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

Luveshan Ramanna

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.

Luveshan Ramanna

Date 14:04:15
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.

_________________________  14:04:15
Signature of Student       Date

_________________________  14:04:15
Signature of Supervisor    Date
Approval

I hereby approve the final submission of the following dissertation.

________________________
Prof. Faizal Bux
Supervisor
Doctoral Degree in Technology: Biotechnology
Durban University of Technology (DUT)

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.
Acknowledgements

Firstly, I would like to thank the omnipresent for giving me the strength to keep going when all I really wanted to do was give up. Thank you for giving me the ability, faith, health and patience to accomplish this goal.

I would like to express sheer gratitude to my family, who have been with me from day one. Thank you for pushing me to pursue my Masters Degree and putting up with me through my trying times. May your lives be ever fruitful. Mr. Deven Padayache, also deserves my whole hearted thanks for assisting and guiding me through this adventure we call life. Mr. Eddie Puckaree for encouraging me to reach this goal. Also, special thanks to Mr. Mervin Allimuthu.

Thanks to Prof. Faizal Bux for believing in my potential and giving me an opportunity to conduct research under his guidance and support. I believe your hard natured personality has moulded me into being a better scientist. I finally know “how to bark with the big dogs and not piss with the puppies”.

Appreciation expressed to Mr. Ismail Rawat for critically reading my research and all the input you have given me throughout this study. My sincere gratitude and appreciation goes to all my friends and colleagues at the Institute for Water and Wastewater Technology for your invaluable help and companionship while performing my research. Thanking you in no specific order Mrs. Trisha Mogany, Mr. Kriveshin Pillay, Dr. Abhishek Guldhe, Mr. Jashan Gokal, Mrs. Virthie Bhola, Dr. Sheena Kumari and Dr. Narendra Sahoo. I would also like to thank Mrs. Sarla Juglal for her words of wisdom in my times of trouble. Knowing your support was behind me has made this possible.

To the Durban University of Technology for funding and providing the facilities to make this study possible. And finally, to all those who have given me advice, showed interest, encouraged, or in some or other way contributed in the completion of my thesis, I give my sincere thanks.
Preface

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


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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<th>Full Form</th>
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<tr>
<td>Acyl Carrier Protein- ACP</td>
<td>Copper- Cu</td>
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Wastewater Treatment Plant- WWTP

Water footprint- WF

Zinc- Zn
List of Mathematical Symbols

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<td>( F'<em>{q}/F'</em>{m} )</td>
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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 (CO₂), 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 (SOₓ) 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 CO₂ 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 CO₂ 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 CO₂ 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).

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

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 microalgal 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 CO₂ 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,
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 by 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 (NH₃), nitrate (NO₃) or urea [CO(NH₂)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 NO₃, nitrite (NO₂), and NH₄ (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 (NH₄NO₃) may be used for microalgal growth. Its production has an embodied energy value of 67 MJ kg⁻¹ N. Also, fertilizer production will release CO₂. 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 NH₃ and NO₂ 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 microalgal 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 CO₂ (Chisti, 2013). Carbon, as CO₂, 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 CO₂ through the activity of the enzyme carbonic anhydrase (Cai et al., 2013a). The use of purchased CO₂ can contribute about 50 % of the total cost for biomass production (Chisti, 2007). Microalgal growth for biodiesel production will not be feasible unless the CO₂ 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 MgSO₄. Domestic WWs are often deficient in Mg. Salts such as MgNO₃, 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.

![Transesterification Reaction Diagram](image)

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$_2$SO$_4$) 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 °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 °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 80i 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 (OD₆₈₀) 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.**
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.

$$\mu = \frac{(ln_{N} - ln_{N_{0}})}{(t - t_{0})}$$

**Equation 3.3: Average Growth Rate (d⁻¹) for microalga.**
Where $\mu$ 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 °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 °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).](image-url)
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.

![pH Graph](image)

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.

![Graph showing total suspended solids in wastewater samples over time for three plants.

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.

![NH$_3$ concentration graph](image)

**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
NO₃ 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 NO₃ 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 plates. 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).

