitive (Govender *et al.*, 2012),

however, the stain has to be optimized for different microalgal strains. The results obtained show that all N sources induced lipid production. Urea produced the highest relative fluorescence per cell lipid of 48.31 % (Figure 4.4). The control, KNO_3 and NH_4NO_3 had lipid concentrations below 35 %. Potassium nitrate showed the lowest relative fluorescence per cell lipid of 23.26 %. A high concentration of NH_4NO_3 inhibited cell growth, and as cell growth declined, the lipids were used up as a form of storage energy.

Lizzul *et al.* (2014), found that *C. sorokiniana* showed a preference for NH_4 as opposed to NO_3 as a N source. The study compared the biomass growth on both WW and commercial medium (Bold's basal medium) whilst assessing the influence of exhaust gas on the process. It was also found that biomass yields and lipid production increased with CO_2 addition. The additional CO_2 dissociated in solution may have had a dual effect by augmenting microalgal growth as well as providing excess C flux towards lipid production. This could be the reason for $CO(NH_2)_2$ proving to be the most effective N source showing the highest biomass concentration of 0.216 gL^{-1} (Arumugam *et al.*, 2013, Lizzul *et al.*, 2014). Ammonium is favoured over NO_3 as a N source by microalgae as there is no redox reaction required for its assimilation and thus it requires less energy. Nitrate utilization only takes place when the NH_4 is completely consumed in the medium (Cai *et al.*, 2013a). For this reason WW with high concentrations of NH_4 or supplemented with $CO(NH_2)_2$ are used to rapidly cultivate microalgae (Cai *et al.*, 2013a).

Wijanarko (2011), worked with *C. vulgaris* Buitenzorg using NH_3 and $CO(NH_2)_2$ as substituted N sources in Benneck medium. The author found that for industrial application purposes, utilization of WW that was supplemented with N sources such as $CO(NH_2)_2$ made biomass production more economical. Amongst the organic N sources tested, $CO(NH_2)_2$ is

preferred for large-scale microalgal cultivation due to the fact that it is less expensive as compared to other N sources (Hsieh and Wu, 2009). Wijanarko (2011), found that $CO(NH_2)_2$ produced a higher cellular lipid content of 33 % when supplemented in WW than in Benneck medium. The same trend can be seen in the present work where $CO(NH_2)_2$ supplemented cultures produced the highest lipid values (48.31% rf/cell).

C. sorokiniana cultivated in WW supplemented with $NaNO_3$ showed its highest growth till day 8 and thereafter culture decline. This may be due to the intercellular conversion of NO_3 via the NO_3 reduction pathway to NO_2. Nitrite is reduced to NH_4 by nitrite reductase and ferredoxin. These two reactions require NADH to proceed. A decrease in cellular NADH would reduce the production of intracellular lipid, protein and chlorophyll formation and which would directly influence to cellular growth (Wijanarko, 2011, Cai *et al.*, 2013b).

Nigam *et al.* (2011), studied *C. pyrenoidosa* which was grown autotrophically in batch culture conditions in Fogg's medium and tested the effect of different concentrations of KNO_3 on growth and lipid content. Their study found an initial concentration of 0.05 gL^{-1} KNO_3 had the highest lipid accumulation of 26 % in the exponential phase of growth. Their study used a commercial media formulation which sustained microalgal growth for a longer period. The authors also state that lipid content increased as KNO_3 concentration decreased. Under N deficient conditions, microalgal cells were able to accumulate C metabolites as lipids. The culture utilized in this study showed just over 20 % rf/cell lipid. As nutrients in the WW start to deplete, microalgae consume stored fats as energy. This is the most likely reason why *C. sorokiniana* showed a low lipid concentration.

Kamyab *et al.* (2014), evaluated the growth and lipid content of *C. sorokiniana* grown in Proteose medium and Bolds Basal medium. The BBM had varying concentrations of $NaNO_3$ (0.3, 0.9, 1.5, 3.0, 6.0, and 11.0 M) and NH_4NO_3 (0.2, 0.7, 1.5, 3.0, and 6.0 M) as N source. Lipid content was analyzed as relative fluorescence by using the Nile red staining method. The authors found that 3.0 M (8.5 gL^{-1}) $NaNO_3$ produced higher biomass and lipid values (2.432 a.u) when compared to other $NaNO_3$ concentrations. According to these authors, the microalgae grew best when there was limited concentration of N. The present findings suggest that a balance of CO_2 and NH_4 sufficiently triggers high amounts of lipid per cell. When using NH_4NO_3 as an N source, 0.2 M (16 gL^{-1}) NH_4NO_3 produced the highest lipid content (3.138 a.u). Nitrogen deprivation induces substantial neutral lipid accumulation, however, it slows the growth rate. The authors equate this as cell to environment adaption, a condition most probably caused by the N starvation theory. Similarly these findings show that, as there was excess N in the medium, lipid production was inhibited. An increase in microalgal biomass may not necessarily mean there is an increase in lipid content of the microalga. As the N limited stress condition will lead to an inhibition of cellular division, the oil production will increase gradually. The results obtained proved that the growth of microalgal cells is influenced by the type of N source used. Lipid production by photosynthetic microalgae is sensitive to a number of environmental factors, including, but not limited to, temperature, N source and concentration and light intensity.

Chapter 5 : Optimisation of biomass and lipid yields of *Chlorella sorokiniana*

5.1 Introduction

Microalgal biodiesel production involves a series of steps including high oil producing strain selection, appropriate media formulation and cultivation (Ramachandra *et al.*, 2013, Pragya *et al.*, 2013), biomass harvesting (Rawat *et al.*, 2013b), and lipid extraction followed by conversion of lipids to biodiesel (Guldhe *et al.*, 2014). Nutrient source selection, harvesting and extraction of microalgal lipids are the most challenging areas in this regard. Lipid extraction needs to be fast, easily scalable, effective and it should not damage or alter the extracted lipids (Pragya *et al.*, 2013). Therefore, microalgal biodiesel production requires an exceptional knowledge and understanding of microalgal downstream processing for successful commercial exploitation.

Lipid production is controlled by cellular mechanisms which are in turn influenced by factors such as the availability of light, temperature and nutrients. Nowadays, we have tools to help us look into these complex metabolic processes on a whole cell level (Jiang *et al.*, 2012). Pulse Amplitude Modulated (PAM) fluorescence measurements are extensively used to routinely assess the photosynthetic electron transport in higher plants, microalgae and cyanobacteria. Moreover, it has been used to examine microalgal physiology owing to its simplicity, rapid and non-destructive nature (Schreiber *et al.*, 2012). Observed changes in PAM fluorescence parameters are regularly used to measure the impacts of varying environmental conditions like nutrient stress, irradiance levels and contamination events. Due to the accuracy associated with fluorescence measurements this potential method for the

assessment of microalgal physiology can be justified (Garrido *et al.*, 2012, Li and Lin, 2012, Schreiber *et al.*, 2012).

This objective focused on identifying the optimal concentration of $CO(NH_2)_2$ supplemented in domestic WW for achieving high biomass levels and oil accumulation by microalgae. This was accomplished through physiological experiments that were monitored using PAM Fluorometry. Finally, elucidation of lipid profiles extracted from *C. sorokiniana* biomass grown on supplemented domestic WW was used to assess its suitability as a biodiesel feedstock.

5.2 Methodology

5.2.1 Microalga and culture conditions

C. sorokiniana was maintained as per section 3.2.2. Microalgal growth was determined as per section 3.2.4, however, specific growth rates were calculated here. The specific growth rate of each microalgal strain was calculated from the slope of the linear regression of time and natural log cell density in exponential growth phase, as specified by (Lizzul *et al.*, 2014, Song *et al.*, 2013) i.e. as per equation 3.3. Where μ is the specific growth rate in the exponential growth phase, N_0 is biomass (gL^{-1}) at the beginning of the exponential phase (t_0) and N represents the biomass at time (t) of the exponential phase.

5.2.2 Urea concentration

After concluding that $CO(NH_2)_2$ was an acceptable N supplement in WW (section 4.4.1). Further experimentation was conducted using filtered WW supplemented with $CO(NH_2)_2$. Urea concentrations were varied (0 gL^{-1}; 0.25 gL^{-1}; 0.5 gL^{-1}; 0.75 gL^{-1}; 1 gL^{-1}; 1.25 gL^{-1}; 1.5 gL^{-1}; 1.75 gL^{-1}; 2 gL^{-1}; 4 gL^{-1}; 6 gL^{-1}; 8 gL^{-1} and 10 gL^{-1}) in order to establish the optimal concentration to produce sufficient biomass as well as high lipid content. The cultivation period was 30 d, for the $CO(NH_2)_2$ optimization experiments, and the culture was analyzed daily for biomass and every 3 d for lipid accumulation. Lipids were quantified as per section 4.2.3. BG11 (contained 1.5 gL^{-1} $NaNO_3$) and un-supplemented WW ([N] = 0.001 gL^{-1}) were used as the PC and the NC respectively.

5.2.3 Pulse amplitude modulated fluorometry

Non-invasive fluorescence measurements were used to determine the effect of nutrient levels on the physiology of *C. sorokiniana* using a Dual-PAM 100 Chlorophyll Fluorometer (Heinz WalzGmbh, Effeltrich, Germany). A 2 mL sample, diluted with distilled water, was decanted into a standard quartz cuvette and left for 15 min in the dark. This dark-adaptation period allowed all the photosystems reaction centers to close. The samples were then placed in the measuring chamber and allowed to stabilize at modulated (non-actinic) light (ML) for a couple of minutes. The use of ML prevents the reduction of the photosystem II (PS II) primary electron acceptor pool (plastoquinones, QA). This is because this light has a very low level of energy. The observed intrinsic fluorescence (F_o) represents the fluorescence given off when all PS II reaction centers are said to be open and are available for electron transport (i.e. photosynthesis). A short saturating pulse (SP) of actinic light (blue

light) (0.6 s at 10 000 μmol photons $m^{-2}s^{-1}$) was then applied to induce the maximal fluorescence yield (F_m) on dark-adapted cells. This triggers the reduction of all QA. Calculating F_o and F_m is done to evaluate the variable fluorescence (F_v), $F_v = F_m - F_o$. This allows the calculation of the maximum efficiency of PS II (F_v/F_m). The F_v/F_m ratio is related to the maximal photochemical efficiency of PS II. This is an indication of the fraction of the absorbed energy that is channelled toward the photosynthetic process by PS II reaction centers (Garrido *et al.*, 2012, Schreiber *et al.*, 2012). The quantum efficiency of PS II charge separation (F_v/F_m) was calculated as (Genty *et al.*, 1989):

$$F_v/F_m = (F_m - F_o)/F_m$$

Equation 5.1: Maximum quantum efficiency of PS II

Rapid light curves were generated by applying a sequence of increasing actinic irradiance in 15 discrete increments. Each period of actinic light lasted for 10 s before a SP of actinic light. This was applied to determine the Electron Transport Rate (ETR) for each irradiance level. Photon irradiance (400 to 700 nm) incident on the sample surface was measured using a Photosynthetically Active Radiation (PAR) micro-sensor (Spherical MicroQuantum Sensor US-SqS/W, Waltz) connected to the PAM fluorometer control unit. Relative Electron Transport Rate (rETR) was calculated as (White *et al.*, 2011):

$$rETR = F'_q/F'_m \text{ X } PPFD$$

Equation 5.2: Relative Electron Transport Rate

Where $F'_q = (F'_m - F')$. Where F'_m is maximum fluorescence in a light adapted sample and F' is dark fluorescence yield. F'_q/F'_m is termed the PS II operating efficiency and estimates the efficiency of light usage absorbed by PS II. 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.

5.2.4 Cell disruption and lipid extraction

Biomass was harvested from both culture media using centrifugation and lyophilized in a freeze dryer (LTE Mini Lyotrap, Lancashire, UK). Samples were analyzed in triplicate. 0.2 g powdered biomass was put into a 50 mL centrifuge tube followed by the addition of 4 mL of chloroform-methanol (2:1 *v*/*v*) and disrupted by sonication for 2 min at 20 MHz (Misonix Ultrasonic Liquid processor XL 2000 series, NY, USA) (Kumari *et al.*, 2011). The mixtures were then centrifuged at 2096 x g for 15 min at 4 ^{o}C. The supernatant was then transferred into 50 mL centrifuge tubes, and the process was repeated for another 3 times. The biomass pellet was then discarded. The supernatant was filtered to remove any remaining biomass. The samples were desolventised in an oven (Labcon, RSA) at 60 ^{o}C for 12 h. The weight of the crude oil attained from each sample was measured gravimetrically (Mettler Toledo, USA). Percentage lipid content was calculated based on per gram dry biomass.

5.2.5 Fatty acid composition analysis

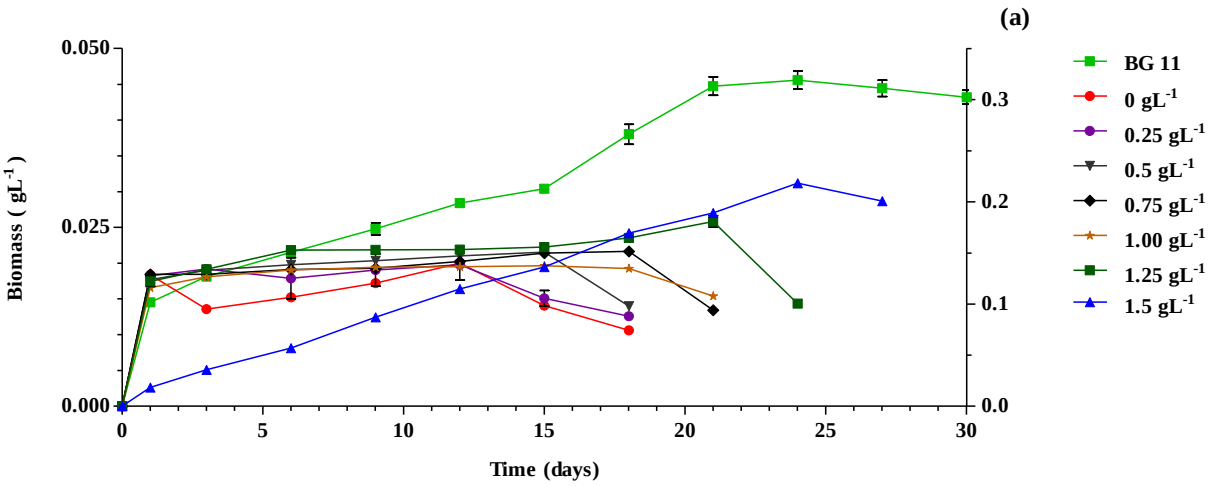
Fatty acid analysis was performed using GC. Lipid samples were placed into capped test tubes and then submitted to methanolysis with 5 % H_2SO_4 and methanol in 30:1 methanol to oil molar ratio at 60 ^{o}C for 4 h in the presence of 1 mL hexane as reaction

solvent. Stirring rate was kept constant at 200 rpm. 200 µL samples were removed and washed with distilled water. Thereafter, the phase containing the FAs was separated and recovered for further analysis. The FAMEs were then analyzed using a Gas Chromatograph (Shimadzu GC-2014, Japan) equipped with a flame ionization detector (GC-FID) and a capillary column (SP2380, Supelco Analytical, USA). The oven temperature was programmed to start at 60 °C and kept at hold for 2 min, then initially increased to 160 °C at a ramp rate of 10 °Cmin^{-1} and then to 240 °C at a ramp rate of 7 °Cmin^{-1} and again kept at hold for 1 min. The injector and detector temperature was 250 °C and N was used as carrier gas (Guldhe *et al.*, 2014). The FA components in lipids extracted from *C. sorokiniana* were identified by comparing their retention times and fragmentation patterns with those for standards. A 37 component fatty acid methyl ester mix Sigma–Aldrich (USA) was used as a standard. All experimentation was carried out in triplicate. All statistical analysis was carried out as per section 3.2.4.

