ABUNDANCE AND DIVERSITY OF MICROALGAE IN FRESHWATER EUTROPHIC SYSTEMS OF NAMIBIA AND OPTIMIZATION OF LIPID AND BIOMASS PRODUCTION IN *NANNOCHLOROPSIS LIMNETICA*

SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF

DOCTOR OF PHILOSOPHY IN SCIENCE (BIOLOGY)

OF

UNIVERSITY OF NAMIBIA

BY

CAROL H. GARUS-OAS

9975306

MARCH 2017

Main Supervisor: Dr. E. Julies

Co-Supervisor: Prof. Dr. C. Posten

ABSTRACT

Namibia is one of the driest countries in Sub-Saharan Africa, and man-made dams play an important role on salvaging water. These dams are however periodically frequented by microalgae blooms that pose devastating effects on the water quality, making water treatment processes troublesome and cost prohibitive. However, these unicellular organisms can be exploited in various ways especially when cultivated in controlled and closed systems under optimal conditions of light, temperature, pH and nutrient concentrations. Biomass can be optimized to produce proteins that can be a source of fertilizers, while lipids can be extracted to refine into various hydrocarbons such as biodiesel and bioethanol. It is therefore essential to study the dynamics of these microalgae and provide necessary guidelines on how to utilize them. The first objective of the current study was to assess the seasonal relative genera abundance, diversity, richness and eveness of microalgae in three fresh water eutrophic systems in central Namibia. The hypothesis was to seek whether there is no significant difference in the seasonal relative genera abundance of microalgae per dam in three freshwater eutrophic systems of Namibia namely, Goreangab dam, Von Bach dam and Swakoppoort dam. Microalgae were enumerated with the use of a haemocytometer and identified to genus level during the dry and the wet seasons. Environmental parameters such as turbidity, pH, temperature, dissolved oxygen, macronutrients (i.e. phosphates, nitrates and ammonia) and dam water volumes, were also measured during this time to assess how they are correlated to the relative genera abundance per dam. All statistical analyses were performed using the SPSS software package (IBM statistics Version 23) and differences in data were considered significant at $p < 0.05$. The Shannon-Weiner diversity index was applied to calculate the microalgae diversity per dam. Evenness Index (J') and Margalef's Index (d) was used to calculate the species evenness and species richness, respectively per dam. Results revealed that seasonal relative genera abundance was not normally distributed ($p = 0.000$) per dam. Von Bach dam seasonal relative genera abundance was statistically not significant ($p = 1.000$). Goreangab dam

seasonal relative genera abundance was statistically not significant (p = 0.652) and Swakoppoort dam relative genera abundance was statistically not significant (p = 0.444). Data presentation of relative genera abundance showed Chlorophyceae as the most dominant class followed by Cyanophycea for Swakoppoort and Von Bach dams for both seasons. Chlorophyceae was the most abundant microalgae class in Goreangab dam during the dry season, while Florideophyceae and Cyanophycea were the second most abundant classes during the wet season. *Microcystis* was the most abundant genus for Swakoppoort and Goreangab dams during dry and wet seasons. However, in Von Bach dam *Aulacoseira* was the most abundant microalgae. The Shannon-Weiner diversity index showed that Von Bach dam had the highest diversity of 3.21 followed by Goreangab dam (2.32) and the lowest diversity was found in Swakoppoort dam (2.13). Species evenness varied between 0.46 - 0.65 for Swakoppoort and Goreangab dams, but was higher for Von Bach dam (0.72 - 0.97). Species richness calculated with the Margaleff's index was around 1 for all the dams and throughout all the seasons, except for Swakoppoort dam during the dry season where it was 1.5. According to the statistical analysis performed, all of the environmental parameters measured for all the dams during the dry and wet seasons were not normally distributed (p = 0.000). Correlation tests showed that all the environmental parameters captured during the time for all the dams were not statistically significant ($p > 0.05$). According to the literature review, the higher relative abundance of the class Chlorophycea observed during the time of sampling for the current study was due to their higher growth rates. The higher relative abundance of the genera *Microcystis* was due to their physiological advantage (i.e. nitrogen fixing, surface to volume ratios, intracellular gas vesicles for buoyancy and slimy undulation to enable photomovement) over other microalgae identified during the time of sampling. *Microcystis* was prominent mostly in Goreangab dam which was characterized by optimum combination of environmental factors such temperature, pH levels and high macronutrients when compared to the other dams. The current study recommends assessment of microalgae diversity and abundance over a one year period with sampling at least every second week. It will be essential to also study other macro-

and micronutrients, trace elements, chlorophyll a along with their cumulative effects on microalgae composition. Furthermore, the study advises assessment of topography and mechanical extractions for water transfer on microalgae composition. Moreover, a variety of mesh sizes will constitute a more diversified pool of microalgae to study. The second objective of the study was to optimize the neutral intracellular lipid and biomass production in *Nannochloropsis limnetica* under variant nitrate concentrations in highly controlled conditions in lab-scaled bioreactors over duration of 14 days. The hypothesis was to seek whether there was no significant difference in growth rate, biomass and neutral intracellular lipid productivity of *Nannochloropsis limnetica* in "normal nitrate" (3.53 M), "nitrate replete" (7.06 M), "moderate nitrate deplete" (1.765 M), and "high nitrate deplete" (0.8825 M) cultivated under the same conditions of light, pH, temperature and CO_2 concentration. The parameters measured throughout this process include optical density to measure growth rates, gravimetrical measurements of bio dry mass/biomass (BDM), ion chromatography measurements for ions, pH as well as sterility tests. All statistical analyses were performed using the SPSS software package (IBM statistics Version 23) and differences in data were considered significant at $p < 0.05$. During the main-cultivation of *Nannochloropsis limnetica*, growth was observed in a characteristic pattern consisting of lag, exponential, stationary and declining phases. The growth rates across all of the Flasks during the main-cultivation were not normally distributed ($p = 0.012$). The statistical tests showed that they were statistically significant ($p = 0.001$). Bio dry mass increased linearly over time for all of the Flasks from 0 day^{-1} to the highest on 14 day^{-1} at the end of the experiment. Bio dry mass was normally distributed ($p = 0.055$). The statistical test showed that it was statistically not significant ($p = 0.939$). Bio dry mass and growth rates were moderate and positively correlated and statistically not significant (r_s (8) = 0.529, $p = 0.077$). Relative fluorescence data recorded for all the Flasks was the highest only on the first couple of days during the lag phase and decelerated towards the end of the cultivation period. Therefore, there was no lipid accumulation. The relative fluorescence data captured for neutral intracellular lipid analysis showed that during the cultivation process all of the

data were not normally distributed (p = 0.000). Statistical analysis showed it to be statistically significant (p = 0.112). Correlation tests performed between BDM and lipids was strong and negatively correlated and statistically significant (r_s (8) = -0.765, p = 0.004). The relationship between lipids and growth rates was moderate, negatively correlated and statistically not significant (r_s (8) = -0.501, p = 0.087). The highest growth rates were under "moderate nitrate deplete" at 6 $days^{-1}$, 11.32 hours at 16.11 OD. The highest BDM value (7780 mg/L) was also captured for "moderate nitrate deplete" at 14 $days^{-1}$, 19.47 hours at 60.27 mg/L nitrate. The highest relative fluorescence (neutral intracellular lipid content) was captured under "nitrate replete" at 2.387 % at 1 day^{-1}, 6.28 hours and 2550.439 mg/L nitrate. The main-cultivation addressed the hypotheses set out. The study was successful in that it exponentially optimized the biomass production of *Nannochloropsis limnetica* in each of the four Flasks during the main-cultivation. These results suggest elaborate research on the biomass composition for various outputs such as proteins, lipids, carbohydrates, pigments et cetera. It was discovered during the current study that the various nitrate concentrations did not optimize lipid productivity. Therefore, it is proposed that other macronutrients such as phosphate concentrations along with variables such as light intensity and CO_2 be manipulated for lipid optimization. Furthermore, in future studies, the Nile red method should be used in conjunction with other conventional gravimetric methods for lipid quantification and validation for the *N.limnetica*.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AA/ ARA	Aarachidonic acid
A.U./a.u.	Astronomical Unit
BDM	Bio dry Mass
DAAD	Deutscher Akademischer Austauschdienst
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulfoxide $(CH_3)_2SO$)
EU	European Union
FACT	Food, Agriculture, Conservation and Trade Act of 1990 (United States)
FAO	Food and Agriculture Organization (of the United Nations)
GHG	Greenhouse Gases
HABs	Harmful Algae Blooms
HRAOPs	High Rate Oxidative Ponds
IPCC	Intergovernmental Panel on Climate Change
KIT	Karlsruhe Institut of Technology
NGWRP	New Goreangab Water Reclamation Plant
NSFAF	Namibia Student Financial Assistance

	Fund
OGWRP	Old Goreangab Water Reclamation Plant
TAG	Triacylglyceride
PUFAs	Polyunsaturated Fatty Acids
SAG	Sammlung von Algenkulturen der Universitat Gottingen
SVBD	Sartorius Von Bach dam
VBD	Von Bach dam
SWKPD	Swakoppoort dam
UK	United Kingdom
UNAM	University of Namibia
UNDP	United Nations Development Program
UNFCCC	United Nations Framework Convention on Climate Change
WCED	World Banks' Sustainable Development
WINGOC	Windhoek Goreangab Operating Company

ACKNOWLEDGEMENTS

This has been by far the most trying time of my life plagued with incessant obstacles. Therefore, it is with the deepest gratitude that I thank those who without their help this would not be possible. First I would like to thank my grandparents (Hanna Garuses, Gisella Harases, Titus ǁGaroeb and Gideon Haraseb) and my parents, Hermann !Garus-oab and Constantia !Garus-oas for bringing me in this world and laying down the foundations for endurance and preservation during this very tumulus time of my life. Further thanks are extended to my Uncle Mr. Hermanus Kasper for his support and kind and encouraging words. Many thanks to my dear friend Christopher !Nowaseb for his patience, tolerance and understanding. Thank you to my sponsors Ministry of Education, NSFAF, DAAD, Atlantic Sea Products for funding the various facets of my research. Dr. Kandawa-Schulz for introducing me to Dr. Julies. Dr. Julies for believing in me enough to provide an opportunity at the University of Namibia to carry out my research along with narrowing down my ideas. Same token goes to Professor Clemens Posten for his opportunity at KIT to carry out my BioProcess Engineering part of my dissertation. Professor Mapaure and Dr. Kwembweya for helping with the stats-thank you very much! The technical staff at the University of Namibia, Department of Biological Sciences (Mr. Asser Katunahange, Ms. Mildred Johnson, Mrs. Mandene Morkel, Mr. Augustinus Mbangu and Ms. Kaveire Kaitjizemine) for your support during field work, chemical acquisition, especially to Mr. Mbangu for staying up late

during my isolation studies. Great thanks to the KIT administrative, technical staff and PhD students (Ms. Luisa Zickwolf, Mr. Christian Steinweg, Ms. Lena Von Riesen, Mr. Andreas Trautmann, Ms Innes Wagner, Dr. Meike Doessel, Dr. Rosa Rossello) as well as the undergraduate and postgraduate students, especially Mr. Lucas Marmiol and co for the endless laughter and support in the lab during those crazy nights. Ms. Innes Wagner, your tremendous support during those sleepless nights will never be forgotten. The Namwater team: Environmental (Mr. N. Du Plessis, Ms Elna Almirall), Hydrological (Mr. Andre Mostert) and GIS (Ms. N. Hatutale and Mr. A. Shinana) for providing needed input to substantiate my findings, the staff at the Von Bach dam Treatment facility for allowing me to carry out my sampling. City of Windhoek Gammams Water Care Works staff, especially Mrs. Immogen Carew and Mrs. Helvi Naluwe for your technical support during my analytical work. Prof. Omoregie, Dr. Hillka Ndjaula, Mr. Martin Dominicus and co, thank you for granting a place to reside during the partial drafting of this dissertation. Thank you to fellow PhD student Natascha Pogori, your encouraging words made the journey worthwhile-thank you!

DEDICATION

To my son Joseph Joshua Merci !Garus-oab and daughter Kenny Uibasen Kamilah !Garus-oas for being my beam of light in this very dark world.

DECLARATION

I, Carol Hermionee Garus-oas, hereby declare that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution of higher education.

……. ……..

Carol.H.Garus-oas-Date

CHAPTER 1

1. INTRODUCTION

1.1 Background

Sub-Saharan Africa where 80 % of the African population is located in the rural areas is the most affected by climate change and global warming (Change, 2007). The region is predicted to face catastrophic events such as reduced agricultural productivity, increased water insecurity, increased exposure to coastal flooding, increased risks to human health and drought. This is further exacerbated by the already existing variability in climate and irregularities in rainfall, especially in semi-arid and hyper-arid countries such as Namibia (Koch, 2004). This predicament should enhance encouragement of efforts to preserve the natural environment through the sustainable use of natural resources as advocated by the World Banks' Sustainable Development (WCED, 1987) regulation which stipulates the conservation and sustainable use of natural resources for the benefit of present and future generations.

The major causative factor of climate change and global warming is the combustion of fossil fuels (Ragauskas et al., 2006; Demirbas & Demirbras, 2007). Fossil fuels when combusted emit greenhouse gases (GHG) like carbon dioxide, nitrogen oxides, methane, sulfur dioxide and volatile organic compounds (Gavrilescu & Chisti, 2005). This combustion is responsible for 73 % of carbon dioxide emissions in the world

(Verma, Mehrotra, Shukla & Mishra, 2010). In addition, crude oil prices have increased astronomically in the past couple of years, coupled with dependence of countries on an increasingly unstable Middle East (Chen, Landsman-Roos, Naughton & Olenyik, 2008). This led the Intergovernmental Panel on Climate Change (Change, 2007) and the rest of the world to strategize and search for low-risk renewable energy sources that can reduce greenhouse gases and subsequently mitigate global warming (Ragauskas et al., 2006; Demirbas & Demirbas, 2007; Parry, 2007; Solomon, 2007; Chen et al., 2008; Peng & Zhou, 2014). Therefore, regulatory requirements were set to substitute fossil diesel fuel with biodiesel as the main alternative (Gouveia & Oliveira, 2009). Biofuel feedstock is distinguished in three generations. First generation feedstocks are food or oil crops like corn, rapeseed, and soy (Patil, Källqvist, Olsen, Vogt & Gislerød, 2007), second generation feedstocks are energy crops like switchgrass, miscanthus and willow (Uellendahl et al., 2008), and third generation feedstocks include microalgae with high biomass and lipid productivity (Chisti, 2008). Over the past decades in the developed world, systems to produce biodiesel and bioethanol from crop plants have been developed and are currently running as profitable businesses. In the EU biodiesel represents 82 % of the total biofuel production (Mata, Martins & Caetano, 2010).

Although contributing a miniscule (1 %) amount to the overall production of liquid fuels, this sector has come under serious international scrutiny and criticism. Criticisms include encroachment of biofuels feedstock production on valuable crop and virgin land, and the subsequent effect they have on food commodity prices. These issues have raised major concerns surrounding social benefits. This is portrayed in the 2008

Gallagher report to the UK House of Commons. The report recommended a slow down on biofuel production until adequate directives are set in place to address displacement effects of the crop feedstock (Gallagher, 2008). Hence feedstock plays a significant role in determining the economic feasibility of the entire process (Ahmad, Yasin, Derek & Lim, 2011).

Namibia has no oil refinery. It imports its entire petroleum products from South Africa. In addition, electricity is a scarce and almost unattainable commodity in Namibia. Schmidt (2009) and Von Oertzen (2012) reported that it has been over three decades since there has been any energy generation facility added to the Namibian national power grid resulting in the country's continued reliance on electricity imports from Southern Africa. Le Fol (2012) along with Von Oertzen (2012) further asserted that in 2010 alone approximately 60 % of Namibia's total electricity supply was generated through the Southern African Power Pool. This reliance on imports and lack of investment in own energy or alternative energy sources is not sustainable for Namibia as South Africa has its own deficiencies regarding energy supply (Inglesi, 2010). Locally most of the electricity is sourced from the Ruacana Power Plant (330 MW) which is powered by hydroelectric means (De Vita et al., 2006). Although environmentally friendly, this support of water catchment of the Kunene River in Angola (Tarr, 2007; Schmidt, 2009) is not sustainable in the long run and may be devastating for Namibia. Other sources of locally generated electricity are the coal-fired Van Eck power station (120 MW), diesel powered Anixas (22 MW) and Paratus (17

MW) which are all detrimental to the environment (Le Fol, 2012).

Several attempts have been made over the years to try and curb the energy sector from depending on foreign exports for fossil fuels by for example cultivation and subsequent biodiesel production from terrestrial plants such as *Jatropha curcus* (Jongschaap, Corré, Bindraban & Brandenburg, 2007). Worldwide this shrub is considered as the best option for sustainable alternative energy (Kumar, Chaube & Jain, 2012). Their oilseed content when compared to non-edible vegetable feedstock have the highest oil yields (i.e. making up 25 – 35 % of the oil content) (Akintayo, 2004; Achten et al., 2008; Srivastava & Narra, 2008; Koh & Ghazi, 2011; Makkar et al., 2011; Basili & Fontini, 2012; Singh, Vyas, Tambunan, Situmorang, Silip, Joelianingsih & Araki, 2012) and they are easily adaptable to a wide array of agro-climatic conditions (Openshaw, 2000; Divakara, Upadhyaya, Wani, & Gowda, 2010). Furthermore, their energy balance and affinity to greenhouse gases is positive (Basili & Fontini, 2012), they provide the cheapest biodiesel feedstock (Mofijur et al., 2012), have similar properties to that of petroleum-based fuels (Koh & Ghazi, 2011), and the carbon lost on the land can be repaired by replacing fossil fuels with *Jatropha*-based biodiesel (Rasmussen, Rasmussen & Bruun, 2012). However, in the Namibian context although seemingly a good idea at the time for energy decentralization, over time experience and research has proven its sustainability incapable of producing any useful results. This is because not only are these specific species exotic and, therefore, invasive by threatening the local biodiversity (Basha & Sujatha, 2007; Jongschaap et al., 2007; Peng & Zhou, 2014), they

also require a lot of land and are therefore, in direct competition for limited arable land (Jongschaap et al., 2007; Peng & Zhou, 2014). Moreover, they take four to five years before harvesting (Peng & Zhou, 2014), whilst requiring at least an optimal rainfall of 1000 to 1500 mm (FACT, 2007; Wan, Sreedevi, Rao & Dixin, 2007). Their lipid or oil extraction methods are also highly labour intensive, requiring up to 12 hours for producing one liter of oil (Jongschaap et al., 2007; Peng & Zhou, 2014). Ideally it would make sense to research and subsequently cultivate and harvest local indigenous species that form natural synergies with the available environment and microalgae provide this opportunity.

1.2 Importance of microalgae

Microalgae exists as primary producers in almost any ecosystem and are the fastest growing aquatic plants on earth, since they self-replicate within 24 hours (Yen et al., 2013). They require little to no land and nutrients for cultivation since they can be cultivated in seawater and waste water, assimilating readily available nutrients like phosphates, nitrates and silicates (Agarwal, 1988; Clarens, Resurreccion, White & Colosi, 2010). Thus, there is no competition with agricultural resources (Schlagermann Göttlicher, Dillschneider, Rosello-Sastre & Posten, 2012). Furthermore, microalgae are easy to cultivate (Li, Horsman, Wang, Wu & Lan, 2008b) and even though there is water demand due to reactor cooling in closed photo bioreactors or due to compensation of evaporation in open systems this process exhibits lower water consumption when compared to crops. Moreover, evaporation losses in open ponds are still one to two

orders of magnitude lower than those of conventional agriculture (Wijffels & Barbosa, 2010).

Microalgae are more flexible in their metabolism when compared to oil plants. They exhibit variation in the biochemical composition with regard to higher lipid, carbohydrate or protein accumulation in the biomass when cultivated under varying conditions (Tredici, 2010). During biosynthesis, many species of microalgae accumulate large amounts of lipids that are largely made up of triglycerides (TAGs) which are preferable sources for biodiesel production (Bowles, 2007) and fatty acids such as long-chain poly-unsaturated fatty acids (PUFAs) which are well known for their dietary importance (Bowles, 2007; Sousa, Gouveia, Batista, Raymundo & Bandarra, 2008). Microalgae have the potential to produce 25 – 220 times more triglycerides and/or fatty acids (Saka & Kusdiana, 2001; Barnwal & Sharma, 2005). Lipid productivity is defined as the mass of lipids produced per unit volume of the microalgae in one day, which relates to the lipid content of the biomass and the microalgal growth rate, otherwise referred to as dry biomass weight (dwt) in percentage (Chisti, 2007; Chen, 2011).

Depending on the microalgae strain and the culture conditions (pH, temperature, nutrients and light) these lipids can be between 20 and 40 % dwt. Chisti (2007) reported a lipid content of as much as 70 % dwt, while Hossain, Salleh, Boyce, Chowdhury & Naqiuddin (2008) recorded lipids composing 90 % dwt when compared to edible or non-edible oil seeds which are about 40 - 50 % dwt (Spolaore, Joannis-Cassan, Duran &

Isambert, 2006). If properly cultivated, microalgae have the potential to completely substitute diesel without competing with food and other supplies of agricultural products (Rajvanshi & Sharma, 2012). Therefore, microalgae to bioenergy solutions (i.e. biodiesel, biomethane, biohydrogen, CO_2-mitigation via CO_2 captured and sequestration) are currently under research and have been studied by several scientists (Xu, Miao & Wu, 2006; Chisti, 2007; Hankamer, Rupprecht, Mussgnug, Posten & Kruse, 2007; Huntley & Redalje, 2007; Li, Xu & Wu, 2007; Ono & Cuello, 2007).

1.3 Namibia and Microalgae

Namibia has two contrasting environments which are the coastal strip along the Atlantic Ocean, stretching for 1572 km and inland areas with supporting freshwater systems. The coastal waters are characterized by the Benguela Current's cold nutrient-rich up-welled waters supporting large quantities of plankton. In addition to sustaining vast populations of commercially exploitable fish and other marine organisms, various commercially inclined maricultural activities were introduced along the coast to exploit the nutrient-rich water since 1984. The aquaculture sector is a prevalent necessity to alleviate the pressure on the capture fishing industry, which is following the worldwide trend of decreasing returns and consolidation. This and Namibias' sub-tropical climate make microalgae cultivation and research a promising entity for oyster farmers and research institutes.

Sam Nujoma Marine and Coastal Resources Research Centre (SANUMARC) has over

the years harvested, cultivated and maintained several species of halophytic or marine microalgae (*Isochrysis* sp., *Pavlova lutheri, Chaetoceros calcitrans* and *Tetraselmis* sp.) for the use as feedstock for rotifers, euphaussids and bivalves (abalones and oysters). Other halophytic microalgae such as *Dunaliella* spp. and *Astermonas* spp. are harvested by SANUMARC and cultivated by National Marine Institute and Research (NatMirc) for the promise of glycerol production. Glycerol is seen as a promising biofuel, especially when sourced from halophytic microalgae herein omitting possible competition of arable land from any other resource. Harvey et al. (2012) harvested and characterized new strains of halophytic microalgae (*Dunaliella* sp.) from Namibian saline water for the feasibility of glycerol production through engineering models and process designs. The study found the most feasible scenario to be an integrated approach of multi product processes and, therefore, including β-carotene extraction in the value chain. Ultimately, the project suggested that the advancement of these technologies is only possible through involvement of and endorsements from various stakeholders such as government subsidies. Other interests in microalgae technologies in Namibia were industrial production of of marine *Nannochloropsis* sp. biomass around the world pitched by Necton to the NatMirc and Sanumarc; Feasibility of biodiesel/biogas and biofertilizer production from wastewater microalgae (Okahandja, Swakopmund and Walvisbay) by Kamol Biofuel Namibia in collaboration with the Municipality of Trelleborg, Sweden; Integrated algae ponds for wastewater treatments by Municipality of Swakopmund; Case study on glycerol production from *Dunaliella* sp. by GlycAL Namibia in collaboration with the Aquaculture Directorate of the

Ministry of Fisheries and Marine Resources. These projects have so far only reached the conception and feasibility phases.

Freshwater microalgae have found popularity due to their blooms in manmade dams meant for potable water. Namwater, the Namibian potable water distributor carries out monitoring programs and have identified various species of trouble causing microalgae. These include blue-green, prokaryotic microalgae such as *Anabeana, Cylindrospermopsis* and *Microcystis* known for blocking filters, causing bad taste and odour in treated water as well as producing toxins. Other microalgae identified are green eukaryotic microalgae such as *Pediastrum* known to cause bad taste and odour in treated water (N. du Plessis, personal communication, 2014). Sirunda & Mazvimavi (2014) studied the effects of water transfer from Swakoppoort and Omatako dams on the water quality of Von Bach dam. In their study, they found that although other factors such as geology of catchment and high evaporation constitute an increase in turbidity, the occurrence of blue-green microalgae notably, *Microcystis* and *Anabaena* are also contributing factors to high turbidity. This was mainly the case at Swakoppoort dam where the water was dominated by *Microcystis.*

The first step in developing a microalgae cultivation process is to choose the microalgae species (Pulz & Gross, 2004). Many microalgae species have been studied over the years for this purpose. Target species are mostly those that are indigenous and in abundance. This strategy is important since the same environmental parameters are applied when cultured in enclosed systems as well as avoiding the introduction of exotic

species that may cause ecological problems (Morweiser et al., 2010). The most sought after microalgae for biofuel and biomass production are from the Chlorophyceae class. These are usually species with known growth cycles and rapid growth rates. Examples include *Tetraselmis suecica*, *Spirulina platensis* for protein production*; Isochrysis galbana, Nannochloropsis oculata* prominent in aquaculture industry (i.e. oyster farming) (Rodolfi et al., 2009) as well as *Haematococcus pluvialis* and *Scenedesmus almeriensis* for metabolite production, synthesis of pigments and food additives (Grewe & Griehl, 2008; Sánchez et al., 2008). Currently world-wide, more than 50 000 species of microalgae are estimated to exist however, only 30 000 of these have been studied and analyzed as potential species for biodiesel production (Richmond, 2004; Rajvanshi & Sharma, 2012).

There is no published research on the identification and assessing the seasonal relative genera abundance, diversity, richness and eveness of microalgae in Namibian freshwater eutrophic systems. In this study three Namibian eutrophic systems (Von Bach, Swakoppoort and Gorengab dams) were selected and studied for the assessment of seasonal relative genera abundance, diversity, richness and eveness of microalgae. Microalgae in these eutrophic systems were sampled during the dry season (14 June 2013 and 4 June 2014) and the wet seasons (14 November 2013 and 28 February 2014). The present study therefore, fills that knowledge gap by investigating and subsequently determining the seasonal relative genera abundance, diversity, richness and eveness of microalgae in these three-fresh water eutrophic systems.

Additionally, this work attempted to increase the neutral intracellular lipid content and biomass production in a known non-motile spherical freshwater picoplankton (2 to 3 micrometers) *Nannochloropsis limnetica*, belonging to the class Eustigmatophyceae (Hibberd, 1981; Andersen, Brett, Potter & Sexton, 1998; Fawley et al., 2004; Fawley & Fawley, 2004; Fietz et al., 2005; Fawley & Fawley, 2007). The manipulation was carried out with variations in nitrogen concentrations in constant light, pH, temperature, and CO_2 supply.The aim was to evaluate the effect of various nitrogen concentrations in the medium on the neutral intracellular lipid and biomass production.

1.4 Statement of the Problem

The Namibian climate is one of the driest in Sub-Saharan Africa with 80 - 92% of the landmass defined as hyper-arid, semi-arid or arid (Koch, 2004; Lahnsteiner & Lempert, 2007). All the rivers and their tributaries in the interior of the country are therefore ephemeral, irregular and water flow is unreliable. Thus, the only way they can be salvaged and fully exploited is through harnessing them in impoundments such as dams and extracting them from groundwater and or aquifers. Additionally, water supply for irrigation purposes is supplemented by reclamation from sewage treatment works.

Windhoek, the capital city of Namibia is situated in the central part of the country and its main water supply is from the Von Bach dam which is fed via pipeline by the Omatako dam and Swakoppoort dam (SWKPD). SWKPD is the largest of the two dams and, therefore, the biggest supplier of water. A recent article by Meding (2016) found

that since 22 February 2016, the water level of Swakoppoort dam decreased from 14.8 to 14.5% that of Von Bach dam decreased from 21.8 to 21.5 % and the Omatako dam level decreased from 7.1 to 6.5 % of their present capacity. Furthermore, the same article stipulated that if the public does not find ways to preserve water, Swakoppoort dam and Von Bach dam will dry up by March 2017 and 3 October 2016, respectively. To add to this water shortage is the devastating effect microalgae blooms pose on the water quality of these dams. This has particularly been a problem over the years in summer whereby at one point pumping water supply from Swakoppoort dam to Von Bach dam (VBD) had to be stopped (Lehmann, 2010).

Microalgae blooms in these dams are due to multiple factors, especially nutrient inflows that have been documented by various scientists over the years (Lehman, 2010; Shinana, 2011). For example, during the rainy season effluents (storm water and rivers) filled with excessive nutrients (mainly nitrates and phosphates) from surrounding waste water treatment facilities, mines, farms and urban areas flow into these dams. This along with little to no mixing or turbulence in the water columns and Namibia's sub-tropical climate provides a good environment that result in microalgae blooms. Many of these blooms are non-toxic, however some blue-green algae species blooms are toxic. During certain stages in their life cycle many of these blue-green microalgae produce an array of toxins from simple ammonia to physiologically active polypeptides and polysaccharides. These toxins are proven to have negative impacts on animals and humans (Annadoter, Cronberg & Jönsson, 2000). Furthermore, the cells of these microalgae contain vesicles inside vacuoles that inflate with gas, thereby regulating

their buoyancy in response to environmental conditions (Sirunda & Mazvimavi, 2014). This gives them an advantage over other microalgae since they can move up and down towards nutrient, temperature and light sufficient zones (i.e. below the euphotic zone) within the water column (Sirunda & Mazvimavi, 2014).

Due to lack of turbulence in the water column, their excessive buoyancy and unreliability to act as fodder to other aquatic organisms (i.e. due to their toxins) allows them to proliferate and form thick layers of scum on the water surface (Steinberg & Hartmann, 1988; Reynolds & Walsby, 2008; Sirunda & Mazvimavi, 2014). This scum blocks sunlight for other photosynthetic organisms (i.e. other microalgae) resulting in mortalities and decomposition by benthic bacteria that in turn deplete oxygen in the water columns. This is fatal for fish due to asphyxiation. Ultimately, these massive losses of life lead to eutrophication or water stagnation. Furthermore, this phenomenon is troublesome and cost prohibitive for water treatment processes by: (a) blockage or clogging of filters and pumps, resulting in the reduction of the carrying capacity of pipelines and canals; (b) odours and bitter taste in treated water; (c) reduced oxygen levels in the water, affecting aquatic biota; (d) increase in the concentration of dissolved organic carbon, iron, ammonia and manganese as well as (e) high turbidity in the water making the treatment of bacteria ineffective (McKendrick & Williams, 1968; Reynolds & Walsby, 2008; Sirunda & Mazvimavi, 2014).

This study addresses the problem of microalgae blooms by studying the types of microalgae that dominate under different environmental conditions in three eutrophic

systems namely, Von Bach, Swakoppoort and Goreangab dams. It is a crucial first step in Namibia to taxonomically identify and assess seasonal relative genera abundance, diversity, richness and eveness of microalgae in these freshwater systems. This will improve understanding of the effects of microalgae blooms on water quality which contributes to the increasing water shortage crisis in Namibia.

The obvious and best way of dealing with the problem of microalgae blooms in freshwater systems is to promote better environmental management of river systems and prevent pollution. However, for eutrophic freshwater systems with uncontrolled pollution and river systems that are difficult to manage, another possible way of dealing with the problem is to turn microalgae blooms into a resource by extracting valuable products from certain microalgae species. This possibility has never been investigated in Namibia and, therefore, this study serves as an initial exploration of this idea. To investigate the feasibility of using freshwater microalgae eventually for valuable product acquisition, this study used the freshwater picoplankton species *Nannochloropsis limnetica* to manipulate its neutral intracellular lipid and biomass productivity.

In biodiesel production, one of the most important criteria is high lipid productivity, which equals the mass of lipids produced per unit volume of the microalgae per day. Lipid production depends on microalgae growth rate and the lipid content of the biomass (Chisti, 2007). Therefore, potential microalgae species for industrial applications are not only selected because of a large amount of fatty acids,

carbohydrates and proteins, but also because of their high biomass productivity which allows for high product productivity or output (Rodolfi et al., 2009; Tabah, 2011).

Nannochloropsis species were considered for this study not only because of the common habitats (Fawley, Fawley & Owen 2005) they share with the microalgae species, but also because this genus is known to have multifarious applications in aquaculture and biotechnology (Watanabe, Kitajima & Fujita, 1983; Rodolfi et al., 2009) due to their rapid growth rates and high lipid content of polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (Volkman, Jeffrey, Nichols, Rogers & Garland, 1989; Sukenik, 1999) in highly controlled conditions in lab-scaled bioreactors. Due to this, their potential and suitability for outdoor cultivation on industrial scale is prominent (Borowitzka, 2016). Several of these species are successfully cultivated at large scale using natural sunlight by companies such as Solix Biofuels, Aurora Algae, Seambiotic and Proviron (Radakovits et al, 2012).

1.5 Research Objectives

The objectives of this research were to assess the seasonal genera abundance, diversity, richness and eveness of microalgae in three fresh water eutrophic systems (Goreangab, Von Bach and Swakoppoort dams) in central Namibia and to optimize the neutral intracellular lipid and biomass production in *Nannochloropsis limnetica* under variant nitrate concentrations.