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

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.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Growth Rate ( (d^{-1}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-chlorinated</td>
<td>0.074</td>
</tr>
<tr>
<td>Post-chlorinated</td>
<td>0.111</td>
</tr>
<tr>
<td>BG 11</td>
<td>0.097</td>
</tr>
</tbody>
</table>
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 °C. All three plant effluents showed temperature drops of below 25 °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 °C- 30 °C (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 °C. 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 °C (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 °C and showed the highest growth rate of 1.60 d$^{-1}$ at 37 °C. 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, NH₃ is a severe problem. Although NH₃ is used as a source for N for microalgal growth, high concentrations of NH₃ (>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 (NH₃-N) has a more toxic form at high pH and a less toxic form at low pH, un-ionized NH₃ and ionized NH₄⁺, respectively. At higher pH values, N in the form NH₃ is frequently found in WW. The presence of un-ionized NH₃, the toxic form, increases as pH rises and decreases as pH falls which causes NH₃ 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 NH₃ 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 NH₃ peak in June. All three plants showed NH₃ 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 (α < 0.05) from May to August. These were the maximum concentrations obtained. Craigieburn WWTP showed a constant NH₃ 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 NH₃ 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$_3$Cl 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$_4^{3-}$. Organic P in WW’s will be hydrolyzed to PO$_4^{3-}$ 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 PO₄ for nutrition; therefore they compete for the available PO₄ (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 \textit{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 \textit{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 \textit{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 \textit{et al.}, 2013b). They may out-compete the target microalgal strain for essential nutrients (Pittman \textit{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 \textit{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\textsubscript{3} and NH\textsubscript{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 \textit{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

\textit{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\textsuperscript{-1}), sodium nitrate (NaNO\textsubscript{3}) (1.5 gL\textsuperscript{-1}), NH\textsubscript{4}NO\textsubscript{3} (1.5 gL\textsuperscript{-1}) and potassium nitrate (KNO\textsubscript{3}) (1.5 gL\textsuperscript{-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-difluoro-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 NaNO₃ showed a steady increase in growth up to day 9, thereafter leading to the decline or death phase, as opposed to CO(NH₂)₂, KNO₃ and NH₄NO₃ 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(NH₂)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(NH₂)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.

<table>
<thead>
<tr>
<th>N source</th>
<th>Growth Rate (d$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-0.014</td>
</tr>
<tr>
<td>NaNO$_3$</td>
<td>0.075</td>
</tr>
<tr>
<td>CO(NH$_2$)$_2$</td>
<td>0.116</td>
</tr>
<tr>
<td>KNO$_3$</td>
<td>0.102</td>
</tr>
<tr>
<td>NH$_4$NO$_3$</td>
<td>0.065</td>
</tr>
</tbody>
</table>

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$_4$NO$_3$, exhibited a trend. These supplemented cultures showed a slight increase and subsequent decrease after day 10. pH of the NH$_4$NO$_3$ supplemented culture decreased steadily after day 5. On day 21 the NH$_4$NO$_3$ 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, KNO₃ and NH₄NO₃ 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 NH₄NO₃ 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).](image-url)
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 NH₄NO₃ dissociates in solution to form NH₄ and NO₃. 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 NH₄NO₃ as a supplement source. This could be due to the fact that microalgal cells are unable to control passive diffusion of NH₃, which is in equilibrium with NH₄ 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 NH₄NO₃ 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 NH₄NO₃ 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 NH₄NO₃ 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$_4$NO$_3$ 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$_4$NO$_3$ 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$_4$NO$_3$ had lipid concentrations below 35 %. Potassium nitrate showed the lowest relative fluorescence per cell lipid of 23.26 %. A high concentration of NH$_4$NO$_3$ 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$_4$NO$_3$ (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$_4$NO$_3$ as an N source, 0.2 M (16 gL$^{-1}$) NH$_4$NO$_3$ 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 $\mu$ 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) \( (0.6 \text{ s at } 10\ 000 \mu\text{mol photons m}^{-2}\text{s}^{-1}) \) 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 \textit{et al.}, 2012, Schreiber \textit{et al.}, 2012). The quantum efficiency of PS II charge separation \( (F_v/F_m) \) was calculated as (Genty \textit{et al.}, 1989):

\[
F_v/F_m = (F_m - F_o)/F_m
\]

\textit{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 \textit{et al.}, 2011):

\[
rETR = F'_q/F'_m \times \text{PPFD}
\]

\textit{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 °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 °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$_2$SO$_4$ and methanol in 30:1 methanol to oil molar ratio at 60 °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(NH₂)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.