5.3 Results

5.3.1 Urea concentration optimization

Figure 5.1 shows the biomass concentrations of *C. sorokiniana* under varying $CO(NH_2)_2$ concentrations. The PC, (BG11), showed the highest biomass concentration of 0.319 gL^{-1} and grew till day 30, when compared to the NC which showed a biomass concentration of 0.02 gL^{-1} and declined at day 12 (Figure 5.1a). The fastest growth rate of 0.108 d^{-1} (table 5.1) was achieved by 1.5 gL^{-1} $CO(NH_2)_2$ supplemented WW which had the second highest biomass concentration of 0.218 gL^{-1}.



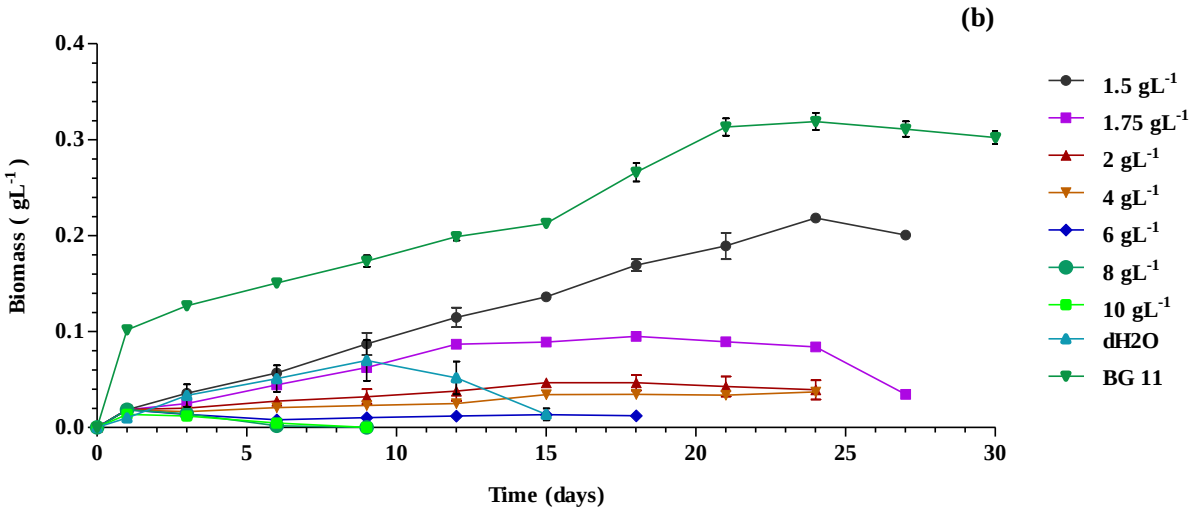


Figure 5.1: **Growth curve of *C. sorokiniana* biomass concentrations resulting from varying urea concentrations. (a) BG11 (on the secondary axis) to 1.5 gL^{-1} (on the secondary axis), (b) BG11 to 10 gL^{-1}. Data are expressed as a mean ± SEM (n = 3).**

Concentrations of 0 gL^{-1} to 1.25 gL^{-1} had biomass concentrations of below 0.05 gL^{-1}. They all had growth rates of below 0.02 d^{-1}. Urea supplementations of 1.75 gL^{-1} to 10 gL^{-1} showed biomass concentrations of below 0.1 gL^{-1}. It was found that cultures supplemented with $CO(NH_2)_2$ at 8 gL^{-1} and 10 gL^{-1} failed to grow past 10 d (Figure 5.1b), and 6 gL^{-1} - 10 gL^{-1} had low biomass concentrations which resulted in negative growth rates.

Table 5.1: **Specific Growth rates of *C. sorokiniana* cultivated in wastewater with varying urea concentrations.**

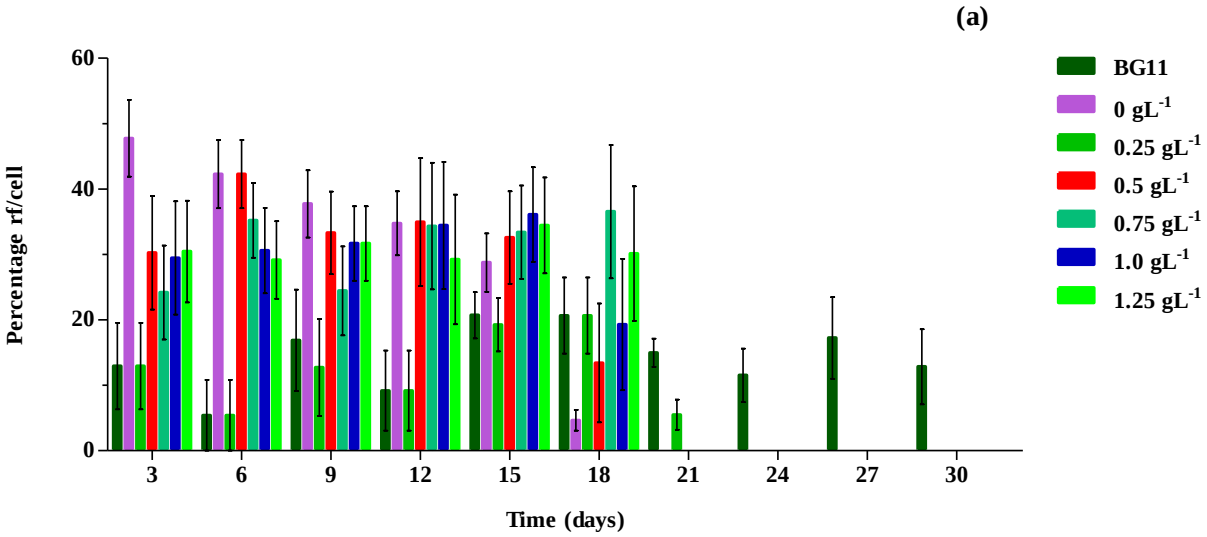
Urea Concentration	Growth Rate (d^{-1})
BG11	0.057
0 gL^{-1}	0.010
0.25 gL^{-1}	0.010
0.5 gL^{-1}	0.017
0.75 gL^{-1}	0.008
1 gL^{-1}	0.012
1.25 gL^{-1}	0.019
1.5 gL^{-1}	0.108
1.75 gL^{-1}	0.098
2 gL^{-1}	0.069
4 gL^{-1}	0.039
6 gL^{-1}	*n/a*
8 gL^{-1}	*n/a*
10 gL^{-1}	*n/a*

n/a- not applicable.

The pH of all cultures ranged between 6 at the start of experimentation to around 10 at the end of experimentation.

5.3.2 Semi quantitative lipid determination

At the start of experimentation, all cultures showed an initial lipid concentration of 19.23 % rf/cell. The 1.5 gL^{-1} $CO(NH_2)_2$ supplemented culture showed the highest rf/cell lipid (≤ 60 % rf/cell) throughout the experimentation as compared to the other concentrations (Figure 5.2b). 0.25 gL^{-1} - 1.75 gL^{-1} $CO(NH_2)_2$ supplementations showed lipid percentages below 45 % rf/cell throughout the study and by day 18 showed no lipid content. From Figure 5.2a, a fairly consistent lipid concentration was observed for the PC up to day 30 (12.84 % rf/cell). This was in contrast to the lipid concentration achieved for the NC which showed a concentration of 47.75 % rf/cell lipid (day 3) and decreased consecutively till day 18 of cultivation to 4.65 % rf/cell. A similar trend was observed with 4 gL^{-1} $CO(NH_2)_2$ supplemented cultures from day 3 to day 21. Even though the 1.75 gL^{-1} $CO(NH_2)_2$ supplemented culture showed the highest biomass concentrations the lipid concentration was lower at 38.51 % rf/cell lipid on day 15.



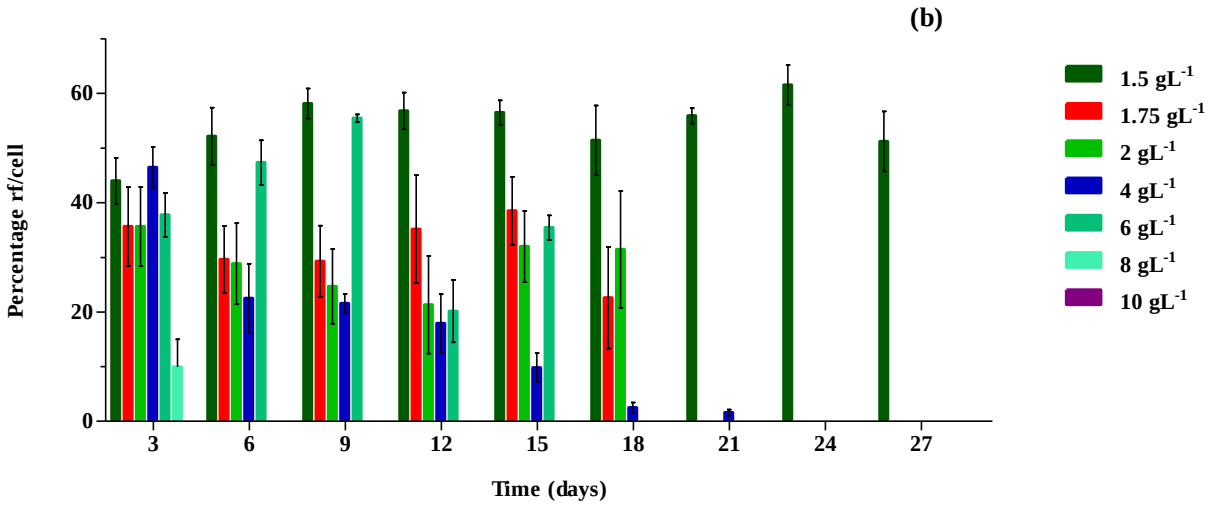


Figure 5.2: **Lipid content of microalgae under varying urea concentrations as determined by relative fluorescence. (a) BG11- 1.25 gL^{-1} urea,(b) 1.5 gL^{-1} - 10 gL^{-1} urea. Data are expressed as a mean ± SEM (n = 10).**

Figure 5.3 shows the lipid content of the microalgal cells grown in WW with the optimal $CO(NH_2)_2$ concentration (1.5 gL^{-1}) from start to the end of experimentation.

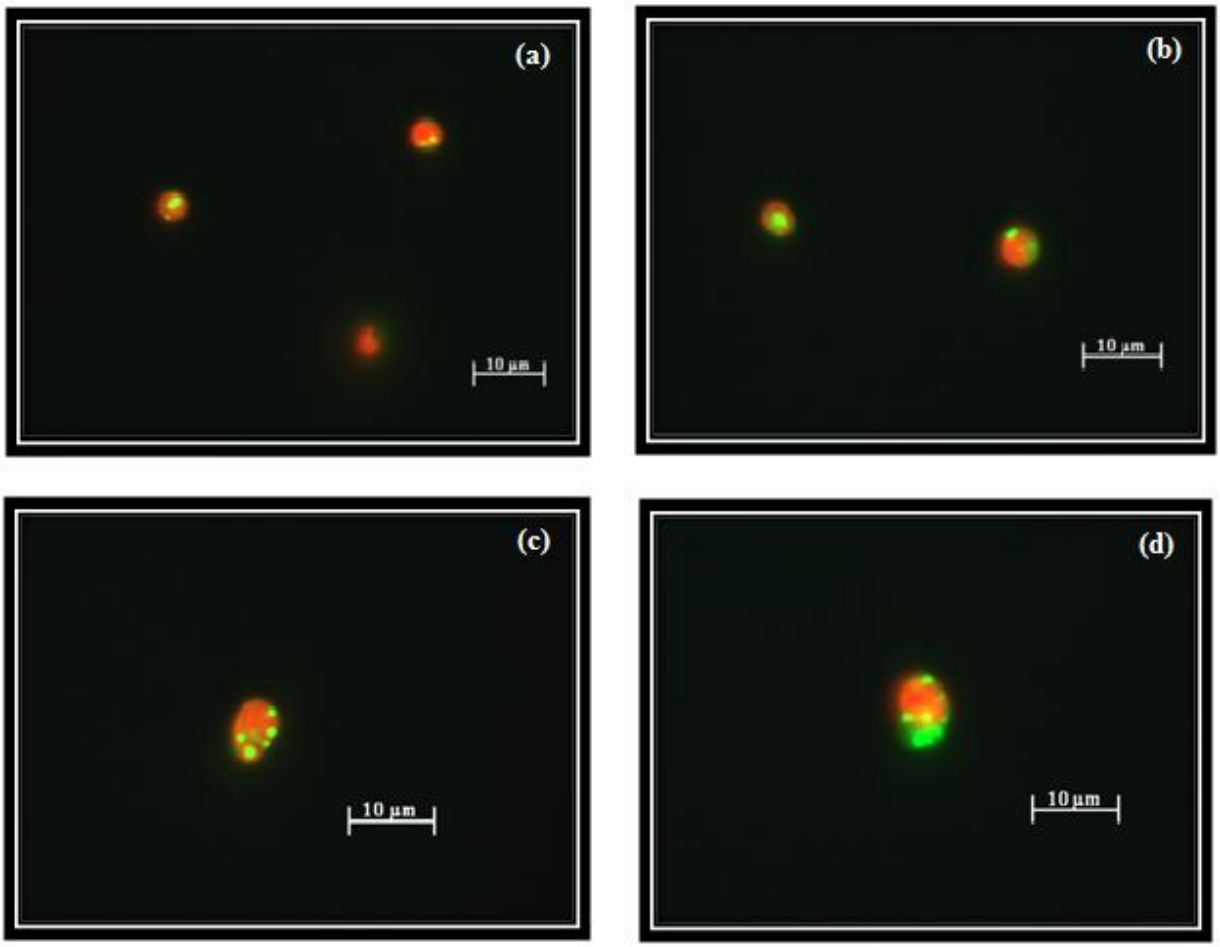


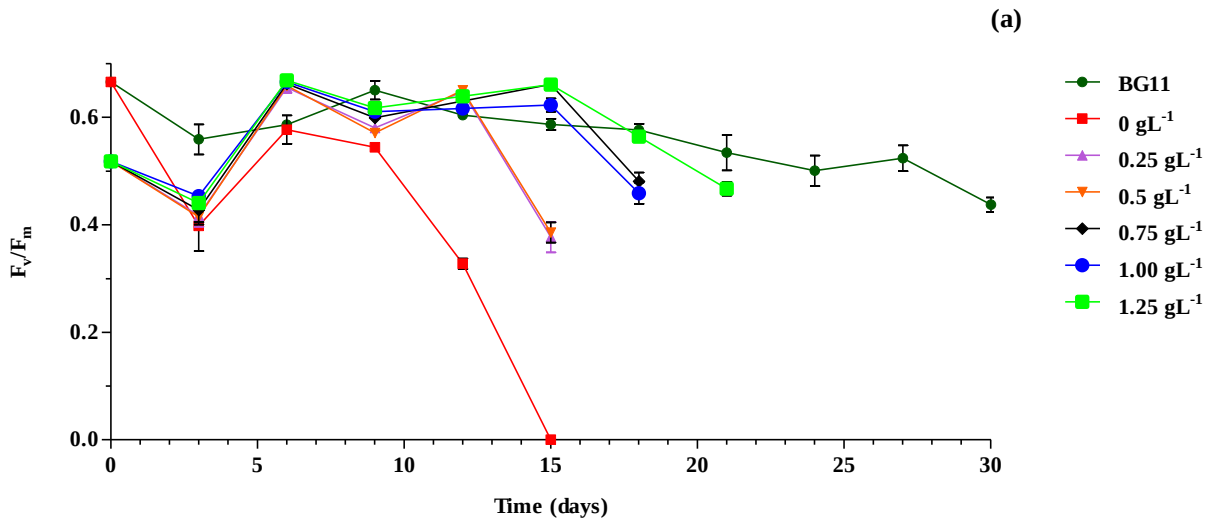
Figure 5.3: **Lipid percentage/content/fraction in cell grown at the optimal urea concentration (1.5 gL^{-1}). (a) At day 0, (b) at day 3, (c) at day 9 and (d) at day 24 of experimentation. Data are expressed as a mean ± SEM (n = 10).**

The microalgal cells were stained red and the lipid droplets within the cell were stained green. Figure 5.3a was captured at day 0 of the experiment and showed a lipid concentration for all cultures of approximately 19.23 % rf/cell. At day 3 the lipid concentration increased to 43.97 % rf/cell (Figure 5.3b) and day 9 to 58.14 % (Figure 5.3c), whilst the highest lipid concentration of 61.53 % rf/cell at day 24 was seen in Figure 5.3d.