The specific objectives of this research were:

1. Determination of seasonal (dry and wet seasons) relative genera abundance, diversity, richness and eveness of microalgae in three freshwater eutrophic systems of Namibia.
2. Assessing potential effects of various environmental factors (pH, temperature, dissolved oxygen, dam water volumes, turbidity, nitrate, phosphate and ammonia) on the seasonal relative genera abundance of microalgae in these three freshwater eutrophic systems of Namibia.
3. Comparison of growth rates of *Nannochloropsis limnetica* under the same light intensity, CO_2, pH and variant nitrate concentrations.
4. Optimization and comparison of neutral intracellular lipid and biomass productivity of *Nannochloropsis limnetica* cultured under variant nitrate concentrations.

1.6 Null Hypotheses

1. There is no significant difference in the seasonal relative genera abundance of microalgae in the three freshwater eutrophic systems of Namibia.
2. There is no correlation between environmental parameters (i.e. concentrations of nitrate, phosphate, ammonia, pH levels, dissolved oxygen, temperature, dam water volumes and turbidity) and the seasonal relative abundance of microalgae in the three freshwater eutrophic systems of Namibia.
3. There is no significant difference in growth rate, biomass and neutral intracellular lipid productivity of *Nannochloropsis limnetica* in normal nitrate (3.53 M), nitrate replete (7.06 M), moderate nitrate deplete (1.765 M), and high nitrate deplete

(0.8825 M) cultivated under the same conditions of light, pH, temperature and CO_2 concentration.

1.7 Significance of Study

The current study is a quintessential first step to introduce a catalogue of freshwater species of microalgae in the three selected eutrophic systems of Namibia. The study assesses the seasonal relative genera abundance of microalgae as well as their correlations to the environmental parameters. Additionally, the study guides through literature review on ways to harvest and explore these species for various outputs. At the same time the research on optimization of neutral intracellar lipid and biomass production of highly productive *Nannochloropsis limnetica* provides guidance on cultivation and maximization of outputs.

The results of this study will help propose suitable applications for especially nuisant blue-green microalgae. Namibia provides an ideal environment for microalgae project implementation due to the sustainable benefits such projects offer. The production systems do not compete with arable land or water resources. Such projects when realized will feed not only to the aquaculture industry but the fertilizers produced will provide the needed input to the agricultural industry that employs 70 % of the Namibian workforce. Furthermore, the production of hydrocarbons from microalgae lipids can help decentralize energy via electricity and heat to the rural community. This is evident in producing glycerol from halophytic microalgae. This carbon neutral fuel can be used directly since it does not require chemical or molecular modification and reformation

(Harvey et al., 2012). This is especially important since Namibia ratified the United Nations Framework Convention on Climate Change (UNFCCC) in 1995 and became legally obligated to adopt and implement policies and measures designed to mitigate the effects of climate change. Additionally, the Kyoto protocol which Namibia is also a party to, promotes the use of greener technologies to lower carbon emissions.

CHAPTER 2

2. LITERATURE REVIEW

Microalgae are a group of polyphyletic, photosynthetic ambiguous prokaryotic or eukaryotic unicellular and sometimes multicellular (Li et al., 2008a; Li, Horsman, Wu, Lan & Dubois-Calero, 2008b) microorganisms (in this study the term microalgae also refers to cyanobacteria). Prokaryotic microalgae are classified in two groups, namely the classes Cyanobacteria and Cyanophyceae, while eukaryotic microalgae occur in the classes Chlorophyta, Chrysophyta and Bacillariophyta. They are photo-, auto- or heterotrophic microorganisms present in all ecosystems (Richmond, 2004). Many species are known to switch from phototrophic to heterotrophic growth (Bowles, 2007). During the phototrophic phase they use light, nutrients and atmospheric CO_2 for energy and carbon metabolism, whilst in the heterotrophic phase in the absence of light, glucose and or other carbon sources are utilized (Ward & Singh, 2005; Bowles, 2007). Some are also known to carry out mixotrophic growth which is a combination of the two growth phases (Bowles, 2007).

2.1 Classification of microalgae

Microalgae classification is based on their life cycle, pigmentation, chemical nature of photosynthetic storage product, organization of photosynthetic membranes, cellular structure and other morphological features (Bowles, 2007; Kumar & Sharma, 2014). Their cellular sizes vary from a few micrometers to more than 100 μm (Ren, 2014).

Table 1 summarises the major classes i.e. diatoms, flagellated, chlorococcalean green microalgae, and filamentous blue-green microalgae along with their genera (Laven & Sorgeloos, 1996; Kumar & Sharma, 2014; Ren, 2014). Autotrophic microalgae produce polysaccharides, proteins, and lipids that are promising in applications of food, medicine, genetic engineering and biodiesel (Lavens & Sorgeloos, 1996; Ren, 2014). Currently more than 40 different species of microalgae are studied for multiple purposes such as fresh water environmental protection and biomass production content analysis (Ren, 2014).

In terms of abundance Bacillariophyta or diatoms represent the largest group of biomass producers on earth and, therefore, the most dominant life forms in phytoplankton and are estimated to be more than 100 000 (Bowles, 2007) (Table 1). Their cell walls contain polymerised silica, and they can accumulate lipids and chrysolaminarin (Bowles, 2007). The second most dominant group are the Chlorophyceae or green microalgae that are the most abundant in freshwater (Bowles, 2007) and their storage product is mostly starch, although lipds can also be produced (Bowles, 2007). For example the fresh water green microalgae *Haematococcus pluvialis* is a commercially important source of astaxanthin, *Chlorella vulgaris* provides an essential supplementary food product, and the halophilic *Dunaliella* species is a source of β-carotene (Bowles, 2007). The third most abundant microalgae are Cyanobacteria and Cyanophyceae or the blue-green microalgae found in a variety of habitats often forming toxic microalgal blooms (Bowles, 2007). The fourth most abundant microalgae are the Chrysophyceae or

golden microalgae which are similar to diatoms and are known to produce lipids and carbohydrates (Bowles, 2007).

In the order of commercial importance the most frequently applied microalgae is firstly Cyanophyceae (blue-green microalgae), secondly the Chlorophyceae (green microalgae), thirdly the Bacillariophyceae (including the diatoms) and fourthly Chrysophyceae (including golden microalgae) (Bowles, 2007).

Table 1: Classes of microalgae and storage material (Laven & Sorgeloos, 1996; Kumar & Sharma, 2014; Ren, 2014).

Class of Microalgae	**Genus**	**No. of species known**	**Storage material**	**Habitat**
Diatoms (Bacillariophyceae)	*Skeletonema, Thalassiosira, Phaeodactylum, Chaetoceros, Cylindrotheca, Bellerochea, Actinocyclus, Nitzchia, Cyclotella,*	100 000	Chyrsolaminarin; TAGs	Marine, fresh and brackish water
Green microalgae (Chlorophyceae)	*Dunaliella, Carteria*	8000	Starch; TAGs	Freshwater
Blue-green microalgae (Cyanophyceae)	*Spirulina*	2000	Starch; TAGs	Marine, fresh and brackish water
Golden microalgae (Chrysophyceae)	*Monochrysis (Pavlova)*	1000	TAGs; Carbohydrates	Freshwater
Haptophyceae / Prymnesiophyceae	*Isochrysis Pseudoisochrysis Dicrateria*	unknown	TAGs; Carbohydrates, Pigments (Fucoxanthin)	Marine and Freshwater
Prasinophyceae	*Tetraselmis (Platymonas), Pyramimonas, Micromonas,*	unknown	Carbohydrates	Marine and some Freshwater representatives
Cryptophyceae	*Chroomonas, Cryptomonas, Rhodomonas, Chlamydomonas*	unknown	Starch; TAGs	Marine, fresh and brackish water
Xanthophyceae	*Olisthodiscus*	600	Chrysolaminarin	Freshwater, marine and soil
Eustigmatophyceae	*Nannochloropsis*	12	PUFAs	Marine, freshwater and soil

2.2 Life cycle of microalgae

Microalgae form symbiotic relations with bacteria in various ecosystems (Cole, 1982; Gast, Sanders, & Caron, 2009) and live in harsh conditions due to their unicellular and multicellular structures (Richmond, 2004; Mata et al., 2010). They have the highest growth rate when compared to other plants (Andersen, 2005; Demirbas, 2008) in that they effectively self-replicate and complete an entire growth cycle within 24 hours (Sheehan, Dunahay, Benemann & Roessler, 1998) and have high photosynthetic rates in that they can efficiently convert solar energy into biomass (Bowles, 2007) due to their simple cellular structures (Lee, 1980). In particular, when submerged in water their large surface to volume ratio enables efficient access to water, CO_2 and uptake of large amounts of nutrients (Bowles, 2007; Kumar & Sharma, 2014).

Figure 1 (A & B) show the various growth phases (growth curve/life cycle) of microalgae. The lag, adaptation and induction phases represents the physiological adaptation of the organisms' cellular metabolism to growth, including increase in the levels of enzymes and metabolites involved in cell division and carbon fixation, under a combination of various cultivation conditions such as temperature, pH, nutrients, aeration, light intensity as well as the age of the inoculum. The condition and age of the inoculum has an effect on the length and/or duration of this phase and is proportional to the length of time the inoculum has been in phases 3 - 5 (Spencer, 1954). For example when the inoculum under the same cultivation conditions is retrieved from a healthy exponentially growing pre-culture into the Main-cultivation there is unlikely to be a lag phase (Fogg & Thake, 1987). However, a lag phase may occur if the inoculum is

transferred from one set of growth conditions to another. Therefore, the cells of the inoculum should already be in the exponential phase to ensure the shortest possible lag phase which can reduce the time required for up scaling (Becker, 2008).

The second phase which is the exponential growth phase or log phase shows the microalgae to be optimally adapted to the cultivation conditions hence maximum growth is observed (Figure 1 A). The growth rate also known as exponential growth rate or specific growth rate of microalgae dependent on the microalgae species is determined from this phase. It is defined as the measure of the increase in biomass or change in cellular mass over time. This phase is also an important indicator expressing the relative ecological success of a species or strain in adapting to its natural environment or the experimental environment imposed upon it (Fogg & Thake, 1987). According to Figure 1 (B) the exponential growth or specific growth rate is determined by: $K' = \frac{Ln\left(N_1/N_2\right)}{t_2 - t_1}$ where K' = exponential growth rate; N_1 and N_2 = biomass at time (t1) and (t2), respectively (Levasseur, Thompson & Harrison, 1993). Once the specific growth rate is known, divisions per day and the generation or doubling time can also be calculated as follow:

Div.day^{-1} = $\frac{K'}{Ln2}$ and Generation time: Gen't = $\frac{1}{Div.day^{-1}}$ (Levasseur et al., 1993).

The third phase called the linear growth phase represents the linear curve on the graph (Figure 1 A & B). During this phase the reproduction of microalgae is inhibited resulting in the deceleration of the growth rate and, therefore, cell division. This is caused by the limitations of the surrounding conditions in the substrate like for instance

nutrients, light, pH, and CO_2 needed for cell metabolism (Fogg & Thake, 1987). Since the linear phase is directly after the exponential phase the growth is eventually balanced out at some point due to the constant supply of air (inclusive of CO_2). This causes any further increase in cell density to be linear rather than exponential (with respect to time) and proportional to the input of CO_2 (Fogg & Thake, 1987). However, during low cell densities too much CO_2 lowers the pH and ultimately depresses the growth rates (Fogg & Thake, 1987). Light limitation at high cell density is caused by self-shading when the cells absorb most of the incoming irradiation and individual cells shade each other (Fogg & Thake, 1987). In most cases the growth of microalgae is saturated at relatively low irradiances of 50 - 200 μmol. photons m^{-2} s^{-1} (Fogg & Thake, 1987). Therefore, microalgae can adapt well and survive conditions of low incident light (Fogg & Thake, 1987).

The transitioning or deceleration phase between the linear and stationary phase is the phase in which the limiting factor and the growth rate reach an equilibrium resulting in constant cell density (Fogg & Thake, 1987). Here the nutrients are almost depleted due to high cell density and light (influenced by self-shading on the unlit side of the culture). This nutrient depletion results in the stagnation of the growth rate leading to the transitioning into the stationary phase. During the stationary phase the nutrient supply is limited, resulting in the equilibrium of the proliferation stage and the death of microalgae cells. Here, the cell density is constant and the net growth rate is zero, causing the cells to go through drastic biochemical changes dependent on the growth limiting factor (Fogg & Thake, 1987). For example nitrogen limitation can result in the

reduction of protein content as well as changes in lipid and carbohydrate content, whilst light limitations may lead to increasing pigment content and shifts in fatty acid composition (Fogg & Thake, 1987). Furthermore, although light intensities are adequate for growth in the first 4 phases, they decrease with increasing biomass during this phase due to self-shading. The cells become stressed leading to photo inhibition which along with nutrient depletion may result in the death of cells (Fogg & Thake, 1987). Therefore, it makes sense to reduce the light intensities when cultures enter stationary phase (Fogg & Thake, 1987).

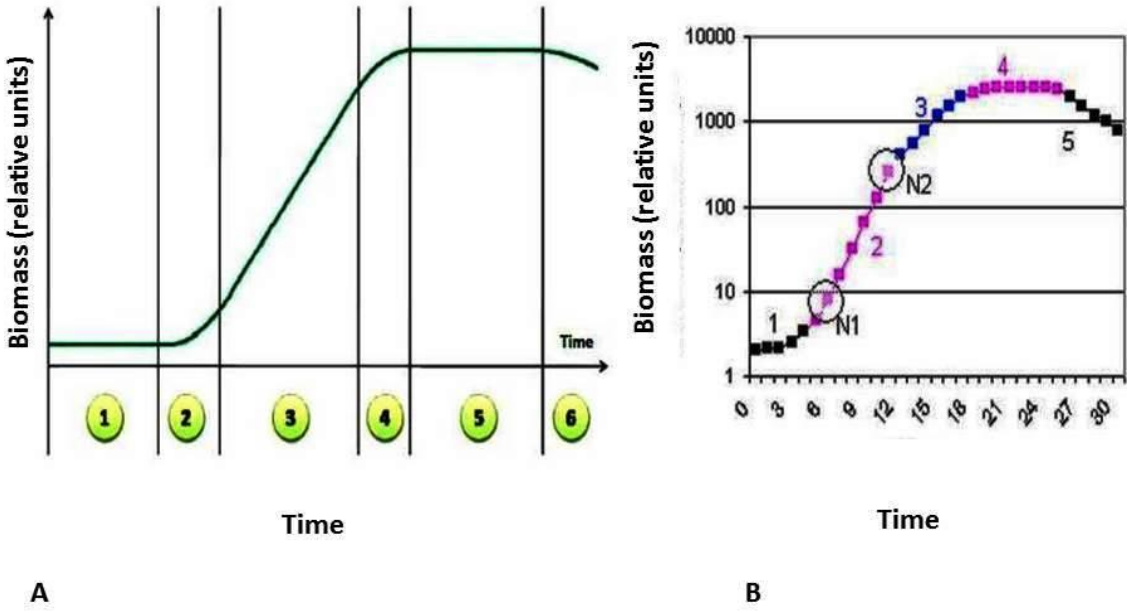

Figure 1: (A) The life cycle (Growth Curve) of batch culture of microalgae expressed as biomass (cells per mL) over time in six phases (B) The life cycle (Growth Curve) of batch culture of microalgae expressed as biomass (cells per mL) over time in five phases i.e. **(1)** Lag/Adaption/Induction phase **(2)** Exponential phase **(3)** Linear Growth Phase **(4)** Transitioning phase **(5)** Stationary Phase **(6)** Death Phase (Fogg & Thake, 1987; Rether, 2011).

Evidence shows that some Chlorophyceae and Cyanobacteria can survive the vegetative state for over 6 - 12 months under very low light intensity (Fogg & Thake, 1987). Moreover, other parameters such as temperature when lowered in conjunction with reduced light intensity can lower stress of cells since survival is inversely proportional to temperature, but only in darkness (Fogg & Thake, 1987). Some

microalgae resort to forming cysts or temporary resting cysts with reduced metabolism under different conditions of stress (Fogg & Thake, 1987). Shutting down these biochemical pathways during stationary phase by altering the growth conditions will indefinitely result in the longer lag phase (Fogg & Thake, 1987).

Finally, the death phase represented by the steep decline on the growth curve (Figure 1) is due to the vegetative metabolism of the cell that can no longer be maintained by the conditions in the substrate (Fogg & Thake, 1987). In this case the cell density decreases exponentially at a greater rate than it could ever recharge. During this phase the substrate that normally allows optimal growth reaches a level incapable of sustaining growth causing the culture to collapse (Fogg & Thake, 1987). This collapse or "culture crush" is led by a combination of factors such as water quality deterioration, nutrients depletion, oxygen deficiency, overheating, pH disturbance, contamination, amongst other factors (Fogg & Thake, 1987). Empirical observation in some species in this phase shows the loss of pigmentation resulting in discolouration making them appear washed out or cloudy (Fogg & Thake, 1987). However, this should not be considered as an indication or definite sign of an unhealthy or healthy culture, since other species cells although lysing can maintain the colour of the culture (Fogg & Thake, 1987). Furthermore, regarding contamination, bacteria that may have been controlled during the exponential and early stationary phase may proliferate as cell membrane integrity become progressively compromised or leaky and a carbon source for bacterial growth is released (Fogg & Thake, 1987). Moreover beyond exponential and early stationary phase, representations of the health of the culture by using turbidity and or optical

density measurements (fluorescence) become unreliable and inaccurate due to the free pigment and bacterial growth in the substrate that act as additions and or substitutes of the biomass (Fogg & Thake, 1987).

Some species regenerate to secondary growth from the remaining vegetative cells or germinate from cysts or temporary cysts (Fogg & Thake, 1987). This growth is supported by the release of nutrients from bacteria into the substrate (Fogg & Thake, 1987). Once the culture reaches this stage it is usually discarded since its nutritional value is far more inferior when compared to other phases (i.e. beyond phase 4) due to reduced digestibility, deficient composition, and possible production of toxic metabolites (Fogg & Thake, 1987). Thus, it is more ideal and preferable to maintain the cultures in the exponential phase (Fogg & Thake, 1987).

2.3 Abiotic and biotic factors affecting microalgae

Microalgae growth and, therefore, metabolic products (lipids, proteins, and carbohydrates) are affected by abiotic, biotic and operational factors (Juneja, Ceballos & Murthy, 2013; Cheng & He, 2014; Singh & Singh, 2015). Abiotic factors include local climatic or seasonal conditions (i.e. temperature and light), pH, dissolved oxygen, dissolved CO_2, salinity, nutrients and toxic compounds (Moheimani, 2005; Griffiths & Harrison, 2009; Kumar et al., 2010; Mata et al., 2010; Juneja et al., 2013).

Biotic factors include competition with other microalgae, bacteria and fungi as well as predation from protozoa, fish and viruses (Moheimani, 2005; Griffiths & Harrison, 2009; Kumar et al., 2010; Mata et al., 2010; Juneja et al., 2013). Operational factors are

dilution rates, mixing rates, harvesting frequency and depth (Moheimani, 2005; Griffiths & Harrison, 2009; Kumar et al., 2010; Mata et al., 2010; Juneja et al., 2013; Singh & Singh, 2015).

Non-mineral nutrients are carbon, hydrogen, and oxygen (Juneja et al., 2013). Their abundance and availability in media for microalgae cultures is not limiting for cellular growth or metabolism (Juneja et al., 2013). However, carbon from the three is an essential nutrient that must be supplied in microalgae culture (Juneja et al., 2013). Macronutrients include nitrogen, phosphorus, sulfur, potassium, and magnesium, whilst micronutrients or essential trace metals such as iron and manganese are required in small amounts (30 - 2.5 ppm) and non-essential trace metals (i.e. cobalt, zinc, boron, copper and molybdenum) are needed in minute quantities (i.e. 2.5 - 4.5 ppm) (Juneja et al., 2013). These nutrients play a pivotal role in various physiological processes and, therefore, metabolism for daily existence not only for microalgae, but also for other aquatic organisms (Juneja et al., 2013; Cheng & He, 2014; Ren, 2014; Sirunda & Mazvimavi, 2014). However, the excessive influx of some of these macronutrients lead to proliferations of microalgae blooms in natural environments that lead to toxicity, oxygen deprived layers and ultimately eutrophication (Donato-Rondón, Morales-Duarte & Castro-Rebolledo, 2010; Sirunda & Mazvimavi, 2014). Therefore, in order to determine the causative agents of these blooms there should be a thorough investigation regarding the nutrients and sources that lead to these blooms.

Collectively abiotic and biotic factors affect photosynthesis by altering carbon fixation and the allocation of carbon into different types of cellular macromolecules that in turn

influence the biochemical composition of microalgae (Juneja et al., 2013). These influences determine the allocation and usefulness of microalgae species in various product applications (Juneja et al., 2013). Therefore, one can strategically alter and manipulate biochemical properties in microalgae cultures to produce various valuable metabolic products such as lipids, carbohydrates, proteins, pigments et cetera (Juneja et al., 2013; Cheng & He, 2014; Ren, 2014) (Figure 2). This is especially true to influence the lipid content needed to produce biofuels by controlling growth and nutrient conditions (Sforza, Bertucco, Morosinotto & Giacometti, 2010; Tredici, 2010). The same is true for microalgae cultured either through autotrophic or heterotrophic production (Chen & Chen, 2006; Liang, Sarkany & Cui, 2009; Xiong, Gao, Yan, Wu & Wu, 2010). Furthermore, it has been established that a combination of environmental stresses and not only one contributes to the accumulation of metabolic products (Cheng & He, 2014). Since these factors are additive, they may have an effect on the yield of biofuel products (Cheng & He, 2014). Several studies demonstrated this, for example Pal, Khozin-Goldberg, Cohen & Boussiba (2011) found that the maximum productivity of total lipids was reached when high light and high salinity stress were simultaneously applied to *Nannochloropsis* cultures. Sun et al. (2014) demonstrated these effects by using nitrogen starvation in conjunction with high light to achieve the maximal triacylglyceride and carbohydrate production in *Neochloris oleoabundans* HK-129 (Cheng & He, 2014; Li et al., 2008a). Table 2 shows some of these factors and their implications.

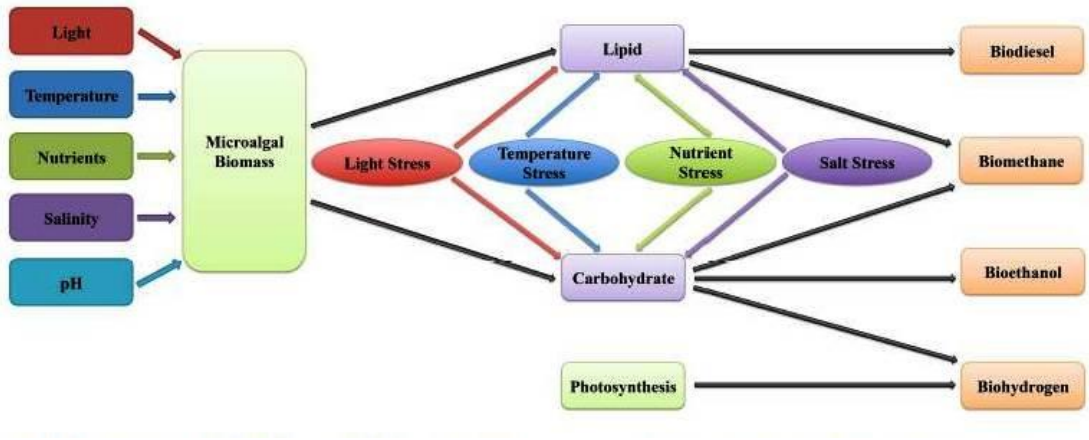

Figure 2: Effects of environmental factors on microalgae biomass (Cheng & He, 2014).

Table 2: Impact of various environmental factors on the biochemical composition of some microalgae (Juneja et al., 2013).

Factor	Organism	Conditions	Biochemical changes observed	References
Temperature	*Botryccoccus braunii*	Increased from 25 to 32 °C	Decrease in intracellular lipid content from 22 to 5 % wt. Accumulation of polysaccharides	Kalacheva, Zhila, Volova and Gladyshev (2002)
	Chlorella vulgaris	Increased from 20 to 38 °C	Decrease in starch resulting in increase in sucrose	Nakamura and Miyachi (1982)
		Increased from 10 to 38 °C	Transformation of L-starch (high molecular weight) to S-starch (low molecular weight). Reversible with temperature	Nakamura and Imamura (1983)
	Haematococcus pluvialis	Increase from 20 to 30 °C	3-fold increase in astaxanthin formation	Tjahjono et al. (1994)
	Chlorococcum	Increase from 20 to 35 °C under nitrogen deprivation	Two fold increase in total carotenoid content	Liu and Lee (2000)
	Nitella mucronata	Increased from 5 to 20 °C	Increase in velocity of cytoplasmic streaming	Raven and Geider (1988)
Light	*Dunaliella virdis*	Darkness (No light)	Increase in total lipid content. Decrease in free fatty acids, alcohol, sterol	Gordillo, Goutx, Figueroa and Niell (1988a)
	Nannochloropsis	Light limited conditions	Increase in lipid content. Increase in *EPA proportions	Sukenik, Carmeli and Berner (1989)
	Porphyridium cruentum	Red light	Enhanced Photosystem II relative to Photosystem I and phycobilisome	Cunningham, Dennenberg, Jursinic and Gantt (1990)
	Chlorella vulgaris	Red light	Increase in sucrose and starch formation	Miyachi, Miyachi and Kamiya (1978)
		Blue light	Increase in lipid fraction and	Miyachi et al. (1978)

			alcohol-water insoluble non carbohydrate fraction	
pH	*Chlamydomonas acidophila*	pH 4.4	Denaturation of V-lysin	Visviki and Palladino (2001)
	Coccochloris peniocystis	pH decreased from 7.0 to 5.0 and 6.0	Decrease in total accumulated carbon and oxygen evolution	Coleman and Colman (1981)
Nitrogen	*Nannochloropsis oculata*	75 % decrease in Nitrogen	Increase in lipid synthesis from 7.9 to 15.31 %	Converti, Casazza, Ortiz, Perego and Del Borghi (2009)
	Phaeodactylum tricornutum	Nitrogen limitation	Increase in lipid synthesis; Decrease in protein content	Morris, Glover and Yentsch (1974)
	Chlorella vulgaris	75 % decrease in Nitrogen	Increase in lipid synthesis from 5.9 to 16.41 %	Converti et al. (2009)
	Haematococcus pluvialis	Nitrogen limitation	Increase in carotenoid formation (13 % *w/w*)	Borowitzka, Huisman and Osborn (1991)
Phosphorous	*Chlamydomonas reinhardtii*	Limitation	Decrease in phosphatidylglycerol	Sato, Hagio, Wada and Tsuzuki (2000)
	Ankistrodesmus falcatus	Limitation	Decrease in chl *a* and protein; Increase in carbohydrate and lipids	Kilham, Kreeger, Goulden and Lynn (1997); Healey (1982); Healey and Hendzel (1979)
	Selenastrum minutum	Starvation	Reduced rate of respiration; Decreased photosynthetic CO_2 fixation	Theodorou, Elrifi, Turpin and Plaxton (1991)
Iron	*Dunaliella tertiolecta*	Limitation	Decrease in cellular chlorophyll concentration	Greene, Geider, Kolber and Falkowski (1992)
	Chlorella vulgaris	High concentration of iron.	Increase in lipid content	Liu, Wang and Zhou (2008)
	Haematococcus pluvialis	High concentration of iron	Increase in carotenoid formation	Kobayashi, Kakizono and Nagai (1993)

Carbon	*Chlamydomonas reinhardtii*	pH exceeding 9.0	Inefficient accumulation of carbon High supply of carbonates required to maintain photosynthetic activity	Moroney and Tolbert (1985)
	Dunaliella salina	CO_2 concentration increased from 2 to 10 % for 1 day	30 % increase in amount of fatty acid (dry weight basis)	Muradyan, Klyachko-Gurvich, Tsoglin, Sergeyenko and Pronina (2004)
		CO_2 concentration increased from 2 to 10 % for 7 days	2.7 fold increase in fatty acid	
	Spirulina platensis	Elevated CO_2 concentrations	Increase in carbohydrate content; Decrease in proteins and pigments	Gordillo, Jiménez, Figueroa and Niell (1998b)

*EPA- eicosapentaenoic acid

2.3.1 Effect of Carbon on microalgae

Carbon is an important non-mineral nutrient required for microalgae reproduction, growth and, therefore, biomass production (Juneja et al., 2013; Usher, Ross, Camargo-Valero, TomLin & Gale, 2014). It is either garnered inorganically in the form of atmospheric CO_2, carbonate, or bicarbonate by autotrophic or photosynthetic microalgae via carboanhydrase and in forms of acetate or glucose by heterotrophic microalgae (Juneja et al., 2013; Usher et al., 2014). Therefore, the carbon metabolic mode (i.e. autotrophic, mixotrophic, or heterotrophic) affects the growth rates of microalgae (Chojnacka & Marquez-Rocha, 2004; Cheng & He, 2014). Since heterotrophic microalgae are not able to assimilate atmospheric carbon they garner sources from substrates such as wastewater streams in forms of by-products from bacterial degradation of organic matter (i.e. acetate, humic substances, partly degraded plant and animal materials), or other highly biodegradable organic compounds such as sugars from industrial sources, such as wastewater from food or drink industries (Juneja et al., 2013; Sirunda & Mazvimavi, 2014; Usher et al., 2014).

Carbon fixed from the atmosphere by microalgae is utilized in three ways: (1) respiration; (2) as an energy source, and (3) as a raw material in the formation of additional cells (Berman & Dubinsky, 1999; Juneja et al., 2013). The empirical formula for this fixation is as follows: $CO_2 + H_2 \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3 - \leftrightarrow 2H^+ + CO_3^{2-}$ (Chenl & Celia, 1994; Juneja et al., 2013). CO_2 in water is therefore, present in any of these forms in this equation depending on the pH, temperature and nutrient content

(Juneja et al., 2013). Reduction in carbon fixation rate is directly proportional to microalgae growth which also declines accordingly (Juneja et al., 2013).

Since there is only 300 - 600 ppm CO_2 in the atmosphere, its low solubility in water and the poor net mass transfer from the atmosphere suppresses microalgae growth rates and makes it a limiting nutrient for microalgae cultivation (Mata et al., 2010; Usher et al., 2014). To eliminate this bottleneck, the use of flue gas has been suggested as an alternative. However, this raised concerns on aquatic environments due to the dissolution of other pollutants (Usher et al., 2014). Furthermore, although CO_2 is essential for photosynthesis, it can also be harmful when in excess since it lowers dissolved oxygen concentration needed by other aquatic organisms (Sirunda & Mazvimavi, 2014). In contrast, photo bioreactors for example, CO_2 is considered for gas exchange, but is a limiting factor since additional carbon supply is needed (Bibeau, 2009; Posten, 2009). Additional supply can be provided by constantly supplying CO_2 either with enriched gas bubbles or pure CO_2, to the reactor (Posten, 2009). CO_2 can also be used as a control mechanism for unstable pH, which is common during the growth of microalgae (Bibeau, 2009). Another problem is the formation of a gradient of pO_2 and pCO_2 that lead to photosynthetic inhibition due to photooxidative damage (Bibeau, 2009; Posten, 2009).

Carbon influences the activity of nitrogenase and, therefore, the nitrogenase-dependent hydrogen production (Dutta, De, Chaudhuri & Bhattacharya, 2005; Cheng & He, 2014). Different amounts and sources of carbon have been shown to affect both the content and the composition of lipids in microalgae cells (Cheng & He, 2014). It has been reported

that high concentrations of CO_2 brings about the accumulation of saturated fatty acids, whereas low concentration of CO_2 facilitates the production of unsaturated fatty acids (Tsuzuki, Ohnuma, Sato, Takaku & Kawaguchi, 1990; Riebesell, Revill, Holdsworth & Volkman, 2000; Hu & Gao, 2003; Cheng & He, 2014). For example, a study carried out on heterotrophically grown *Chlorella* cells found that they synthesized about 280 % more lipids and 45 % more carbohydrates compared to autotrophically grown cells (Miao & Wu, 2006; Cheng & He, 2014). Another study by Riebesell et al. (2000) on *Emiliania huxleyi*, to demonstrate the effect of CO_2 concentration on lipid distribution showed a significant effect of CO_2 on the composition of the PUFAs and alkenones. The study concluded that lower CO_2 concentrations led to an increase in 22:6 (n−3) PUFA, whereas 14:0 fatty acids were found to be predominant at higher CO_2 concentrations (Cheng & He, 2014). A study on *Dunaliella salina,* showed the increase in CO_2 resulted in a higher amount of fatty acid accumulation, along with the change in composition (Muradyan et al., 2004; Cheng & He, 2014). Tsuzuki et al. (1990) reported a similar increase in fatty acid content and unsaturation with increase in CO_2 concentrations. Another study by Gordillo et al (1998b) with the cyanobacterium *Spirulina platensis* reported that elevated CO_2 concentrations decrease relative concentrations of proteins and pigments in the cells, but increase carbohydrate content. This change in the cell composition was accompanied by reduction in the maximum biomass yield (Cheng & He, 2014).