<table>
<thead>
<tr>
<th>Urea Concentration</th>
<th>Growth Rate (d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BG11</td>
<td>0.057</td>
</tr>
<tr>
<td>0 gL⁻¹</td>
<td>0.010</td>
</tr>
<tr>
<td>0.25 gL⁻¹</td>
<td>0.010</td>
</tr>
<tr>
<td>0.5 gL⁻¹</td>
<td>0.017</td>
</tr>
<tr>
<td>0.75 gL⁻¹</td>
<td>0.008</td>
</tr>
<tr>
<td>1 gL⁻¹</td>
<td>0.012</td>
</tr>
<tr>
<td>1.25 gL⁻¹</td>
<td>0.019</td>
</tr>
<tr>
<td>1.5 gL⁻¹</td>
<td>0.108</td>
</tr>
<tr>
<td>1.75 gL⁻¹</td>
<td>0.098</td>
</tr>
<tr>
<td>2 gL⁻¹</td>
<td>0.069</td>
</tr>
<tr>
<td>4 gL⁻¹</td>
<td>0.039</td>
</tr>
<tr>
<td>6 gL⁻¹</td>
<td>n/a</td>
</tr>
<tr>
<td>8 gL⁻¹</td>
<td>n/a</td>
</tr>
<tr>
<td>10 gL⁻¹</td>
<td>n/a</td>
</tr>
</tbody>
</table>

*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 (\(\leq 60\ %\) 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(NH₂)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).

(a)
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).

![Graph](image)

**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(NH₂)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(NH₂)₂ was 15.23 on day 6. In Figure 5.5a, the 1.25 gL⁻¹ CO(NH₂)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(NH₂)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(NH₂)₂ concentrations. In Figure 5.5b, 8 gL⁻¹ and 10 gL⁻¹ CO(NH₂)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(NH₂)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.

![Fatty acid profile graph](image)

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 hypersaline 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. quadricula 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\textsubscript{v}/F\textsubscript{m} values at all CO(NH\textsubscript{2})\textsubscript{2} concentrations declined considerably thereafter, with the PC; 1.5; 4 and 6 gL\textsuperscript{-1} showing a 34.00; 81.50; 98.50 and 93.68 % decline respectively. Changes in F\textsubscript{v}/F\textsubscript{m} provided essential information on the effects of the microalgal physiology under different CO(NH\textsubscript{2})\textsubscript{2} concentrations (Garrido et al., 2012, White et al., 2011). The PC showed a declining yet continuous F\textsubscript{v}/F\textsubscript{m} value up till day 30 (Figure 5.4a). Although the culture grown on 1.5 gL\textsuperscript{-1} CO(NH\textsubscript{2})\textsubscript{2} supplemented WW showed a high initial F\textsubscript{v}/F\textsubscript{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\textsubscript{v}/F\textsubscript{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\textsubscript{v}/F\textsubscript{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\textsuperscript{-1} and 10 gL\textsuperscript{-1} CO(NH\textsubscript{2})\textsubscript{2} supplemented cultures showed a F\textsubscript{v}/F\textsubscript{m} value of 0 at day 6. This was because the culture declined after 3 d as these CO(NH\textsubscript{2})\textsubscript{2} concentrations proved too toxic for the microalgae to handle. Lower CO(NH\textsubscript{2})\textsubscript{2} concentrations (Figure 5.4a) show higher F\textsubscript{v}/F\textsubscript{m} values whilst higher CO(NH\textsubscript{2})\textsubscript{2} concentrations had lower F\textsubscript{v}/F\textsubscript{m} values, attributed to NH\textsubscript{4} toxicity.

Lower F\textsubscript{v}/F\textsubscript{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\textsuperscript{-1} CO(NH\textsubscript{2})\textsubscript{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).
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 (α < 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 NOx 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 KNO₃, NaNO₃ and NH₄NO₃.

- 1.5 gL⁻¹ CO(NH₂)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


Department of Water Affairs and Forestry. 1999.