5.3.3 Pulse amplitude modulated fluorometry

5.3.3.1 The quantum efficiency of PS II charge separation (F_v/F_m)

Pulse amplitude modulation fluorometry together with the SP method is now a widely accepted method for non-invasive assessment of the photosynthetic electron transport in microalgae and cyanobacteria (Garrido *et al.*, 2012, Schreiber *et al.*, 2012). This technique measures the fluorescence emitted by the pigments present in PS II which is a direct representation of the photochemical energy conversion of PS II reaction centers (Schreiber *et al.*, 2012, Garrido *et al.*, 2012, Jiang *et al.*, 2012).



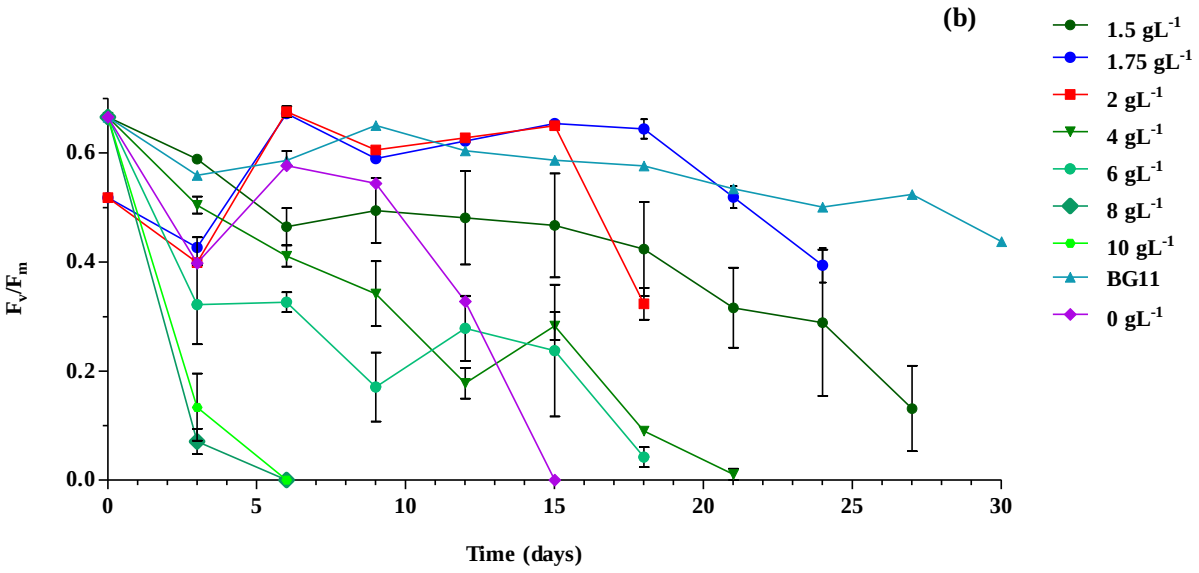


Figure 5.4: **The maximum quantum yield of PS II (F_v/F_m) recorded under the different urea concentrations. (a) BG 11- 1.25 gL^{-1}. (b) 1.5 gL^{-1}- 10 gL^{-1}. Data are expressed as a mean ± SEM (n = 3).**

At the start of experimentation all cultures showed F_v/F_m values below 0.7 (Figure 5.4). It can be observed from Figure 5.4a the PC had stable F_v/F_m values of 0.665 to 0.437 throughout the experiment. The 1.5 gL^{-1} $CO(NH_2)_2$ supplemented culture showed a high initial F_v/F_m of 0.665 it slowly decreased till day 27; this showed the culture reaching death phase. The decrease in the F_v/F_m values on day 3 in both graphs (Figures 5.4a and b) can be attributed to culture acclimation. In Figure 5.4b, 8 and 10 gL^{-1} $CO(NH_2)_2$ supplemented cultures show values of 0 at day 6 as the culture declined after 3 d.

5.3.3.2 Photosystem II operating efficiency (rETR)

Relative Electron Transport Rate was used to calculate the rate of linear electron transport through PS II. This can be correlated with the overall photosynthetic performance of the microalga (White *et al.*, 2011).

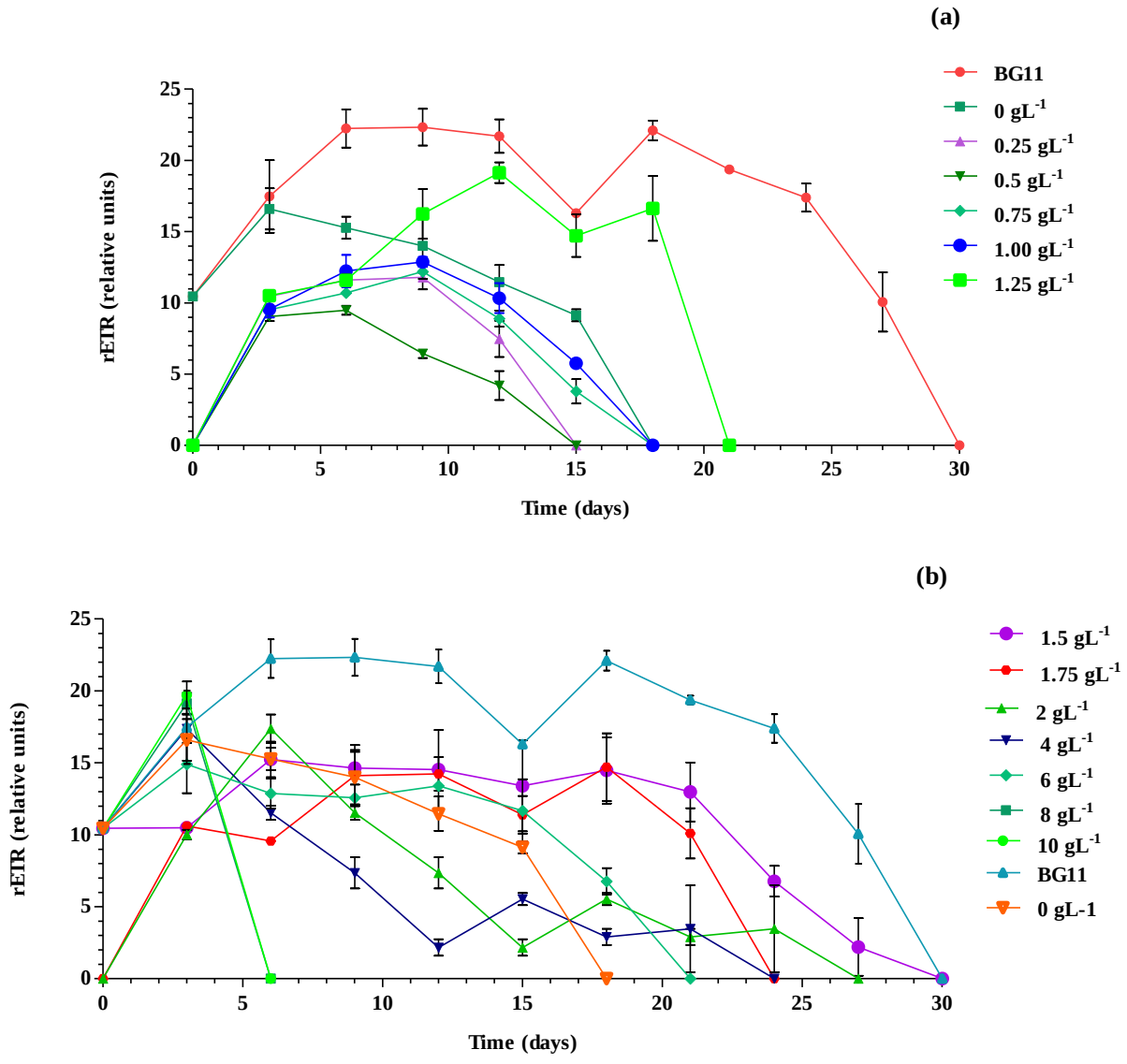


Figure 5.5: Relative electron transport rate recorded under the different urea concentrations. (a) BG11- 1.25 gL^{-1}. (b) 1.5 gL^{-1}- 10 gL^{-1}. Data are expressed as a mean ± SEM (n = 3).

The strain showed an increase in rETR within the first 3 d (Figure 5.5). At a $CO(NH_2)_2$ concentration of 1.5 gL^{-1}, rETR showed a steady state up to day 21, thereafter decreasing. The highest rETR value reached by cultures growing in 1.5 gL^{-1} $CO(NH_2)_2$ was 15.23 on day 6. In Figure 5.5a, the 1.25 gL^{-1} $CO(NH_2)_2$ supplemented culture showed the highest rETR value of 19.13 on day 12. The lowest rETR value was seen for 0.5 gL^{-1} $CO(NH_2)_2$ supplemented culture between 6-12 d and eventually decline to an ultimate rETR value of 0 showing culture death. The PC maintained a higher rETR as opposed to other $CO(NH_2)_2$ concentrations. In Figure 5.5b, 8 gL^{-1} and 10 gL^{-1} $CO(NH_2)_2$ supplemented cultures showed high rETR values of 19.3 and 19.7 respectively at day 3 and thereafter show a 0 value at day 3 indicating culture death. Cultures growing in $CO(NH_2)_2$ concentrations of 0 gL^{-1}; 2 gL^{-1}; 4 gL^{-1} and 6 gL^{-1} declined at days 18; 27; 24 and 21.

5.3.4 Lipid Analysis and FAME conversion

Gas Chromatography analysis was carried out for FA profiling of the extracted FAs from *C. sorokiniana* cultivated in WW and BG11. From Figures 5.6a and b, major peaks observed were palmitic acid, oleic acid and linoleic acid.

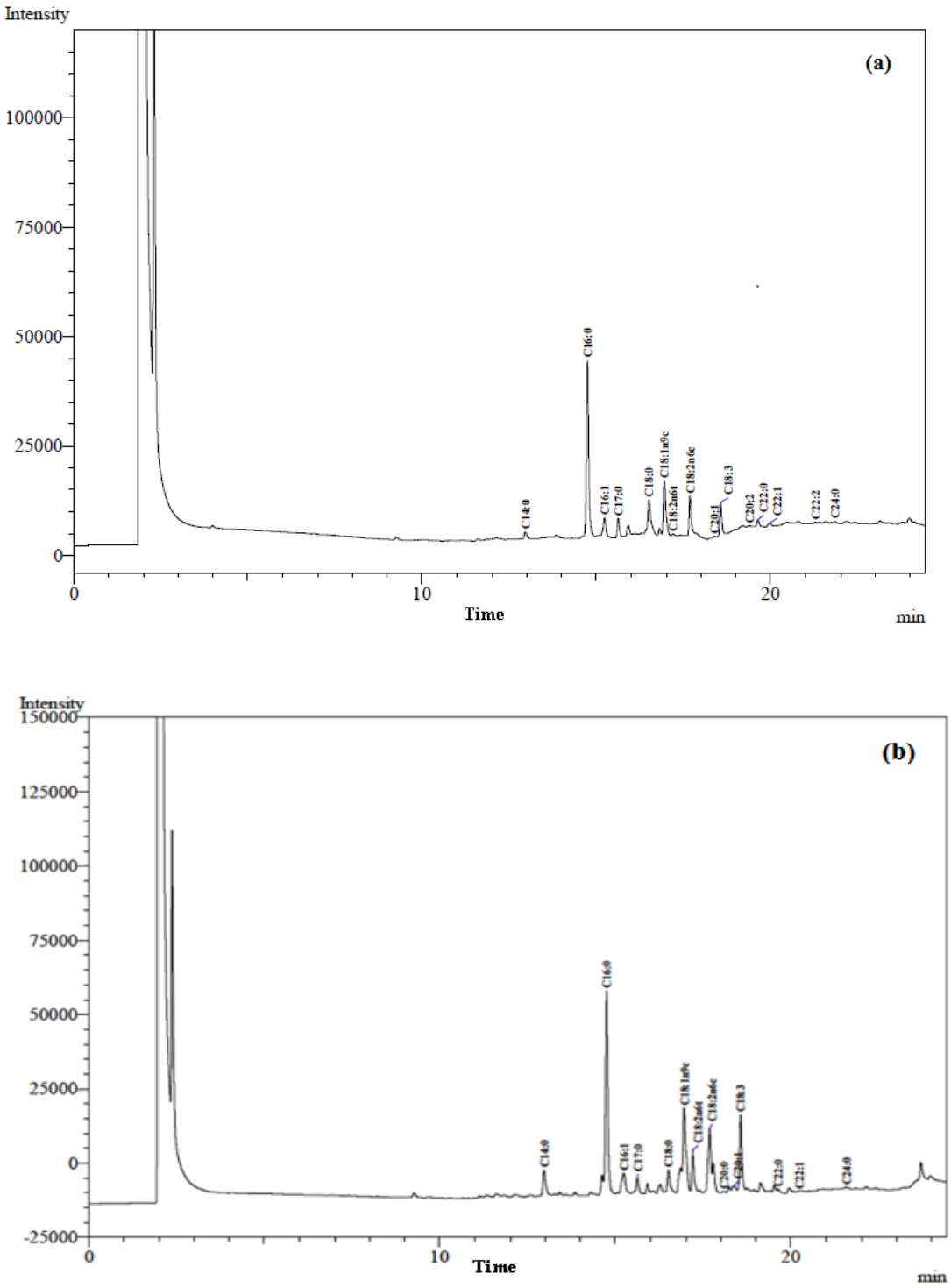


Figure 5.6: **Gas Chromatographs of FAME produced from fatty acids extracted from *C. sorokiniana* grown in (a) BG11 media and (b) domestic wastewater (1.5 gL^{-1} urea). Data are expressed as a mean ± SEM (n = 3).**

The FA profile of *C. sorokiniana* consisted of FAs with C chains ranging from C12:0-C24:0 (Figures 5.6 and 5.7). Of these FAs C16:0, C18:0 C18:1, C18:2 and C18:3, which were the major contributing FAs, are most suitable for obtaining ideal biodiesel properties.