2.3.2 Effect of Phosphorous on microalgae

Phosphorus is an essential inorganic macronutrient playing a vital role in a variety of cellular or energy metabolic processes in aquatic organisms (Sirunda & Mazvimavi, 2014; Usher et al., 2014). Its limitation affects the growth and development of microalgae and, therefore, even contributes to microalgae blooms (Hu, 2004; Richmond, 2004; Juneja et al., 2013; Cheng & He, 2014; Sirunda & Mazvimavi, 2014). Furthermore, it was demonstrated that phosphorus is the least abundant amongst the nutrients needed in large quantity by photosynthetic organisms, and so it and not nitrogen, is the primary limiting nutrient for microalgae growth in many freshwater environments (Anderson, Glibert & Burkholder, 2002; Juneja et al., 2013; Usher et al., 2014). Therefore, excess phosphorus can lead to eutrophication in freshwater, compared to marine environments where nitrogen is the growth limiting nutrient (Usher et al., 2014). Furthermore, phosphorous can also limit or co-limit microalgae growth in estuarine and marine environments that are sustaining high nitrogen inputs (Anderson et al., 2002). Therefore, although phosphorus constitutes only about 1 % of dry weight of microalgae (Juneja et al., 2013), it may be required in excess amounts since not all of it is available due to formation of complexes with metal ions (Chisti, 2007; Juneja et al., 2013). Barsanti and Gualtieri (2014) studied the immediate effects of phosphorus limitation. The results of this study showed the reduction in the synthesis and regeneration of substrates in the Calvin-Benson cycle and ultimately the reduction in the rate of light utilization required for carbon fixation (Juneja et al., 2013). There are three benchmarks to be considered for microalgae growth under phosphorous concentrations:

(1) when Total Phosphorus (TP) ≤ 0.045 mg/L, the microalgae growth will be prohibited; (2) During high concentration of phosphorus (i.e. TP ≥ 1.65 mg/L), growth is not significantly promoted (Dortch, 1990); (3) when TP = 0.02 mg/L, microalgae can grow well and (4) at TP ≥ 0.2 mg/L the concentration of phosphorus has no promotion of growth rate of microalgae (Xu, Miao & Wu, 2006; Ren, 2014).

It has been shown that microalgae select different sources of phosphate for their daily metabolism (Ren, 2014). The most consumed by microalgae for growth are dipotassium hydrogen phosphate and orthophosphate (Ren, 2014; Sirunda & Mazvimavi, 2014). In freshwater aquatic systems, orthophosphate is the most readily available form of phosphorus for microalgae uptake during photosynthesis (Sirunda & Mazvimavi, 2014). It is the measure of the inorganic oxidized form of soluble phosphorus (Sirunda & Mazvimavi, 2014). The increase and excessive amount of orthophosphate causes blooms of blue-green microalgae (Sirunda & Mazvimavi, 2014). Major sources of orthophosphate are sewage treatment plant effluent, agriculture, urban development and industrial effluents (Dallas & Day, 2004; Reynolds & Walsby, 2008; Sirunda & Mazvimavi, 2014).

Phosphorus limitation leads to accumulation of lipids in microalgae cells (Juneja et al., 2013; Cheng & He, 2014; Ren, 2014). A study on *Scenedesmus* sp. LX1 showed the total lipid content increased from 23 to 53 % under phosphorus-limiting conditions, with a reduction in initial total phosphorus concentration of 0.1 from 2.0 mg/L, whilst only containing 25 - 28 % lipid under phosphorus-replete conditions (Xin, Hong-ying, Ke & Ying-xue, 2010; Juneja et al., 2013; Cheng & He, 2014;). Another study by Markou,

Chatzipavlidis and Georgakakis (2012) showed that the carbohydrate content in *Arthrospira platensis* (*Spirulina*) increased from 11 to 67 % after transfer to phosphorous-limiting medium (Cheng & He, 2014). Furthermore, Sato et al. (2000) observed a decrease with phosphorus limitation in *Chlamydomonas reinhartdtii* (Juneja et al., 2013). It is said that phosphatidylglycerol (PG), is one of the four major glycerolipids constituting membrane lipids in chloroplasts (Juneja et al., 2013). This is essential for cell growth, the maintenance of chlorophyll-protein complex levels, and normal structure-function of the PSII complex (Juneja et al., 2013). Total acidic lipid (i.e. sulphoquinovosyldiacylglycerol and PG) content of the chloroplast was observed not to change significantly since a decrease in one acidic lipid was accompanied by an increase in another acidic lipid (Sato et al., 2000; Juneja et al., 2013). Reitan, Rainuzzo and Olsen (1994) observed that phosphate limitation also reduces the synthesis of n-3 PUFA (Juneja et al., 2013). Furthermore, phosphorus depletion reduces chlorophyll *a* and protein content hence, increasing the relative carbohydrate content in microalgal cells (Juneja et al., 2013). In accumulation of astaxanthin for example, phosphate deprivation has been demonstrated to result in an overall reduction in cell growth (Juneja et al., 2013). Moreover, a decrease in cellular phycobilisome under conditions of phosphorus deprivation due to cellular division and the cessation of phycobilisomes synthesis was also observed (Collier & Grossman, 1992; Juneja et al., 2013). Additionally, Theodorou et al. (1991) observed that phosphorus starvation in *Selenastrum minutum* reduces respiration rate (Juneja et al., 2013).

2.3.3 Effect of Nitrogen on microalgae

Nitrogen is an essential constituent in all structural and functional proteins of microalgae cells (Juneja et al., 2013). In its organic and inorganic form it is an important nutrient for growth of microalgae and is usually the limiting nutrient in many temperate and polar marine systems as well as in estuaries (Anderson et al., 2002; Juneja et al., 2013; Sirunda & Mazvimavi, 2014). It accounts for 7 - 20 % of microalgae cell dry weight (Juneja et al., 2013). Inorganic nitrogen is assimilated into biochemically active compounds and recycled within cells as a necessity for changing physiological needs (Juneja et al., 2013). This assimilation is required for the formation of genetic material, energy transfer molecules, proteins, enzymes, chlorophylls and peptides (Usher et al., 2014).

There are several forms of nitrogen (NH_4^+N, NH_3–N, NO_3^-, NO_2^- and NH) available for microalgae use (Ren, 2013; Usher et al., 2014). Although some species of microalgae can fix nitrogen directly from the atmosphere (Ren, 2014), it is stated that ammonium nitrogen (NH_4^+–N) is easier for microalgae to absorb, especially when compared to ammonia nitrogen (NH_3–N) (Ren, 2014). If these two nitrogen species are not available or limited in the substrates microalgae have the ability to utilize other inorganic nitrogen species such as nitrate (NO_3^-) or nitrite (NO_2^-) (Usher et al., 2014). Sources of total nitrogen include sewage treatment plant effluent, agriculture, urban development, paper plants, recreation, mining (blasting residuals), and industrial effluents (Sirunda & Mazvimavi, 2014). Excessive nitrogen content in growing conditions will increase the growth of microalgae (Ren, 2014). This can lead to rapid proliferation of microalgae

blooms of especially toxic blooms (i.e. toxins from cyanobacteria), free ammonia which is toxic to fish and low oxygen concentrations as a result of eutrophication (Sirunda & Mazvimavi, 2014; Usher et al., 2014).

Nitrogen is said to be the most important nutrient affecting lipid metabolism in microalgae (Cheng & He, 2014). Under nitrogen deprivation, the majority of the carbon fixed in photosynthesis is used to synthesize lipids (Converti et al., 2009; Wang, Ullrich, Joo, Waffenschmidt & Goodenough, 2009; Demirbas, 2010, Juneja et al., 2013), triacylglycerols (Takagi, Watanabe, Yamaberi & Yoshida, 2000; Stephenson, Dennis, Howe, Scott & Smith, 2010; Juneja et al., 2013) and carbohydrates (Hu, 2004), instead of proteins (Lynn, Kilham, Kreeger & Interlandi, 2000; Heraud, Wood, Tobin, Beardall & McNaughton, 2005; Juneja et al., 2013; Cheng & He, 2014). Since this deprivation reduces the synthesis of photosynthetic proteins and pigments, microalgae growth, biomass yield and lipid accumulation may be affected (Converti et al., 2009; Lardon, Helias, Sialve, Steyer & Bernard, 2009; Mata et al., 2010; Chen & Walker, 2011; Juneja et al., 2013; Cheng & He, 2014; Usher et al., 2014). Moreover, the effects of nitrogen deficiencies also include the reduction in oxygen evolution, carbon dioxide fixation and tissue production (Juneja et al., 2013).

Several scientists have reported that a variety of microalgae species increase the accumulation of lipids under nitrogen limitation (Cheng & He, 2014). Li et al. (2008a) and Rodolfi et al. (2009) demonstrated that the lipid content in *Neochloris oleoabundans* and *Nannochloropsis* sp. F&M-M24 increased twice and once, respectively, after

nitrogen deprivation (Cheng & He, 2014). To demonstrate the biosynthesis of carbohydrates under nitrogen deplete conditions, Ji et al. (2011) and ShihHsin ChunYen and JoShu (2012) reported a four times increase in carbohydrate content in *Tetraselmissub cordiformis*, and a 29 % increase in carbohydrate content in *Scenedesmus obliquus* CNW-N (Cheng & He, 2014). Other studies (Das & Veziroğlu, 2001; Dutta et al., 2005; Abed, Dobretsov & Sudesh, 2009) showed that, many cyanobacteria can produce hydrogen as a byproduct of nitrogen fixation when grown under nitrogen limiting conditions (Cheng & He, 2014). Regarding protein synthesis, a study by Holm-Hansen, Nishida, Moses and Calvin (1959) revealed an increase in amino acid content of *Chlorella pyrenoidosa* to the detriment of sugar phosphates (i.e. glucose-6-phosphate, fructose-6-phosphate) with addition of ammonium (nitrogen source) to the growing culture (Juneja et al., 2013). Regarding the effects of nitrogen deprivation on pigments, Collier & Grossman (1992) found the degradation of phycolbillisomes (i.e. light harvesting antennae of photosystem II in microalgae) in cyanobacteria and red algae (Juneja et al., 2013). A study on *Spiriluna platensis* under nitrogen deficient conditions, showed cells to exhibit reduced carbon fixation capacity under normal to high available CO_2 concentrations (Gordillo et al., 1998b; Juneja et al., 2013). According to Round (1984), nitrogen deprivation can alter the enzyme balance of cells, leading to the synthesis of lipids and decrease in chlorophyll synthesis resulting in abundance of carotenoids in cells (Juneja et al., 2013). This was confirmed by several scientists (Ben-Amotz & Avron, 1990; Borowitzka et al., 1991; Harker, Tsavalos & Young, 1996) during a study on *Dunaliella* sp. and *Haematococcus pluvialis* that

accumulated large amounts of carotenoids, astaxanthin and acylesters (up to 13 % *w/w*), when grown under nitrogen deplete conditions (Juneja et al., 2013). Moreover, Zhekisheva, Boussiba, Khozin-Goldberg, Zarka and Cohen (2002) found that, *Haematococcus pluvialis* produced fatty acids and astaxanthin in a 5:1 ratio under nitrogen depleting conditions (Juneja et al., 2013).

2.3.4 Effect of Sulphur on microalgae

Sulphur is a macronutrient important for photosynthesis, protein synthesis, and lipid metabolism in microalgae (Cheng & He, 2014). It is also a nutrient known to affect biohydrogen production in microalgae (Cheng & He, 2014). Sulphur limitation reduces cellular division in microalgae (Ariño, Ortega-Calvo, Hernandez-Marine & Saiz-Jimenez, 1995; Cheng & He, 2014). Additionally, it has also been reported that, this limitation causes an anaerobic environment inside microalgae cells, thus induces the activity of hydrogenase and the release of hydrogen (Dutta et al., 2005; Esquível, Amaro, Pinto, Fevereiro & Malcata, 2011; Cheng & He, 2014). This phenomenon was studied in various microalgae species, including *Gloeocapsaalpicola*, *Synechocystis* sp. PCC 6803, *Chlamydomonas reinhardtii*, *Chlamydomonas noctigama* and *Chlamydomonas euyale* (Antal & Lindblad, 2005; Laurinavichene, Fedorov, Ghirardi, Seibert & Tsygankov, 2006; Skjånes, Knutsen, Källqvist & Lindblad, 2008; Cheng & He, 2014). Total fatty acid content in *Chlamydomonas reinhardtii* doubled after exposure to sulphur limitation (Matthew et al., 2009; Cheng & He, 2014). Another study by Brányiková et al. (2011) reported that *Chlorella vulgaris* cells synthesized 50 % more

starch under sulphur-limiting conditions than under sulphur-replete conditions (Cheng & He, 2014).

2.3.5 Effect of Trace metals on microalgae

Micronutrients or essential trace metals including iron (Fe), manganese (Mn), cobalt (Co), zinc (Zn), copper (Cu) and nickel (Ni) are present in small quantities (< 4 ppm), while non-essential trace metals such as cadmium (Cd), lead (Pb) and chromium (Cr) are present in smaller quantities (i.e. 2.5 - 4.5 ppm) in microalgae cells (Juneja et al., 2013; Sirunda & Mazvimavi, 2014). These metals are essential components of microalgae physiology and, therefore, play important roles in the cellular/metabolic processes of microalgae (Kennish, 1992; Juneja et al., 2013; Sirunda & Mazvimavi, 2014). Furthermore, some trace metals such as iron, nickel, magnesium, molybdenum, and zinc are also essential for nitrogenase-catalyzed hydrogen production (Horner, Heil, Happe, & Embley, 2002; Lin & Lay, 2005; Carrieri, Ananyev, Costas, Bryant & Dismukes, 2008; Cheng & He, 2014). In natural systems, trace metal availability to microalgae is highly dependent on speciation (i.e. free ion concentration) (Parent et al., 1996; Juneja et al., 2013). Limitations of these metals can inhibit metabolic processes, even in small quantities and, therefore, reduce microalgae growth, whilst excess concentrations may inhibit growth, impair photosynthesis, deplete antioxidants, and damage the cell membrane (Juneja et al., 2013; Cheng & He, 2014).

Iron is essential for normal microalgae growth and functioning of photosynthesis (i.e. photosynthetic electron transport chain) and respiration (Juneja et al., 2013). Its

limitation compromises photosynthetic electron transfer, ultimately leading to the reduction in NADPH (Nicotinamide adenine dinucleotide phosphate-oxidase) formation (Van Oijen, Van Leeuwe, Gieskes & de Baar, 2004; Liu, Wang & Zhou, 2008; Juneja et al., 2013; Cheng & He, 2014). Similarly, the absence of manganese in freshwater systems depresses photosynthesis (Sirunda & Mazvimavi, 2014). Iron and Manganese are both used as indicators during freshwater water quality monitoring, since they show the presence and decay of microalgae (Sirunda & Mazvimavi, 2014). The demand for iron in the water increases during microalgae blooms, whereas it is released back into the water column during death and decay of blooms (Sirunda & Mazvimavi, 2014).

Trace metals affect the accumulation of lipids and carbohydrates in numerous microalgae cells (Cheng & He, 2014; Sirunda & Mazvimavi, 2014). During a study by Liu et al. (2008) on cultures of *Chlorella vulgaris*, excess concentrations of iron were observed to increase (up to seven fold) the lipid content (Juneja et al., 2013). Similarly, another study by Griffiths & Harrison (2009), showed that silicon limitation resulted in increased lipid content in several diatom species with 89, 110, and 104 % lipid content in *Chaetoceros muelleri*, *Cyclotella cryptica*, and *Navicula saprophila*, respectively (Cheng & He, 2014). Regarding carbohydrates, a study reported that glucose in *Agmenellum quadruplicatum* increased from 5 to 45 % during iron limitation (Hardie, Balkwill & Stevens, 1983; Juneja et al., 2013). As for pigments, iron reduces cellular chlorophyll concentration (Greene et al., 1992) as well as carotenoid composition (Kobayashi et al., 1993; Van Leeuwe & Stefels, 1998; Juneja et al., 2013).

Some essential trace elements (e.g. Zn, Cu, Ni and Fe) are known to cause toxicity to microalgae when in large amounts in natural systems (Campanella, Cubadda, Sammartino & Saoncella, 2001; Juneja et al., 2013). This toxicity is largely pH dependent due to the competition between H^+ and free metal cations for cellular binding sites and depending on whether the metal can inhibit carbon fixation as well as delay nutrient uptake (Rai, Mallick, Singh & Kumar, 1991; Juneja et al., 2013). For example, excessive iron may result in oxidative damage of the cells (Choudhary, Jetley, Khan, Zutshi & Fatma, 2007; Cheng & He, 2014). Copper (Cu) being one of the most toxic of these metals (Juneja et al., 2013) inhibits both cell division and photosynthesis in *Asgerionella glacialis* (marine diatom) and *Chlorella pyrenoidosa* (freshwater microalgae) (Juneja et al., 2013). Furthermore, effects of pH on copper toxicity carried out on the green microalgae *Scenedesmus quadricauda* showed it to increase 76-fold under a pH of 5.0 – 6.5 (Peterson, Healey & Wagemann, 1984; Juneja et al., 2013). Although not carrying any biological importance in a living cell, cadmium (Cd) is said to be toxic to microalgae cells (Juneja et al., 2013). It is assimilated by marine cells in the form of complexes with organic matter and absorbed onto organic matter and inorganic matter in ionic form (Wong, Burnison, & Chau, 1979; Juneja et al., 2013). It inhibits phosphorus uptake, which is also pH dependent (i.e. increases in the pH range of 5.5 – 8.5) (Peterson et al., 1984; Juneja et al., 2013). Wong and Chau (1990) reported that zinc is also a toxic metal which is rapidly taken up by the microalgae and is incorporated into polysaccharide and nucleic acid fractions (Juneja et al., 2013).

2.3.6 Effect of Light on microalgae

Microalgae along with plants and some bacteria are oxygenic photoautotrophs that convert light energy from the sun into chemical energy (Janssen, 2002). This light driven, biomass generating process is called photosynthesis (Janssen, 2002). Both plants and microalgae do not completely absorb the photosynthetic active radiation (PAR) with wavelengths between 400 and 700 nm to convert light energy into biomass. This is due to the weak absorbance of chlorophyll in what is called the green band. Therefore, enzymatic steps for the fixation of CO_2 into carbohydrate only constitute about 35 % effectiveness and the theoretical maximum of photosynthetic conversion efficiency is estimated to be 12.4 % or in other words the maximum interception of 400 - 700 nm light is limited to 90 %, so that about 10 % of the PAR is rejeccted as energy loss (Zhu, Long, & Ort, 2008; Tredici, 2010). Thus, 55 % or over loss of the total incident radiation occurs (Lehr & Posten, 2009). Even so, microalgae are different from other photosynthetic organisms since they have specialized structures for phototaxis, which is the bodily movement of motile organisms in response to light, which can either be towards the source of light (positive phototaxis) or away from it (negative phototaxis) that allows them to find the most suitable light conditions and hence optimize photosynthesis (Foster & Smyth, 1980). For example, to influence light on cellular behavior, flagellated microalgae found a way to adapt to sunlight conditions by phototaxis. They carry this out by using their flagellates to orient themselves depending on light direction (Foster & Smyth, 1980). Light conditions therefore, directly affect the

growing and photosynthesis of microalgae (Al-Qasmi, Raut, Talebi, Al-Rajhi & Al-Barwani, 2012).

The process of photosynthesis can be divided into two groups: (1) Photochemical reactions (the photolysis of water) and (2) biochemical reactions of the Calvin cycle. In this sense, microalgae need a light phase which involves the photochemical reactions to produce Adenosine triphosphate (ATP) and Nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) and the dark phase which is for biochemical reactions to synthesize essential molecules for growth (Al-Qasmi et al., 2012; Cheirsilp & Torpee, 2012).

Figure 3 provides a schematic diagram of photosynthesis for chloroplast containing organisms. Here sunlight is captured through photons inside carotenoids or chlorophyll pigments (of the photosystem (I and II) antenna system. The energy and reducing power from ATP and NADPH are then used to fix or metabolise CO_2 from the atmosphere through the action of ribulose-biphosphate carboxylase (Rubisco) into phosphoglyceraldehyde (triose P) (Janssen, 2002). Triose P is further used to synthesise building blocks for fats, fatty acids, amino acids and carboxylic acids (Janssen, 2002). Additionally, triose P is the starting point of synthesis of hexose P, which is followed by carbohydrate synthesis (i.e. starch). The carbohydrates produced are stored as energy sources for respiration (Janssen, 2002). Water is used as a substrate, whilst molecular oxygen is released as a by-product into the atmosphere (Janssen, 2002).

In eukaryotic photoautotrophs like plants and microalgae this process takes place in the photosynthetic apparatus or the photosystem which is situated in the aqueous region i.e.

stroma or thylakoid membranes inside organelles called chloroplasts whereas in prokaryotic photoautotrops, such as cyanobacteria it takes place inside the cytoplasm, plasma membranes or the membranes derived from these (Janssen, 2002).

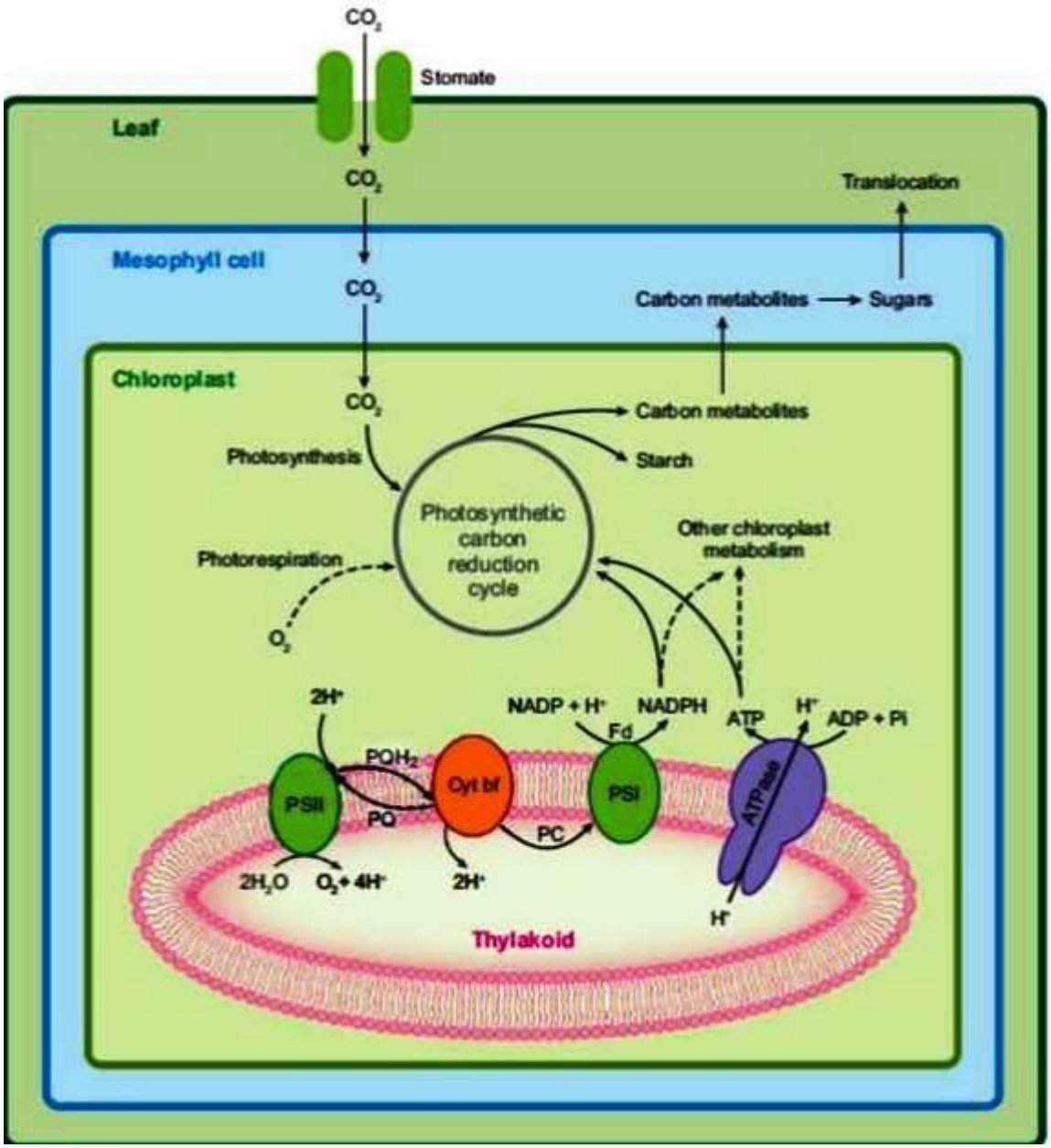

Figure 3: Photosynthesis in leafy plants (Baker, 2008).

Figure 4 represents the PI curve (photosynthesis-irradiance) which is the relationship between light intensity I (irradiation) and photosynthetic activity (P) of phototrophic organisms. This is measured in oxygen production (i.e. growth rate) versus Irradiance I as the light kinetic curve (Richmond, 2008; Tredici, 2010). The curve is divided into three sections which are photo limitation, photo saturation and photo inhibition. Photo limitation shows the part on the curve without light, but respiration does occur, whilst the increase in light/irradiation shows the increase in photosynthetic activity until photosynthetic oxygen production and respiration are balanced at the irradiance compensation or Ic point. Further increase in light leads to the increase in photosynthetic rate. This increase is linear until the photosynthetic rate slowly decreases resulting in the photo limitation section to end at Ik. This is the irradiance level at which control passes from light absorption and photochemical energy conversion to reductant utilization (Tredici, 2010). This is followed by the photo saturation section, where the photosynthetic rate does not increase and remains stationary. Here, the photosynthetic rate is at its maximum value which is referred to as Pmax on the curve. During this time, the enzymatic reactions of the Calvin-Cycle are at full capacity; therefore, absorbed photons are no longer necessary and no longer utilized. Hence enzymatic reactions are the limiting factor of photosynthetic activity (Tredici, 2010). Furthermore, increased light energy reaches a point (Ih) at which the photosynthetic rate starts to decrease. This is referred to as photo inhibition where the light starts to damage the chloroplast.

Photo inhibition not only depends on irradiance, but also on the duration of its exposure (Tredici, 2010). This over excitation and saturation of light can lead to the formation of toxic species (i.e. singlet oxygen) or reactive oxygen species (ROS) that can cause damage to the photosystem (Janssen, 2002; Cheng & He, 2014). These are harmful to microalgae cells resulting in photo inhibition which in turn decreases the biomass productivity (Cheng & He, 2014).

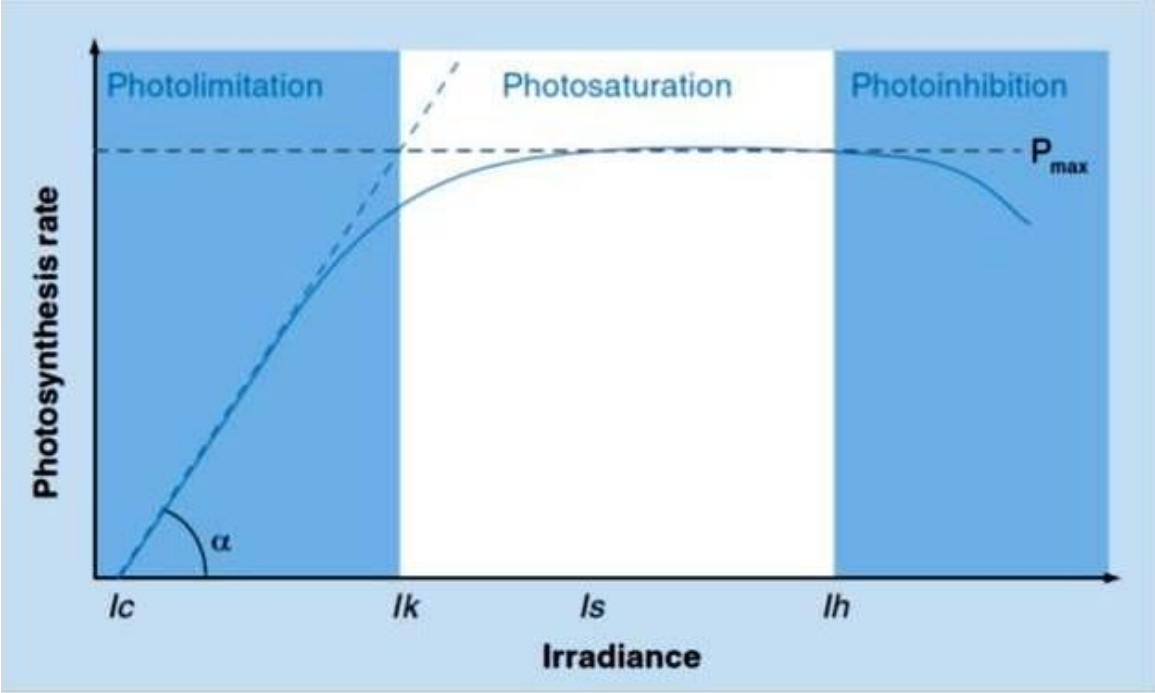

Figure 4: PI-Curve showing the relation between photosynthetic rate P and irradiance I (Tredici, 2010).

Increased light intensities and duration is directly proportional to the increase of cultivated microalgae biomass until light saturation (i.e. 200 - 400 mE) is reached

(Radakovits, Jinkerson, Darzins & Posewitz, 2010; Al-Qasmi et al., 2012). In addition, high light intensities result in light limitations due to mutual shading of cells for dense microalgal cultures. To avoid cell stagnation in the dark or light zones of the culture proper mixing is recommended (Bibeau, 2009). Mixing is an important parameter in cell culture because it homogenizes cell distribution, heat, metabolites, as well as facilitates the transfer of gases (Mata et al., 2010). Benefits of mixing include productivity by reducing cell boundary effects and improving mass transfer in cultures of high microalgal density (Qiang & Richmond, 1996). This is why microalgae generally grow well in lakes or streams because of turbulence and movement of the water (Wang, 2006). Furthermore, the beneficial effect of mixing as an improvement in the light and dark cycle to which cells are exposed as a result of mutual shading was demonstrated by Richmond and Vonshak (1978) as well as Hu, Guterman and Richmond (1996).

In cultures maintained at optimal cell concentration, beneficial effects of mixing on productivity and photosynthetic conversion efficiency (PCE) relate to the improvements on the mode of light availability for the individual cells (i.e. the light regime). Qiang and Richmond (1996) further demonstrated how to obtain high photo conversion efficiency in outdoor cultivations under strong saturation of PFD (i.e. photo flux density; the number of photons per second per unit area). In this case reactors of a narrow light path should be employed to maintain the culture at very high microalgal density by providing proper mixing at the maximal rate without causing cell damage. Similarly, turbulence is used in microalgal culture to promote the fast circulation of microalgae cells from the dark to the light zone of the reactor in especially a large-scale production (Barbosa,

2003; Mata et al., 2010). However, care should be taken to avoid high liquid velocities and turbulence caused by mechanical mixing that could lead to the damage of microalgae due to shear stress (Eriksen, 2008; Mata et al., 2010). The optimum level of turbulence above which cell death occurs is strain dependent and should be investigated in order to avoid decline in productivity (Barbosa, 2003; Mata et al., 2010). The combination of a narrow light path and intensive mixing, in outdoor facilities allows individual cells to be provided with a suitable light regime or 'light field' (Grobbelaar, 1994). In those optimized systems light saturation is not a problem since light is intermittent and optimal for cells and it could only be limiting if it was too high and harmful to the photosynthetic apparatus of cells (Qiang & Richmond, 1996). Bibeau (2009) studied the impact of the flashing light effect i.e. the use of high frequencies (> 10 Hz) of short flashes of high intensity light followed by a longer dark period. The study concluded that biomass productivity is not reduced when compared to continuous illumination of cultures.

Microalgae appear coloured because their respective pigments absorb light at the visible range, depending on the absorbed wavelengths. This is especially true for green microalgae and plants containing chlorophyll in abundance and using chlorine to absorb mostly red and blue wavelengths of light, whilst reflecting green light (Baker, 2008). Figure 5 shows the characteristic absorption spectra of three different classes of microalgae: green microalgae (Chlorophyta), a diatom (Chrysophyta) and a cyanobacterium (Cyanophyta) (Janssen, 2002). Chlorophyll absorption takes place in the

red region with absorption peaks between 650 and 700 nm, whilst carotenoids or accessory pigments are absorbed in the blue region (400 - 500 nm) (Janssen, 2002). The carotenoids transfer the excitation energy to the chlorophylls, making photosynthesis efficient over a wide range of wavelengths (Janssen, 2002). During over excitation and oversaturation of light when microalgae are exposed to high light intensities, carotenoids become useful since they are capable of scavenging these toxic photoproducts and, therefore, protect the photosynthetic system in the situation (Janssen, 2002). They do this by dissipating heat in the antenna complex preventing their formation (Jansenn, 2002). Furthermore, caretonoids that portray additional capacity can be induced after long-term exposure to high light intensity (Janssen, 2002). Moreover, carotenoids like astaxanthin can accumulate and act as a sun shade during high-light exposure in combination with other stress factors (Janssen, 2002).

Some microalgae have additional pigments in addition to chlorophylls and carotenoids. For example, cyanobacteria additionally contain other pigments called phycobilisomes that make them absorb a part of the solar spectrum (600 - 650 nm) more strongly than the other microalgae (Janssen, 2002).