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 NH₃ 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 NH₃ 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 NH₃ free deionised water. It was then cooled. 1.0 g of Sodium Dichloroisocyanurate was dissolved and made up to 500 mL with NH₃ 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 NH₄ chloride was dissolved in 1000 mL of NH₃ 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 NO₂ by hydrazine under alkaline conditions. The total NO₂ ions are then reacted with sulphanilamide and N-1-naphthylethlenediamine 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 NO₂ 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 KNO₃ 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 NH₄ 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 H₂SO₄ 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 NH₄ Molybdate was added to 250 mL of dilute H₂SO₄. 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

<table>
<thead>
<tr>
<th>Component</th>
<th>1 litre stock solution gL⁻¹</th>
<th>Quantity (mLL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO₃</td>
<td>15.0</td>
<td>100.0</td>
</tr>
<tr>
<td>di-potassium hydrogen orthophosphate</td>
<td>4.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>7.5</td>
<td>10.0</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>3.6</td>
<td>10.0</td>
</tr>
<tr>
<td>Citric acid</td>
<td>0.06</td>
<td>10.0</td>
</tr>
<tr>
<td>Ferric ammonium citrate</td>
<td>0.06</td>
<td>10.0</td>
</tr>
<tr>
<td>EDTA (disodium salt)</td>
<td>0.01</td>
<td>10.0</td>
</tr>
<tr>
<td>Sodium carbonate</td>
<td>0.2</td>
<td>10.0</td>
</tr>
<tr>
<td>Trace metal solution</td>
<td></td>
<td>1.0</td>
</tr>
</tbody>
</table>

Add components to 1 L⁻¹ distilled water. The pH should be 7.1 after sterilization.

**Trace metal solution:**

<table>
<thead>
<tr>
<th>Components</th>
<th>gL⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boric acid</td>
<td>2.86</td>
</tr>
<tr>
<td>Manganese chloride</td>
<td>1.81</td>
</tr>
<tr>
<td>Zinc sulphate</td>
<td>0.222</td>
</tr>
<tr>
<td>Sodium molybdate</td>
<td>0.39</td>
</tr>
<tr>
<td>Copper sulphate</td>
<td>0.079</td>
</tr>
<tr>
<td>Cobalt nitrate</td>
<td>49.4 mg</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Biomass (gL(^{-1}))</th>
<th>Optical Density (680 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000000</td>
<td>0.000000</td>
</tr>
<tr>
<td>0.004800</td>
<td>0.151000</td>
</tr>
<tr>
<td>0.007400</td>
<td>0.298000</td>
</tr>
<tr>
<td>0.009800</td>
<td>0.364500</td>
</tr>
<tr>
<td>0.011700</td>
<td>0.467500</td>
</tr>
<tr>
<td>0.013500</td>
<td>0.493000</td>
</tr>
<tr>
<td>0.015200</td>
<td>0.603000</td>
</tr>
<tr>
<td>0.017800</td>
<td>0.819000</td>
</tr>
</tbody>
</table>
## Raw data for the standard Curve of BG11

<table>
<thead>
<tr>
<th>Biomass (gL(^{-1}))</th>
<th>Optical Density (680 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0000</td>
<td>0.0000</td>
</tr>
<tr>
<td>0.0059</td>
<td>0.2770</td>
</tr>
<tr>
<td>0.0065</td>
<td>0.3035</td>
</tr>
<tr>
<td>0.0137</td>
<td>0.5055</td>
</tr>
<tr>
<td>0.0184</td>
<td>0.6540</td>
</tr>
<tr>
<td>0.0197</td>
<td>0.7070</td>
</tr>
<tr>
<td>0.0265</td>
<td>0.8865</td>
</tr>
<tr>
<td>0.0350</td>
<td>1.1310</td>
</tr>
</tbody>
</table>
Appendix G: Publication: The optimization of biomass and lipid yields of \textit{Chlorella sorokiniana} when using wastewater supplemented with different nitrogen sources

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**HIGHLIGHTS**

- Growth of \textit{Chlorella} varies with addition of different nitrogen sources.
- \(1.5 \text{ g L}^{-1}\) urea produced 0.218 g L\(^{-1}\) biomass and 51.52\% 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 \textit{C. sorokiniana} in wastewater.

**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 \textit{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 \(1.5 \text{ g L}^{-1}\) 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 0.631. Gas chromatography analysis revealed that C16:0, C18:0, C18:1, C18:2 and C18:3 were the major fatty acids produced by \textit{C. sorokiniana}. Urea proved to be an effective nitrogen supplement for cultivation of \textit{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
Appendix H: Publication: Bodipy staining, an alternative to the Nile Red fluorescence method for the evaluation of intracellular lipids in microalgae.

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 microalgae 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 primaevia and Chlorella calcicola. 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.

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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; Cooke 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 protozoa (Carman et al., 1991; Pitscu et al., 1990). Cooke 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 photo-stability and interference chlorophyll (Laurens and Wolfrum, 2010).