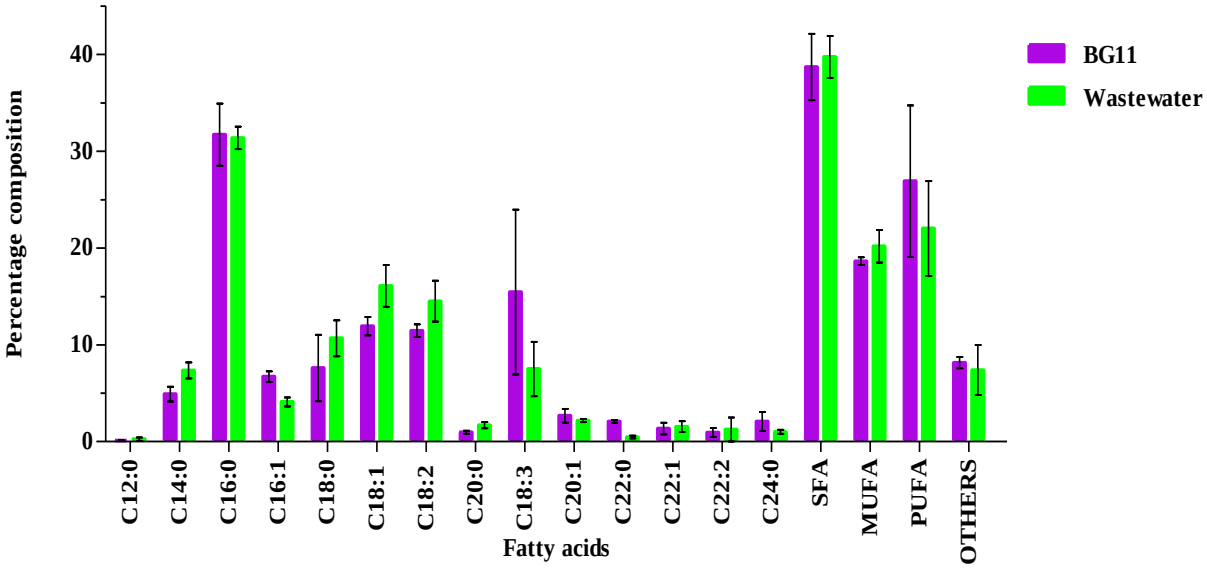


Figure 5.7: **Fatty acid profile of *C. sorokiniana* grown in BG11 media and wastewater (1.5 gL^{-1} urea) at day 16. Data are expressed as a mean ± SEM (n = 3).**

Microalgae grown in BG11 had a higher polyunsaturated fatty acid (PUFA) percentage than WW which was 26.9 ± 11.09 % and 22.01 ± 6.96 % respectively, although the WW grown culture had higher saturated fatty acid (SFA), and monounsaturated fatty acid (MUFA) percentages.

5.3.5 FAME conversion

After the completion of the reaction, samples were taken and analysed by GC for percentage FAME conversion.

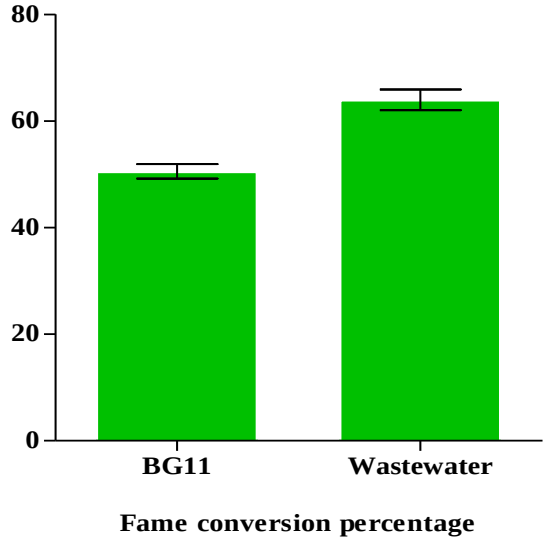


Figure 5.8: **Biodiesel conversion of fatty acids from *C. sorokiniana* grown in BG11 and wastewater (1.5 gL^{-1} urea). Data are expressed as a mean ± SEM (n = 3).**

It was found that the biodiesel conversion from the BG11 extracted lipid culture was 62.97 %, whereas the culture growing on WW had a 50.56 % biodiesel conversion. Wastewater grown cultures had a 12 % higher biodiesel conversion than the cultures grown on BG11. There was a significant difference between BG11 and WW cultures ($\alpha < 0.05$).

5.4 Discussion

5.4.1 *C. sorokiniana* biomass and lipid optimization

It was found that 8 gL^{-1} and 10 gL^{-1} $CO(NH_2)_2$ supplemented WW cultures ceased to grow within a day of experimentation (Figure 5.1). This could be attributed to the oversaturation of NH_4 in solution, which was toxic to the microalgae. An excess of intracellular NH_4 is known to inhibit the formation of ATP in the chloroplast following substrate activation, leading to growth inhibition (Wijanarko, 2011). Wijanarko (2011), found that a 500 mgL^{-1} $CO(NH_2)_2$ concentration slowed the growth of *C. vulgaris* Buitenzorg when grown on Benneck medium. The present findings are in contrast with these in that $CO(NH_2)_2$ at 1.5 gL^{-1} proved to be optimal for biomass growth and lipid accumulation. This can be attributed to the differences in strains as their strain was obtained from a culture collection as opposed to our indigenous wild-type strain. Microalgae have the ability to adapt to different environments, some to extreme conditions; *Dunaliella salina* was able to grow in hyper saline conditions (Wu *et al.*, 2014). Due to the seasonal variation in composition and complexity of domestic WW, many microalgal species may not adapt when cultivated in WW. In the research conducted by Xin *et al.* (2010a), eight out of twelve microalgal species evaluated showed almost no growth in domestic secondary effluent. These species, although highlighted in literature for high biomass and lipid production, could not fully adapt to the environment. From these observations it can be seen that specific microalgal species can only adapt to specific growth conditions.

Widjaja *et al.* (2009), investigated factors influencing lipid production on the freshwater microalgae *C. vulgaris.* The results suggested that lipid concentration was higher

at day 20 than at day 15 of incubation because of N starvation. This resulted in higher accumulation of intracellular lipid. The present findings showed that there was no significant difference ($\alpha > 0.05$) from day 9 to day 24 and biomass can be harvested for lipid extraction on day 9, as in the case of 1.5 gL^{-1} $CO(NH_2)_2$. This shorter cultivation period could allow for higher overall productivity. Wijanarko (2011), reported that *Chlorella vulgaris* Buitenzorg cultivated in diluted $CO(NH_2)_2$ concentrations induce higher lipid formation. This is in accordance with the present findings as a lower $CO(NH_2)_2$ concentration of 1.5 gL^{-1} accumulated higher lipid content in the present *Chlorella* strain. This was due to the fact that $CO(NH_2)_2$ metabolism did not consume NADH, which is also essential for intracellular lipid formation. Hsieh and Wu (2009), cultivated *Chlorella* sp. in batch mode on Walne's nutrient medium made with artificial seawater supplemented with $CO(NH_2)_2$ to assess biomass and lipid productivity. It was found that an increase in the $CO(NH_2)_2$ concentration led to a decrease in lipid content of cells. This is in accordance with the present findings where a lower $CO(NH_2)_2$ concentration ($\leq$1.5 gL^{-1}) induced higher lipid formation ($\leq$ 60 rf/cell) and 1.75 gL^{-1} or higher $CO(NH_2)_2$ supplementations showed lower lipid percentages per cell (1.6- 38.51 rf/cell).

The microalgal culture produced and accumulated more lipid toward the end of the study (Figure 5.2c and 5.2d) which was in correlation with Figure 5.3c and 5.3d. As per literature it was deduced that as N concentrations decrease in the culture medium an increase in lipid and TAG content in microalgal cells can be observed. This is characteristic of most oleaginous microalgae (Xin *et al.*, 2010b, Singh *et al.*, 2014, Lizzul *et al.*, 2014). In work done by Han *et al.* (2014), four microalgal species including *Chlorella* sp. SDEC-10, *C. ellipsoidea* SDEC-11, *S. bijuga* SDEC-12 and *S. quadricauda* SEDC-13 were isolated from a local lake and all four were used to investigate growth and lipid accumulation in synthetic

sewage. The commercial BG11 medium was set up as the control group. The authors found that the growth cycles of the four isolated microalgae were relatively short in general, as opposed to BG11 where they could live up to 40 d. BG11 medium comprises of all the nutritional components. Although the BG11 cultivated microalgae had longer growth cycles, they showed reduced biomass productivity. The maximum microalgal biomass in synthetic sewage was found to be between 0.4 and 0.5 gL^{-1}, while microalgal biomass ranged between 0.2 to 0.3 gL^{-1} when grown in BG11. The relatively high biomass can be attributed to the mixotrophic growth mode for assimilating the organic C, provided by $CO(NH_2)_2$, as well as autotrophic fixing of CO_2. The lipid content of all four microalgal species grown in synthetic sewage ranged from 25.92 to 27.76 %, which was higher than that achieved by the microalgae grown in BG11 medium (> 20 %). The lipid content of our locally isolated microalga was relatively higher when cultivated in WW than BG11.

5.4.2 Pulse amplitude modulated fluorometry

5.4.2.1 The quantum efficiency of PSII charge separation (F_v/F_m)

Photosynthetic parameters were tested to determine the physiology of the strain at different $CO(NH_2)_2$ concentrations. These fluorescence measurements provide information on how the light that is absorbed is utilized by the PS II reaction centers. In this way, it is possible to characterize the physiological response of microalgal cells to changes in external environments (Jiang *et al.*, 2012).

At the start of experimentation all cultures had F_v/F_m ratios of 0.7 (Figure 5.4). Microalgal strains with F_v/F_m ratios > 0.5 are accepted to be physiologically acclimated to the environmental conditions (Schreiber *et al.*, 2012, Jiang *et al.*, 2012), therefore, at the start of

experimentation the strain under study was adequately acclimatized (Garrido *et al.*, 2012). F_v/F_m values at all $CO(NH_2)_2$ concentrations declined considerably thereafter, with the PC; 1.5; 4 and 6 gL^{-1} showing a 34.00; 81.50; 98.50 and 93.68 % decline respectively. Changes in F_v/F_m provided essential information on the effects of the microalgal physiology under different $CO(NH_2)_2$ concentrations (Garrido *et al.*, 2012, White *et al.*, 2011). The PC showed a declining yet continuous F_v/F_m value up till day 30 (Figure 5.4a). Although the culture grown on 1.5 gL^{-1} $CO(NH_2)_2$ supplemented WW showed a high initial F_v/F_m of 0.665 it slowly decreased as the experiment proceeded to day 27, this was seen as the culture reaching death phase. The culture was dying, as evident by both growth curve and F_v/F_m values. Mutual shading does not factor in here as cultures were diluted before running PAM analysis, however, light penetration due to shading has been known to reduce the growth of microalgae. The decrease in the F_v/F_m values on day 3 in both graphs (Figure 5.4a and b) can be attributed to culture acclimation. In Figure 5.4b, 8 gL^{-1} and 10 gL^{-1} $CO(NH_2)_2$ supplemented cultures showed a F_v/F_m value of 0 at day 6. This was because the culture declined after 3 d as these $CO(NH_2)_2$ concentrations proved too toxic for the microalgae to handle. Lower $CO(NH_2)_2$ concentrations (Figure 5.4a) show higher F_v/F_m values whilst higher $CO(NH_2)_2$ concentrations had lower F_v/F_m values, attributed to NH_4 toxicity.

Lower F_v/F_m values have been associated with stress to the physiology of the microalgae due to nutrient limitation (Jiang *et al.*, 2012). Nutrient stressed conditions alter the C utilization pathway from production of cellular components to energy storage components such as lipids. Nitrogen limitation decreases the protein content whilst increasing lipid accumulation and storage. Nitrogen limitation also results in a decrease of the photosynthetic efficiency of the microalgae (Jiang *et al.*, 2012, Wijanarko, 2011, Garrido *et al.*, 2012). This was evident in Figure 5.2b as on day 24, the 1.5 gL^{-1} $CO(NH_2)_2$ supplemented culture

showed the highest lipid content as well as F_v/F_m values showed a 43.3 % decline on day 24. A drastic decline in the normal functioning of the cell induced the formation and accumulation of intracellular lipids (White *et al.*, 2011) which was seen in Figures 5.2, 5.3 and 5.4. The PC maintained F_v/F_m values above 0.5 throughout the duration of this study, and only showed a decrease on day 27 which was considered the death phase for this strain. Lowered $CO(NH_2)_2$ concentrations showed higher F_v/F_m values (0.25-1.75 gL^{-1}). Higher $CO(NH_2)_2$ concentrations (4- 10 gL^{-1}) were also found to have lower F_v/F_m values as NH_4 toxicity leads to reduced ATP formation. Lowered ATP concentrations affect the efficiency of PS II, primarily as a result of reduced photosynthetic pigments, which ultimately decrease the rate of photosynthesis and cellular death (White *et al.*, 2011). A decline in photosynthetic efficiency was shown by a decrease in the F_v/F_m values.

5.4.2.2 Photosystem II operating efficiency (rETR)

Relative Electron Transport Rate is the rate of linear electron transport through PS II, which can be directly related to the overall photosynthetic performance of the microalgae (White *et al.*, 2011). There was an increase in rETR within the first 3 d of growth (Figure 5.5). This may be due to the acclimatization of the microalgae to the new environment (Li and Lin, 2012). At the optimal $CO(NH_2)_2$ concentration (1.5 gL^{-1}), rETR showed a steady state up to day 21, thereafter decreasing. This suggested that PS II dependent electron transport at this $CO(NH_2)_2$ concentration was not completely inhibited when compared to the other concentrations (Hsieh and Wu, 2009). This finding also suggested that the culture supplemented with 1.5 gL^{-1} $CO(NH_2)_2$ concentration by this point in the experiment had used almost if not all of the $CO(NH_2)_2$. The PC retained a higher rETR as opposed to the other

concentrations (Figure 5.5). This was attributed to the fact that the PC was BG11 media which had all the necessary nutrients to support microalgal growth.

White *et al.* (2011), used PAM fluorometry to assess microalgal nutrient stress and observe cellular lipid formation. The authors found that with a depletion of N the microalgae showed a significant change in the physiological parameters measured. A 75 % decrease in the maximum rETR value was noted when compared to the control. The present findings showed that as the $CO(NH_2)_2$ concentration increased, the rETR decreased. Under stressful conditions [>1.5 gL^{-1} $CO(NH_2)_2$] microalgal growth rates decrease (table 5.1). Microalgae then remobilize C to producing energy storage products, like lipids and/or starch in order to acclimatize to the environment. This was evident in Figure 5.2b where a high concentration of lipids was observed between day 3 and 18. These N stressed environments reduced the ability of the microalga to utilize photosynthetically fixed C for protein synthesis, which aided the formation of photosynthetic storage products, resulting in the accumulation of lipids during this period (Jiang *et al.*, 2012).

Another explanation for the decrease in rETR was likely due to nutrient toxicity. Uptake of NH_4 reduced the efficiency of PS II, which decreased the rate of photosynthesis, leading ultimately to microalgal death (Hulatt *et al.*, 2012, Schreiber *et al.*, 2012). The present findings showed that excess NH_4 led to growth inhibition, maybe by disrupting the normal production of ATP in the cell. This was evident by the drop in the rETR and F_v/F_m values for all the concentrations tested. These conditions reduced regular cellular functions like photosynthesis (rETR), respiration and enzyme activities (Schreiber *et al.*, 2012).

5.4.3 Lipid Analysis and FAME conversion

There was not much variation in the FA composition using BG11 or WW as a medium in this study. Generally microalgae amass lipid under nutrient deprivation, when light and a C source are accessible and when the cellular mechanisms for the photosynthesis are active. Lipid composition depends on the species and culture conditions: microalgae modify their metabolic pathways to produce FAs as a result of different environmental conditions to which they are exposed (Chia *et al.*, 2013, Hidalgo *et al.*, 2013).