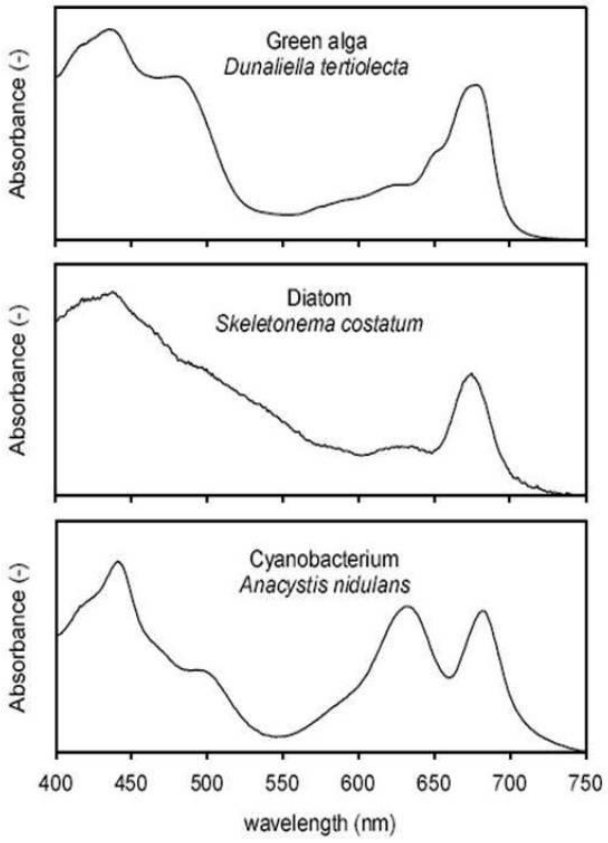

Figure 5: Characteristic absorption spectra of three microalgae classes (i.e. a green microalgae; *Dunaliela tertiolecta*, a diatom; *Skeletonema costatum* and cyanobacterium; *Anacystis nidulans* (Kromkamp & Limbeek, 1993; Janssen, 2002).

Several scientists have studied the biochemical influence of different spectra on microalgae. According to Richardson, Beardall and Raven (1983), blue and green light promote protein synthesis, whilst white light plays a major role in carbohydrate formation for *Cyclotella nana*. Furthermore, blue light with cultures of *Phaeodactylum tricornutum* show higher growth rate when compared to using white light with the same photon flux density (Holdsworth, 1985). Moreover, studies carried out on *Chlorella* spp.

have shown that growth is enhanced using only red light, whereas partial exposure to blue light does not have a significant improvement in biomass production (Das, Lei, Aziz & Obbard, 2011).

Other scientists (Eberhard, Finazzi & Wollman, 2008; Bräutigam et al., 2009; Khoeyi, Seyfabadi & Ramezanpour, 2012) demonstrated that the capacity for an effective and prompt acclimation to light is an important parameter to be considered in the characterization of microalgae species for biomass production. In addition to its metabolic role, light is also known to play a significant role in signaling and intensifying effects of many cellular processes. Therefore, microalgae grown under different light conditions demonstrate extensive changes in their chemical composition (Cheng & He, 2014).

Lipid production being a direct result of PCE decrease is well documented. The amounts of polyunsaturated fatty acids increase under low light conditions, whereas high light promotes the accumulation of saturated and mono-unsaturated fatty acids (storage lipids) (Cheng & He, 2014). This is exacerbated by nutrient limitation, of mainly nitrogen, in which case microalgae are prevented from continuing to grow and their metabolism is induced into the synthesis of energy storage products (Schlagermann et al., 2012). For example during biodiesel production, high light intensities are required to enhance saturated and mono-unsaturated fatty acids (Cheng & He, 2014). Damiani Popovich, Constenla and Leonardi (2010) suggested that light intensity and or strong illumination influences microalgae capacity for lipid accumulation, whilst Fisher, Berner, Iluz and Dubinsky (1998) and Solovchenko, Khozin-Goldberg, Recht and Boussiba (2011)

observed some *Nannochloropsis* species to induce higher levels of lipid accumulation when exposed to high light intensity (Tables 2 & 3). To show the effect of light on carbohydrate synthesis in *Scenedes musobliquus* CNW-N, a study by Ho et al. (2012) reported that the carbohydrate content increased from 16.3 to 22.4 % after exposure to high light.

2.3.7 Effect of Temperature on microalgae

After light energy, temperature is one of the most important environmental factors required for every species of microalgae (Mata et al., 2010; Juneja et al., 2013; Singh & Singh, 2015). It influences microalgae growth rate (Renaud, Thinh, Lambrinidis & Parry, 2002), cell size (Rhee, 1982; Juneja et al., 2013), cellular biochemical composition as well as availability and, therefore, uptake of various nutrients within the substrate (Darley & Darley, 1982; Juneja et al., 2013).

In nature for example, thermal stratification in the water column results in the formation of different densities that float on top of each other because of altering temperatures (Sirunda & Mazvimavi, 2014). The resulting water quality varies with the densities so that bottom layers have much lower oxygen content than upper layers (Sirunda & Mazvimavi, 2014). Oxygen gets depleted in the bottom layers during stratification causing substances such as ammonia, phosphate, sulphide, silicate, iron, and manganese compounds to be released and diffuse from the sediments into the lower water layers (Sirunda & Mazvimavi, 2014). However, most of these nutrients like phosphorous are oxidized and do not really add to the process of microalgae blooms and eutrophication

(Sirunda & Mazvimavi, 2014). Iwkume and Rasuno (1985) studied the effects of temperature on the maximum rate of photosynthesis on microalgae in Lake Kasumigaura (Ren, 2014). They found that when the temperature of water was lower than 4 °C the photosynthesis of microalgae was completely inhibited, whilst when the temperature was between 4 - 11 °C, photosynthesis was substantially inhibited (Ren, 2014). Eventually, after the temperature became higher than 11 °C, the relationship between temperature and growth of microalgae became linear (Iwkume & Rasuno, 1985; Ren, 2014). Therefore, temperature determines the activity and reaction rates of intracellular enzymes in microalgae, and these in turn influence photosynthesis, respiration intensity, growth rates and ultimately the microalgae distribution (Xiao, FanXiang, Yang, XiaoLi, & Min, 2009; Ren, 2014).

Temperature effects are specific to the composition of the microalgal culture medium and the species cultured (Ras, Steyer & Bernard, 2013). Ideal growth temperatures allow the cells to undergo photosynthesis without modifying any inherent biochemical or physiological parameters (Ras et al., 2013). Optimal growth temperature for mesophilic microalgae (i.e. *Chlorella*) is between 20, 25 and up to 40 °C for thermophilic species (i.e. *Chaetoceros* and *Anacystis nidulans*) and as low as 17 °C for psychrophilic species (e.g. *Asterionella formosa*) (Singh, Pant, Olsen & Nigam, 2012; Ras et al., 2013). Many species are able to tolerate up to 15 °C of temperature difference lower than their optimum, while increasing temperature a few degrees above that (exceeding the optimum temperature by only 2 - 4 °C) may have fatal consequences that can result in

the total loss of the culture (Mata et al., 2010; Wijffels & Barbosa, 2010; Cheng & He, 2014;). Biomass loss attributed to temperature is also associated with overheating when temperature inside the reactor reach 55 °C, especially in closed culture systems during hot days (Mata et al., 2010). Additionally, biomass losses have been reported at high temperatures during dark cycles (Bibeau, 2009). To solve this problem in closed culture systems, evaporative water cooling systems may be employed to decrease the temperature to about 20 - 26 °C (Moheimani, 2005; Mata et al., 2010). However, in the case of dark cycles, loss of biomass is imminent, because of respiration (Raven & Geider, 1988; Chisti, 2007; Cheng & He, 2014). Nevertheless, optimal temperature range rise leads to improved microalgae biomass production (Cheng & He, 2014). Therefore, high biomass accumulation can be achieved by increasing the temperature to optimal in the morning to enhance productivity during the day and decreasing the temperature at night to avoid biomass loss (Hu, 2004; Cheng & He, 2014). Table 3 shows cardinal temperatures and optimal growth rates for 14 microalgae species (Ras et al., 2013).

Low temperatures tend to increase the unsaturation of fatty acids, whereas high temperatures improve the saturation of fatty acids (Renaud et al., 2002; Liu et al., 2005; Juneja et al., 2013; Cheng & He, 2014). Therefore, a suitable high temperature promotes the production of high quantity total lipid yield and high quality (high saturation degrees) of fatty acids suitable for biodiesel production (Juneja et al., 2013; Cheng & He, 2014). In several studies, some microalgae, including *Ochromonas danica* and

Nannochloropsis oculata, have been found to increase their lipid content to 37 and 89%, respectively with increasing temperature (Converti et al., 2009; Cheng & He, 2014).

Table 3: Cardinal temperatures (Tmin - minimum temperature, Topt - optimum temperature, Tmax - maximum temperature) and optimal growth rates (μopt) for 14 microalgae species (Ras et al., 2013).

Species	**Reference**	**Tmin**	**Topt**	**Tmax**	**μopt**
Asterionella formosa	Butterwick, Heaney and Talling (2005)	-7.3	20.1	29.8	1.6
Ceratium furca	Baek, Shimode, Han and Kikuchi (2008)	8.4	24.4	32.1	0.6
Ceratium furcoides	Butterwick et al. (2005)	6.9	22.3	30	0.3
Ceratium fusus	Baek et al. (2008)	4.2	26.5	30.7	0.5
Chlorella pyrenoidosa	Sorokin and Krauss (1962)	5.2	38.7	45.8	2.0*
Cryptomonas marssonii	Butterwick et al. (2005)	-2.4 15.9 30.3 0.8	15.9	30.3	0.8
Dinobryon divergens	Butterwick et al. (2005)	-5.8	17	28.4	0.7
Dunaliella tertiolecta	Eppley and Sloan (1966); Eppley (1972); Ukeles (1961)	5.0	32.6	38.9	3.9*
Nannochloropsis oceanica	Sandnes, Källqvist, Wenner and Gislerød (2005)	-0.2	26.7	33.3	1.8*
Phaeodactylum tricornutum	Fawley (1984)*; Kudo Miyamoto, Noiri and Maita (2000);	-27.7	22.5	25.2	1.8*
Porphyridium cruentum	Dermoun, Chaumont, Thebault and Dauta (1992)	5.8	19.1	30	1.3
Scenedesmus	Xin, Hong-Ying and Yu-Ping (2011)	3.1	26.3	32.7	0.8
Skeletonema costatum	Butterwick et al. (2005)	8	24.5	33	1
Tychonema bourrelyi	Butterwick et al. (2005)	0.4	21.8	30	1

* Condition providing highest growth rate for this species Bernard and Remond (2012).

A study on *Nannochloropsis oculata* and *Chlorella vulgaris* both with the optimum growth temperature of 25 °C, showed that increasing the growth temperature from 20 to 25 °C doubled the lipid content from 7.90 to 14.92 % in *Nannochloropsis oculata* and that the increase of temperature from 25 to 30 °C in *Chlorella vulgaris* decreased the lipid content from 14.71 to 5.90 % (Converti et al., 2009). Furthermore, it was demonstrated that temperature also influences the level of carbohydrates, proteins and pigments in microalgae. A study by Ogbonda, Aminigo and Abu (2007) demonstrated a 50 % increase in the carbohydrate content of *Spirulina* when the temperature was increased from 25 to 40 °C (Cheng & He, 2014). Konopka and Brock (1978) found that increasing temperature beyond the optimum reduced protein synthesis consequently resulted in decreased growth rates (Juneja et al., 2013). A study on a marine diatom *Phaeodactylum tricornutum,* by Morris et al. (1974) showed a considerable increase in protein synthesis rates at night with lower temperatures (Juneja et al., 2013). Accessory pigments, such as carotenoid in microalgae species increase with temperature because of the increased oxidative and photo-damaging effects noted at elevated temperatures (Tjahjono et al., 1994; Liu & Lee, 2000; Tripathi, Sarada & Ravishankar, 2002; Juneja et al., 2013). Tjahjono et al. (1994) studied the increase of astaxanthin formation in the green alga *Haematococcus pluvialis and* reported a three times increase with an increase in cultivation temperature of 20 to 30 °C (Juneja et al., 2013). Similarly, a study on a different green alga (*Chlorococcum* sp.) confirmed a two times increase in total carotenoid content by raising the temperature from 20 to 35 °C under conditions of nitrogen deprivation (Liu & Lee, 2000; Juneja et al., 2013). Increase in carotenoid

formation with increasing temperature is generally attributed to cellular response to enhanced active free oxygen radical formation (Tjahjono et al., 1994) or increased biosynthetic enzyme activity (Liu & Lee, 2000; Juneja et al., 2013).

2.3.8 Effect of pH on microalgae

pH is regarded as an indicator of water quality and, therefore, microalgae blooms (Sirunda & Mazvimavi, 2014). This is because it facilitates the solubility and availability of CO_2, essential nutrients such as ammonia, heavy metals as well as salts that affect metabolism and growth of microalgae as well as other aquatic organisms (Junenja et al., 2013; Cheng, & He, 2014; Ren, 2014; Sirunda & Mazvimavi, 2014). In natural waters free from pollution the pH range is generally from 6.5 to 8.5 (Shinana, 2011). However, in waters affected by pollution, mostly due to anthropogenic activities such as mining, agriculture, industrial effluents and acidic precipitation pH may fluctuate and can be as high as 9.0 (Shinana, 2011; Sirunda & Mazvimavi, 2014). The lethal effect of pH on aquatic organisms occurs when the pH is below 4.5 and when it is above 9.5 (Sirunda & Mazvimavi, 2014). The effect of pH is strain or species specific in microalgae cultures with an optimal range for most growth occurring between 8.2 and 8.7 (Pedersen & Hansen, 2003; Havlik, Lindner, Scheper & Reardon, 2013; Cheng, & He, 2014). However, Juneja et al. (2013) suggested it to be around neutral pH (7.0–7.6) for species such as *Ceratium lineatum*, *Heterocapsa triquetra* and *Prorocentrum minimum* (Hansen, 2002) and *Chlamydomonas applanata* (Visviki & Santikul, 2000).

pH is the major determining factor of relative concentrations of the carbonaceous species in water (Azov, 1982; Juneja et al., 2013). Due to the uptake of inorganic carbon (atmospheric CO_2) by microalgae during the day for photosynthesis and release of CO_2 by respiration by other aquatic organisms at night, pH tends to rise significantly in microalgal cultures and in nature (Juneja et al., 2013; Cheng, & He, 2014; Ren, 2014; Sirunda & Mazvimavi, 2014). This increase of carbon leads to the precipitation of carbonate salts, whilst low pH increases the concentration of carbon dioxide and carbonic acid concentration (Sirunda & Mazvimavi, 2014). Higher pH limits the availability of carbon from CO_2, which, in turn, suppresses microalgal growth (Azov, 1982; Chenl & Celia, 1994; Juneja et al., 2013). Therefore, since microalgae capture CO_2, the growing condition becomes alkaline (Ren, 2014). Alkaline pH increases the flexibility of the cell wall of mother cells, which prevents its rupture and inhibits autospore release, thus increasing the time for cell cycle completion which allows microalgae to produce more biomass (Guckert & Cooksey, 1990; Zang et al., 2011; Juneja et al., 2013; Ren, 2014). Increase in pH allows the carbon for the microalgae to be available in the form of carbonates (Juneja et al., 2013), since dissolved CO_2 in the water transitions to HCO_3^-, which is a weak acid (Ren, 2014). However, according to Liu et al. (2005), the chlorophyll concentration of microalgae will decrease when the pH value rises from 8.5 to 9.5 (Ren, 2014). Higher pH also lowers the affinity of microalgae to free CO_2 (Azov, 1982; Rotatore & Colman, 1991; Juneja et al., 2013).

In photoautotrophic cultures for example replacement of CO_2 taken up for photosynthesis is slower resulting in a decrease of CO_2 partial pressure and thus leading

to an increase in pH (Pruder & Bolton, 1979; Juneja et al., 2013). Therefore, change in pH of media may limit microalgal growth via metabolic inhibition (Juneja et al., 2013). Furthermore, alkaline pH indirectly results in an increase in triacylglycerol accumulation, but a decrease in membrane-associated polar lipids because of cell cycle inhibition (Juneja et al., 2013). For example in *Chlorella* the membrane lipids were observed to be less unsaturated under conditions of alkaline pH (Guckert & Cooksey, 1990). Under alkaline conditions when the extracellular pH is higher than intracellular pH, the cell must rely on active transport of HCO_3^- and not on passive flux of CO_2 for inorganic carbon accumulation (Azov, 1982; Moazami-Goudarzi & Colman, 2012; Juneja et al., 2013). A study on the effects of pH on carbon uptake of *Chlamydomonas reinhardtii* reported an efficient utilization of CO_2 for photosynthesis at lower pH (< 6.95) (Moroney & Tolbert, 1985; Juneja et al., 2013). However, at high external pH (6.95 – 9.5), where HCO_3^- dominates, microalgae cannot efficiently accumulate carbon and require high supply of carbonates for maintaining photosynthetic activity (Juneja et al., 2013).

There is an affinity of microalgae for CO_2 increases at lower pH (Azov, 1982; Rotatore & Colman, 1991; Juneja et al., 2013) and, therefore, acidic conditions can also alter nutrient uptake (Gensemer, Smith & Duthie, 1993; Juneja et al., 2013) or induce metal toxicity (Sunda, 1975; Anderson & Morel, 1978) and, therefore, affect microalgal growth. Carbon accumulation is thought to be accomplished by the passive movement of CO_2 along a pH gradient into the cell or chloroplasts in more acidic environments. This is where the internal pH exceeds that of the surrounding medium and where CO_2

comprises a major portion of the total external inorganic carbon (Colman, Huertas, Bhatti & Dason, 2002; Juneja et al., 2013; Ramanan et al., 2012). Studies by Hargreaves and Whitton (1976) showed the effects of low pH on the morphology of five microalgal species (Juneja et al., 2013). These showed that acidic conditions (pH 1.3 - 1.5) limit the motility of cells in *Chlamydomonas applanata* var. *acidophila* and *Euglena mutabilis* (Juneja et al., 2013).

A study on the effect of external pH on photosynthesis of *Coccochloris peniocystis* found a significant decrease in total accumulated carbon and oxygen evolution at pH 5.0 and 6.0 (Juneja et al., 2013). This decrease suggested the reduction in photosynthesis in this cyanobacterium at these pH ranges (Coleman & Colman, 1981; Juneja et al., 2013). Acid tolerant microalgae such as *Chlorella saccharophila* (Gehl & Colman, 1985) and *Euglena mutabilis* (Lane & Burris, 1981) tend to change their intracellular pH in response to changing external pH (Juneja et al., 2013). For example, *Chlorella saccharophila* maintained an internal pH of 7.3 when the external pH range was 5.0 - 7.5. Nevertheless, decreasing the pH further to 3 caused a decrease in cellular pH to 6.4 (Gehl & Colman, 1985; Juneja et al., 2013). Similar results were observed for *Euglena mutabilis* (Lane & Burris, 1981).

Halophyte microalgae like *Dunaleilla acidophila* adapt to acidic conditions in growth media by accumulating glycerol to prevent the osmotic imbalance caused by high concentrations of sulphuric acid (Fuggi, Pinto, Pollio & Taddei, 1988), whilst other species such as *Chlamydomonas* sp. and *Pinnilaria braunii* var. *amplicephala,* an acidophilic diatom, accumulate storage lipids such as triacylglycerides under highly

acidic conditions (pH 1) (Tatsuzawa, Takizawa, Wada & Yamamoto, 1996; Juneja et al., 2013). Another observation under acidic conditions is an increase in saturated fatty acid content, which reduces membrane fluidity and inhibits high proton concentrations (Tatsuzawa et al., 1996; Juneja et al., 2013). This was observed in a *Chlamydomonas* sp. in which total fatty acid content increased from 2 % at pH 7 to 2.4 % at pH 2.7 (Poerschmann, Spijkerman & Langer, 2004; Juneja et al., 2013).

Several scientists over the years studied the implication of pH changes on marine microalgae growth rates. A study on marine centric diatom, *Thalassiosira pseudonana* cells usually adapted to a low pH of 6.5 showed lower growth rates at sub-optimal pH of 8.8 (Pruder & Bolton, 1979). The pH had to be readjusted and ultimately lowered by adding HCl to restore its normal growth rate (Juneja et al., 2013). Similarly, a study by Chenl and Celia (1994) confirmed this by showing that photosynthetic rate and microalgae growth was minimal at pH 9.0, but carbon uptake rates were enhanced when the pH was lowered to 8.3 (Juneja et al., 2013). Another study by Visviki and Santikul (2001) showed that the growth of *Chlamydomonas applanata* within a pH range 1.4 to 8.4 with 1 point increments had no growth from pH 1.4 to 3.4, above which tolerance of pH in *Chlamydomonas applanata* was observed with optimum growth observed at 7.4 (Juneja et al., 2013). Exponential growth was observed for up to five days at pH 5.4 to 8.4, but maximum growth was achieved at pH 7.4 (Juneja et al., 2013). In a study on *Chlamydomonas acidophila* at pH 4.4, it was observed that hydrogen ions denature V-

lysin, a proteolytic enzyme that facilitates releasing of daughter cells from within the parental wall (Visviki & Palladino, 2001; Juneja et al., 2013).

2.3.9 Effects of Salinity on microalgae

Salinity tolerance of microalgae is species dependent (Ren, 2014). However, since all microalgae species have their optimal growth salinity that can increase during hot weather conditions due to high evaporation, exposure to lower or higher salinities can change growth rates. Excess salinity inhibits photosynthesis, thus reduces the yield and alters cell composition (Mata et al., 2010; Cheng & He, 2014; Ren, 2014). Salinity changes generally affect microalgae in three ways: (1) osmotic stress, (2) ion (salt) stress, and (3) changes of the cellular ionic ratios due to the membrane selective ion permeability (Moheimani, 2005; Mata et al., 2010). Salinity can be controlled in various ways in microalgae cultures. Under low salinity it is advised to add NaCl and Na_2SO_4 (Ren, 2014) and during high salinity to simply add freshwater as required (Mata et al., 2013; Ren, 2014). However, when the salinity is higher than 6 g/L, the growth rate of microalgae will indefinitely be prohibited (Liu et al., 2008; Ren, 2014).

Higher salinity increases microalgae lipid content (Fábregas, Abalde, Herrero, Cabezas & Veiga, 1984; Ben-Amotz, Tornabene & Thomas, 1985; Renaud et al., 1994; Zhila, Kalacheva & Volova, 2011; Juneja et al., 2013). In a marine microalga *Dunaliella,* an increase in saturated and monounsaturated fatty acids was observed with an increase in NaCl concentration from 0.4 to 4 M (Xu & Beardall, 1997). An increase in intracellular lipids (60 to 67 %) and triacylglycerol concentration (40 to 56 %) with an increase in

NaCl concentration from 0.5 (freshwater) to 1.0 M was observed in *Dunaliella tertiolecta* (Takagi & Yoshida, 2006). In a study with the fresh water microalgae *Botryococcus braunii*, increasing the NaCl level of cultures showed an increase in growth rate, carbohydrate content and lipid content (Juneja et al., 2013). These cultures exhibited the greatest biomass concentration at the lowest salinity level (Rao, Dayananda, Sarada, Shamala & Ravishankar, 2007). A similar study confirmed these results in the same microalgae when the lipid content of microalgae grown in 0.5 M NaCl was higher compared to media without NaCl addition (Juneja et al., 2013). However, protein, carbohydrates, and pigments levels were lower (Ben-Amotz et al., 1985). For the same microalgae in a different study, increased salinity resulted in a decrease in protein content and unchanged carbohydrate and lipid content (Vazquez-Duhalt & Arredondo-Vega, 1991).

2.3.10 Effects of Biotic factors on microalgae

Microalgae are affected by various biotic factors throughout their life-cycle within their respective ecosystems. These factors directly impact on species abundance and diversity. Therefore, it is important to consider the type of species, their abundance and respective interconnectivity they assume with each other (intra-specific interactions) as well as other microalgae and organisms (inter-specific interactions) within an ecosystem. Grazers as well as inter- and intraspecific competition are important in determining the temporal variability of individual species.

Viruses exist everywhere in the natural environment and affect and control microalgae populations (Bergh, Børsheim, Bratbak & Heldal, 1989; Fuhrman & Suttle, 1993; Beltrami & Carroll, 1994; Usher et al., 2014). Viruses can have adverse effects on microalgal commercial entities such as aquaculture in two ways: (1) lead to a population collapse, resulting in loss of the product and (2) have a knock on effect on the supply chain for which the microalgal produce was intended (Usher et al., 2014). However, these viruses can still be utilized in permissible quantities in microalgal cultures to control blooms (Usher et al., 2014). Other pathogens such as bacteria can coexist with microalgae and sometimes even form symbiotic relationships (Ramanan, Kim, Cho, Oh & Kim, 2016). However, they can be problematic in effluents entering open pond microalgal cultures such as waste streams, particularly municipal or animal waste, where they can enter the harvested biomass and, therefore, affect the final effluent process and end product (Curtis, Mara & Silva, 1992; Usher et al., 2014). This will affect the end use of the microalgae product and require post-treatment before it can be used in any product due to potential health risks (Usher et al., 2014). There are also occupational health hazards for those managing the microalgal farms when bioactive or toxin producing cyanobacteria are present (Usher et al., 2014). There may also be an element of parasite (i.e. *Amoeboaphelidium protococcarum*) infestation in microalgal culture that may compromise its health (Usher et al., 2014). These parasites are diverse and, therefore, require further research to protect microalgal cultures (Letcher et al., 2013; Usher et al., 2014).

Most problems experienced in both fresh and marine natural waters are the development of blooms by unwanted or toxic species such as the blue-green microalgae and dinoflagellates (red tides) (Mata et al., 2010). The resulting damage inflicted by these upon other aquatic organisms and humans is by means of producing high level biotoxins that are detrimental in various ways (Mata et al., 2010) (Tables 10 & 11). These examples include the development of massive green chlorococcalean microalgae (i.e. *Synechocystis* and *Microcystis)* in freshwater and marine diatom *Phaeodactylum* which is unpalatable for bivalve molluscs (De Pauw, Morales & Persoone, 1984; Mata et al., 2010).

Freshwater cyanobacteria or blue-green algae blooms (BGA) such as *Microcystis* and *Anabaena* or as they are commonly known, have advantages over other microalgae in that they have vesicles within their cells that allow them to move up and down to access more favorable growth conditions in the euphotic zone (Sirunda & Mazvimavi, 2014). Additionally they require only very little nutrients since they are able to easily fix nitrogen from the atmosphere and require only about 0.001 mg/L of phosphate to form a bloom (Sirunda & Mazvimavi, 2014). However, during turbulence these cyanobacteria are outcompeted and outnumbered by other microalgae due to the deep mixing that surpasses the euphotic zone (Sirunda & Mazvimavi, 2014).

Attempts made to cultivate some microalgae species in raceway ponds have failed, since cultures collapse due to predation by protozoa and contamination by other microalgal species (Mata et al., 2010). The most feasible way to culture microalgae to avoid predation, contamination and competition would be in highly controlled closed systems

i.e. high nutritional, pathogen free feedstock with reduced risk of introducing unwanted pathogens (Richmond, 2004; Mata et al., 2010). However, monocultures are susceptible to infestation by viruses, bacteria, fungi, and exposure to predators such as protozoan, rotifers, crustaceans, and even microplantonic larvae of benthic organisms (Mata et al., 2010).

There are numerous ways to deter microalgae collapse from these unpredictable factors (Mata et al., 2010). One way would be to maintain cultures in duplicates (Borowitzka, 1997). Another would be to induce and manage microalgal blooms completely separated from their consumers, which involves firstly inducing a bloom of natural phytoplankton and then following that by the introduction of consumers (De Pauw et al., 1984; Mata et al., 2010). The third would be to directly use live microalgae or indirectly in a concentrated form after harvesting and preserving (i.e. as additives in pelleted food) (Mata et al., 2010). Fourthly, Moheimani (2005) and Mata et al. (2009) suggested a way to decrease contaminants and improve yield by temporarily exposing the culture to extreme change of environmental factors such as temperature, pH, or light after removing the unwanted organism (Mata et al., 2010). Finally, Kan and Pan (2010) advised the use of antibiotics, including ampicillin (against red bacteria), Cefotaxine (against white bacteria) and Carbendazim (against fungal infections), especially when working with wild strains. However, this method may deem futile as some bacteria and fungi form resistance towards these antibiotics.

2.4 Microalgae blooms and eutrophication

Eutrophication is a natural process over geological time scale associated with richness of nutrients in water bodies. These nutrients are normally present in low quantities and accumulate gradually over time (Mukherjee, Nivedita & Mukherjee, 2010; Ralph & JI-Dong, 2010). However, over the past 50 years this process accelerated due to anthropogenic carbon enrichment and nutrient influx of mostly phosphorus and nitrogen from industrial, agricultural and domestic wastewater runoff (Andersen, Conley & Hedal, 2004; Fang, 2004; Nhapi & Tirivarombo, 2004, Ralph & JI-Dong, 2010). These excessive influxes of nutrients lead to the proliferation of microalgae which is referred to as microalgae blooms. During a microalgae bloom, many of these species are non-toxic, whilst only a few dozen species are known to produce toxins (Anderson, 2004). The toxicity and array of microalgae toxins (i.e. hepatotoxins, cytotoxins or neurotoxins) produced throughout microalgal life-cycles depends on the type of microalgae species and environmental conditions (Anderson, Glibert & Burkholder, 2002; Shaw, Moore & Garnett, 2003). Due to these environmental spatial variability and type of species present in alternating seasons the presence or absence of these toxins are difficult to predict (Rellán, Osswald, Saker, Gago-Martinez & Vasconcelos, 2009).

Variations in chemical compositions (i.e. inorganic nutrients) are considered indicators in the regulation of abundance, composition, geographical and periodical distribution of microalgae (Yang Yang, Wu, Hao & He, 2008). Redfield's ratio reflects the atomic ratio of C:N:P in microalgae cells and is equal to 106:16:1 (Redfield, 1963; Ren, 2014). Thus,

when the N:P ratio exceeds 16, the concentration of phosphorus is considered as a limiting factor and when nitrogen is less than 16 it needs to be controlled to ensure an optimal growing condition for microalgae. However, different species of microalgae have different atomic ratios in cells, therefore, requirement for nitrogen and phosphorus will be species dependent (Sun et al., 2006; Ren, 2014). Although nitrogen has received great attention due to the large amounts used in fertilizers (Dolan et al., 2007), phosphorus is the most pivotal nutrient in microalgal cell physiology and its increase will undoubtedly lead to increased microalgal growth (Thomas, 1969). Studies by Schindler et al. (2008) concluded that eutrophication cannot be controlled by reducing nitrogen inputs, unless these concentrations are of concern to humans. Scientists examined the relationships between harmful microalgae blooms (HABs) and inorganic nutrient ratios (i.e. N:P and N:Si) (Kuo, 2010). It has been said that growth of these microalgae is influenced by dissolved silicate-Si (DSi) concentration in water and its ratio to nitrate (Yang et al., 2008). Especially since food webs directly linked to them can be compromised (Yang et al., 2008). Dortch, Rabalais, Turner and Qureshi (2001) carried out such a study and concluded that areas with high silica concentrations were found to have *Rhopalodia gibberula* and *Nitzschia palea* as the primary dominant species whereas blue-green microalgae abundance were observed in marine areas with less sewage pollutants.

Blooms of Cyanophyceae or Cyanobacteria genera are the most prolific, notorious and problematic freshwater group because of their advantages over other microalgae (Paerl

Fulton, Moisander & Dyble, 2001). These consist mostly of *Microcystis aeruginosa*, *Oscillatoria rubescens* and *Anabaena flosaquae* (O'Neil, Davis, Burford & Gobler, 2012) (Tables 4 & 5). At high cell densities, these blooms along with blooms of other genera (both toxic and non-toxic) rapidly deplete nutrients, increase turbidity and deplete inorganic carbon (CO_2) supplies and other essential resources, causing a sudden decline in biomass, which is referred to as a "crash" (Paerl, et al., 2001). The crashing of these blooms is followed by decaying, odoriferous, unsightly scums (Paerl et al., 2001). These scums contain a variety of microbial pathogens, causing significant chemical and biological changes, including hypoxia anoxia, release of toxic hydrogen sulfide as a by-product of bacterial decomposition, and accelerated release of nutrients from sediments leading to remineralization which in turn reactivates or causes blooms which further aggravates eutrophication (Paerl et al., 2001; Anderson, 2004; Hudnell, 2008). The resulting hypoxia and anoxia in the water column is stressful to most fauna and fatal to most finfish and shellfish (Steinberg & Hartmann, 1988; Schindler et al., 2008; Donato-Rondon, Morales-Duarte & Castro-Rebolledo, 2010). Ultimately this rapid biological degradation proves fatal and, therefore, leads to large-scale mortalities of other aquatic organisms (Anderson, 2004; Ralph & JI-Dong, 2010).

Furthermore, accumulations and decaying of these microalgae blooms also lead to adverse tastes and odors of affected waters (Paerl et al., 2001). Even after water treatments the presence of microalgae result in unpalatable tastes in potable water and the high load of organic detritus react with the chlorine to form carcinogens known as trihalomethanes (Carpenter et al., 1998; Izaguirre, 2008). The resulting smell and

discolouration in potable and non-potable water have detrimental effects on recreational activities and tourism (Rabalais, Turner, Justic, Dortch & Wiseman 1999). In Namibia, freshwater, manmade dams, including Von Bach, Swakoopoort and Goreangab dams for potable water are the most susceptible to eutrophication. This is due to their lack of turbulence and surrounding perplexities to farms and mines (Shinana, 2011). Therefore, harmful microalgae blooms (HABs) (particularly cyanobacteria) and eutrophication are not only considered as indicators of nutrient over-enrichment (Paerl & Fulton, 2006; Lopez, Jewett, Dortch, Walton & Hudnell, 2008), but also of low water quality (Vezjak, Savsek & Stuhler 1998; Biggs, 2000).