Most microalgae have FA chains ranging from C11 to C26, although chain lengths of C14 to C22 are typically observed (Singh *et al.*, 2014). Singh *et al.* (2013) found that the majority of FAs in *Chlorella* sp. comprise of C14–C18 chain lengths. Fatty acids can be classified as saturated (cannot chemically add H) or unsaturated (UFA); the latter may vary in the number of double bonds as well as the position of where they are located on the C chain. Environmental conditions like nutrient concentrations and microalgal physiological state affect the degree of saturation in microalgal lipids (Chia *et al.*, 2013, Giakoumis, 2013).

It was shown that the majority of the FAME was derived from palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid, the last three being the primary unsaturated acids (Giakoumis, 2013, Islam *et al.*, 2013). Singh *et al.* (2013) grew *C. minutissima* in 100 % WW from a local hotel sewage drain. Upon GC analysis, the extracted oil mainly consisted of small chain FAs (C16:0, C18:1, C18:2, and C18:3). Even though this study worked with *C. sorokiniana* the resulting FAs extracted were similar. In most studies palmitic acid has consistently been the major FA produced and identified (Han *et al.*, 2014, Singh *et al.*, 2013, Ramachandra *et al.*, 2013). Palmitic acid makes up the most part (40 – 70 %) of the lipid

composition in microalgae (Han *et al.*, 2014). Palmitic acid is produced by microalgae when a C source in the medium is in excess. After normal cellular functions are completed the excess C source is converted to palmitic acid, it is also the first FA produced during FA synthesis and a precursor to longer chain FAs (Guldhe *et al.*, 2014, Han *et al.*, 2014).

Apart from palmitic acid, stearic, oleic and linoleic acid also make up the lipid composition of many different species microalgae. Ramachandra *et al.* (2013) conducted a study that focused on the lipid prospects of three different species of microalgae grown in WW systems. GC–MS analyses were used for the comparison of the FAs extracted from *Euglena* sp., *Spirogyra* sp. and *Phormidium* sp. The results are in agreement with the present findings as palmitic acid, stearic acid, oleic acid and linoleic acid were found to be the prominent FAs from growing the microalgae on WW. The authors concluded that the composition of the FA profiles from the three species will provide a reasonable balance of biodiesel properties.

The WW culture in the present study showed higher percentages of all the above mentioned FAs except C18:3. The BG11 cultured microalga showed a higher linolenic acid concentration than the WW cultured microalga, 15.45 % and 7.51 % respectively. When statistically analyzed with a 95 % confidence interval there was a significant difference between the means ($\alpha < 0.0001$). Linolenic acid is a major component of the photosynthetic apparatus of microalgae, and it is relatively susceptibility to oxidation. Prior to the microalga reaching death phase, as evident by the lower F_v/F_m values (Figure 5.4), the FAs where extracted. A possible explanation for the 7.95 % difference in linolenic acid could be that the culture growing in BG11 media had enough nutrients to sustain growth till the end of experimentation, thus being able to produce more linolenic acid to prevent photo-oxidation.

The quantity of linolenic acid needs to be under 12 % to satisfy the quality standards of biodiesel from European standards (EN, 2008, Rawat *et al.*, 2013b). In the present study the concentration of linolenic acid of the microalgae grown in WW was favourable for biodiesel production. A high concentration of linolenic acid, in the BG11 grown microalgae (15.45 %) will lower the oxidation stability of the biodiesel produced and will lead to its rancidity, if the fuel is not supplemented with antioxidants (Guldhe *et al.*, 2014).

The FA composition will have a profound effect on the fuel property of the resulting biodiesel. There is an inverse correlation between oxidation stability and cold flow properties of biodiesel. High SFA concentrations are desirable for better oxidation stabilities of the fuel. This will ensure for longer fuel storage. High UFA concentrations will be beneficial for cold flow properties (cloud point, cold filter plugging point [CFPP], and pour point) of the biodiesel. This provides an added advantage as the fuel can be used in countries with colder climates. It is desirable to have a mixture of both saturated and unsaturated FAs in the lipid extracts to balance out both the oxidation stability and cold flow properties (Guldhe *et al.*, 2014). A recent study was conducted by Han *et al.* (2014) to assess nutrient removal and the lipid accumulation properties in four strains of microalgae. *Chlorella* sp. SDEC-10, *C. Ellipsoidea* SDEC-11, *S. bijuga* SDEC-12 and *S. quadricauda* SEDC-13 were isolated from a local lake grown in synthetic sewage using BG11 as a control. The lipid content of the four microalgae in synthetic sewage was found to be higher than that obtained in BG11 medium. This confirms the results obtained in the present study (Figure 5.7), as the BG11 grown culture had lower lipid content than the culture grown on WW. Also, the authors found that a locally isolated strain obtained relatively high lipid content when cultivated in WW. Analysis of FAME profiles are in conformity with the present study as the main composition was C16–C18 reaching 80 % of the SFA concentration. The dominant components in their study

were found to be palmitic acid and linolenic acid. *C. sorokiniana* in the present study had roughly equal quantities of SFAs and UFAs, 39.74 % and 42.20 % respectively. Unsaturated FAs include MUFAs and PUFAs. Oleic acid (16.09 %), linoleic acid (14.51 %) and linolenic acid (7.51 %) were the main compositions of UFAs in these findings. From the study it was concluded that *C. sorokiniana* was an ideal microalgal candidate for biodiesel production.

A high PUFA will lead to satisfactory cold flow properties of biodiesel at low temperatures (Zheng *et al.,* 2013). An excess of UFA will adversely affect the oxidative stability of the resulting biodiesel, lowering the cetane numbers (CN) that tend to increase gaseous and particulate exhaust emissions attributed to incomplete combustion. Microalgae grown in BG11 showed a higher PUFA percentage than the microalgae grown on WW. This was 26.9 ± 11.09 % and 22.01 ± 6.96 % respectively, although the WW culture had higher SFA, and MUFA percentages. This was attributed to the fact that N concentrations in the WW were minimal by the end of the study.

Wastewater grown *C. sorokiniana* had a suitable lipid profile to be used as a biodiesel feedstock, however, it is known that microalgal lipids have high FFA content. Free FAs generally should only be compromised of about 1–2 % of the lipids in microalgae. Most of the FAs are bounded to glycerol molecules forming acylglycerols. Among these lipids, only TAGs are easily converted into biodiesel. Thus, for pure ASTM grade biodiesel, it is the FA composition rather than the lipid content that must be considered for potential biodiesel production (Nascimento *et al.*, 2013, Guldhe *et al.*, 2014).

In the present study a mixture of methanol and sulphuric acid, a homogeneous acid catalyst, was used for the conversion of *C. sorokiniana* lipids. Acid catalysts may be used

when the FFA content in lipids is higher than 1 % (Hidalgo *et al.*, 2013). After the completion of the reaction, samples were taken and analysed by GC for percentage FAME conversion. The methanol/ sulphuric acid mixture played a positive role on the FFA reduction (Hidalgo *et al.*, 2013). Using acid catalysts may promote both transesterification and esterification reactions of microalgae lipids (Hidalgo *et al.*, 2013). The FAME production from both the BG11 and WW grown microalgal lipid was attributed to conversion of FFA and TAG through transesterification. After the consumption and esterification of FFA (60 min), the yield of FAME was attributed largely to the transesterification of TAG (Dong *et al.*, 2013).

The FA C chain length and degree of unsaturation is used to determine biodiesel specifications. There are various physical and thermodynamic properties to consider when producing microalgal biodiesel (Giakoumis, 2013, Islam *et al.*, 2013). Physical properties include physical appearance, biodegradability and odour to name a few. Thermo-physical properties include CN, boiling point range, CFPP, flash point range and heating value etc., of biodiesel. These factors are used for the characterization of microalgal biodiesel (Saxena *et al.*, 2013). High levels of UFAs influence biodiesel properties such as iodine value, CN, oxidative stability, cold flow and viscosity which will cause the resulting biodiesel to be off specification (Giakoumis, 2013, Gimpel *et al.*, 2013). The CN is regarded as a standardized measure and represents the ignitability of the fuel when injected into a compression ignition engine (Islam *et al.*, 2013). Higher CNs are associated with better biodiesel produced in terms of improved combustion, cold starting, reduced noise, hydrocarbon, CO and particulate emissions. Low CNs are associated with long ignition delay, i.e. long time between fuel injection and start of combustion. As the chain length on the FAMEs increase i.e. the degree of unsaturation increases, the quality of the biodiesel decreases (Frampton *et al.*, 2013,

Giakoumis, 2013). Different oil extraction methods, FA composition and transesterification approaches will cause variation in the biodiesel produced affecting viscosity and, consequently, affecting CN values (Nascimento *et al.*, 2013).

In the present study, qualitative analysis of FAME produced from the WW cultured microalga showed that this microalga had a predominance of saturated FAs (39.73 %) and MUFA (below 30 %), which would tend to generate a biodiesel with a good oxidative stability. Saturated FAs will have higher melting points and are less prone to oxidation, which is favourable in terms of biodiesel storage (He *et al.*, 2013, Giakoumis, 2013). On the other hand, when more saturated molecules of FA esters are present in oils, crystallization may occur at temperatures below the normal engine operation range. The resulting biodiesel will have poor CFPP properties. Biodiesels rich in C16:0 and C18:0 have tendencies to present a poor CFPP, because when a liquid biodiesel is cooled, these FAMEs are the first to precipitate. In the present research, the levels of palmitic and stearic acid were generally high. These high values contribute to the lower temperatures of CFPP of the biodiesel (Nascimento *et al.*, 2013). Both the MUFA and PUFA show concentrations of below 30 %. A decrease in N concentration resulted in an increase in UFAs. Polyunsaturated FAs improve the cold-flow owing to their lower melting points, however, they are not desirable when producing biodiesel, so pre-treatment of the FAME mixture is necessary for large scale production (Islam *et al.*, 2013). Also, FA composition may influence the biodiesel emission properties such as NO_x emission. A blend of MUFA and SFA in a balanced proportion will produce biodiesel with the best properties (Singh *et al.*, 2014). Biodiesel may be blended with diesel fuel to form a mixture that can be used in any diesel engine with no or minor modifications. It can be blended from 2 % to 20 %. An example of this is blend ‘B20’ which refers to the

percentage of biodiesel present in the diesel fuel; however the properties of the mixed blend will change with the amount of diesel fuel blended with biodiesel (Saxena *et al.*, 2013).

It is evident from the results obtained in this study that *C. sorokiniana* cultured in domestic WW provided the essential FAs that is suitable for biodiesel production. The study provided vital information about microalgal growth and lipid productivities in domestic WW. FA analysis indicated the existence of SFA and UFA which are efficient biodiesel components, indicating potential utilities of the WW microalgae.

Chapter 6 : Conclusions and Recommendations

6.1 Significant Conclusions from the Study

- The results in this study showed that *C. sorokiniana* can be cultivated on domestic WW effluent. Wastewater as a medium is a promising method to produce microalgal lipids.

- Based on the results obtained, the temperature of the WW effluents should be conducive for microalgal growth. All three WWTP effluents were found to be acceptable for microalgal cultivation in terms of temperature range and TSS.

- Post-chlorinated WW effluent from Kingsburgh WWTP was found to be an acceptable WW stream. Bacteria present in this WW stream may have had an effect on microalgal growth.

- Domestic WW effluents can support microalgal growth for limited periods of time due to diminished nutrients. Supplementation of the WW with an additional N and P source is required to promote and sustain microalgal growth for longer periods.

- Urea proved to be the best N source compared to KNO_3, $NaNO_3$ and NH_4NO_3.

- 1.5 gL^{-1} $CO(NH_2)_2$ was found to be the optimal concentration tested in this study. The physiological responses of *C. sorokiniana* exposed to post-chlorinated WW

supplemented with $CO(NH_2)_2$ demonstrated by PAM fluorometry further justify the efficacy and suitability of this media formulation.

- The main FA present in *C. sorokiniana* lipids: C16:0, C18:0 C18:1, C18:2 and C18:3 are suitable for production of biodiesel with acceptable properties.

6.2 Recommendations

The following are suggestions and implications for further research:

- Future studies need to focus on combining agricultural and industrial WW streams as substrates for microalgal propagation. This may solve the N supplementation problem. Feasibility studies need to be conducted so as to critically analyze biodiesel production from microalgae grown in WW.

- Pharmaceutical and heavy metals degradation products may be commonly found in domestic WW. Domestic WW needs to be further analysed focussing on the effects on the final biomass and products. Studies need to focus on microalgal- bacterial relationships in WW effluents.

- The mechanism of interaction between Cl and microalgal cells needs to be thoroughly investigated. Future research needs to focus on the effect of free Cl on microalgal growth to determine if it should be considered a trace element.

- The current research did not evaluate the protein and carbohydrate content in the spent biomass. With large scale cultivation, spent biomass can be recycled as animal feed, fertilizers or anaerobically co-digested to produce biomethane. This would largely require scrubbing of microalgal biomass to remove heavy metals.

- The viability of microalgal biodiesel rests firmly on the biorefinery process. The specific biorefinery approach adopted is dependent on a number of factors. Therefore, feasibility studies, including microalgal strain/consortium of choice, climatic conditions, existing infrastructure, logistic considerations as well as overall availability of waste resources are required to make this approach viable.

References

Abou-Shanab, R. A., Ji, M. K., Kim, H. C., Paeng, K. J. and Jeon, B. H. 2013. Microalgal species growing on piggery wastewater as a valuable candidate for nutrient removal and biodiesel production. Journal of Environmental Management. **115:**257-264.

Ahmad, F., Khan, A. U., and Yasar, A. 2012. Uptake of Nutrients from Municipal Wastewater and Biodiesel Production by Mixed Algae Culture. Pakistan Journal of Nutrition **11:**550-554.

American Public Health Association, American Water Works Association and Water Environment Federation. 1998. Standard Methods for the Examination of Water and Wastewater, 20th ed, Washington, DC, USA.

Aresta, M., Dibenedetto, A., Carone, M., Colonna, T., and Fragale, C. 2005. Production of biodiesel from macroalgae by supercritical CO_2 extraction and thermochemical liquefaction. Environmental Chemistry Letters **3:**136-139.

Arias-Penaranda, M. T., Cristiani-Urbina, E., Montes-Horcasitas, C., Esparza-Garcia, F., Torzillo, G., and Canizares-Villanueva, R. O. 2013. *Scenedesmus incrassatulus* CLHE-Si01: A potential source of renewable lipid for high quality biodiesel production. Bioresource Technology **140C:**158-164.

Arumugam, M., Agarwal, A., Arya, M. C., and Ahmed, Z. 2012. Influence of organic waste and inorganic nitrogen source on biomass productivity of *Scenedesmus* and *Chlorococcum* sp. International Journal of Energy and Environment **2:**1125-1132.

Arumugam, M., Agarwal, A., Arya, M. C., and Ahmed, Z. 2013. Influence of nitrogen sources on biomass productivity of microalgae *Scenedesmus bijugatus*. Bioresource Technology. **131:**246–249.

Bahadar, A., and Bilal Khan, M. 2013. Progress in energy from microalgae: A review. Renewable and Sustainable Energy Reviews **27:**128-148.

Bhatnagar, A., Bhatnagar, M., Chinnasamy, S., and Das, K. C. 2010. *Chlorella minutissima*- A Promising Fuel Alga for Cultivation in Municipal Wastewaters. Applied Biochemistry and Biotechnology **161:**523-536.

Blatti, J. L., Michaud, J., and Burkart, M. D. 2013. Engineering fatty acid biosynthesis in microalgae for sustainable biodiesel. Current Opinion in Chemical Biology **17:**496-505.

Borowitzka, M. A., and Moheimani, N. R. 2013. Sustainable biofuels from algae. Mitigation and Adaptation Strategies for Global Change **18:**13-25.

Bowman, M., Hilligoss, D., Rasmussen, S., and Thomas, R. 2006. Biodiesel: A Renewable and Biodegradable Fuel. Hydrocarbon Processing**:**103-106.

Brennan, L., and 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.

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

Cabanelas, I. T., Ruiz, J., Arbib, Z., Chinalia, F. A., Garrido-Perez, C., Rogalla, F., Nascimento, I. A., and Perales, J. A. 2013a. Comparing the use of different domestic wastewaters for coupling microalgal production and nutrient removal. Bioresource Technology **131C:**429-436.

Cabanelas, I. T. D., Arbib, Z., Chinalia, F. A., Souza, C. O., Perales, J. A., Almeida, P. F., Druzian, J. I., and Nascimento, I. A. 2013b. From waste to energy: Microalgae production in wastewater and glycerol. Applied Energy **109:**283-290.

Cai, T., Park, S. Y., and Li, Y. 2013a. Nutrient recovery from wastewater streams by microalgae: Status and prospects. Renewable and Sustainable Energy Reviews **19:**360-369.

Cai, W., Zhang, B., Jin, Y., Lei, Z., Feng, C., Ding, D., Hu, W., Chen, N., and Suemura, T. 2013b. Behavior of total phosphorus removal in an intelligent controlled sequencing batch biofilm reactor for municipal wastewater treatment. Bioresource Technology **132C:**190-196.

Campbell, M. N. 2008. Biodiesel: Algae as a Renewable Source for Liquid Fuel. Guelph Engineering Journal **1:**2-7.

Chen, G.-Q., Jiang, Y., and Chen, F. 2008. Variation of lipid class composition in *Nitzschia laevis* as a response to growth temperature change. Food Chemistry **109:**88-94.

Chen, G., Zhao, L., and Qi, Y. 2015. Enhancing the productivity of microalgae cultivated in wastewater toward biofuel production: A critical review. Applied Energy **137:**282–291.

Chen, M., Tang, H., Ma, H., Holland, T. C., Ng, K. Y. S., and Salley, S. O. 2011. Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*. Bioresource Technology **102:**1649-1655.

Chia, M. A., Lombardi, A. T., Melão, M. D. G. G., and Parrish, C. C. 2013. Effects of cadmium and nitrogen on lipid composition of *Chlorella vulgaris* (*Trebouxiophyceae, Chlorophyta*). European Journal of Phycology **48:**1-11.

Chinnasamy, S., Bhatnagar, A., Hunt, R. W. and Das, K. C. 2010. Microalgae cultivation in a wastewater dominated by carpet mill effluents for biofuel applications. Bioresource Technology **101:** 3097-3105.

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.

Chisti, Y. 2013. Constraints to commercialization of algal fuels. Journal of Biotechnology **167:**201-214.

Chisti, Y., and Yan, J. 2011. Energy from algae: Current status and future trends Algal biofuels – A status report. Applied Energy **88:**3277-3279.

Cho, S., Lee, N., Park, S., Yu, J., Luong, T. T., Oh, Y.-K., and Lee, T. 2013. Microalgae cultivation for bioenergy production using wastewaters from a municipal WWTP as nutritional sources. Bioresource Technology **131:**515-520.

Cho, S., Luong, T. T., Lee, D., Oh, Y.-K., and Lee, T. 2011. Reuse of effluent water from a municipal wastewater treatment plant in microalgae cultivation for biofuel production. Bioresource Technology **102:**8639-8645.

Chu, F. F., Chu, P. N., Cai, P. J., Li, W. W., Lam, P. K., and Zeng, R. J. 2013. Phosphorus plays an important role in enhancing biodiesel productivity of *Chlorella vulgaris* under nitrogen deficiency. Bioresource Technology **134:**341–346.

Costa, J. A. V., and de Morais, M. G. 2011. The role of biochemical engineering in the production of biofuels from microalgae. Bioresource Technology **102:**2-9.

Daims, H., Taylor, M. W., and Wagner, M. 2006. Wastewater treatment: a model system for microbial ecology. Trends in Biotechnology **24:**483-489.

Di Iaconi, C., Del Moro, G., De Sanctis, M. and Rossetti, S. 2010. A chemically enhanced biological process for lowering operative costs and solid residues of industrial recalcitrant wastewater treatment. Water Research. **44:**12: 3635-3644.

de-Bashan, L. E., and Bashan, Y. 2010. Immobilized microalgae for removing pollutants: Review of practical aspects. Bioresource Technology **101:**1611-1627.

Department of Water Affairs and Forestry. 1999.

http://hwt.co.za/downloads/NWA%20General%20and%20Special%20Authorisations.pdf

Ding, A., Qu, F., Liang, H., Ma, J., Han, Z., Yu, H., Guo, S., and Li, G. 2013. A Novel integrated vertical membrane bioreactor (IVMBR) for removal of nitrogen from synthetic wastewater/domestic sewage. Chemical Engineering Journal **223:**908–914.

Dong, T., Wang, J., Miao, C., Zheng, Y., and Chen, S. 2013. Two-step *in situ* biodiesel production from microalgae with high free fatty acid content. Bioresource Technology **136:**8-15.

Dou, X., Lu, X.-H., Lu, M.-Z., Yu, L.-S., Xue, R., and Ji, J.-B. 2013. The Effects of Trace Elements on the Lipid Productivity and Fatty Acid Composition of *Nannochloropis oculata*. Journal of Renewable Energy **2013:**1-6.

Ebenezer, V., Nancharaiah, Y. V., and Venugopalan, V. P. 2012. Chlorination-induced cellular damage and recovery in marine microalga, *Chlorella salina*. Chemosphere **89:**1042–1047.

Ebrahimian, A., Kariminia, H-R., Vosoughi, M. 2014. Lipid production in mixotrophic cultivation of Chlorella vulgaris in a mixture of primary and secondary municipal wastewater. Renewable Energy **71:**502-508.

EN. 2008. European Standard EN 14214 2008. Automotive fuels-fatty acid methyl esters (FAME) for diesel engines-requirements and test methods. Management Centre.

Eustance, E., Gardner, R. D., Moll, K. M., Menicucci, J., Gerlach, R., and Peyton, B. M. 2013. Growth, nitrogen utilization and biodiesel potential for two chlorophytes grown on ammonium, nitrate or urea. Journal of Applied Phycology **25:**1663-1677.

Fernandes, H., Jungles, M. K., Hoffmann, H., Antonio, R. V., and Costa, R. H. 2013. Full-scale sequencing batch reactor (SBR) for domestic wastewater: Performance and diversity of microbial communities. Bioresource Technology **132C:**262-268.

Fon Sing, S., Isdepsky, A., Borowitzka, M. A., and Moheimani, N. R. 2011. Production of biofuels from microalgae. Mitigation and Adaptation Strategies for Global Change **18:**47-72.

Frampton, D. M., Gurney, R. H., Dunstan, G. A., Clementson, L. A., Toifl, M. C., Pollard, C. B., Burn, S., Jameson, I. D., and Blackburn, S. I. 2013. Evaluation of growth, nutrient utilization and production of bioproducts by a wastewater-isolated microalga. Bioresource Technology **130:**261-268.

Garrido, M., Cecchi, P., Vaquer, A., and Pasqualini, V. 2012. Effects of sample conservation on assessments of the photosynthetic efficiency of phytoplankton using PAM fluorometry. Deep Sea Research Part I: Oceanographic Research Papers. **71:**38–48.

Genty, B., Briantais, J. M., and Baker, N. R. 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochimica et Biophysica Acta **90:**87-92.

Giakoumis, E. G. 2013. A statistical investigation of biodiesel physical and chemical properties, and their correlation with the degree of unsaturation. Renewable Energy **50:**858-878.

Gimpel, J. A., Specht, E. A., Georgianna, D. R., and Mayfield, S. P. 2013. Advances in microalgae engineering and synthetic biology applications for biofuel production. Current Opinion in Chemical Biology **17:**1-7.

Govender, T., Ramanna, L., Rawat, I., and Bux, F. 2012. BODIPY staining, an alternative to the Nile Red fluorescence method for the evaluation of intracellular lipids in microalgae. Bioresource Technology **114:**507-511.

Guldhe, A., Singh, B., Rawat, I., Ramluckan, K., and Bux, F. 2014. Efficacy of drying and cell disruption techniques on lipid recovery from microalgae for biodiesel production. Fuel **128:**46-52.

Halim, R., Danquah, M. K., and Webley, P. A. 2012. Extraction of oil from microalgae for biodiesel production: A review. Biotechnology Advances **30** 709–732.

Han, L., Pei, H., Hu, W., Han, F., Song, M., and Zhang, S. 2014. Nutrient removal and lipid accumulation properties of newly isolated microalgal strains. Bioresource Technology **165:**38–41.

He, P. J., Mao, B., Shen, C. M., Shao, L. M., Lee, D. J., and Chang, J. S. 2013. Cultivation of *Chlorella vulgaris* on wastewater containing high levels of ammonia for biodiesel production. Bioresource Technology **129:**177-181.\

Hernandez, D., Riano, B., Coca, M. and Garcia-Gonzalez, M. C. 2013. Treatment of agro-industrial wastewater using microalgae-bacteria consortium combined with anaerobic digestion of the produced biomass. Bioresource Technology. **135:**598-603.

Hidalgo, P., Toro, C., Ciudad, G., and Navia, R. 2013. Advances in direct transesterification of microalgal biomass for biodiesel production. Reviews in Environmental Science and BioTechnology **12:**179–199.

Hsieh, C.-H., and Wu, W.-T. 2009. Cultivation of microalgae for oil production with a cultivation strategy of urea limitation. Bioresource Technology **100:**3921-3926.

Huang, M.-h., Li, Y.-m., and Gu, G.-w. 2010. Chemical composition of organic matters in domestic wastewater. Desalination **262:**36-42.

Hulatt, C. J., Lakaniemi, A.-M., Puhakka, J. A., and Thomas, D. N. 2012. Energy Demands of Nitrogen Supply in Mass Cultivation of Two Commercially Important Microalgal Species, *Chlorella vulgaris* and *Dunaliella tertiolecta*. BioEnergy Research **5:**1-16.

Hussain, S., Aziz, H. A., Isa, M. H., Ahmad, A., Van Leeuwen, J., Zou, L., Beecham, S., and Umar, M. 2011. Orthophosphate removal from domestic wastewater using limestone and granular activated carbon. Desalination **271:**265-272.

Islam, M. A., Ayoko, G. A., Brown, R., Stuart, D., Magnusson, M., and Heimann, K. 2013. Influence of Fatty Acid Structure on Fuel Properties of Algae Derived Biodiesel. Procedia Engineering **56:**591-596.

Ji, M. K., Kim, H. C., Sapireddy, V. R., Yun, H. S., Abou-Shanab, R. A., Choi, J., Lee, W., Timmes, T. C., Inamuddin and Jeon, B. H. 2013. Simultaneous nutrient removal and lipid production from pretreated piggery wastewater by Chlorella vulgaris YSW-04. Applied Microbiology and Biotechnology. **97:6:** 2701-2710.

Jiang, Y., Yoshida, T., and Quigg, A. 2012. Photosynthetic performance, lipid production and biomass composition in response to nitrogen limitation in marine microalgae. Plant Physiology and Biochemistry **54:**70-77.

Kamyab, H., Lee, C. T., Md Din, M. F., Ponraj, M., Eva Mohamad, S., and Sohrabi, M. 2014. Effects of nitrogen source on enhancing growth conditions of green algae to produce higher lipid. Desalination and Water Treatment **52:**1-6.

Karya, N. G. A. I., van der Steen, N. P., and Lens, P. N. L. 2013. Photo-oxygenation to support nitrification in an algal-bacterial consortium treating artificial wastewater. Bioresource Technology **134:**244-250.

Khan, S. A., Rashmi, Hussain, M. Z., Prasad, S., and Banerjee, U. C. 2009. Prospects of biodiesel production from microalgae in India. Renewable and Sustainable Energy Reviews **13:**2361-2372.

Khozin-Goldberg, I., Iskandarov, U., and Cohen, Z. 2011. LC-PUFA from photosynthetic microalgae: occurrence, biosynthesis, and prospects in biotechnology. Applied Microbiology and Biotechnology **91:**905-915.

Kim, J., Yoo, G., Lee, H., Lim, J., Kim, K., Kim, C. W., Park, M. S., and Yang, J. W. 2013. Methods of downstream processing for the production of biodiesel from microalgae. Biotechnology Advances **31:**862–876.

Kim, S., Lee, Y., and Hwang, S.-J. 2013. Removal of nitrogen and phosphorus by *Chlorella sorokiniana* cultured heterotrophically in ammonia and nitrate. International Biodeterioration & Biodegradation **85:**511-516.

Kirrolia, A., Bishnoi, N. R., and Singh, R. 2013. Microalgae as a boon for sustainable energy production and its future research & development aspects. Renewable and Sustainable Energy Reviews **20:**642-656.

Kong, Q. X., Li, L., Martinez, B., Chen, P. and Ruan, R. 2010. Culture of Microalgae Chlamydomonas reinhardtii in Wastewater for Biomass Feedstock Production. Applied Biochemistry and Biotechnology. **160:1:** 9-18.

Kumari, P., Reddy, C. R., and Jha, B. 2011. Comparative evaluation and selection of a method for lipid and fatty acid extraction from macroalgae. Analytical Biochemistry **415:**134-144.

Li, P., and Lin, J. 2012. Effect of ultraviolet radiation on photosynthesis, biomass, and fatty acid content and profile of a *Scenedesmus rubescens*-like microalga. Bioresource Technology **111:**316-22.

Li, T., Zheng, Y., Yu, L., and Chen, S. 2013. High productivity cultivation of a heat-resistant microalga *Chlorella sorokiniana* for biofuel production. Bioresource Technology **131:**60-67.

Lin, Q., and Lin, J. 2011. Effects of nitrogen source and concentration on biomass and oil production of a *Scenedesmus rubescens* like microalga. Bioresource Technology **102:**1615-1621.

Lizzul, A. M., Hellier, P., Purton, S., Baganz, F., Ladommatos, N., and Campos, L. 2014. Combined remediation and lipid production using *Chlorella sorokiniana* grown on wastewater and exhaust gases. Bioresource Technology **151:**12-18.

Ma, X., Zhou, W., Fu, Z., Cheng, Y., Min, Min., Liu, Y., Zhang, Y., Chen, P., Ruan, R. 2014. Effect of wastewater-borne bacteria on algal growth and nutrients removal in wastewater-based algae cultivation system. Bioresource Technology **167:**8–13.

MacKellar, N., New, M., and Jack, C. 2014. Observed and modelled trends in rainfall and temperature for South Africa: 1960–2010. South African Journal of Science **110:**1-13.

Mahapatra, D. M., Chanakya, H. N., and Ramachandra, T. V. 2013. Treatment efficacy of algae-based sewage treatment plants. Environmental Monitoring and Assessment **185:**7145-7164.

Makareviciene, V., Andruleviciute, V., and Skorupskaite, V. 2013. Biodiesel fuel from microalgae-promising alternative fuel for the future: a review. Reviews in Environmental Science and Biotechnology **12:**119–130.

Markou, G., and Nerantzis, E. 2013. Microalgae for high-value compounds and biofuels production: A review with focus on cultivation under stress conditions. Biotechnology Advances dx.doi.org/10.1016/j.biotechadv.2013.07.011 **31:**1532–1542.