Table 4: Harmful freshwater microalgae, toxins and adverse impacts (Lopez et al., 2008).

Inland HAB Taxa (Specific organisms of concern)	**Toxins**	**Adverse Impacts**
Cyanobacteria (Table 5)	Hepatotoxins, neurotoxins, cytotoxins, dermatotoxins, respiratory and olfactory irritant toxins	Human and animal health impacts; water discolouration, unsightly and foul-smelling scums; hypoxia from high biomass blooms; taste-and-odour problems in drinking water and in farm raised fish
Haptophytes (i.e. *Prymnesium parvum, Chrysochromulina polylepis*)	Ichthyotoxins	Mortalities of fish and other gill breathing species
Chlorophytes Microalgae (i.e. *Volvox, Pandorina*)	None	Discoloured water, localized hypoxia
Macromicroalgae (i.e. *Cladophora*)	None	Unsightly and foul-smelling mats, localized hypoxia, clogged water intakes
Euglenophytes(*Euglena sanguinea*)	Ichthyotoxin	Discoloured water, mortalities of fish (Zimba, Rowan & Triemer, 2004)
Raphidophytes *Chattonella* (marine but blooms in inland saline waters)	Ichthyotoxins	Fish Kills (SSERG, 2001; Reifel et al., 2002)
Dinoflagellates (i.e. *Peridinium polonicum Glenodinium, Gymnodinium*)	Ichthyotoxins	Mortalities of fish (Nolen, 1989; Oshima, Minami, Takano & Yasumoto, 1989; Roset et al., 2002)
Cryptophytes	None	High biomass blooms can cause discoloured water, localized hypoxia
Diatom (*Didymosphenia geminata*)	None	Produces large amounts of extracellular stalk material resulting in ecosystem and economic impacts

Table 5: Cyanobacterial toxins, the freshwater taxa that produce them (Paerl, 2001; Fristachi et al., 2008), and human health effects (Harrness, 2005; Falconer, 2008; Lopez et al., 2008).

Toxin	**Genera**	**Short Term Health Effects**	**Long Term Health Effects**
Microcystins	*Anabaena, Aphanocapsa, Hapalosphon, Microcystis, Nostoc, Oscillatoria, Planktothrix*	Gastrointestinal, liver inflammation, and hemorrhage, pneumonia, dermatitis	Tumor promoter, liver failure leading to death
Nodularins	*Nodularia spumigena*	Similar to Microcystins	Similar to Microcystins
Saxitoxins	*Anabaena, Aphanizomenon, Cylindrospermopsis, Lyngbya*	Tingling, burning, numbness, drowsiness, incoherent speech,	Respiratory paralysis leading to death
Anatoxins	*Anabaena, Aphanizomenon, Oscillatoria, Planktothri*x	Tingling, burning, numbness, drowsiness, incoherent speech, respiratory paralysis leading to death	Cardiac arrhythmia leading to death
Cylindrospermopsin	*Aphanizomenon, Cylindrospermopsis, Umezakia*	Gastrointestinal, liver inflammation and hemorrhage, pneumonia, dermatitis	Malaise, anorexia, liver failure leading to death
Lipopolysaccharide	*Aphanizomenon, Oscillatoria*	Gastrointestinal, dermatitis	Unknown
Lyngbyatoxins	*Lyngbya*	Dermatitis	Skin tumors (Fujiki et al., 1990), unknown
BMAA	*Anabaena, Cylindrospermopsin, Microcystis, Nostoc, Planktothrix*	Degeneration of hippocampal neurons	Potential link to neurodegenerative diseases

2.4.1 Harvesting microalgae blooms for various product applications

Remedial measures have been put in place in some parts of the world to mitigate the onset of eutrophication and subsequent hypoxia. In Namibia for instance, to eradicate microalgae blooms in manmade dams for potable water, powdered activated carbon is added to the water reservoirs before extraction and granular activated carbon to the pipeline after extraction point for adsorption and filtration (N. du Plessis, personal communication, 2014). Additionally, solar bees were added for the sake of mechanical intervention (Meding, 2016) (Figure 6). The solar bees are designed to float on the surface of the dam by operating 24 hours a day whilst powered by solar power (Meding, 2016). The role of the solar bees is to; (1) suppress growth of blue-green microalgae via habitat disturbance, by artificial circulation and causing turbulence of the upper layer of warm water, (2) create conditions that would result in the growth of desirable forms of microalgae and (3) eliminate toxic blue-green blooms (Lakesteilacoomcom, 2016). These results are still pending (Meding, 2016).

Figure 6: Solar bees floating on Swakkoppoort dam, Namibia (Meding, 2016).

Kuo (2010) suggested a new approach for mitigating hypoxia that has a fast response time with the immediate realization of environmental benefits. This was to harvest microalgae blooms prior to the onset of their death phase (Figure 7). This approach immediately decreases the organic content of the water, thus limiting the heterotrophic bacteria growth that leads to hypoxic conditions (Kuo, 2010). The microalgae harvested can be converted into valuable products like biofuel or extract special proteins, which can offset the cost for harvesting microalgae from a hypoxic zone (Sheehan et al., 1998; Akkerman, Janssen, Rocha & Wijffels, 2002; Kuo, 2010). Miao Wu and Yang (2004) even suggested using microalgae harvested from lakes to produce bio-oil via fast pyrolysis and as an environmental solution to reduce microalgae blooms **(**Bowles, 2007). In fact in the past several scientists studied the ability of cyanobacterial species to

produce lipids for biodiesel production (Costa & De Morais, 2011; Karatay & Donmez, 2011; Taher, Al-Zuhair, Al-Marzouqi, Haik & Farid, 2011). After screening five cyanobacteria strains for lipid production, Silva, DaRos, Silva-Stenico, Fiore and Castro (2011) found that the microalgal bloom causing strain *Microcystis aeruginosa* isolated from a sewage plant was the best source for lipid production.

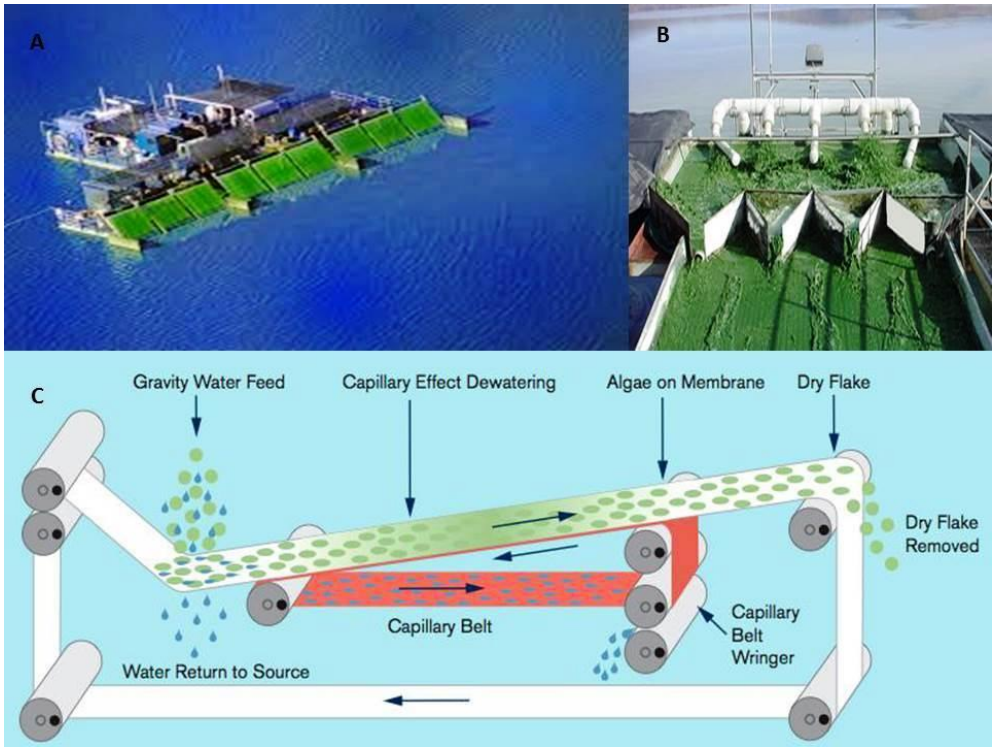

Figure 7: Harvesting models for microalgae (A & B) Microalgae harvester developed by Simplexity Creative on Upper Klamath Lake (Spiritofleadershipinfo, 2016; Powerorganicscom, 2016), (C) AVS-HDD (MicroalgaeVenture systems harvester) (Newenergyandfuelcom, 2016).

In order to understand the effects of harvesting microalgal blooms and converting their biomass into biofuels, Kuo (2010) further evaluated the overall energy balance of the whole process based on a variety of different processing methods, including harvesting methods, conversion processes and end products. The end product was an engineering process model called Harvested Microalgae Biofuel Energy Recovery model (HABER) (Figure 8). This model was developed and implemented in a Microsoft Excel spreadsheet to collect information from a large number of literature sources, calculate the energy balances, and investigate the sensitivity of the energy balance to variations in input parameters (Kuo, 2010). It includes a set of Excel worksheets including input parameters (i.e. microalgae characteristics, harvesting processes, refinery methods and end product values) (Kuo, 2010). These parameter values and ranges have been identified from scientific literature and industrial experience, which draws from a variety of disciplines related to the proposed hypoxia mitigation strategy (Kuo, 2010).

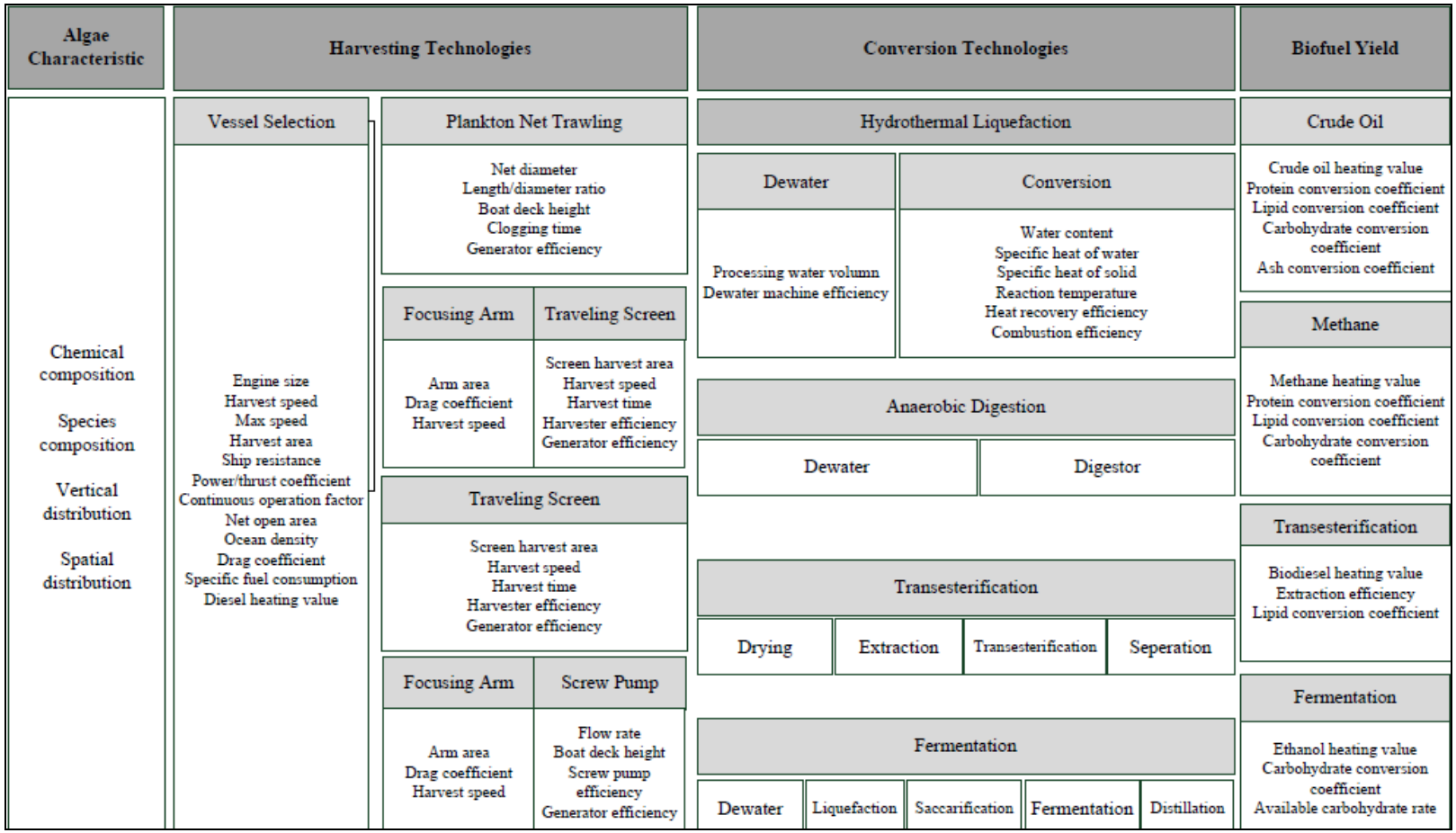

Figure 8: Process scheme of HABER model (Kuo, 2010).

2.5 Microalgae applications

After being exposed to sunlight, inorganic carbon (CO_2 from the atmosphere), organic carbon and nutrients microalgae produce carbohydrates, proteins and lipids (fatty acids) (Metzger & Largeau, 2005; Guschina & Harwood, 2006). Under favorable culture conditions, some microalgae biomasses contain 30 - 50 % proteins, 20 - 40 % carbohydrates and 8 - 15 % lipids (Hu, 2004). Therefore, microalgae to product application have received tremendous attention worldwide (Bowles, 2007). Microalgae assimilate excess nutrients and produce dissolved oxygen for bacteria to degrade hazardous organic pollutants in wastewater (Munoz & Guieysse, 2006; Perales-Vela, Pena-Castro & Canizares-Villanueva, 2006; Bowles, 2007; Hameed, 2007; Shi, Podola & Melkonian, 2007; Hernandez, de-Bashan, Rodriguez, Rodriguez & Bashan, 2009), remove heavy metals from water (Munoz & Guieysse, 2006; Singh, Mehta & Gaur, 2007), remove xenobioticsin from the environment as well as CO_2 from exhaust gases (Bowles, 2007). Due to these abilities microalgae can act as bioremediators in wastewater treatment (Mallick, 2002; Kalin, Wheeler & Meinrath, 2004; Suresh & Ravishankar, 2004; Munoz & Guieysse, 2006), be nitrogen fixing biofertilizers (Vaishampayan et al., 2001; Hernandez et al., 2009) and provide high protein feedstock for livestock as well as fertilizers for agriculture (Schneider, 2006; Haag, 2007). Since microalgal protein has been estimated to be a considerable profile of amino acids, in 2007, 30 % of the global microalgae cultures were cultured for animal feed (Becker, 2007; Gross, 2013). Still under the same token of microalgae usefulness, heterotrophic microalgae although sometimes outnumbered by bacteria because of their lower growth

rates, can be combined in consortia with CO_2 mitigation to clean up wastewaters (Bowles, 2007). Eventually the biomass yield produced from this process can be used to produce high value products such as chemicals, biofuels and biogas as by-products (Munoz & Guieysse, 2006; Bowles, 2007). Table 6 summarizes the various product applications from microalgae.

Table 6: Microalgae species and their commercial applications (Pulz & Gross, 2004; Bowles, 2007).

Species/group	**Product**	***Application Areas**	**Production facilities**	**References**
Spirulina (*Arthrospira platensis)*/Cyanobacteria)	Phycocyanin, *Biomass	Health food, Cosmetics	Open ponds, natural lakes	Lee (2001) ;Costa, Colla and Filho (2003)
Chlorella vulgaris/Chlorophyta	*Biomass	Health food, Food supplement, feed surrogates	Open ponds, basins, glass tube photo bioreactors (PBR)	Lee (2001)
Dunaliella salina/Chlorophyta	Carotenoids, *β*-carotene	Health food, food supplement, feed	Open ponds, lagoons	Jin and Melis (2003); Del Campo, García-González and Guerrero (2007)
Haematococcus pluvialis/Chlorophyta	Carotenoids, Astaxanthin	Health food, Pharmaceuticals, feed Additives	Open ponds, PBR	Del Campo et al. (2007)
Odontella aurita/Bacillariophyta	Fatty acids	Pharmaceuticals, cosmetics,	Open ponds	Pulz and Gross

		baby food		(2004)
Porphyridium cruentum/Rhodophyta	Polysaccharides	Pharmaceuticals, cosmetics, nutrition	Tubular PBR	Fuentes et al. (1999)
Isochrysis galbana/Chlorophyta	Fatty acids	Animal Nutrition	Open ponds, PBR	Grima, Pérez, Camacho, Sevilla and Fernandez (1994); Pulz and Gross (2004)
Phaedactylum Tricornutum/Bacillariohyta	Lipids, fatty Acids	Nutrition, fuel Production	Open ponds, basins, PBR	Yongmanitchai and Ward (1991); Fernández et al. (2003)
Lyngbya Majuscule/Cyanobacteria	Immune Modulators	Pharmaceuticals, Nutrition		Singh, Kate and Banerjee (2005)
Muriellopsis/Chlorophyta	Carotenoids, Lutein	Health food, food supplement, feed	Open ponds, PBR	Blanco Moreno, Del Campo, Rivas and Guerrero (2007); Del Campo et al. (2007)

*All application areas in the table are for humans, unless stated otherwise. * Biomass on the table refers to the overall organic material generated by microalgae at a given time. This includes a composite of lipids, carbohydrates, proteins, pigments and or any other material it comprises of.

2.5.1 Microalgae to biofuels

Literature portrays clean-burning fuels produced from grease, vegetable oils, or animal fats as alternatives for the worlds' diminishing petroleum reserves (Srivastava & Prasad, 2000; Hossain et al., 2008; Vasudevan & Briggs, 2008; Gouveia & Oliveira, 2009). Although progress is being made in the production of alternatives, crude oil from fossil fuels still represent 70 % of total global energy requirements in transportation, manufacturing and household heating and thus remain the main source of liquid fuels (Gouveia & Oliveira, 2009). The lipids produced during biosynthesis throughout the microalgae growth process can be refined into any type of hydrocarbon such as petrol or diesel (Banerjee, Sharma, Chisti & Banerjee, 2002; Metzger & Largeau, 2005; Guschina & Harwood, 2006). Some theories even attribute the origin of petroleum, oxygen and natural gas to diatoms (Zahng, 2010). Studies by Demirbas and Demirbas (2011) confirmed 50 % lipid of total dry weight of microalgae, while Amin (2009) demonstrated 80 % lipid content (Table 7).

Microalgae lipids have been considered as a potential substitute for diesel fuel due to their lower degree of unsaturation and accumulation in microalgae cells at the end of the growth stage (Malapascua, Chou, Lu & Lan, 2012). The emphasis is placed on neutral intracellular lipids which accumulate in lipid vesicles called oil bodies in the cytoplasm (De la Pena, 2007). These are either composed of a glycerol molecule bound to three fatty acids, known as triacylglycerol (TAG), or to two fatty acids with the third position taken up by a phosphate (phospholipids) or carbohydrate (glycolipids) group (Abdo, Ahmed, El-Enin, El Din & Ali12, n.d). The triacylglycerols serve as fatty acid energy

reserves and the cholesteryl esters act as storage depots for excess cellular cholesterol (Abdo et al., n.d). These lipids have been observed to show great potential at values of 75 000 and 60 000 kg ha^{-1} $year^{-1}$ for oil and biodiesel yield, respectively, when compared to plant lipid sources (Moazami, Ranjbar, Ashori, Tangestani & Nejad, 2011). Moazami et al. (2011) successfully demonstrated a large-scale biodiesel production using microalgae in a three raceway ponds of 2000 L capacity each.

Table 7: Lipid productivity of various microalgae species (Hu et al., 2008; Kumar & Sharma, 2014)

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

The proverbial as to why microalgae are considered to be an ideal source for biofuel production is primarily due to their multifarious advantages over other plants. Compared to other plants or feedstock of agriculture origin microalgae require much less land for cultivation. Microalgae require 49 to 132 times less land when compared to rapeseed or

soya crops for the production of 30 % (w/w) of lipid content in microalgae biomass (Chisti, 2007). For example, microalgae have the potential to give the highest yield of approximately 1 36 900 L/ha/yr on 2 Mha land when compared to terrestrial plants like *Jatropha* that have a lipid productivity of 1892 L/ha/yr on 140 Mha of land (Kumar & Sharma, 2014) (Table 8).

Chemical composition or fatty acid profile of bio crude oil vary depending on the type of renewable feedstock (i.e. microalgae and crops), technology and operating conditions (culture conditions, the growth stage of the culture or the species) (Chen et al., 2008; Li et al., 2008b). Chen, Landsman-Roos, Naughton and Olenyik, 2008 and Li et al (2008b) reported that lipids and or bio crude oil produced from microalgae are better in quality by application when compared to lipids produced from any other terrestrial crop. This is especially the case in internal combustion application where it can be blended with fossil diesel or natural gas, in diesel and gas-turbine engines, and external combustion applications in steam cycles, organic rankine cycle as well as stirring engines. Additionally, optimal culture conditions for the desired fatty acid profile can be easier regulated during the microalgae growth in photo bioreactors than for oil crops (Schlagermann et al., 2012). A number of microalgae strains have been studied and tested throughout the years to demonstrate this ability (Dunahay, Jarvis, Dais & Roessler, 1996; Sheehan, Dunahay, Benemann & Roessler, 1998; Banerjee et al., 2002; Gavrilescu & Chisti, 2005) (Table 9).

Table 8: Lipid productivity of microalgae and other feedstock for biodiesel production (Nielsen, 2008; Reijnders & Huijbregts, 2008; Kheira & Atta, 2009; Cenciani, Bittencourt-Oliveira, Feigl, & Cerri 2011; Rajvanshi & Sharma, 2012; Kumar & Sharma, 2014).

Lipid feedstocks	**Lipid content (% dry wt. biomass)**	**Lipid yield (L oil/ha/year)**	**Land use (m^2/year/L biodiesel)**	**Biodiesel productivity (L biodiesel/ha/year)**
Microalgae (low lipid content)	30	58 700	0.2	61 091
Microalgae (medium lipid content)	50	97 800	0.1	1 01 782
Microalgae (high lipid content)	70	1 36 900	0.1	1 42 475
Corn/Maize (*Zea mays* L.)	44	172	56	179
Hemp (*Cannabis sativa* L.)	33	363	26	378
Soyabean (*Glycine max* L.)	18	636	15	661
Jatropha (*Jatropha curcas* L.)	28	741	13	772
Camelina (*Camelina sativa* L.)	42	915	10	952
Canola/Rapseed (*Brassica napus* L.)	41	974	10	1 014
Sunflower (*Helianthus annuus* L.)	40	1070	9	1113
Caster (*Ricinus communis*)	48	1307	8	1360
Palm oil (*Elaeis guineensis*)	36	5366	2	5585

Table 9: Microalgae species selected for biodiesel production (Bowles, 2007).

Species	**Lipid content (%dw)**	**Reference**
Ankistrodesmus TR-87	28 - 40	Ben-Amotz et al. (1985)
Botryococcus braunii	29 - 75	Sheehan et al. (1998); Banerjee et al. (2002); Metzger & Largeau (2005)
Chlorella sp.	29	Sheehan et al. (1998)
Chlorella protothecoides (autotrophic/ heterothrophic)	15 - 55	Xu et al. (2006)
Cyclotella DI-35	42	Sheehan et al. (1998)
Dunaliella tertiolecta	36 - 42	Kishimoto et al. (1994); Tsukahara & Sawayama (2005)
Hantzschia DI-160	66	Sheehan et al. (1998)
Isochrysis sp.	7 - 33	Sheehan et al. (1998); Valenzuela-Espinoza, Millán-Núñez and Núñez-Cebrero (2002)
Nannochloris	31(6 - 63)	Ben-Amotz and Tornabene (1985); Negoro, Shioji, Miyamoto and Micira (1991); Sheehan et al. (1998)
Nannochloropsis	46 (31 - 68)	Sheehan et al. (1998); Hu and Gao (2006)
Nitzschia TR-114	28 - 50	Kyle and Gladue (1991)
Phaeodactylum tricornutum	31	Sheehan et al. (1998)
Scenedesmus TR-84	45	Sheehan et al. (1998)
Stichococcus	33 (9 - 59)	Sheehan et al. (1998)
Tetraselmis suecica	15 - 32	Chisti (2007); Sheehan et al. (1998); Zittelli, Rodolfi, Biondi and Tredici (2006)
Thalassiosira pseudonana	(21 - 31)	Brown, Dunstan, Norwood and Miller (1996)

2.5.2 *Nannochloropsis* species

Nannochloropsis limnetica belongs to the phylum Heterokontophyta, class Eustigmatophyceae, family Eustigmataceae and species *Nannochloropsis*. So far, it is the only known freshwater species of this group. The rest are marine and comprise of *Nannochloropsis gaditana*, *Nannochloropsis granulata*, *Nannochloropsis oceanica*,

Nannochloropsis oculata and *Nannochloropsis salina* (Hibberd, 1981). The marine species have garnered great interest in research and outdoor microalgae cultivation due to their rapid growth rates and high amounts of PUFAs of especially EPA (De Pauw & Persoone, 1988; Dragone, Fernandes, Vicente & Teixeira, 2010). The fatty acid compositions found in these species are not found in any other types of phytoplankton (Apt & Behrens, 1999). These can be increased and accumulated under stressful conditions (Rodolfi et al., 2009).

Since fish are unable to synthesize omega 3 fatty acids they consume and accumulate these from such microalgae (Kanazawa & Gordon, 1985; Pigott, Pigott, Pigott & Pigott, 1989). Therefore, these species serve as an important food source for an array of organisms. This is portrayed in their use as feedstock in mariculture (i.e. rotifers and fish hatcheries). Other applications include hens fed on *Nannochloropsis* sp. showing an increase in the omega three fatty acid content in their egg yolk (Nitsan, Mokady & Sukenik, 1999). Due to this, research on this group of species has beens mostly focused on the lipid and biomass optimization of marine *Nannochloropsis* spp.. Section 2.3 summarizes the wealth of this research.

N.limnetica is fairly knewly discovered picoplankton (< 3 mm in diameter) in this group. It was first reported in highly productive lakes in Germany (Krienitz, Hepperle, Stich & Weiler, 2000). Their high abundance and contribution to primary production of stagnant inland lakes and ponds at different trophic level is due to their effective volume-to-surface ratio (Stockner, 1991; Hepperle & Krienitz, 2001). They were discovered to be the most dominant picoplankton taxon in the hypertropic village pond Dorfteich

Schwarz, the polytrophic Lake Roter See in Germany (Krienitz et al., 2000), the mesotrophic Lake Itasca, Minnesota, USA (Fawley et al., 2005) and the oligotrophic Lake Baikal, Russia (Fietz et al., 2005). Freire et al (2016) carried out a study comparing the nutritional value of *Nannochloropsis limnetica* to that of *Nannochloropsis gaditana* and *Chlorela vulgaris* to the productivity of rotifer *Brachionus plicatilis*. The study found that although *Nannochloropsis limnetica* and *Nannochloropsis gaditana* shared an almost identical fatty acid profile, cultures with high microalgae rations, growth and egg-ratios of the rotifer *B. plicatilis* cultured with *N. limnetica* were more than twice that with the same doses of *N. gaditana*. The results further deliberated that *N. limnetica* has the potential to substitute freshwater *Chlorella* in live-feed production protocols, due to its better fatty acid profile. Krienitz & Wirth (2006) studied and compared the fatty acid composition of *Nannochloropsis limnetica* against other highly abundant and productive green microalgae picoplankton species from freshwater lakes such as *Choricystis minor* and *Pseudodictyosphaerium jurisii* and three key nanoplanktonic green microalgae taxa of importance for biotechnology and aquaculture (*Chlorella vulgaris*, *Scenedesmus obtusiusculus*, *Monoraphidium braunii*). It was found that the sums of n-6 and n-3 fatty acids in *N. limnetica* were ten times higher than that of the picoplankton, and higher than in the nannoplankton.

2.5.3 Production systems

There are several modes of growing, cultivating and harvesting microalgae depending on the purpose of the production facility (Bowles, 2007). Lab scale photo bioreactors (PBRs) or closed systems used to cultivate microalgae (Richmond, 2004) will be utilized for this study (Figure 9). Although cost prohibitive when considering commercialization due to high capital costs (Borowitzka, 1999), they eliminate many problems experienced with open ponds and raceway systems (Pulz, 2001; Bowles, 2007) (Table 10). They are ideal for monocultures or axenic cultures for producing tailored or specific products. Water, nutrients and CO_2 are provided in a controlled way, whilst oxygen is removed (Bowles, 2007). Sunlight is provided either directly through the transparent container walls or via light fibers or tubes that channel it from sunlight collectors (Bowles, 2007).

In contrast to PBRs open pond systems and raceways are shallow ponds in which one or more microalgae strains are cultivated (Bowles, 2007) (Figure 10). Light in this case is provided directly from the sun, CO_2 is consumed from the atmosphere and nutrients are provided from run off or via sewage water. Uniform aeration and mixing is controlled from fitting paddles and or rotating structures to these systems (Chaumont, 1993; Borowitzka, 1999; Bowles, 2007). It was found that production of photosynthetic oxygen by microalgae reduces the need for external aeration of the wastewater, which is useful when volatile pollutants must be biodegraded aerobically, but should not evaporate due to mechanical aeration (Olguin, Galicia Mercado & Pérez, 2003; Munoz & Guieysse, 2006; Bowles, 2007).

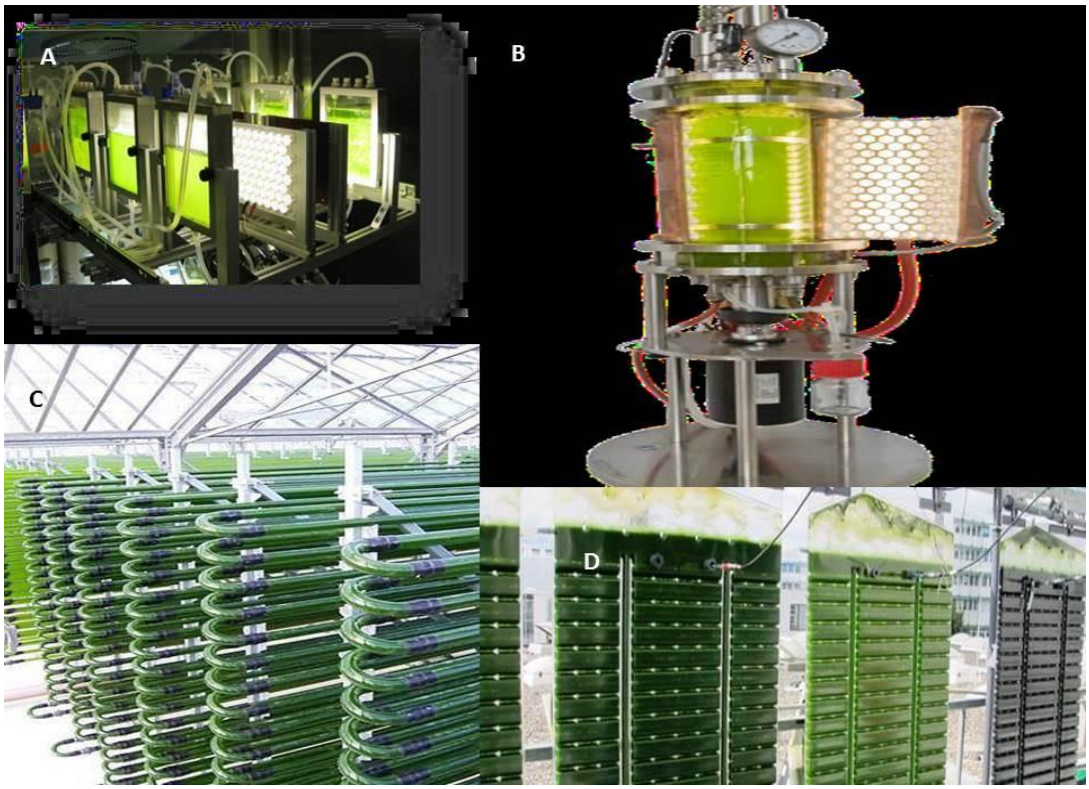

Figure 9: Various photo bioreactors designs. (A) Mini-plate reactor (KIT), (B) Stirring round glass reactor with LED lightning ring (KIT), (C) 30 -liter flat -panel airlift reactors at FraunhoferInstitutin, Stuttgart, Germany, (D) Tubular reactors in Klötze (Germany).

Since there is only 300 - 600 ppm CO_2 in the atmosphere, microalgae growth rates are suppressed because of low carbon source availability (Mata et al., 2010). Heterotrophic microalgae that rely on and utilize other sources of carbon for metabolism (Ward & Singh, 2005) are recommended to be cultivated in conventional fermenters (Bowles, 2007).

Table 10: Open ponds/race ways versus photo bioreactors (PBRs) (Pulz 2001; Bowles, 2007).