Markou, G., Vandamme, D. and Muylaert, K. 2014. Microalgal and cyanobacterial cultivation: The supply of nutrients. Water Research **65:**186- 202.

Mata, T. M., Martins, A. A., and Caetano, N. S. 2010. Microalgae for biodiesel production and other applications: A review. Renewable and Sustainable Energy Reviews **14:**217-232.

Meher, L. C., Sagar, D. V., and Naik, S. N. 2006. Technical aspects of biodiesel production by transesterification—a review. Renewable and Sustainable Energy Reviews **10:**248-268.

Mohan, S. V. Rohit, M.V. Chiranjeevi, P. Chandra, R. Navaneeth, B. 2014. Heterotrophic microalgae cultivation to synergize biodiesel production with waste remediation: Progress and perspectives. Bioresource Technology, doi:10.1016/j.biortech.2014.10.056.

Moheimani, N. R., and Borowitzka, M. A. 2006. The long-term culture of the coccolithophore *Pleurochrysis carterae* (Haptophyta) in outdoor raceway ponds. Journal of Applied Phycology **18:**703-712.

Muñoz, R., and Guieysse, B. 2006. Algal–bacterial processes for the treatment of hazardous contaminants: A review. Water Research **40:**2799-2815.

Mutanda, T., Karthikeyan, S., and Bux, F. 2011a. The Utilization of Post-chlorinated Municipal Domestic Wastewater for Biomass and Lipid Production by *Chlorella* spp. Under Batch Conditions. Applied Biochemistry and Biotechnology **164:**1126-1138.

Mutanda, T., Ramesh, D., Karthikeyan, S., Kumari, S., Anandraj, A., and Bux, F. 2011b. Bioprospecting for hyper-lipid producing microalgal strains for sustainable biofuel production. Bioresource Technology **102:**57-70.

Nascimento, I. A., Marques, S. S. I., Cabanelas, I. T. D., Pereira, S. A., Druzian, J. I., Souza, C. O., Vich, D. V., Carvalho, G. C., and Nascimento, M. A. 2013. Screening Microalgae Strains for Biodiesel Production: Lipid Productivity and

Estimation of Fuel Quality Based on Fatty Acids Profiles as Selective Criteria. BioEnergy Research **6:**1-13.

Nigam, S., Rai, M. P., and Sharma, R. 2011. Effect of Nitrogen on Growth and Lipid Content of *Chlorella pyrenoidosa*. American Journal of Biochemistry and Biotechnology **7** 124-129.

Olguin, E. J. 2012. Dual purpose microalgae-bacteria-based systems that treat wastewater and produce biodiesel and chemical products within a biorefinery. Biotechnology Advances. **30:5:**1031–1046.

Park, J. B. K., Craggs, R. J., and Shilton, A. N. 2011. Recycling algae to improve species control and harvest efficiency from a high rate algal pond. Water Research **45:**6637-6649.

Park, K. C., Whitney, C., McNichol, J. C., Dickinson, K. E., MacQuarrie, S., Skrupski, B. P., Zou, J., Wilson, K. E., O'Leary, S. J. B., and McGinn, P. J. 2012. Mixotrophic and photoautotrophic cultivation of 14 microalgae isolates from Saskatchewan, Canada: potential applications for wastewater remediation for biofuel production. Journal of Applied Phycology **24:**339-348.

Peccia, J., Haznedaroglu, B., Gutierrez, J., and Zimmerman, J. B. 2013. Nitrogen supply is an important driver of sustainable microalgae biofuel production. Trends in Biotechnology **31:**134-138.

Pignata, C., Fea, E., Rovere, R., Degan, R., Lorenzi, E., de Ceglia, M., Schiliro, T., and Gilli, G. 2012. Chlorination in a wastewater treatment plant: acute toxicity effects of the effluent and of the recipient water body. Environmental Monitoring and Assessment **184:**2091-2103.

Pittman, J. K., Dean, A. P., and Osundeko, O. 2011. The potential of sustainable algal biofuel production using wastewater resources. Bioresource Technology **102:**17-25.

Pragya, N., Pandey, K. K., and Sahoo, P. K. 2013. A review on harvesting, oil extraction and biofuels production technologies from microalgae. Renewable and Sustainable Energy Reviews **24:**159-171.

Raja, R., Shanmugam, H., Ganesan, V., and Carvalho, I. S. 2014. Biomass from Microalgae: An Overview. Oceanography **2:**1-7.

Ramachandra, T. V., Durga Madhab, M., Shilpi, S., and Joshi, N. V. 2013. Algal biofuel from urban wastewater in India: Scope and challenges. Renewable and Sustainable Energy Reviews **21:**767-777.

Rawat, I., Bhola, V., Kumar, R. R., and Bux, F. 2013a. Improving the feasibility of producing biofuels from microalgae using wastewater. Environmental Technology **34:**1765-1775.

Rawat, I., Ranjith Kumar, R., Mutanda, T., and Bux, F. 2011. Dual role of microalgae: Phycoremediation of domestic wastewater and biomass production for sustainable biofuels production. Applied Energy **88:**3411-3424.

Rawat, I., Ranjith Kumar, R., Mutanda, T., and Bux, F. 2013b. Biodiesel from microalgae: A critical evaluation from laboratory to large scale production. Applied Energy **103:**444-467.

Resurreccion, E. P., Colosi, L. M., White, M. A., and Clarens, A. F. 2012. Comparison of algae cultivation methods for bioenergy production using a combined life cycle assessment and life cycle costing approach. Bioresource Technology **126:**298-306.

Sahu, A. K., Siljudalen, J., Trydal, T., and Rusten, B. 2013. Utilisation of wastewater nutrients for microalgae growth for anaerobic co-digestion. Journal of Environmental Management **122:**113-120.

Saxena, P., Jawale, S., and Joshipura, M. H. 2013. A Review on Prediction of Properties of Biodiesel and Blends of Biodiesel. Procedia Engineering **51:**395-402.

Schreiber, U., Klughammer, C., and Kolbowski, J. 2012. Assessment of wavelength-dependent parameters of photosynthetic electron transport with a new type of multi-color PAM chlorophyll fluorometer. Photosynthesis research **113:**127-144.

Shao, Y., Pei, H., Hu, W., Chanway, C. P., Meng, P., Ji, Y. and Li, Z. 2014. Bioaugmentation in lab scale constructed wetland microcosms for treating polluted river water and domestic wastewater in northern China. International Biodeterioration & Biodegradation. **95:**151-159.

Singh, A., Nigam, P. S., and Murphy, J. D. 2011. Renewable fuels from algae: An answer to debatable land based fuels. Bioresource Technology **102:**10-16.

Singh, B., Guldhe, A., Rawat, I., and Bux, F. 2014. Towards a sustainable approach for development of biodiesel from plant and microalgae. Renewable and Sustainable Energy Reviews **29:**216-245.

Singh, S. K., Bansal, A., Jha, M. K., and Dey, A. 2012. An integrated approach to remove Cr(VI) using immobilized *Chlorella minutissima* grown in nutrient rich sewage wastewater. Bioresource Technology **104:**257–265.

Singh, S. K., Bansal, A., Jha, M. K., and Jain, R. 2013. Production of biodiesel from wastewater grown *Chlorella minutissima*. Indian Journal of Chemical Technology **20:**341-345.

Soares, B. M., Vieira, A. A., Lemoes, J. S., Santos, C. M., Mesko, M. F., Primel, E. G., Montes D'Oca, M. G., and Duarte, F. A. 2012. Investigation of major and trace element distribution in the extraction-transesterification process of fatty acid methyl esters from microalgae *Chlorella* sp. Bioresource Technology **110:**730–734.

Song, M., Pei, H., Hu, W., and Ma, G. 2013. Evaluation of the potential of 10 microalgal strains for biodiesel production. Bioresource Technology **141:**245-251.

Subashchandrabose, S. R., Ramakrishnan, B., Megharaj, M., Venkateswarlu, K., and Naidu, R. 2011. Consortia of cyanobacteria/microalgae and bacteria: biotechnological potential. Biotechnology Advances **29:**896-907.

Suberu, M. Y., Mustafa, M. W., Bashir, N., Muhamad, N. A., and Mokhtar, A. S. 2013. Power sector renewable energy integration for expanding access to electricity in sub-Saharan Africa. Renewable and Sustainable Energy Reviews **25:**630-642.

Van Wagenen, J., Miller, T. W., Hobbs, S., Hook, P., Crowe, B., and Huesemann, M. 2012. Effects of Light and Temperature on Fatty Acid Production in *Nannochloropsis salina*. Energies **5:**731-740.

VanGerpen, J., Shanks, B., Pruszko, R., Clements, D., and Knothe, G. 2004. Biodiesel Production Technology NREL/SR-510-36244. National Renewable Energy Laboratory. U.S. Department of Commerce.

Velasquez-Orta, S. B., Lee, J. G. M., and Harvey, A. P. 2013. Evaluation of FAME production from wet marine and freshwater microalgae by *in situ* transesterification. Biochemical Engineering Journal **76:**83-89.

Viswanath, B., Mutanda, T., White, S., and Bux, F. 2010. The Microalgae: A Future Source of Biodiesel. Dynamic Biochemistry, Process Biotechnology and Molecular Biology **4:**37-47.

Wang, H., Zhang, W., Chen, L., Wang, J., and Liu, T. 2013b. The contamination and control of biological pollutants in mass cultivation of microalgae. Bioresource Technology **128:**745-750.

Wang, L., Min, M., Li, Y., Chen, P., Chen, Y., Liu, Y., Wang, Y., and Ruan, R. 2009a. Cultivation of Green Algae *Chlorella sp.* in Different Wastewaters from Municipal Wastewater Treatment Plant. Applied Biochemistry and Biotechnology **162:**1174-1186.

Wang, T., Yabar, H., and Higano, Y. 2013a. Perspective assessment of algae-based biofuel production using recycled nutrient sources: the case of Japan. Bioresource Technology **128:**688-696.

Wang, Z. T., Ullrich, N., Joo, S., Waffenschmidt, S., and Goodenough, U. 2009b. Algal Lipid Bodies: Stress Induction, Purification, and Biochemical Characterization in Wild-Type and Starchless *Chlamydomonas reinhardtii*. Eukaryotic Cell **8:**1856-1868.

Watanabe, M. M., Kawachi, M., Hiroki, M., and Kasai, F. 2000. NIES-Collection list of strains, microalgae and protozoa. Microbial Culture Collections 6th ed. National Institute for Environmental Studies. , Tsukuba, Japan.

Wessels, K. J., Colgan, M. S., Erasmus, B. F. N., Asner, G. P., Twine, W. C., Mathieu, R., van Aardt, J. A. N., Fisher, J. T., and Smit, I. P. J. 2013. Unsustainable fuelwood extraction from South African savannas. Environmental Research Letters **8:**1-10.

White, S., Anandraj, A., and Bux, F. 2011. PAM fluorometry as a tool to assess microalgal nutrient stress and monitor cellular neutral lipids. Bioresource Technology **102:**1675-1682.

Widjaja, A., Chien, C.-C., and Ju, Y.-H. 2009. Study of increasing lipid production from fresh water microalgae *Chlorella vulgaris*. Journal of the Taiwan Institute of Chemical Engineers **40:**13-20.

Wijanarko, A. 2011. Effect of the Presence of Subtituted Urea and also Ammonia as Nitrogen Source in Cultivied Medium on *Chlorella*'s Lipid Content, p. 273-282. *In* S. S. Shaukat (ed.), Progress in Biomass and Bioenergy Production. InTech.

Wu, Y-H., Hu, H-Y., Yu, Y., Zhang, T-Y., Zhu, S-F., Zhuang, L-L., Zhang, X., and Lu, Y. 2014. Microalgal species for sustainable biomass/lipid production using

wastewater as resource: A review. Renewable and Sustainable Energy Reviews **33:**675-688.

Wu, L. F., Chen, P. C., Huang, A. P. and Lee, C. M. 2012. The feasibility of biodiesel production by microalgae using industrial wastewater. Bioresource Technology. **113:** 14-18.

Xin, L., Hong-ying, H., and Jia, Y. 2010a. Lipid accumulation and nutrient removal properties of a newly isolated freshwater microalga, *Scenedesmus* sp. LX1, growing in secondary effluent. New Biotechnology **27:**59-63.

Xin, L., Hong-ying, H., Ke, G., and Ying-xue, S. 2010b. Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga *Scenedesmus* sp. Bioresource Technology **101:**5494-5500.

Yuan, X., Kumar, A., Sahu, A. K., and Ergas, S. J. 2011. Impact of ammonia concentration on *Spirulina platensis* growth in an airlift photobioreactor. Bioresource Technology **102:**3234-3239.

Yuan, Z., Pratt, S., and Batstone, D. J. 2012. Phosphorus recovery from wastewater through microbial processes. Current Opinions in Biotechnology **23:**878-883.

Zamyadi, A., Fan, Y., Daly, R. I., and Prevost, M. 2013. Chlorination of *Microcystis aeruginosa*: toxin release and oxidation, cellular chlorine demand and disinfection by-products formation. Water Research **47:**1080-1090.

Zhang, Q. L., Liu, Y., Ai, G. M., Miao, L. L., Zheng, H. Y., and Liu, Z. P. 2012. The characteristics of a novel heterotrophic nitrification-aerobic denitrification bacterium, *Bacillus methylotrophicus* strain L7. Bioresource Technology **108:**35-44.

Zheng, Y., Li, T., Yu, X., Bates, P. D., Dong, T., and Chen, S. 2013. High-density fed-batch culture of a thermotolerant microalga *Chlorella sorokiniana* for biofuel production. Applied Energy **108:**281-287.

Zhou, W., Chen, P., Min, M., Ma, X., Wang, J., Griffith, R., Hussain, F., Peng, P., Xie, Q., Li, Y., Shi, J., Meng, J., and Ruan, R. 2014. Environment-enhancing algal biofuel production using wastewaters. Renewable and Sustainable Energy Reviews **36:**256–269.

Zhou, X., Ge, H., Xia, L., Zhang, D. and Hu, C. 2013. Evaluation of oil-producing algae as potential biodiesel feedstock. Bioresource Technology **134:**24–29.

Zhou, W., Li, Y., Min, M., Hu, B., Chen, P., and Ruan, R. 2011. Local bioprospecting for high-lipid producing microalgal strains to be grown on concentrated municipal wastewater for biofuel production. Bioresource Technology **102:**6909-6919.

Zhu, L. 2015. Biorefinery as a promising approach to promote microalgae industry: An innovative framework. Renewable and Sustainable Energy Reviews **41:**1376–1384.

Zuka, Z. McConnell, B. Farag, I. 2012. Comparison of Freshwater and Wastewater Medium for Microalgae Growth and Oil Production. Journal of American Science **8:(**2)392-398.

Appendix A: Ammonia Reagent Preparation

Wastewater samples were first filtered through a 0.45 µ filter paper. 3 mL samples were put into Gallery cuvettes. The instrument was calibrated before samples were analyzed. Upon sample entry, 20 µL of NH_3 Reagent 1 was added to 120 µL of sample. Samples were incubated for 18 s. Following this, 20 µL of NH_3 Reagent 2 was added to the sample and incubated for 600 s. Samples were read at a wavelength of 660 nm.

Principle

Ammonia reacts with hypochlorite ions generated by the alkaline hydrolysis of sodium dichloroisocyanurate to form monochloramine. This reacts with salicylate ions in the presence of sodium nitroprusside at around pH 12.6 to form a blue compound. The absorbance of this compound is measured spectrophotometrically at wavelength 660 nm and is related to the NH_3 concentration by means of a calibration curve.