Parameter	**Open ponds and raceways**	**Photo bioreactors (PBR)**
Required space	High	PBR itself low
Water loss	Very high may also cause salt precipitation	Low
CO_2-loss	High, depending on pond depth	Low
Oxygen concentration	Usually low enough because of continuous spontaneous outgassing	Build-up in closed system requires gas exchange devices (O_2 must be removed to prevent inhibition of photosynthesis and photo oxidative damage)
Temperature	Highly variable, some control possible by pond depth	Cooling often required (by spraying water on PBR or immersing tubes in cooling baths)
Shear	Low (gentle mixing)	High (fast and turbulent flows required for good mixing, pumping through gas exchange devices)
Cleaning	Low	Required (wall-growth and dirt reduce light intensity), but causes abrasion, limiting PBR lifetime
Contamination risk	High (limiting the number of species that can be grown)	Low
Biomass quality	Variable	Reproducible
Biomass concentration	Low, between 0.1 and 0.5 g/L	High, between 2 and 8 g/L
Production flexibility	Only few species possible, difficult to switch	High, switching possible
Process control and reproducibility	Limited (flow speed, mixing, temperature only by pond depth)	Possible within certain tolerances
Weather dependence	High (light intensity, temperature, rainfall)	Medium (light intensity, cooling required)
Start-up	6 - 8 weeks	2 - 4 weeks
Capital costs	High ~ US $ 100,000 per hectare	Very high ~ US $ 1,000,000 per hectare (PBR plus supporting systems)
Operating costs	Low (paddle wheel, CO_2 addition)	Very high (CO_2 addition, pH-control, oxygen removal, cooling, cleaning,

		maintenance)
Harvesting cost	High, species dependent	Lower due to high biomass concentration and better control over species and conditions
Current commercial applications	5000 t of microalgal biomass per year	Limited to processes for high added value compounds or microalgae used in food and cosmetics

Several scientists (Choi et al., 2003; Janssen et al., 2003; Carvalho et al., 2006; Bowles, 2007; Hankamer et al., 2007) have reviewed photo bioreactors over the years for optimization. The advantages of photo bioreactors over other modes of microalgal culture have been demonstrated in several studies by various scientists. Fernandez et al. (2000) demonstrated that photo bioreactors can overcome the problems of contamination and evaporation encountered in open ponds. Chisti (2007) also demonstrated that the biomass productivity of photo bioreactors can be 13 times more than that of traditional raceway ponds. Despite closed systems offering no advantage in terms of areal productivity, photo bioreactors surpass conventional ponds in terms of volumetric productivity (8 times higher) and cell concentration (about 16 times higher) (Richmond, 2004; Mata et al., 2009). Harvesting of biomass from photo bioreactors is also less expensive when compared to raceway ponds, since the typical microalgal biomass is about 30 times more concentrated than the biomass found in raceways (Chisti, 2007).

Harvesting methods of microalgae biomass depend on the species, cell density, culture conditions and purpose of harvesting (Bowles, 2007). Several conventional harvesting methods include planktonic nets in eutrophic lakes (Lavens & Sorgeloos, 1996) (Figure 11), drying, centrifugation (Heasman, Diemar, O'connor, Sushames & Foulkes, 2000),

foam fractionation (Csordas & Wang, 2004), flocculation (Knuckey, Brown, Robert & Frampton, 2006; Poelman, De Pauw & Jeurissen, 1997), membrane filtration (Rossignol, Lebeau, Jaouen & Robert, 2000), ultrasonic separation (Bosma, Van Spronsen, Tramper & Wijffels, 2003), sedimentation, filtration and ultra-filtration with additional flocculation (Li et al., 2008b; Mata et al., 2010). Many innovative alternatives exist for drying the biomass, but the costs can become prohibitive i.e. contributing 20 – 30 % to the total cost of microalgal biomass (Grima, Belarbi, Fernández, Medina & Chisti, 2003). For instance Weissman and Goebel (1987) studied micro-straining, belt filtering, and flotation with float collection as well as sedimentation. Most common techniques for oil extraction are with organic solvents such as hexane and chloroform, in the presence of ultrasound waves (Craigie & Hellebust, 1978). The biggest drawback of microalgae cultivation in comparison to traditional oil crops is that energy and CO_2 demands may be higher (Schlagermann et al., 2012). However, future research and development efforts are now proposed to mainly focus on the improvement of both the economic feasibility and sustainability to produce biofuels from microalgae (Schlagermann et al., 2012).

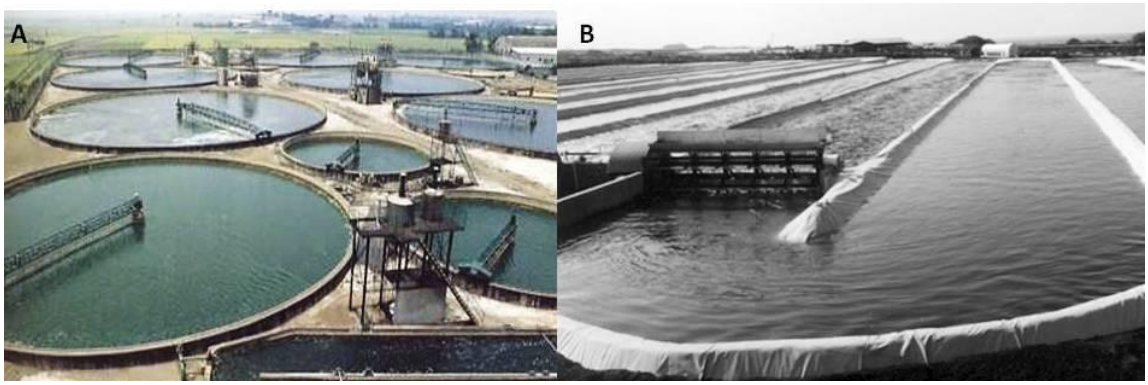

Figure 10: (A) Open ponds (Sucurinet, 2016) and (B) Raceways (Janssen, 2002).

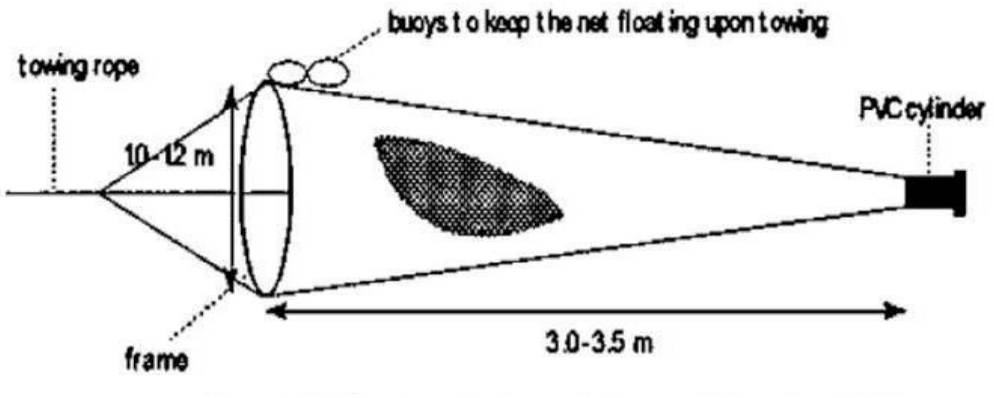

Figure 11: Plantonic net scheme (Lavens & Sorgeloos, 1996).

2.5.4 Economics of microalgae applications

Currently the cost of microalgae lipid production surpasses that of petroleum products (Chisti, 2007). Hannon, Gimpel, Tran, Rasala and Mayfield (2010) elucidated that although biofuels from microalgae provide a viable alternative to fossil fuels, the industry has to overcome economic hurdles to compete with fossil fuels. Capital costs include strain identification, growth rates, biomass processing (i.e. harvesting,

dewatering), lipid content and extraction from biomass as well as the scale of production systems (Chisti, 2007; Harvey et al., 2012). Other costs include operations and maintenance (i.e. expenses for nutrients (i.e. N-P-K), CO_2 distribution, water replenishment (due to evaporation losses), utilities, components replacement, and labour costs) (Harvey et al., 2012). Additional costs are spread across engineering, permitting, infrastructure preparation, balance of plant, installation, integration, and contractor fees (Harvey et al., 2012). Therefore, the future economy of the microalgae biofuel depend on implementation of multi-product processes, genetic engineering on promising strains, biorefineries, advancement in engineering of photo bioreactors and petroleum product prices (Chisti, 2007; Harvey et al., 2012).

Chisti (2007) estimated the production cost of microalgae oil from a photo bioreactor with an annual production capacity of 10 000 tonnes of biomass. The author suggested that if the said species has 30 % oil content a production cost of $2.80/L ($10.50/gallon) of microalgae oil can be produced. This estimation excludes the costs of transeserification (converting microalgae oil to biodiesel), distribution, marketing and taxes. Compared to that and still higher is the petroleum price which at that point was $2.00 to $3.00 per gallon. Chisti (2007) eventually came up with an equation to estimate how the cost of microalgae can compete against or substitute petroleum crude oil. The equation is $C_{microalgae\ oil} = 25.9 \times 10^{-3} C_{petroleum\ oil}$, where $C_{microalgae\ oil}$ is the price of microalgal oil in $/gallon, and $C_{petroleum}$ is the price of crude oil in $/barrel. The equation assumes that microagae oil has 80 % of the caloric energy value that of petroleum crude oil. For instance, when the petroleum price is $100/barrel, microalgae oil should cost

$2.59/gallon in order to be competitive with petroleum crude oil. Table 11 by Sun et al. (2011) summarises the assumptions of various players/scenarios in the microalgae industry on the mode of cultivation, estimated oil and biomass production and cost over a period of time.

It is essential that a thorugh economic feasibility study is conducted prior to embarking on biofuel production from microalgae in Namibia. However, this was not the focus of this study.

Table 11: Cost assumptions of various microalgae industries/sources on cultivation, lipid and biomass production over a given of time (Sun et al., 2011).

Sources	**Scenario**	**Cultivation**	**Cost (USD gal^{-1})**	**Lipid yield (wt.% of dry mass)**	**Areal Dry Algae Mass Yield (gm m^{-1} day^{-1})**	**Loan Period (yrs)**
Benemann	Baseline	Open pond	$1.7	50%	30	5
Benemann	Maximum growth	Open pond	$1.2	50%	60	5
NREL	Current	Open pond	$10.6	25%	20	15
NREL	Aggressive	Open pond	$3.5	50%	50	15
NREL	Maximum growth	Open pond	$2.4	60%	60	15
NMSU	Current, 1 acre	Open pond	$38.7	35%	35	20
NMSU	Highest yield, 1 acre	Open pond	$13.9	60%	58	20
NMSU	Current, 2000 hectare	Open pond	$25.2	35%	35	20
NMSU	Highest yield, 2000 hectare	Open pond	$9.7	60%	58	20
Solix	Current	Hybrid	$31.8	16 - 47%	0 - 25	unknown
Solix	Phase I	Hybrid	$2.6	16 - 47%	30 - 40	unknown
Solix	Phase II	Hybrid	$0.9	16 - 47%	30 - 40	unknown
Seambiotic/IEC, Israel	Best yield	Open pond	$24.9	35%*	20	Unknown

Sandia	Current	Open	$15.7	35%	30	10
Sandia	Current	PBR	$33.2	35%	30	10
Bayer Tech Services	Optimistic	PBR	$14.3	33%	52	10
General Atomic	Low	Open/hybrid	$20.0	unknown	unknown	Unknown
General Atomic	High	Open/hybrid	$32.8	unknown	unknown	Unknown
California Polytech, Pomona	Waste treatment	Open pond	$16.8	25%	20	8
Tapie & Bernard	Tubes on ground	PBR	$40.6	35%*	20	5
Tapie & Bernard	Double tubular bioreactor	PBR	$43.1	35%*	20	5

* Assumed quantity required to convert from weight to oil.

CHAPTER 3

3. MATERIAS AND METHODS

3.1 Description of Study Area

The Namibian climate is considered to be one of the driest in Sub-Saharan Africa with 80 - 92 % of the landmass defined as hyper-arid, semi-arid or arid (Koch, 2004; Lahnsteiner & Lempert, 2007). This predicament leaves the country with scarce and unpredictable rainfall with spells of long severe droughts (Lahnsteiner & Lempert, 2007). The country is divided into two distinctive seasons which are the dry (end of April to end of November) and wet or rainy (end of November to the end of March) seasons (N. du Plessis, personal communication, 2014).

Average rainfall is 250 mm of which approximately 83 % evaporates (Cashman et al., 2014). Fourteen percent of the total rainfall is available to support vegetation, but most of this is lost through evapotranspiration, leaving only 2 % available as surface run-off, whilst less than 1 % recharges groundwater (Du Pisani, 2006). This results in the flow of the rivers in the interior of the country to be ephemeral, irregular and unreliable. Consequently, the surface water sources are limited and the only way they can be salvaged and fully exploited is through harnessing and storing them in impoundments, extracting them from groundwater/aquifers and through water reclamation from treated domestic wastewater (Van der Merwe & Menge, 1996; Metcalf et al., 2007; Conway et

al., 2009; Du Pisani, 2006; Rodriguez et al., 2009; Lehmann, 2010; Cashman, Foster, McCluskey & Zhang, 2014).

The Swakop River is located alongside numerous mines, farms and towns and is considered as one of the largest ephemeral rivers in Namibia (Lehmann, 2010) (Figure 12). It is 460 km long, rises in the Khomas Highland and drains westwards into the Atlantic Ocean, south of Swakopmund (Lehmann, 2010). It lies in the catchment of two tributary rivers which are the Klein Windhoek River in the North of Windhoek, the capital city of Namibia, and the Otjiseru River situated in the South-West (Figure 12). The Klein Windhoek River flows into the Otjiseru River which eventually reaches the Swakop River between Okahandja and the Swakoppoort dam (Lehmann, 2010) (Figure 12). Other sources of water supply are the country's six perennial rivers situated alongside its borders (i.e. the Orange River makes up the southern border, whilst the Kunene, Kavango, Kwando, Zambezi and Chobe rivers make up the northern and north - eastern borders) (Conway et al., 1995; Heyns, 1998). However, these perennial rivers originate in neighboring countries (Angola, Zambia, Botswana and South Africa) and are governed by international water laws which require entering into trans-boundary agreements which are at times unobtainable (Ashton, 2000; Sirunda & Mazvimavi, 2014).

Windhoek the capital city has a population of about 350 000 inhabitants (Wood, 2014) with a yearly increase of 4.4 % (Statistic Agency, 2011). It is situated in the catchment

of the Swakop River, is 750 km to the closest perennial river (the Okavango River) and 300 km from the Atlantic Ocean (Lahnsteiner & Lempert, 2007; Lehmann, 2010) (Figures 12 & 13). Annual average maximum day time temperatures are between 30 and 20 °C in January and July, respectively, whilst the annual average minimum temperatures are 17 °C in January and 7 °C in June (Info-namibiacom, 2016). Annual rainfall is 350 mm (Magnusson, 2005) whilst water demand per day is over 30 mm^3 (Jacobsen, Webster & Vairavamoorthy, 2012). Of this 8 % is supplied from groundwater extracted from 50 municipal production boreholes, 26 % is from both the New Goreangab Water Reclamation Plant (NGWRP) and the Old Goreangab Water Reclamation Plant (OGWRP) and 66 % is from the Satorious Von Bach dam , Swakoppoort dam and Omatako dam (Van der Merwe, 2000; Menge, 2010; Jacobsen, Webster & Vairavamoorthy, 2012). Although these three dams are designed to store up to three times the mean annual runoff and at times contribute 95 % (approximately 20 Mm^3/yr) of Windhoek's drinking water supply, their inflow and capacity are still dependent on the amount of precipitation. Therefore, the central Namibian reservoirs and wells cannot be guaranteed to produce enough water to satisfy the demand of the rapidly increasing Windhoek population (Lahnsteiner & Lempert, 2007; Sirunda & Mazvimavi, 2014).

Water quality in the three dams are affected by long retention periods, high evaporation rates and intermittent inflow from the catchments resulting in high organic and inorganic nutrient offloads mostly from human activities, animals (through fecal

contamination), and sediments (Goel, 2006; World Health Organization, 2010; Sirunda & Mazvimavi, 2014; Wang et al., 2014). It is therefore, important to rectify water quality issues which can lead to producing much needed water to the Windhoek community (Lahnsteiner & Lempert, 2007).

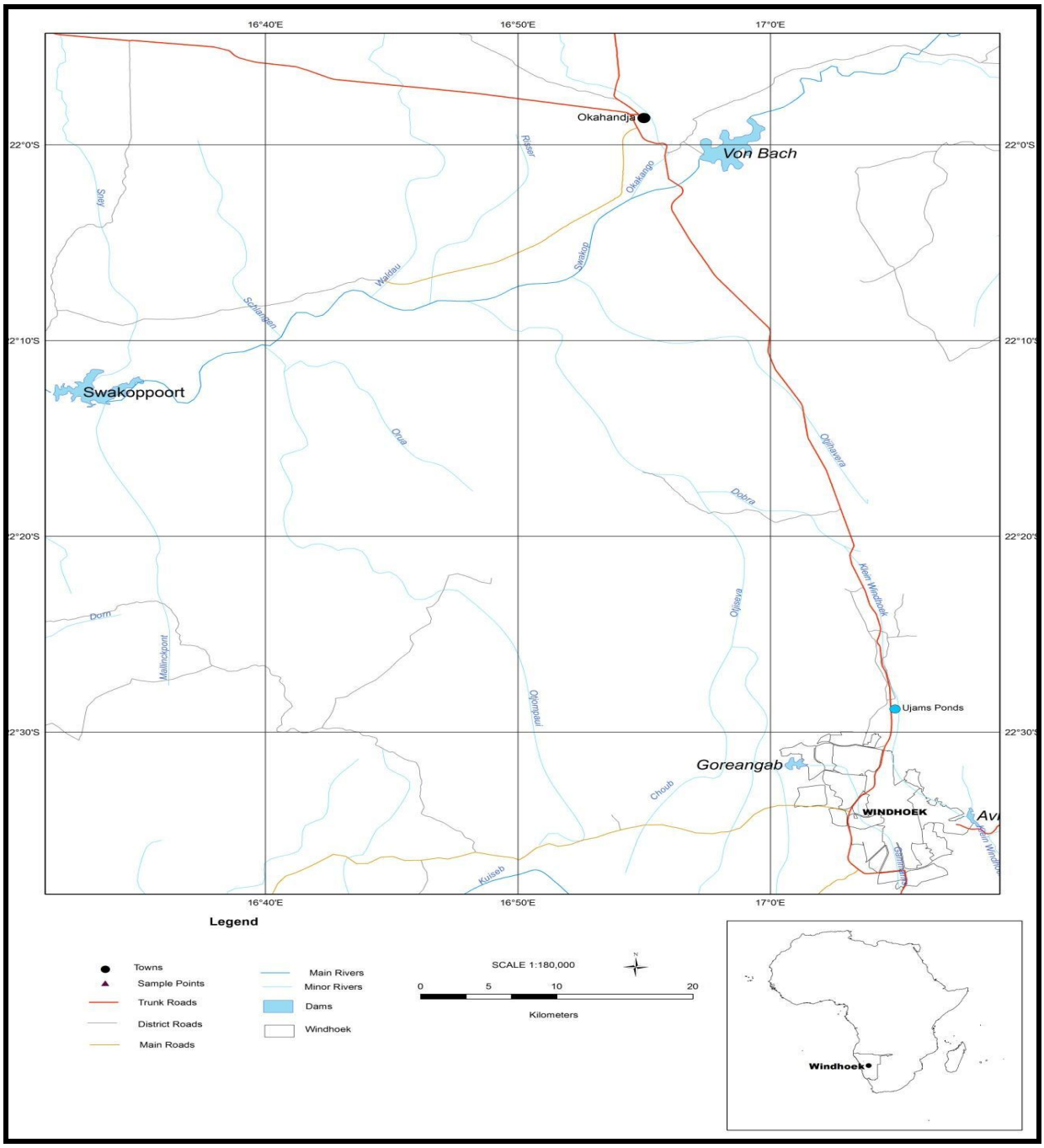

Figure 12: Swakop catchment; Swakoppoort dam, Von Bach dam, Goreangab dam and catchment areas (Shinana, 2011).

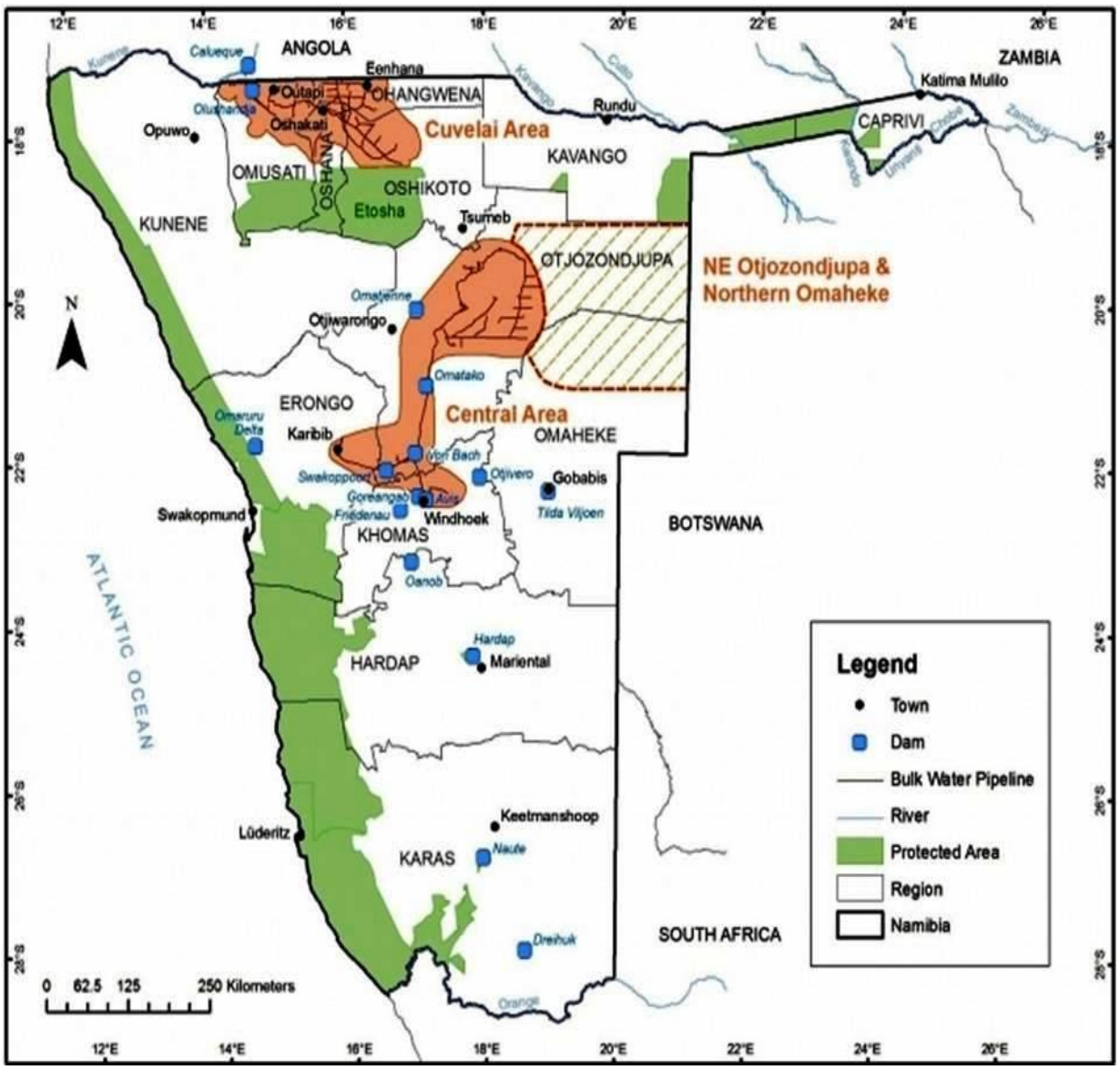

Figure 13: Namwater dams (Lcecomna, 2016).

3.1.1 Swakoppoort dam

Swakoppoort dam is a concrete arch dam with a carrying capacity of 63 489 Mm^3 and surface area of 7.808 km^2 (Sirunda & Mazvimavi, 2014). It is located 50 km outside Okahandja and 100 km from Windhoek. It was constructed in 1978 to supply water to

the town of Karibib (Navachab Gold Mine) and downstream augmentation to Von Bach dam for water supply to urban settlements such as Okahandja, Windhoek as well as cattle and game farming on commercial scale (Sirunda & Mazvimavi, 2014). By covering up to 75 % of the capital's water demand along with the Sartorious Von Bach and Omatako dams and having the largest capacity (Meding, 2016) of the three dams, Swakoppoort dam constitutes an interconnected water supply system that is very crucial (Lehmann, 2010).

Figure 14: Swakoppoort dam (Tixikcom sro, 2017; Neweracomna, 2016a).

The catchment area of Swakoppoort dam is mostly surrounded by savanna shrub and broadleaved trees (Sirunda & Mazvimavi, 2014) (Figure 14). It is fed by the Swakop River east in the upper part of the Swakop catchment. A small part of the flow to the dam is contributed by the Sney River from the North (Lehmann, 2010) (Figures 12 & 13). Over the years causative factors of water quality deterioration at Swakoppoort dam have been well documented. During the dry season effluent streams like Otjiseru River and Klein Windhoek River dry up before reaching the dam and, therefore, have little

influence on the water quality of the dam. However, during the rainy season, these along with discharges from urban areas of especially sewage treatment facilities like Ujams ponds have an effect on the water quality of the dam.

Ujams ponds are wastewater treatment facilities build in 1973 to treat wastewater originating from Windhoek. They are located alongside the Klein Windhoek River that supplies Swakop River with water. At some point these facilties were not treating wastewater according to the Namibian Water Reource Management Act (Act No. 11 of 2013) and therefore the oxidative ponds were shut down to make way for new and better technology (i.e. membrane bioreactors). Currently the facility treats industrial sewage effluent originating from Northern Industrial and Lafrenz Industrial areas from small food and beverage industries in Windhoek (Shinana, 2011; Cashman et al., 2014). Despite this, the effluent of the Ujams ponds produce treated wastewater with a substantial nutrient content which eventually drain into Swakoppoort dam via Swakop River during the rainy season (Koch, 2004; Lehmann, 2010; Shinana, 2011; Sirunda & Mazvimavi, 2014). Van Zyl (2011) also reported some industries not linked to this facility discharing wastewater into the streams joining Klein Windhoek River. Adding to this effluent is domestic wastewater treated at the Gammams Care Works that also flows into the SWKPD during the rainy season (Lehmann, 2010). This high influx of phosphorous concentrations as well as traces of nickel and other trace metals that can be traced back to the Ujams ponds lead to microalgae blooms of especially blue-green microalgae (Lehmann, 2010). Eventually these blooms form thick mats on the water surface that lead to low dissolved oxygen layers which constitute a eutrophic system. A

study by Sirunda & Mazvimavi (2014) claimed that excess nutrients in Swakoppoort dam are now slowly infecting the Von Bach dam. This was particularly a concern in November 2010 summer, where at one point water transfer to the SVBD (Figure 15) had to be stopped (Lehmann, 2010; Shinana, 2011; Meding, 2016).

Figure 15: (A) Microalgae blooms (New era publication corporation, 2016) and (B) scum floating on the surface of Swakoppoort dam (Lehmann, 2010).

3.1.2 Von Bach dam

Von Bach dam formally known as Sartorius Von Bach dam (SVBD) is a rock-filled embankment dam located near the town of Okahandja, 70 km from Windhoek, in the Otjozondjupa Region of Namibia (Olivier & Olivier, 1976) (Figures 12 & 13). It was built in 1968 and commissioned in 1970 to supply water to Okahandja as well as most of Windhoek's water demand. It has a carrying capacity of 48.56 Mm^3 and is fed via a pipeline by Swakoppoort and the Omatako dams. Its surface area is 4.9 km^2 and it has lower evaporation losses (2254 mm per year) when compared to Swakoppoort and Omatako dams which have much greater surface areas (Sirunda & Mazvimavi, 2014). Like Swakoppoort dam, Von Bach dam is located on the Swakop River and its catchment areas are mostly surrounded by shrub savanna and broadleaved trees (Sirunda & Mazvimavi, 2014) (Figure 16). Its land use services range from commercial livestock farming to game farming and villages (Sirunda & Mazvimavi, 2014).

Figure 16: Von Bach dam (Tungenicom, 2016; Namwater, 2016).

Sirunda and Mazvimavi (2014) revealed that the transfer of Omatako and Swakoppoort to Von Bach dam have negative effects on the water quality of Von Bach dam, especially since the two tributaries, the Otjiseru and Klein Windhoek rivers are draining contaminants into the Swakop River.

3.1.3 Goreangab dam

Windhoek relied on groundwater as the sole source of freshwater until 1933 when Avis dam with the capacity of 2.4 Mm^3 was constructed. However, this dam was built upstream of Windhoek with a very small catchment area and, therefore, could not sufficiently supply the growing population of Windhoek with water (Du Pisani, 2006). This led to the establishment of Goreangab in 1958 as the closest source of water with a larger catchment area downstream of Windhoek (Ogunmokun, Mwandemele & Dima, 2000; Cisneros & Rose, 2009; Cashman et al., 2014).

Goreangab dam is located northwest of Windhoek on the outskirts of the city (Shinana, 2011) (Figure 17). It has a carrying capacity and catchment of 3.6 Mm^3 and 150 km^2, respectively. It is fed by two ephemeral rivers, Arebbusch River and its tributary and the Gammams River which both run across Windhoek (Ogunmokun et al., 2000; Cashman et al., 2014) (Figure 12 & 13). Goreangab dams' land use is predominantly urban, with a significant proportion dominated by low cost and informal residential areas with an estimated population size of about 7000 people with a gross density estimated to be in excess of 80 people/km^2 in 2000 (NRC, 2000; Ogunmokun et al., 2000).

Figure 17: Goreangab dam (Neweracomna, 2016b).

The main source of pollution around Goreangab dam is human feces originating from the use of river beds close to the dam as toilets due to a lack of sanitation in the surrounding informal settlements (Ogunmokun et al., 2000). The evidence of these nutrient supplies is confirmed by empirical observations of scum forming microalgae. Apart from this the area has a high risk of sediment generation due to deforestation for fire wood. Eventually this leads to erosion which forces large particles of sediments deposited into the dam with the storm water (Ogunmokun et al., 2000). This ultimately results in remineralization and or addition of inorganic and organic nutrients into the system.

Goreangab dam has an overflow that discharges into a tributary to Swakop River. A study by Lehmann (2010) and Shinana (2011) revealed that the elevation of phosphates and pollutants such as heavy metals, phenol and formaldehyde in this outflow has implications on the water quality of the downstream tributaries and eventually Swakoppoort dam. Shinana (2011) established that the highest concentrations of nitrogen and phosphate originate from Ujams ponds as well as Goreangab dam. The deterioration of water quality at Goreangab dam has been well documented since 1990 to the extent that the organic content was equal to or at times more than that of the treated wastewater effluent (Ogunmokun et al., 2000; Menge, 2010). Therefore, because of these high levels of pollution in the runoff (Figure 18), the NGWRP is no longer able to treat water from the dam to potable standards. The City of Windhoek is now forced to mix its reclaimed water with water piped in from other reservoirs (Cashman et al., 2014).

Figure 18: Pollution at Goreangab dam (Musheko, 2015).

3.2 Sampling for ecological study

Microalgae from three eutrophic systems: Swakoppoort, Goreangab and Von Bach dams were sampled during the dry season (14 June 2013 and 4 June 2014) and the wet season (14 November 2013 and 28 February 2014).

The sampling strategy followed convenient sampling and a ski boat was used for sampling at sites that were pre-determined and as close as possible to the dam wall, which are the deepest sites at all the dams. Sampling locations were marked using a portable GPS (Table 12). The geographical maps for the positions were generated from coordinates recorded during sampling at Namwater GIS Head office, Windhoek. The coordinates were entered onto Microsoft Excel Spreadsheets, Windows 97. Thereafter images were extracted from google earth, georeferenced in Arcmap (Figures 19, 20 & 21).

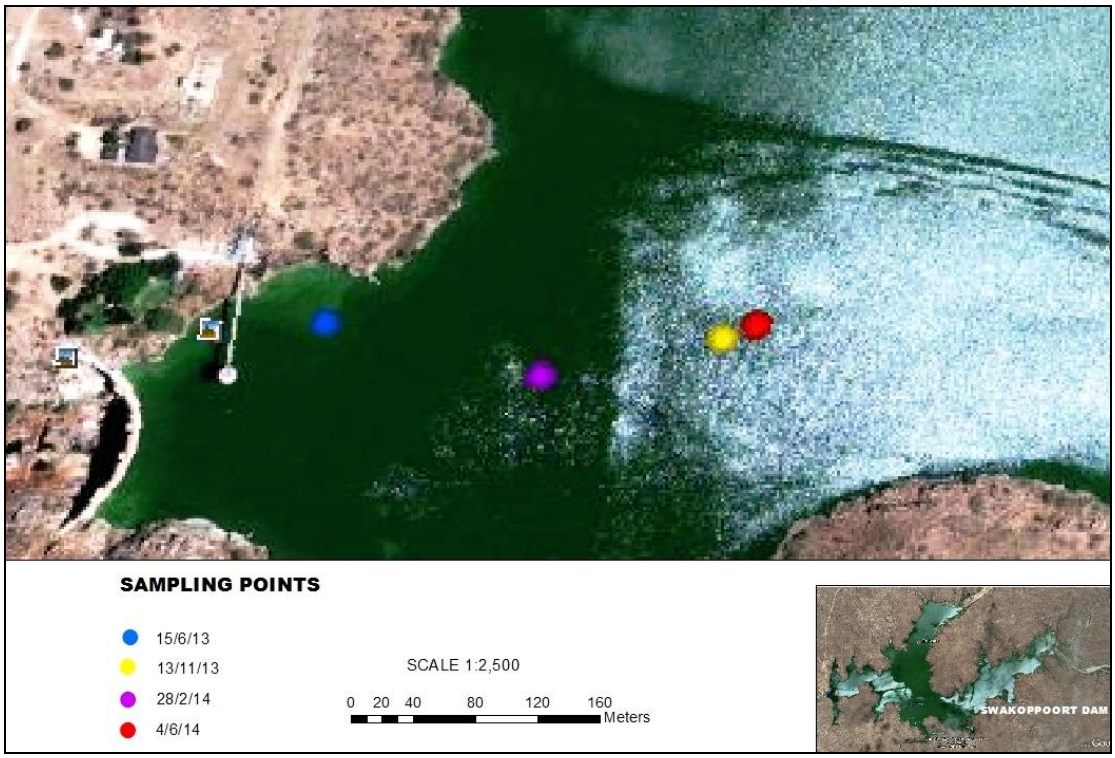

Figure 19: Swakoppoort dam sampling points.

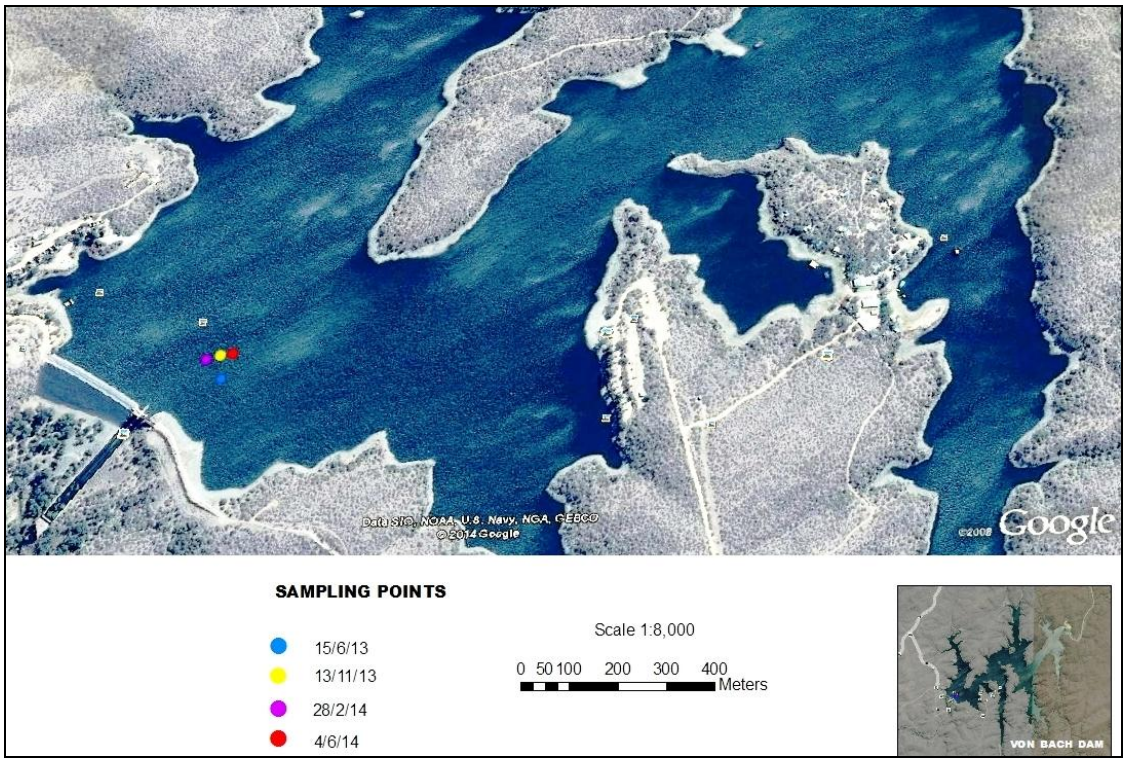

Figure 20: Von Bach dam sampling points.

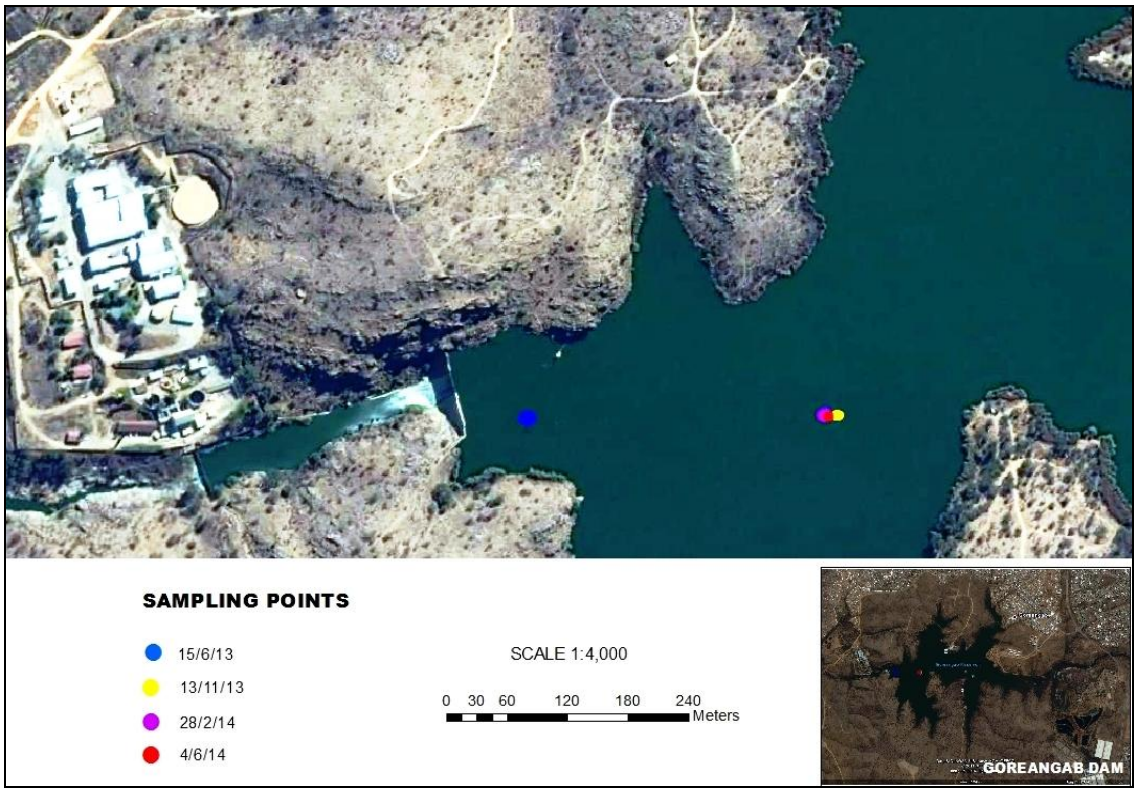

Figure 21: Goreangab dam sampling points.

Three groups of parameters: physical, chemical and biological were measured to determine the water quality of the three dams. Once at the sampling site the engine was shut down to commence sampling. After determining the depth of the euphotic zone with the secchi disk, physical parameters such as temperature, dissolved oxygen and pH were collected using a portable Eutech multi probe. All of these data were recorded in the site logbook and later onto a field data sheet (Figure 22). Thereafter, two 500 mL black labeled polyethylene bottles per dam were used to collect the samples for macronutrient analysis at a depth of 30 cm below the surface.

Figure 22: Sampling Procedure. (A) Site data on log book, (B) 63µm phytoplankton net for microalgae sampling, (C) Storing sample bottles in a cooler box for analysis.

To avoid chemical and biological changes that continue in the sample after collection, the samples were immediately preserved with 0.5 mL H_2SO_4 (Stednick, 1991). Maximum recommended storage at 4 °C for nitrates, phosphates, and nitrites is 48 hrs and for ammonia 7 days (Greenberg, Clesceri & Eaton, 1992). After preservation the samples were transferred on ice to the laboratory (Figure 22C).

At the same time whilst in the field, biological sampling took place for the taxonomic identification, abundance and diversity of microalgae species. This was done by first filtering the water through a 63 µm phytoplankton net and pouring the filtered water in three black labeled polyethylene bottles of 125 mL for each dam (Figure 22B). The samples were thereafter placed on ice (Figure 22C) and immediately transferred to the laboratory for analysis. Dam water volume data was obtained from logged data from the Hydrological station at Namwater in Windhoek (Table 12).

Table 12: Field parameters.

	Swakoppoort dam				**Von Bach dam**				**Goreangab dam**			
	Dry season		**Wet season**		**Dry season**		**Wet season**		**Dry season**		**Wet season**	
Date	14/06/2013	4/06/2014	14/11/2013	28/02/2014	14/06/2013	4/06/2014	14/11/2013	28/02/2014	14/06/2013	4/06/2014	14/11/2013	28/02/2014
Time	11:00	12:56	13:46	12:45	14:25	16:04	15:00	15:13	16:50	08:56	10:21	09:12
GPS	22° 21' 11"S; 16° 52' 44"E	22° 21' 11"S; 16° 52' 480000 "E	22° 21' 11"S; 16° 52' 56"E	22° 21' 12"S; 16° 52' 51"E	22°01' 22"S; 16°95' 30"E	22°01 '108000"S; 16°95 66000"E	22° 01' 19"S; 16° 95' 29"E	22° 01' 20"S; 16° 19' 328140"E	22° 52' 52860 "S; 17° 00' 56400 "E	22°52' 474000"S; 17° 01' 582000"E	22° 52' 53"S; 17° 01' 12"E	22° 52' 172"S; 17° 01' 12"E
Dam water volume (Mm^3)	37.49	38.54	29.7	29.97	33.29	27.87	26.61	22.78	3.63	3.63	3.62	3.63
% of full capacity	59	60.7	46.8	47.2	68.5	57.4	54.8	46.9	100.2	100.3	100	100.2

3.3 Laboratory analysis

3.3.1 Macronutrient analysis

Macronutrients (ammonia, phosphate and nitrate) analyses were carried out at Gammams Water Care Works in Windhoek, Namibia. The analyses were done using ion chromatography following the Standard Methods by Greenberg et al. (1992) via the Continuous Flow Analyser, (Figure 23).

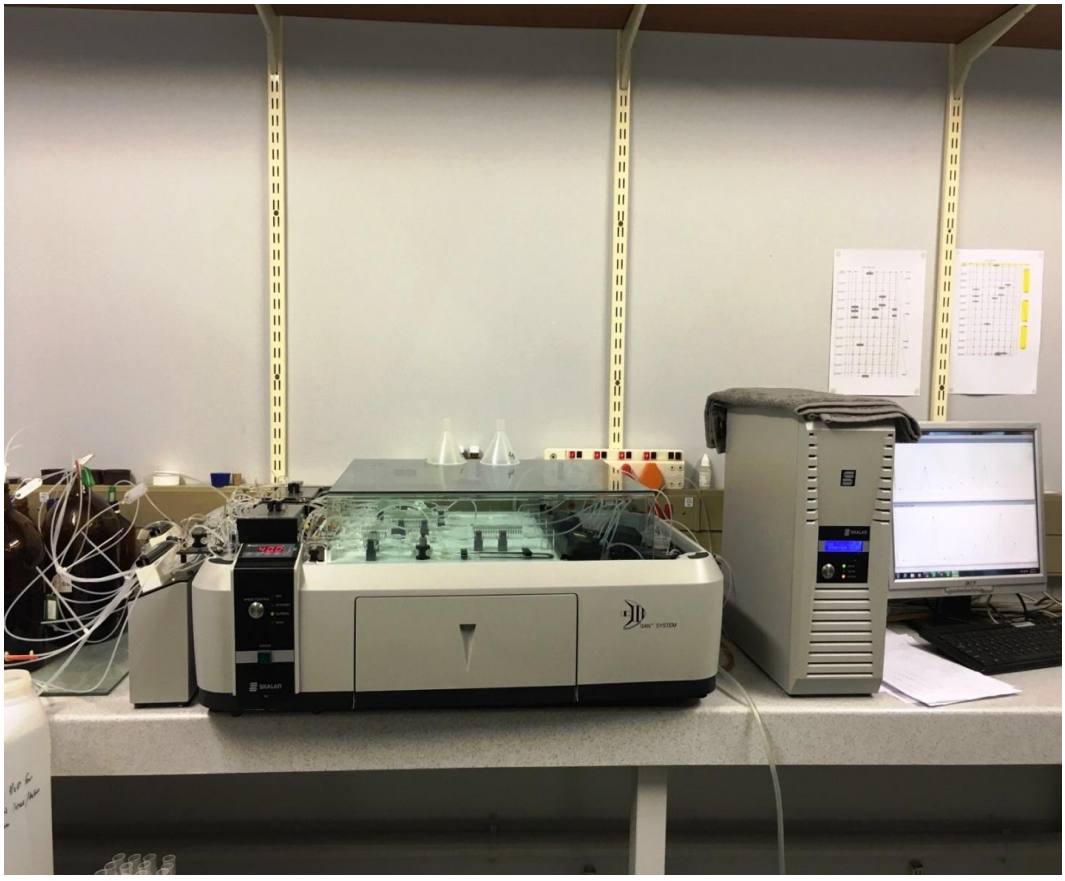

Figure 23: Continuous Flow Analyzer (Carew, January 7, 2016).

The Skalar system is based on the principles of continuous flow analysis which enables it to perform a wide variety of wet biochemical determinations. Therefore, more than one determination of various tests was carried out simultaneously. Samples were placed on a sample tray and were sequentially aspirated into a continuous flow analytical stream. A wash solution was introduced between each sample for sample separation. The various outcomes are continuous and automatic. The spectrophotometric output of the Skalar system produces a curve that is converted to concentration units by the use of FlowAccess Software. Prior to analysis various reagents/stock solutions were produced for each nutrient to be analyzed.

3.3.2 Determination of relative genera abundance and diversity of microalgae

After sampling, taxonomic identification and subsequent abundance and diversity analysis was carried out within 48 hours to minimize cell lysing. The 3 samples collected were simultaneously analyzed three times using an improved Hirschmann-Laborgerate Haemocytometer at 100 and 400 magnification using a compound microscope. To accomplish an even distribution of cells on the haemocytometer the slide was cleansed and rinsed with 70 % ethanol, air dried and wiped with lens paper. To cover reflective surfaces a coverslip was placed on top and the cells were examined by adding 10 μL of the cell suspension via a micropipette to the H-shaped groove on each side of the haemocytometer. A tally counter system was used to count the cells that were entered into a logbook then transferred to a Microsoft Excel Worksheet, Windows 97,

where the data was later extrapolated. The formula to calculate the cell density were as follows: Number of cells$/mL = \frac{Number\ of\ cells\ in\ sample}{Volume\ of\ haemocytometer}$.

The species present were identified and enumerated to genus level with the aid of various taxonomic keys (Belcher & Swale, 1976; Ettl & & Gaertner, 1988; Staley, Bryant & Holt, 1989; Huynh & Serediak, 2006; Bellinger & Sigee, 2010; Edward & David, 2010; Barsanti & Gualtieri, 2014).

3.4 Optimization of neutral intracellular lipid and biomass production in *Nannochloropsis limnetica* SAG 18.99

Experiments on microalgal cultures were carried out at the Karlsruhe Institute of Technology, Germany at the Department of Chemical and Process Engineering, Institute of Process Engineering in Life Sciences, Section III Bioprocess Engineering from 21 May - October 2015. Axenic cultures of *Nannochloropsis limnetica* (*N.limnetica* SAG 18.99) were obtained from the Culture Collection of microalgae, University of Göttingen, Germany and maintained in the incubation chamber at 21 °C on permanent LED illumination (Figure 24).

Figure 24: Strain maintenance. (A) Department of Chemical and Process Engineering, Institute of Process Engineering in Life Sciences, Section III Bioprocess Engineering, (B) Laboratory set up at the Karlsruhe Institute of Technology (C) Axenic cultures of *Nannochloropsis limnetica* (*N.limnetica* SAG 18.99) were obtained from Culture Collection of Microalgae, University of Göttingen, Germany, (D & E) Maintained in incubation chamber at 21 °C on permanent LED illumination (Rost GMBH; Kalte Klima Schank-anglagebau).

3.4.1 Strain screening and medium preparation

Pre-cultures were cultured and maintained in sterilized freshwater based modified BG_{11}^{0} growth medium in triplicate 500 mL Erlenmeyer Flasks enclosed with ventilated closures, which in this case were cellulose stoppers. The BG_{11}^{0} medium was modified from a protocol by Rippka, Deruelles, Waterbury, Herdman and Stanier (1979) (Figure

25C). The three Erlenmeyer Flasks in this study are referred to as contact or incubation chambers (Figure 25A & F). There was no external CO_2 supply provided during culture maintenance, therefore, carbon dioxide to support growth was derived from the atmosphere (approx. 300 ppm) (Figure 25F).

The medium was produced by adding 5 mL each of various strength stock solutions to 500 mL deionized water. The solution was mixed and was added to a 1L Duran Pressure Resistant Schott bottle containing 500 mL of deionized water to make up a 1L BG_{11}^{0} stock solution. To stabilize the pH throughout the experiment at pH 7.5 to 8.3 two buffers were prepared. $NaHCO_3$ was acting as a carbon source and HEPES as a zwitterion ion providing supplementary buffering without membrane permeability interference of biochemical reactions in the cells in the culture medium (Good et al., 1966).

The three 500 mL Erlenmeyer Flasks were filled with 5 mL deionized water to promote maximum sterilization. The stoppers were covered with aluminum foil that deterred water from perspiration entering the Flasks during and after autoclaving (Figure 25A). The 1 L BG_{11}^{0} stock solution, three 500 mL Erlenmeyer Flasks, two buffers solutions and pipette tips enclosed in autoclavable bags were autoclaved at 121 °C for 20 min at 118 kPa (Figure 25B).

Figure 25: Experimental set up for pre-cultures. (A) Empty 500 mL Erlenmeyer Flasks with cellulose stoppers and aluminum foil, (B) Autoclaving Empty 500 mL Erlenmeyer Flasks with cellulose stoppers and aluminum foil, (C) $BG_{11}{}^{0}$ medium and two ($NaHCO_3$ and HEPES) buffer stock solutions under the Thermo Scientific Biological Safety Cabinet, (D) Inoculation of 3 Erlenmeyer Flasks to an Optical Density (OD) of 0.1 at 750 nm, (E) 500 mL Erlenmeyer Flasks covered with Parafilm, (F) Erlenmeyer Flasks set on permanent illumination of 180 µmol/m2/ s (LED) at room temperature of 25 ± 1 °C, agitated at 100 rpm for 14 days.

After cooling to approx. 60 °C on the bench all the Schott bottles, the three Erlenmeyer Flasks and Eppendorf pipettes were wiped with 76 % ethanol then transferred to the Thermo Scientific Biological Safety Cabinet which was also disinfected with Kohrsolin and 76 % ethanol before any work resumed (Figure 25C, D & E).

Once in the Thermo Scientific Biological Safety Cabinet, the deionized water was removed from the contact chambers. Thereafter, the 1 L Schott bottles containing the $BG_{11}{}^{0}$ stock solution were used to fill the 3 contact chambers. This was followed by adding 5 mL each from the two buffers ($NaHCO_3$ and HEPES) to each contact chamber. The content of all the contact chambers for this experiment were diluted, using 1:20 dilution factor to an optical density (OD) value at 750 nm equal to 0.1, to obtain a final volume of 200 mL at exponential phase. After inoculation and flaming the necks of the Flasks using a Bunsen burner, the openings of the Flasks were sealed with Parafilm that were previously treated with 76 % ethanol (Figures 25E). Thereafter, the Flasks were removed from the Thermo scientific biological safety cabinet and transferred to the shaking incubator/station (Rost GMBH; Kalte Klima Schank-anglagebau) where they were set on permanent illumination of approximately 180 μmol/m^2/s^1 (LED) at room temperature of 25 ± 1 °C and agitated at 100 rpm to assure continuous gas exchange for 14 days. Light intensity was measured by placing a photo probe on the LED lights via an empty 500 mL photometry Erlenmeyer Flask with a quantum meter (Model LI 250 light meter) (Figures 25F). Table 13 shows the initial parameters of the Preculture Flasks.

Table 13: Parameters for pre-culture Flasks.

Flask	**Light intensity (μmol quanta/m^2/s^1)**	**Energy charge/Voltage (mA)**	**pH before Inoculation**	**Inoculum (mL) (1:20 dilution factor)**
1	178.9	100	7.5	5
2	179	100	7.5	5
3	180	100	7.5	5

Growth rates, cell concentrations, pH and microscopy were recorded throughout this experiment on a daily basis by sampling 5 mL from each Flask under the Thermo Scientific Biological Safety Cabinet (Figure 25E & Figure 26C, D, E & F). At the end of the 14 days, gravimetric dry biomass (BDM) measurements were carried out in triplicates of 30 mL sample per Flask. To appraise the quantity of nutrient uptake (in this case anions; SO_4^{2-}, NO_3^-, PO_4^{3-}) at the end of the fourteen days ion chromatography was conducted and the results compared to the initial molecular mass (concentration) in the BG_{11}^0 stock solution (Figure 30F, G & H). Sterility tests were also carried out to ensure the axenic cultures were not contaminated (Figure 29). All chemicals used were purchased from Carl Roth and were of analytical grade (p.a.).

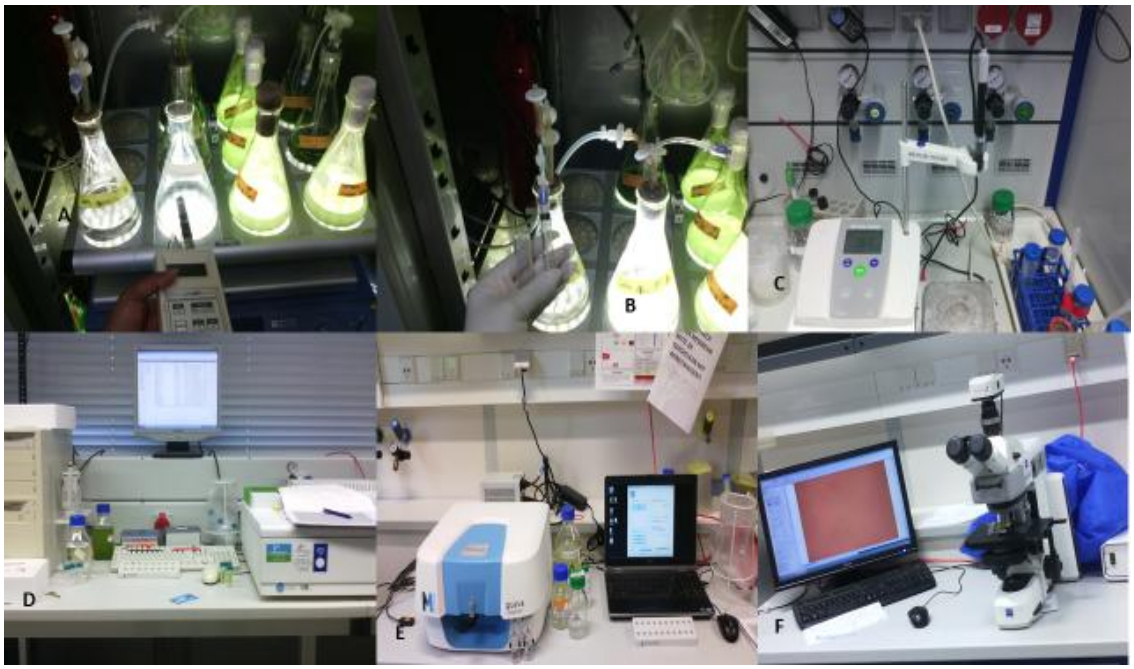

Figure 26: Monitoring instruments. (A) Measuring Light intensity using a quantum meter (Model LI 250 light meter), (B) Sampling via Braun Leur Lock Omnifix syringe via Straight Male threaded swabable valve, (C) Measuring pH via Mettler Toledo, (D) Perkin Elmer Lambda 35 ES UV/VIS Spectrophotometer for measuring Optical Density, (E) Flow-cytometry via Guava easyCyte for measuring cell concentrations, (F) Bio-AG Zeiss Axioscope A.1 for microscopy.

3.4.2 Main-cultivation

This work represents an attempt to increase the neutral intracellular lipid content of *Nannochloropsis limnetica* (*N. limnetica* SAG 18.99) as a result of nitrate replete and deplete concentrations in identical culturing conditions (pH, light, CO_2, temperature). Other variables considered and monitored were growth rates, cell concentration, bio dry

mass (BDM) productivity and morphological changes of the cells throughout the experiment via microscopy.

3.4.2.1. Experimental set-up

3.4.2.1.1 Make-shift apparatus

Nannochloropsis l. was simultaneously cultivated and monitored in 1 L Erlenmeyer Flasks in four batch reactors which as before were used as, and would be referred to, as contact or incubation chambers in this experiment. Each of the four treatments was replicated twice. The difference in this experiment was that these Flasks were fitted with bungs containing make-shift holes fitted to glass, rubber hoses as well as detachable stainless steel needles (Figure 27 & Figures 30A & B). The rubber bungs contained two holes drilled into the top for the outflow and inflow hoses and another pierced by a stainless-steel needle for sample recovery and buffer injection. Two glass hoses (6 mm x 4 mm diameter) were inserted into both the inflow and outflow holes. The inflow hose (22.8 cm length) extended to the mid of the container to provide surface aeration, whilst the outflow hose (8.5 cm length) only extended a short way into the container to promote flushing out of the O_2 build up in the chamber. On the inflow glass hose, one silicon hose (3 mm x 6 mm diameter with 5.5 cm length) was fitted and attached to a sterile micro filter (0.20 μm) and again another silicone hose (4 cm length) attached to yet another sterile micro filter with the same diameter and pore size, respectively. The outflow glass hose only had one silicon hose (5 cm length) fitted to it and subsequent micro filter attached to it with the same measurements as above. The stainless-steel

needle (2.1 x 80 mm BC/SB) was inserted a quarter way into the Flasks and fitted to a silicon hose (3 mm x 6 mm diameter and 23.4 cm length) which reached to the bottom of the Flask. On the outside, the needle was attached to a hose connector that was fitted to another silicon hose (10.7 cm length) with the same diameter as above which was in turn fitted to a Leur Lock fitted to the Straight Male threaded swabable valve. To avoid leakage of any kind all of the above connections were tightened with cable ties (TYB23M, TY-RAP CABLE TIE, 2 mm x 16 mm).

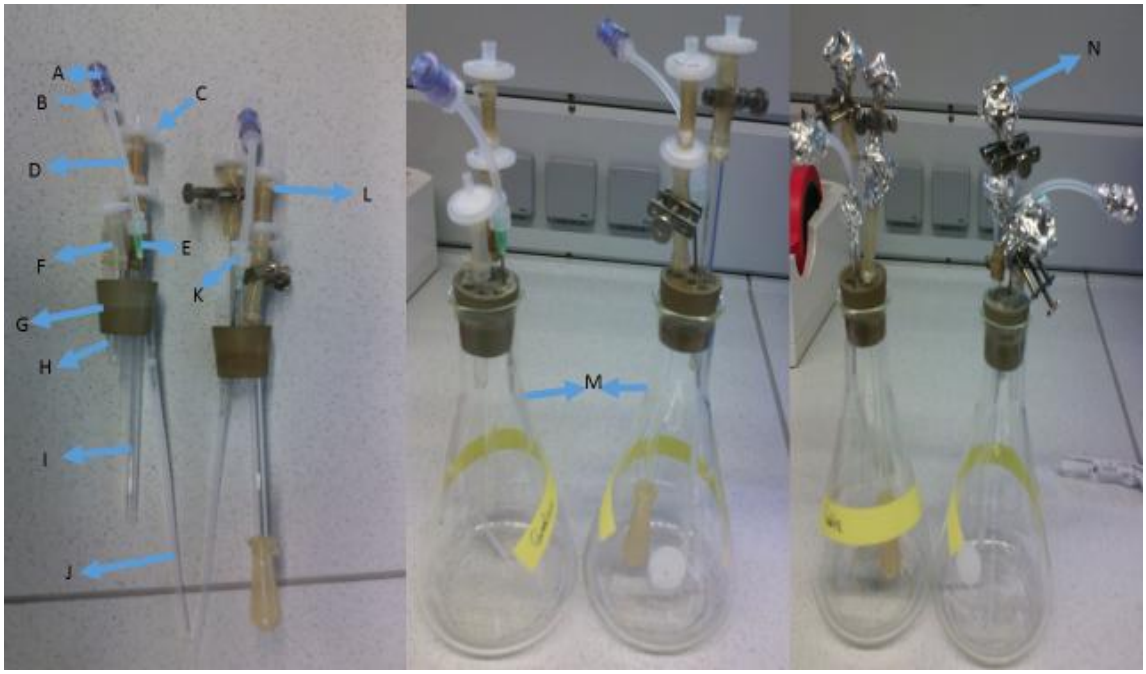

Figure 27: Make-shift apparatus. (A) Straight Male Threaded Swabable Valve; (B) Leur Lock (LL); (C) 0.2 µm Sterile micro filter, (D & F) 3 mm x 6 mm diameter Silicon hose, (E) Braun Detachable stainless steel needle (2.10 x 80 mm BC/SB), (G) Rubber Bung, (H) 0.5 mm Glass hose for Inflow, (I) 6 mm x 4 mm diameter glass hose for outflow, (J)

3 mm x 6 mm diameter Silicon hose for sample collection and buffer injection, (K) Hose/Needle connector, (L) Cable tie, (M) 1000 mL Erlenmeyer Flasks, (N) All openings were covered with aluminum foil before autoclaving.

3.4.2.1.2 $NaNO_3$ concentration variations in BG_{11}^0 stock solution

$NaNO_3$ was used as the only source of nitrogen in this cultivation. The $NaNO_3$ concentrations in the BG_{11}^0 stock solution varied per batch throughout this experiment. Normal $NaNO_3$ concentration referred to as A1 and A2 of 3.53 M according to the BG_{11}^0 solution was entered into one batch to simulate "unstressed" growth of *Nannochloropsis limnetica*. The second batch referred to as B1 and B2 contained double of this concentration (7.06 M) to simulate nitrate replete. The third batch referred to as C1 and C2 contained half of the original/normal $NaNO_3$ concentration which was 1.765 M and the fourth batch referred to as D1 and D2 contained half the concentration (0.8825 M) of the third batch. The third and fourth batches were employed to simulate moderate and high nitrate stress. The varied $NaNO_3$ in the BG_{11}^0 stock solution were prepared for each batch via graduated volumetric Flask to the volume of 750 mL and transferred to 1 L pressure resistant Schott bottles per batch.

$NaHCO_3$ buffer was removed from the original medium composition because it is a potential carbon source for heterotrophic organisms (Fábregas, Vázquez, Cabezas & Otero, 1993) and also to restrict the double dose of CO_2 that may reduce the pH throughout the experiment due to possible production of carbonic acid ($CO_2 + H_2O \rightarrow$

$H_2CO_3 \rightarrow H^+ + HCO_3^- \rightarrow 2H^+ + CO_3^{2-}$). However, 1 M HEPES buffer as before along with 1 M of NaOH and 0.1 M of HCL was prepared to keep the pH constant at pH 7.5 to 8.3 when needed throughout this experiment.

3.4.2.1.3 CO_2, light, pH and temperature

All of the cultivation parameters (CO_2, light, pH and Temperature) were kept identical throughout this experiment. pH was set at approx. 7.5 - 8.3 (Krienitz & Wirth, 2006; Krienitz et al., 2000; Sukenik et al., 1989), light intensity was set at approx. ±290 μmol quanta/m^2/s (Sukenik et al., 1989) and the cultures were aerated via surface aeration with sterile air containing 2 % (v/v) CO_2 at 0.05 vvm which equal to 50 mL/min flow rate per 1 L Erlenmeyer Flask (Figure 28A & B). The temperature was set at 25 ºC (Converti et al., 2009).

3.4.2.1.4 Culturing procedure

All of the varied BG_{11}^0 stock solutions made up to 750 mL contained in 1 L pressure resistant Schott bottles per batch, 1 M HEPES buffer, 1 M NaOH buffer, 0.1 M HCl stock solution along with eight 1 L Erlenmeyer Flasks filled with 5 mL deionized water (closed loosely to avoid pressure), makeshift rubber bungs attached to hoses, filters and Braun Detachable stainless steel needles (2.10 x 80 mm BC/SB) (all outlets covered with aluminum foil), pipette tips and Braun Leur Lock syringes (2.5 mL, 3 mL, 5 mL, 12 mL, 60 mL) enclosed in autoclavable bags were autoclaved at 121 °C for 20 min at

118 kPa. Thereafter, they were left cooling on a bench for 30 minutes to approximately 60 °C and then later transferred to the Thermo scientific biological safety cabinet.

After the 5 mL deionized water was removed from each contact chamber in the Thermo Scientific Biological Safety Cabinet, 12 mL Braun Leur Lock syringes were attached to Braun Detachable stainless steel needles (2.10 X 80 mm BC/SB) and used to draw 10 mL from each of the two buffer stock solutions (HEPES, NaOH). These were later transferred to the varied eight 1 L Erlenmeyer Flasks via the Braun Leur Lock Omnifix syringe through the straight male threaded swabable valve. The Flasks were then transferred to the strain maintenance chamber (Rost GMBH; Kalte Klima Schank-anglagebau), set on agitation of 100 rpm and fitted to silicon tubing for CO_2 surface aeration at 25 ± 1 °C. To establish the pH of approximately 7.5 - 8.3 in the medium as well as achieve CO_2 saturation throughout the experiment, 2 % (v/v) CO_2 was aerated in all of the eight Flasks for 24 hours (Figure 28). The pH was adjusted during this time and not after inoculation, because an increase in addition of the HCl stock solution and or any other buffering agent when carried out after inoculation will affect cell osmolarity, ultimately resulting cell shrinking. Prior to this the irradiation was set at 290 µmol quanta/ m^2/s after careful calibration by measuring the light intensity via photo probe on the LED lights via an empty 500 mL photometry Erlenmeyer Flask using a quantum meter (Model LI 250 light meter).