Interference

Magnesium forming a precipitate of Mg hydroxide at high pH values (> 12). The trisodium citrate is used to prevent this interference and the method should tolerate Mg at concentrations normally found in most non-saline waters.

Sodium Salicylate Solution (Reagent 1)

65 g of Sodium Salicylate and 65 g of tri-Sodium Citrate was dissolved in 400 mL NH_3 free deionised water, pH was adjusted to 8.0 with 0.4 % Nitric acid. 0.49 g of Sodium Nitroprusside was dissolved in 10 mL of free deionised water. The solution was made up to 500 mL with NH_3 free deionised water. This reagent was stored at 8 °C and had a shelf life of 1 month.

D.I.C Solution (Reagent 2)

16 g of sodium hydroxide was dissolved in 250 mL NH_3 free deionised water. It was then cooled. 1.0 g of Sodium Dichloroisocyanurate was dissolved and made up to 500 mL with NH_3 free deionised water. This reagent was stored at 8 °C and had a shelf life of 1 month.

Ammonia Standard Solution – 1000mgL^{-1} as N

3.819 g of dried NH_4 chloride was dissolved in 1000 mL of NH_3 free water. This solution was stored between 2 – 8 °C and was stable for 1 month.

Appendix B: Nitrite Reagent Preparation

Wastewater samples were first filtered through a 0.45 µ filter paper. 3 mL samples were put into Gallery cuvettes. The instrument was calibrated before samples were analyzed. Samples were first incubated for 18 s. 20 µL of TON Reagent 3 was added to the sample and incubated for 360 s. Samples were read at a wavelength of 540 nm.

Principle

Diazotization of sulphanilamide by nitrite in the presence of Phosphoric acid, at 1.9 pH and the subsequent formation of an azo dye with N-1-naphthylethylenediamine (NEDD). The absorbance of this compound is measured spectrophotometrically at 520 nm and is related to the nitrite by means of a calibration curve.

Interference

Oxidising agents, amines, chloramines, thiosulphate, hexametaphosphate, alkalies and ferric iron may cause interferences.

Colour Reagent

50 mL of concentrated Phosphoric acid was carefully added to 500 mL of distilled water. 5 g of sulphanilamide was added and dissolved completely before adding 0.25 g N-(1-naphthyl)-ethylenediaminedihydrochloride. It was then diluted to 1000 mL with distilled water and stored in an amber bottle between 2 - 8 °C. This reagent was stable for 1 month.

Nitrite Standard Solution-100 mgL^{-1}

0.493 g of dried sodium nitrite was dissolved in 1000 mL distilled water.

Appendix C: Total Oxidised Nitrogen Reagent Preparation

Wastewater samples were first filtered through a 0.45 µ filter paper. 3 mL samples were put into Gallery cuvettes. The instrument was first calibrated, and then read. Upon sample entry, 50 µL of TON Reagent 1 was added to 120 µL of sample. Samples were incubated for 240 s. Following this, 50 µL of TON Reagent 2 was added to the sample and incubated for 420 s. Thereafter, 30 µL of TON Reagent 3 was added to the sample and incubated for 300 s. Samples were read at a wavelength of 660 nm.

Principle

Nitrate is reduced to NO_2 by hydrazine under alkaline conditions. The total NO_2 ions are then reacted with sulphanilamide and N-1-naphthylethylenediamine dihydrochloride under acidic conditions to form a pink azo-dye. The absorbance is measured at 540 nm and is related to the total oxidised N concentration by means of a calibration curve. Nitrate was calculated by subtracting NO_2 from total oxidised N.

Interference

No interferences were identified.

Sodium Hydroxide (Reagent 1)

0.8 g sodium hydroxide was dissolved in 100 mL of distilled water. This solution was stable for 1 day.

Reductant (Reagent 2)

0.325 g of hydrazine sulphate was dissolved in 400 mL distilled water. 0.75 mL of stock Cu sulphate solution and 5 mL of zinc sulphate was added and made up to 500 mL with distilled water. This solution was stable for 1 month.

Stock solutions required for reductant

Copper sulphate solution

0.78 g Cu sulphate was dissolved in 200 mL of distilled water. This solution was stable for 1 month.

Zinc sulphate solution

9.0 g Zn sulphate was dissolved in 200 mL distilled water. This solution was stable for 1 month.

Colour reagent (reagent 3)

50 mL of concentrated phosphoric acid was carefully added to 500 mL of distilled water. 5 g of sulphanilamide was added and dissolved completely before adding 0.25 g n-(1-naphthyl)-ethylenediaminedihydrochloride. This was diluted to 1000 mL with distilled water and stored in an amber bottle between 2 – 8 °C. This solution was stable for 1 month.

Total Oxidised Nitrogen standard solution

1.6306 g of dried KNO_3 was dissolved in a 1000 mL of distilled water. This was stored between 2 - 8 °C, this solution was stable for 1 month.

Appendix D: Phosphate Reagent Preparation

Principle

Wastewater samples were first filtered through a 0.45 µ filter paper. 3 mL samples were put into Gallery cuvettes. The instrument was calibrated before samples were analyzed. Samples were first incubated for 18 s. 14 µL of Reagent 1 was added to 120 µL of sample and incubated for 120 s. Following this, 6 µL of Reagent 2 was added to the sample and incubated for 540 s. Samples were read at a wavelength of 880 nm.

Interference

Silica forms a pale blue complex which absorbs at 880 nm. This interference is insignificant as to produce a positive 1 mgL^{-1} error in orthophosphate would require a silica concentration of approximately 4000 mgL^{-1}. The determination is sensitive to variations in acid concentrations, the higher the acidity the lower the sensitivity.

Stock Solutions

Antimony Potassium Tartrate solution (Reagent 1)

0.3 g of antimony K tartrate was dissolved in 50 mL distilled water and diluted to 100 mL. This reagent was stored in an amber bottle between 2 – 8 °C**.**

Ammonium Molybdate (Reagent 2)

4.0 g of NH_4 molybdate was dissolved in 100 mL distilled water. The reagent was stored in a plastic container.

Dilute Sulphuric acid (Reagent 3)

140 mL of concentrated H_2SO_4 was very slowly added to 1000 mL distilled water.

Ascorbic Acid Solution (Reagent 4)

1.76 g ascorbic acid was dissolved in 100mL distilled water. This solution was stable for 5 days when refrigerated between 2 – 8 °C.

Working Solutions

Reagent 1

75 mL of stock NH_4 Molybdate was added to 250 mL of dilute H_2SO_4. 25 mL of stock antimony K tartrate was added to this mixture.

Reagent 2

Stock Ascorbic acid solution.

28 mL of Reagent 1 was mixed with 12 mL of Reagent 2 for a single shot assay. This was prepared daily.

Appendix E: BG11 Medium

	1 litre stock solution gL^{-1}	Quantity (mLL^{-1})
$NaNO_3$	15.0	100.0
di-potassium hydrogen orthophosphate	4.0	10.0
Magnesium sulphate	7.5	10.0
Calcium chloride	3.6	10.0
Citric acid	0.06	10.0
Ferric ammonium citrate	0.06	10.0
EDTA (disodium salt)	0.01	10.0
Sodium carbonate	0.2	10.0
Trace metal solution		1.0

Add components to 1 L^{-1} distilled water. The pH should be 7.1 after sterilization

Trace metal solution:

Components	gL^{-1}
Boric acid	2.86
Manganese chloride	1.81
Zinc sulphate	0.222
Sodium molybedate	0.39
Copper sulphate	0.079
Cobalt nitrate	49.4 mg

Each element was dissolved in 1 L^{-1} distilled water, thereafter sterilized.

Appendix F: Raw Data for the Standard Curves

Raw data for the standard Curve of WW

Biomass (gL^{-1})	Optical Density (680 nm)
0.000000	0.000000
0.004800	0.151000
0.007400	0.298000
0.009800	0.364500
0.011700	0.467500
0.013500	0.493000
0.015200	0.603000
0.017800	0.819000

Raw data for the standard Curve of BG11

Biomass (gL^{-1})	Optical Density (680 nm)
0.0000	0.0000
0.0059	0.2770
0.0065	0.3035
0.0137	0.5055
0.0184	0.6540
0.0197	0.7070
0.0265	0.8865
0.0350	1.1310

Appendix G: Publication: The optimization of biomass and lipid yields of *Chlorella sorokiniana* when using wastewater supplemented with different nitrogen sources

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The optimization of biomass and lipid yields of *Chlorella sorokiniana* when using wastewater supplemented with different nitrogen sources

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HIGHLIGHTS

- Growth of *Chlorella* varies with addition of different nitrogen sources.
- 1.5 g L^{-1} urea produced 0.218 g L^{-1} biomass and 61.52% lipid by relative fluorescence.
- Fatty acids produced were predominantly 16 and 18 carbon chains in length.
- Urea is an effective nitrogen supplement for growth of *C. sorokiniana* in wastewater.

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ABSTRACT

The potential of nitrogen sources supplementing domestic wastewater for the cultivation of microalgae was assessed. Urea, potassium nitrate, sodium nitrate and ammonium nitrate were evaluated for their effect on cultivation and lipid production of *Chlorella sorokiniana*. Urea showed the highest biomass yield of 0.220 g L^{-1} and was selected for further experimentation. Urea concentrations (0–10 g L^{-1}) were assessed for their effect on growth and microalgal physiology using pulse amplitude modulated fluorometry. A concentration of 1.5 g L^{-1} urea produced 0.218 g L^{-1} biomass and 61.52% lipid by relative fluorescence. Physiological stress was evident by the decrease in relative Electron Transport Rate from 10.45 to 6.77 and quantum efficiency of photosystem II charge separation from 0.665 to 0.131. Gas chromatography analysis revealed that C16:0, C18:0, C18:1, C18:2 and C18:3 were the major fatty acids produced by *C. sorokiniana*. Urea proved to be an effective nitrogen supplement for cultivation of *C. sorokiniana* in wastewater.

1. Introduction

The development and production of biodiesel is becoming increasingly important as an alternative to fossil fuels (Lin and Lin, 2011). Microalgae have shown immense potential as a feedstock for biodiesel due to their ability to synthesize and accumulate high amounts of lipids (Rawat et al., 2013). Biodiesel production from microalgae is dependent on the microalgal growth rate and lipid productivity (Chen et al., 2011). Lipid accumulation in microalgae can be enhanced by changing cultivation conditions. Ammonia, urea and nitrate are often selected as the nitrogen (N) sources for the mass cultivation of microalgae (Hsieh and Wu, 2009; Kim et al., 2013; Lin and Lin, 2011). These N sources are frequently found in domestic wastewater. Microalgae have been previously cultivated in domestic wastewater streams, as it is a readily available and cost-effective substrate, and have proven to be efficient at removing and accumulating nutrients, trace metals and vitamins thereby providing additional tertiary treatment of wastewater (Rawat et al., 2011). Despite final effluent discharged from the wastewater treatment processes having lower concentrations of nutrients, it still proves to be an effective growth substrate (Lizzul et al., 2014). Supplementing wastewater with N fertilizer for optimal growth is too expensive which proves to be unfeasible thus giving the requirement for cheaper sources of N to be investigated (Borowitzka and Moheimani, 2013).

Nitrogen is crucial to the cultivation of microalgae. Organic N may be found in numerous biological substances which include peptides, proteins, enzymes, chlorophylls, energy transfer molecules (ADP, ATP), and genetic materials (RNA, DNA) (Cai et al., 2013). The most appropriate N source and concentration must be identified for each microalgal species as this is a vital factor which affects growth and lipid regulation (Chia et al., 2013; Hsieh and Wu, 2009). Nitrogen is available in several forms. The form of N that is supplied to the microalgae may affect the

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Appendix H: Publication: Bodipy staining, an alternative to the Nile Red fluorescence method for the evaluation of intracellular lipids in microalgae.

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BODIPY staining, an alternative to the Nile Red fluorescence method for the evaluation of intracellular lipids in microalgae

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ABSTRACT

In order to develop feasible production processes for microalgal biodiesel, the isolation of high neutral lipid producing microalgae is crucial. Since the established Nile Red (NR) method for detection of intracellular lipids has been successful only for some microalgae, a more broadly applicable detection method would be desirable. Therefore, BODIPY 505/515, a lipophilic bright green fluorescent dye was tested for detection of intracellular lipids in *Chlorella vulgaris*, *Dunaliella primolecta* and *Chaetoceros calcitrans*. An optimum concentration of 0.067 μg ml^{-1} was determined for lipid staining in the microalgae. Compared to NR, BODIPY 505/515 was more effective in staining microalgae and showed resistance to photobleaching, maintaining its fluorescence longer than 30 min.

1. Introduction

Microalgae have been recognized as a feedstock for biodiesel production because of their high photosynthetic efficiency, rapid growth rates and ability to accumulate significant amounts of lipids (Huang et al., 2010; Li et al., 2008; Mutanda et al., 2011). These organisms have great diversity in that more than 3000 species have been described; however this comprises only 10% of the total number of species that have been investigated (Sheehan et al., 1998). In order to develop a feasible commercial production process for biodiesel, microalgal strains that are high in biomass and lipid productivity need to be selected (Griffiths and Harrison, 2009). Therefore, large numbers of isolates have to be screened or genetic modification of already cultivated isolates has to be carried out. In each case, screening of large numbers of microalgae using a rapid, accurate and reliable method for detection and quantification of lipids produced is required (Elsey et al., 2007). The quantification of microalgal lipids is usually achieved by gravimetric means after solvent extraction (Bligh and Dyer, 1959; Lee et al., 1998) which requires about 3–4 days and needs a minimum of 10–15 mg wet weight of cells (Elsey et al., 2007). Gas chromatography–mass spectrometry (GC–MS) analysis of fatty acid methyl esters (FAME) requires specialized equipment. Therefore, detection of microalgal lipid is most often accomplished by staining techniques employing fluorescence microscopy (Chen et al., 2009; Cooksey et al., 1987; Elsey et al., 2007).

NR staining has been used for staining of lipid droplets in smooth muscle cells, macrophages, (Greenspan and Fowler, 1985), yeasts, fungi (Kimura, 2004), single-cell eukaryotes including microalgae and ciliated protozoan (Carman et al., 1991; Priscu et al., 1990). Cooksey et al. (1987) proposed using the dye as a vital stain for the detection of intracellular lipid by fluorescence microscopy, spectrofluorometry and flow cytometry. Huang et al. (2009), Elsey et al. (2007) and Lee et al. (1998) described semi-quantitative techniques for microalgal lipid staining. According to Chen et al. (2009) and Huang et al. (2009) the concentration of NR required for staining of difference microalgae varies considerably (0.01–100 μg/ml) between the different classes of algae. The major disadvantage using NR for microalgal staining is that the accuracy and precision of staining differs depending on strain of algae and is greatly affected by uneven dye uptake due to the polarity of solvent used to dissolve the dye (Chen et al., 2009). Uptake of NR is also affected by cell wall structure and composition, growth conditions and environment (Gao et al., 2008; Laurens and Wolfrum, 2010). NR dye is unable to efficiently penetrate the cell walls of microalgae with thick, rigid cell walls. Thus, NR lipid staining requires a combination of grinding, microwave and solvent treatments to assist penetration of the dye across the cell membrane and to decrease hydrophobicity. An improvement of the NR staining technique was carried out by Chen et al. (2011) who used dimethyl sulfoxide (DMSO) as stain carrier and microwave irradiation to increase staining efficiency. Nevertheless, the major drawbacks of NR are its limited photostability and interference chlorophyll (Laurens and Wolfrum, 2010).

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