After 24 hours of CO_2 aeration and confirmation of the pH at 7.5 - 8.3 the Flasks were inoculated with the pre-cultures diluted to 1:20 to an optical density (OD) value of 0.1 at

750 nm. To accomplish this, pre-cultures were transferred to the Thermo Scientific Biological Safety Cabinet and samples were collected via Braun Leur Lock Omnifix syringe attached to a Braun Detachable stainless steel needle (2.10 X 80 mm BC/SB) and samples were transferred to the eight 1 L Erlenmeyer shaking Flasks via the straight male threaded swabable valves. Sampling was carried out daily by collecting samples with the Braun Leur Lock Omnifix syringes via the Straight Male threaded swabable valves. Before each sampling 1 mL was retrieved to deter examination of old cells that may be in the sampling silicon tube (Figure 26B). All the experiments were conducted for 14 days. Table 14 shows the initial parameters for the main-cultivation.

Table 14: Parameters for main-culture Flasks.

Flask	**Light intensity (µmol quanta/m^2/s)**	**Energy charge/voltage (mA)**	**pH before aeration and Inoculation**	**pH after 24 hours aeration and before Inoculation**	**Inoculum (mL) (1:20 dilution factor)**
A1	294.1	301	8.60	7.53	8
A2	293.4	285	8.65	7.51	8
B1	290.5	289	8.56	7.58	8
B2	292.5	270	8.67	7.51	8
C1	294.2	294	8.78	7.55	8
C2	294.1	289	8.73	7.62	8
D1	292.2	290	8.74	7.64	8
D2	294.2	294	8.69	7.66	8

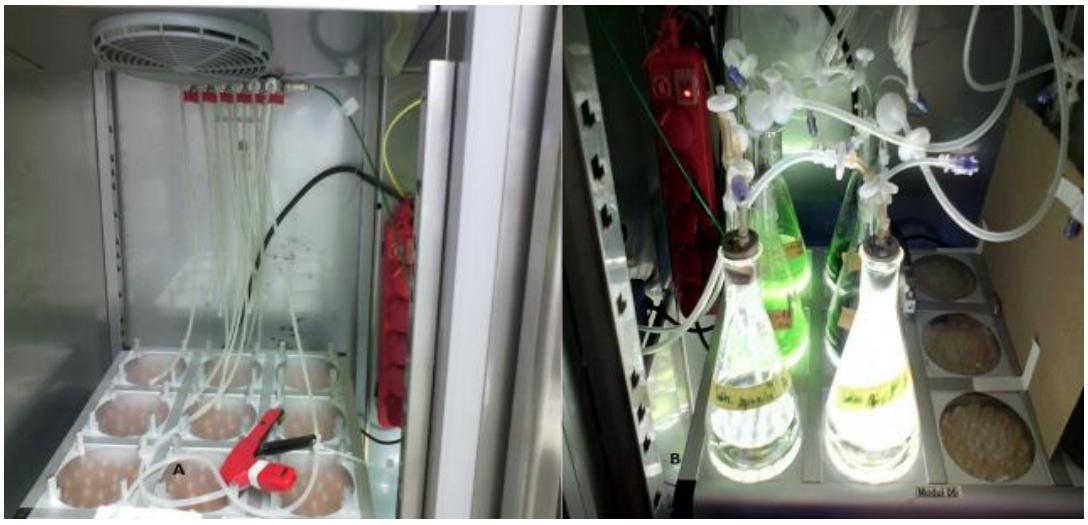

Figure 28: CO_2, light and temperature. (A) Silicon aeration tubes attached to aeration valves for CO_2 consumption, (B) pH adjustment for 24 hours via CO_2 saturation.

3.4.3 Analytical methods

3.4.3.1 Sterility tests

To ensure axenic conditions in all of these cultures sterility tests were performed regularly. For this purpose two separate agar culture media favoring bacterial and fungal growth were prepared by adopting modified versions of the media described in Madigan, Martink and Parker (1996). A sample of 1 mL was collected from the pre-cultures and spread evenly on the two separate agar plates under the Thermo Scientific biological safety cabinet (Figure 29). Sampling from the main-culture was done by retrieving 1 mL with a Braun Leur Lock Omnifix syringe via the straight male threaded swabable valves. The plates were left in the incubater at 31°C for 24 and 48 hours to test for bacteria and fungi, respectively. At the same time other modes of testing sterility were facilitated via

Optical Density (OD), Flow-cytometry as well as microscopy which are good observatory procedures to detect contamination.

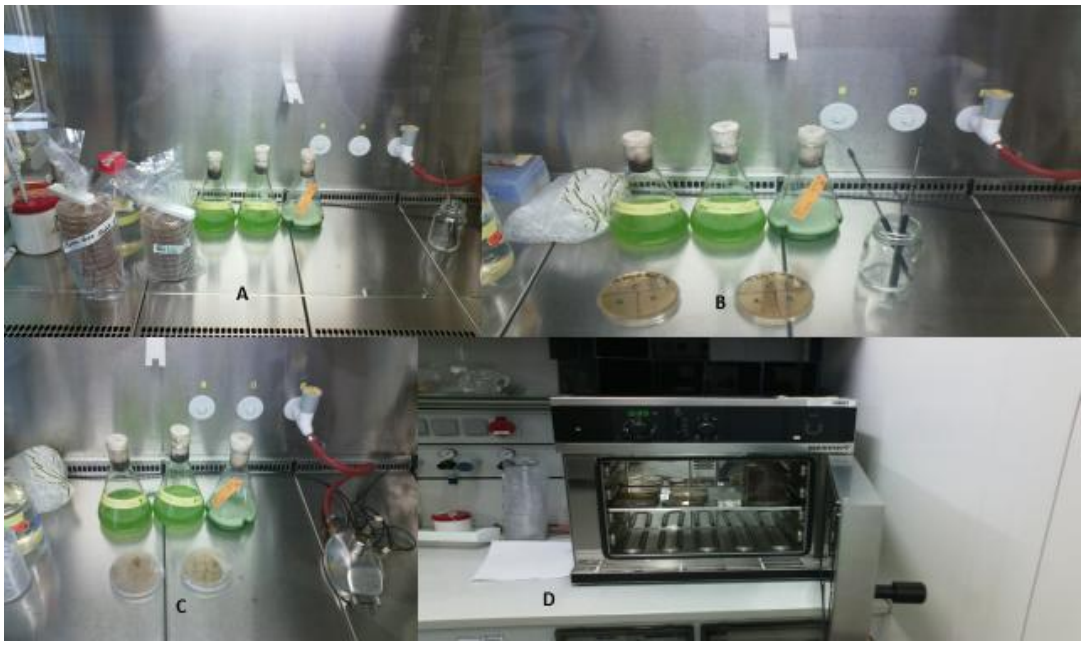

Figure 29: Sterility tests. (A) Three Pre-cultures with fungi and bacterial agar plates in the Thermo Scientific biological safety cabinet, (B) Agar plates marked and inoculated, (C) Agar plates sealed with Parafilm M (Laboratory Film, NEENAH, WI 54956), (D) Agar plates transferred to oven at 31 °C.

3.4.3.2 Optical density, Flow-cytometry, pH and microscopy

To follow the growth rates and cell concentrations throughout these cultivations Optical Density (OD) was measured using Perkin Elmer Lambda 35 ES UV/VIS spectrophotometer at 750 nm and Flow-cytometry using Guava easyCyte, respectively. During pre-culturing 5 mL was collected daily from each Flask in 5 mL Eppendorf tubes

via Eppendorf-Research-Physio Care concept pipettes under the Thermo Scientific biological safety cabinet. Two millitre aliquots of these were used to measure pH. As for the Main-cultivation 2 mL of samples were collected from each Flask daily from the straight male swabable valve via Braun Leur Lock syringe and 1 mL of this was set aside for pH measurements (Figure 26B). Optical Density measurements were repeated three times in separate cuvettes as technical and biological triplicates to avoid errors and achieve precision. Cells for both optical density and flow cytometry analysis were mixed thoroughly via up and down suction motion with the Eppendorf-Research-Physio Care concept pipettes to avoid settling errors (Harris, 2009). Flow-cytometry only required a single sample per Flask as the machine was already set on high precision. pH was measured daily for both cultivations using the Mettler Toledo pH meter. The probe was inserted into the separated sample/aliquot and when the reading stopped changing after 10 minutes this value was recorded and considered as the equilibrium (Figure 26C). Photomicrographs were captured regularly by placing 10 μL on a slide covered with a cover slip and examined at 100 and 400 magnification for pre-cultures and 400 and 1000 magnification for main-cultures using the Bio-AG Zeiss Axioscope A.1, equipped with Neofluar objectives, under bright field, differential interference contrast (DIC) and phase contrast illumination (Figure 26F). The specific growth rates, reflected in the measured optical density and cell concentrations during the cultivations were calculated by the slope of the logarithmic phase for number of cells in correlation to the cultivation process time (Barsanti & Gualtieri, 2006; Rether, 2011).

3.4.3.3 Bio dry mass (BDM)

Gravimetric dry biomass (Falkowski, Dubinsky & Wyman, 1985; Rai, Mallick, Singh & Kumar, 1991) measurements were carried out on the first, fourth and final days by initially collecting duplicates of 30 mL and then 20 mL samples, consecutively from each Flask via the straight male swabable valve into 50 mL Falcon tubes for the main-cultures (Figure 30F). For the pre-cultures it was carried out at the end of the cultivation (after 14 days) in 30 mL triplicates per Flask into stainless steel cylinders.

Thereafter, the samples were centrifuged at 4500 rpm for 30 minutes at 4 °C using the Rotina 420R, Hettich. The supernatant was decanted and samples of 4 mL per Flask for pre-cultures and 2 mL per Flask for main-culture were stored at -20 °C until further analysis for Cl^-, SO_4^{2-}, and NO_3^- determination. The micro-pellet left in the cylinder or falcon tube was resuspended in 30 mL of deionized water which was used to wash the pellet twice and simultaneously centrifuge after every wash at 4500 rpm for 30 minutes at 4 °C. Finally, the last bit of deionized water was discarded and the metal and falcon tubes were left in the oven for 48 hours to dry at 80 °C in a SLE-500, 35 Memmert drying oven. Thereafter, the cylinders containing the pellets were weighed. The BDM was determined using the BDM formula which equates the difference between the mass of the empty cylinder or falcon tubes and the mass containing the pellet divided by the sample volume. For example: $cx = (m2 - m1) \div Vs$

Where, cx = bio dry mass concentration (g/L)

m2 = mass of the cylinder with the biomass (g)

m1 = mass of the cylinder without the biomass (g)

Vs = sample volume (L)

Figure 30: Main-culture set up and procedure. (A & B) Make shift main-culture bioreactors, (C & D) Aeration and inoculation of main-cultures, (E, F, G & H) Centrifugation for BDM and Ion Chromatography, (I) Medium for lipid analysis (J & K) DMSO and Nile red method.

3.4.2.4 Ion chromatography (IC)

To appraise the quantity of macronutrient uptake, in this case the anions Cl^-, SO_4^{2-}, NO_3^- and PO_4^{3-} throughout these cultivations ion chromatography was applied using 822 Compact IC plus, Metrohm equipped with a conductivity detector (Metrohm) (Dillschneider, Steinweg, Rosello-Sastre & Posten, 2013). As for the pre-cultures

sampling was carried out at the end of the fourteen days via 4 mL per Flask as described above to compare with the anion concentrations at the start in the $BG_{11}{}^{0}$ stock solution. During the Main-cultivation the initial sampling was carried out right before CO_2 aeriation and inoculation from the straight male swabable valve via Braun Leur Lock syringe into 2 mL Eppendorf tubes per Flask, all without centrifugation since there was no cell suspension. Thereafter, sampling was carried out every day by retrieving 2 mL from each Flask from samples containing microalga suspension by centrifuging for 10 minutes at 11 000 rpm and the supernatant stored in the freezer at -20° C (Figure 30G). At the end of the cultivation the samples were defrosted, and 2 mL of the sample was diluted (1:10) according to the initial media, and injected by the professional sample processor 858 autosampler unit (Figure 30H). The ions bind on the stationary phase of the column and elute after a specific time depending on the exchange capacity of each macronutrient. The stationary phase was a Metrosep A Supp 5 column (Metrohm) consisting of polyvinyl-alcohol with quaternary ammonium groups. The elution buffer consisted of 3.2 mM Na_2CO_3, 1.0 mM $NaHCO_3$ and 12.5 % (v/v) acetonitrile in water (Dillschneider et al., 2013).

3.4.3.5 Neutral intracellular lipid quantification with Nile red fluorescence

This Nile red (9- diethylamino-5H-benzo[a]phenoxazine-5-one, Sigma–Aldrich) staining protocol was based on the methods published by Chen, Zhang, Song, Sommerfeld and Hu (2009) and Dillschneider et al. (2013). This is a popular and useful method for determining neutral intracellular lipids in microalgae and is applied to cells in an

aqueous medium (Abdo et al., n.d). The outcome of the neutral intracellular lipid accumulation is reported as gram lipids per gram biomass or percentage in the cells over cultivation time.

Sampling was carried out daily via the straight male swabable valve, via Braun Leur Lock syringe. Two millitres was sampled and transferred into Eppendorf tubes. The microalgae suspension was centrifuged for 10 minutes at 11 000 rpm (Figure 30G). The supernatant was decanted and the micro pellets stored at -20 °C. The samples were later defrosted and resuspended with 2 mL each of previously produced media with corresponding varied $NaNO_3$ concentrations (Figure 30I). After vortexing and to adjust the calibration range 1 mL from the samples were retrieved and diluted to an optical density (OD750 nm) of 0.15 in 1.5 mL Eppendorf tubes.

The preparation of Dimethyl sulfoxide (DMSO) stock solution was as follows: 50 mg of Nile red was dissolved into 50 mL DMSO. This was further diluted by adding 10 mL of it to 100 mL DMSO. Thereafter under the Thermo scientific biological safety cabinet, 625 µL DMSO, 25 µL Nile red working solution and 850 µL double distilled water were added in cuvettes to the previously diluted 1 mL sample for each treatment. The total 2.5 mL volume was homogenized by up and down motion using a pipette (Figure 30J & K). After 10 min of incubation in darkness and at room temperature the Aminco Bauman Series 2 (Thermo Electron Corporation) fluorescence spectrophotometer was used to measure the fluorescence intensity (a.u). The excitation and emission levels were set throughout this experiment by adopting previous recommendations on *Nannochloropsis*

by Greenspan, Mayer and Fowler (1985). Sensitivity was set at 700 V, excitation wavelength was set between 488 nm and 490 nm, the emission wavelength was in the range of 500 and 700 nm and the emission slits were set at 5 nm (Dillschneider et al., 2013; Elsey, Jameson, Raleigh & Cooney, 2007). The relative fluorescence of Nile Red for the lipids was obtained after subtraction of the auto fluorescence of microalgae cells and Nile Red alone. Auto fluorescence was also measured for a microalgae suspension without Nile Red.

3.5 Data analysis

All statistical analyses were performed using the SPSS software package (IBM statistics Version 23). Differences in data were considered significant at $p < 0.05$.

3.5.1 Relative genera abundance of microalgae

Even though, the relative genera data were analysed three times in the laboratory per specimen, per month, per season, the data was eventually pooled. The values of relative abundance were first classified in genera which were presented in bar charts and thereafter classes which were presented in pie charts using Windows Microsoft Excel 2007 spreadsheet. Since these were all absolute values, standard errors were not required.

To test for normality of data, the Shapiro-Wilk test was used. If the data were normally distributed independent T-test was used and when the data were not normally distributed

the Mann Whitney test was used to test for significant differences in relative genera abundance between the dry and wet seasons per dam.

The environmental data for correlation analysis were also pooled. Since there is no true independent and dependent relationship between relative genera abundance and environtmental parameters (Wojciechowski & Padial, 2015), correlations analysis was used to determine their relationships during the time of sampling. Pearson's product moment test was used if the data were normally distributed, whereas the Spearman's rank correlation test was performed when the data were not normally distributed.

3.5.1.1 Diversity, Evenness and Richness of microalgae

Indices were used to determine diversity, richness and evenness. These were displayed by means of bar charts using Windows Microsoft Excel 2007 spreadsheet. These were absolute values and therefore, do not recquire standard errors. The Shannon-Weiner diversity index was applied to calculate the relative genera diversity (Boyce, 2005; Morgado, Antunes & Pastorinho, 2003; Shannon & Weaver, 1948). Evenness Index (J') and Margalef's Index (d) was used to calculate the relative genera evenness (i.e. how evenly individuals are distributed among species) and relative genera richness respectively (Cao et al., 2007; Chen et al 2011; Boyce, 2005). The Shannon-Weiner diversity index was calculated as follows:

$$H' = -\sum_{i=1}^{5} p_i \ln(p_i)$$

Where (H') = Shannon-Wiener index

S= total number of species

i = the proportion of species

(p_i)= proportion of all observations in the i^{th} species category

Ln(p_i) = the natural logarithm of this proportion

The resulting product is summed across species, and multiplied by -1.

Evenness Index (J') is the ratio of Shannon-Wiener Diversity Index (H') to the maximum H' value; J' = $\frac{H'}{H'max}$

Where, H' = Shannon-Wiener Diversity Index

H' max = $\log_2 S$

S = total number of species

Margalef's Index (d) was used to calculate species richness. Margalef Index (d) = $\frac{S-1}{\ln(N)}$

Where, S = number of species in a community

N = total number of individuals per species

3.5.2 Optimization of neutral intracellular lipid and biomass production in *Nannochloropsis limnetica* SAG 18.99

All volumetric productivities (bio dry mass productivity and volumetric lipid productivity) were calculated by linear regression as the slopes of curves of biomass concentration and lipid concentration over cultivation process time after onset of various macronutrient concentrations and especially nitrate variations (Han, Li, Miao & Yu, 2012). Additionally, the effects of variant nitrate concentrations and pH on growth rates

(OD value), cell density, bio dry mass and lipid production were also calculated via linear regression over the cultivation process time. On the days BDM was not determined gravimetrically, the correlation factor between OD and BDM was calculated individually for every Flask i.e. BDM (initial) ÷ OD (initial) = Correlation Factor and, therefore, BDM = the Correlation factor x OD for that specific day.

In order to determine whether nitrate concentrations had a significant effect on cell growth, biomass and lipid productivity during the Main-cultivation a Shapiro-Wilk test was first used to test for normality. Thereafter, if the data were normally distributed one way ANOVA test was used and when the data were not normally distributed Kruskal Wallis test was carried out. The same procedure as before during relative genus abundance was followed to assess the correlations between various macronutrients in the BG_{11}^{0} stock solution (i.e. Cl^{-}, SO_4^{2-}, NO_3^{-} and PO_4^{3-}) against cell growth, BDM and lipid production.

CHAPTER 4

4. RESULTS

4.1 Relative genera abundance of microalgae

Genera relative abundance for all the dams was not normally distributed (p = 0.000). Von Bach dam seasonal relative genera abundance was statistically not significant (p = 1.000). Goreangab dam seasonal relative genera abundance was statistically not significant (p = 0.652) and Swakoppoort dam relative genera abundance was statistically not significant (p = 0.444). Therefore, the null hypotheses were accepted.

4.1.1 Swakoppoort dam

During the dry season the most abundant class was Chlorophyceae (41 %), followed by Cyanophyceae (30.8 %) and Bacillariophyceae (7.7 %). The rest of the classes contributed 5.1 %. During the wet season, Chlorophyceae still remained the most abundant class (57.1 %) followed by Cyanophyceae (22.5 %) and the rest of the classes had a relative abundance of 4.18 % (Figure 31).

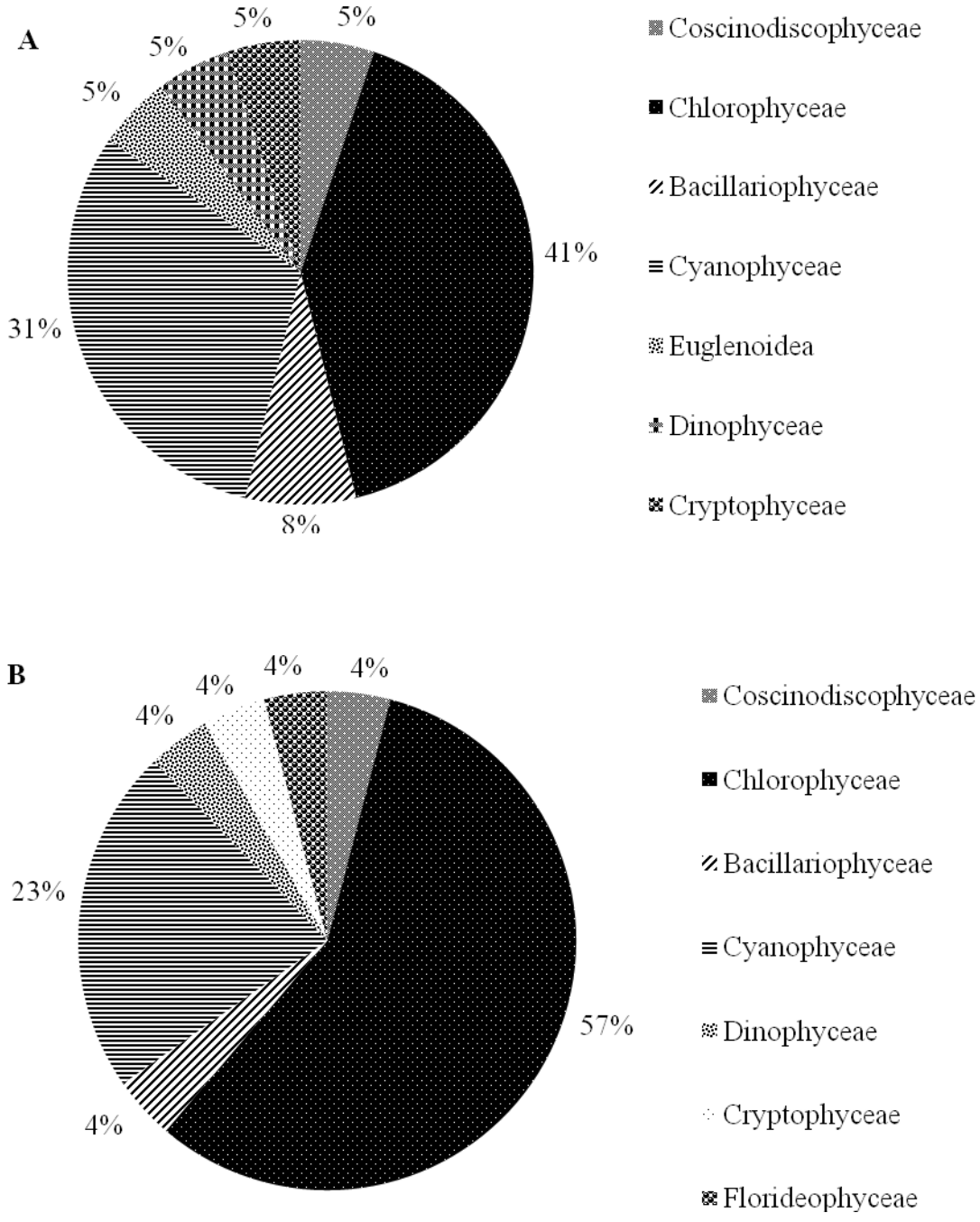

Figure 31: Relative abundance of microalgae classes in Swakoppoort dam during the (A) dry and (B) wet seasons.

Figure 32 shows the relative abundance of the microalgae classified to genus level sampled during the dry and the wet season in Swakoppoort dam. The genus *Microcystis* had the highest relative abundance during the dry season (39.8 %), followed by *Closteriopsis* (12.5 %) and then *Scenedesmus* (11.8 %). During the wet season the genus *Microcystis* was also the most abundant genus (60 %) followed by *Anabaena* (26.3 %) and thereafter *Chlamydomonas* (5.9 %).

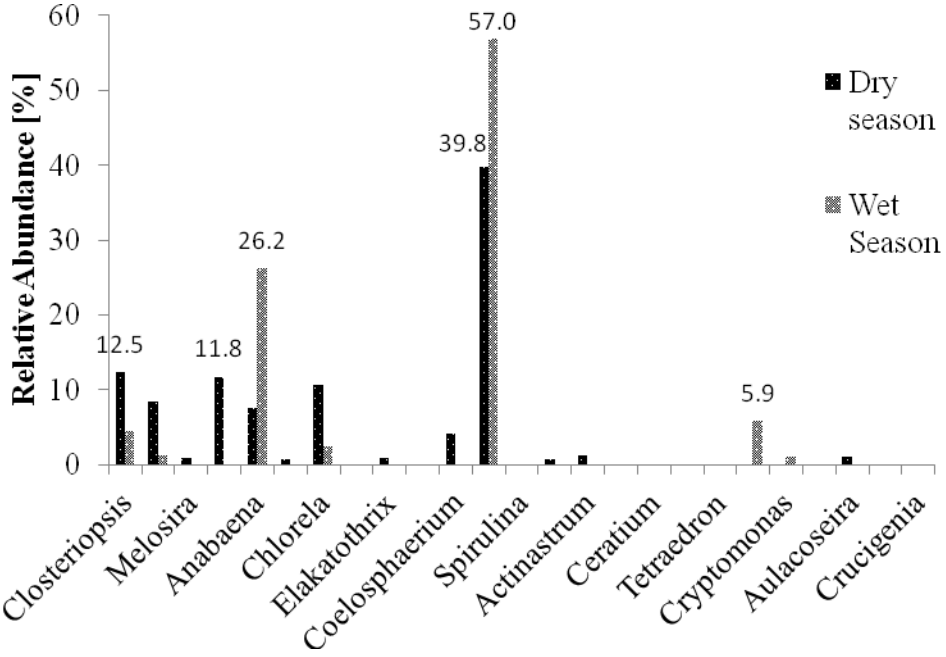

Figure 32: Relative abundance of microalgae genera in Swakoppoort dam during the dry and wet seasons.

4.1.2 Von Bach dam

During the dry season the class Chlorophyceae was the most abundant group (45.5 %), followed by Cyanophyceae (18.2 %) and then Bacillariophyceae (12.1 %). The remainder of the classes contributed to 6.1 % of the microalgae abundance. During the wet season the most abundant class was also Chlorophyceae (46.7 %), followed by Cyanophyceae (20 %), whilst the remainder of the classes contributed to 6.7 % of the microalgae abundance (Figure 33).

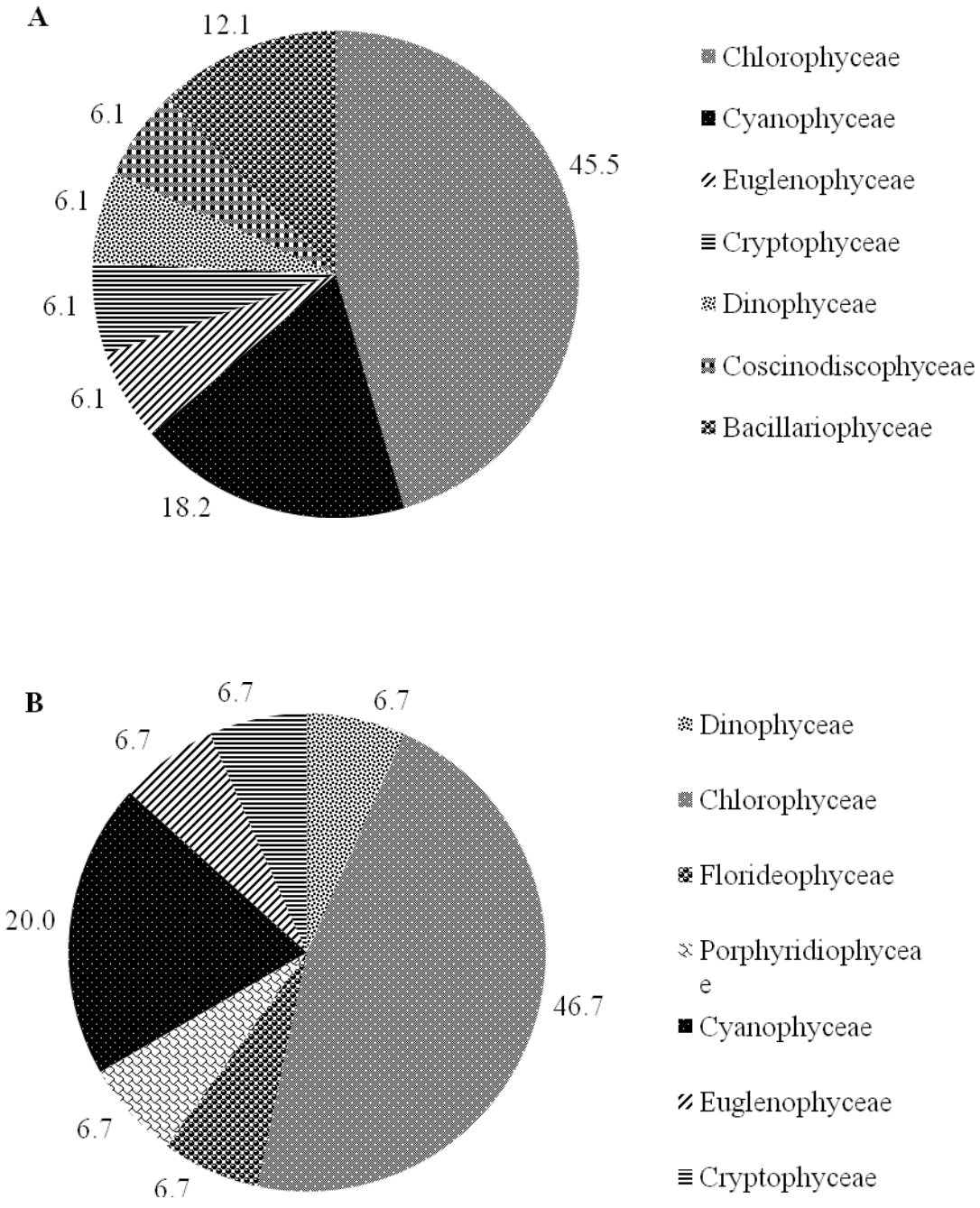

Figure 33: Relative abundance of microalgae classes in Von Bach dam during the (A) dry and (B) wet seasons.

During the dry season the genus *Aulacoseira* had the highest relevant abundance (85.1 %), followed by *Coelosphaerium* (6.8 %) and then *Chlorela* (6 %) (Figure 34). As for the wet season, *Ceratium* was the most abundant (78.36 %), followed by *Closteriopsis* (63.9 %) and then *Peridinium* (19.5 %) (Figure 34).

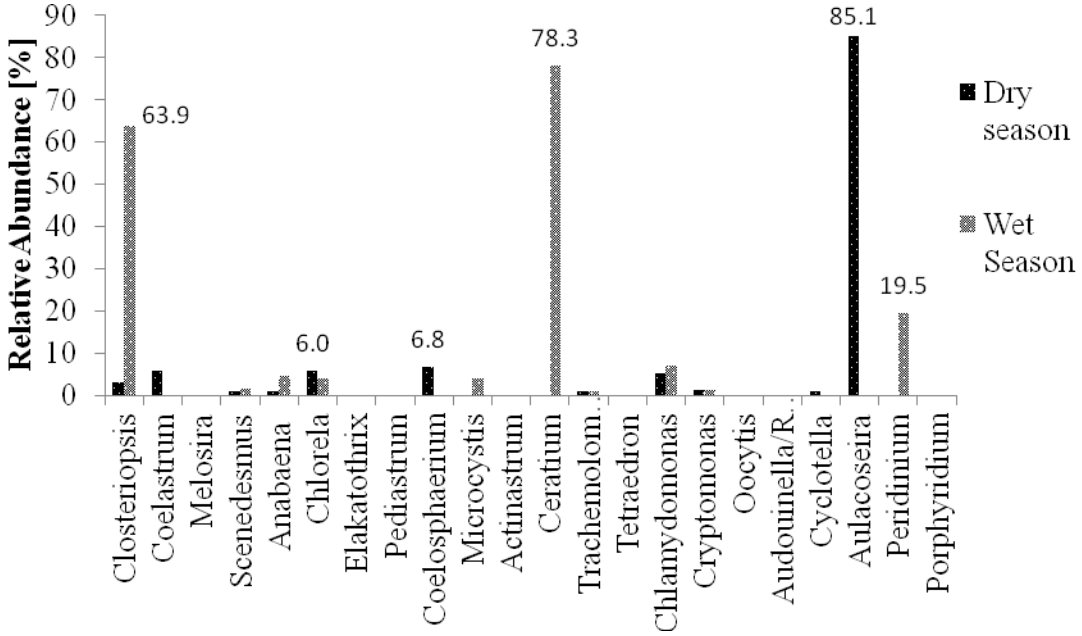

Figure 34: Relative abundance of microalgae genera in Von Bach dam during the dry and wet seasons.

4.1.3 Goreangab dam

Figure 35 shows the classes sampled at Goreangab dam during the dry and wet seasons. During the dry season the class Chlorophyceae had the highest relative abundance (34.4 %), followed by Cyanophyceae at 21.9 %, whilst the rest of the classes contributed 6.3 % to the total microalgae abundance. During the wet season, Chlorophyceae also had the highest abundance (26.7 %), followed by Florideophyceae and Cyanophyceae (13.3 % each), while the remainder of the other classes contributed to 6.7 % of the microalgae abundance.

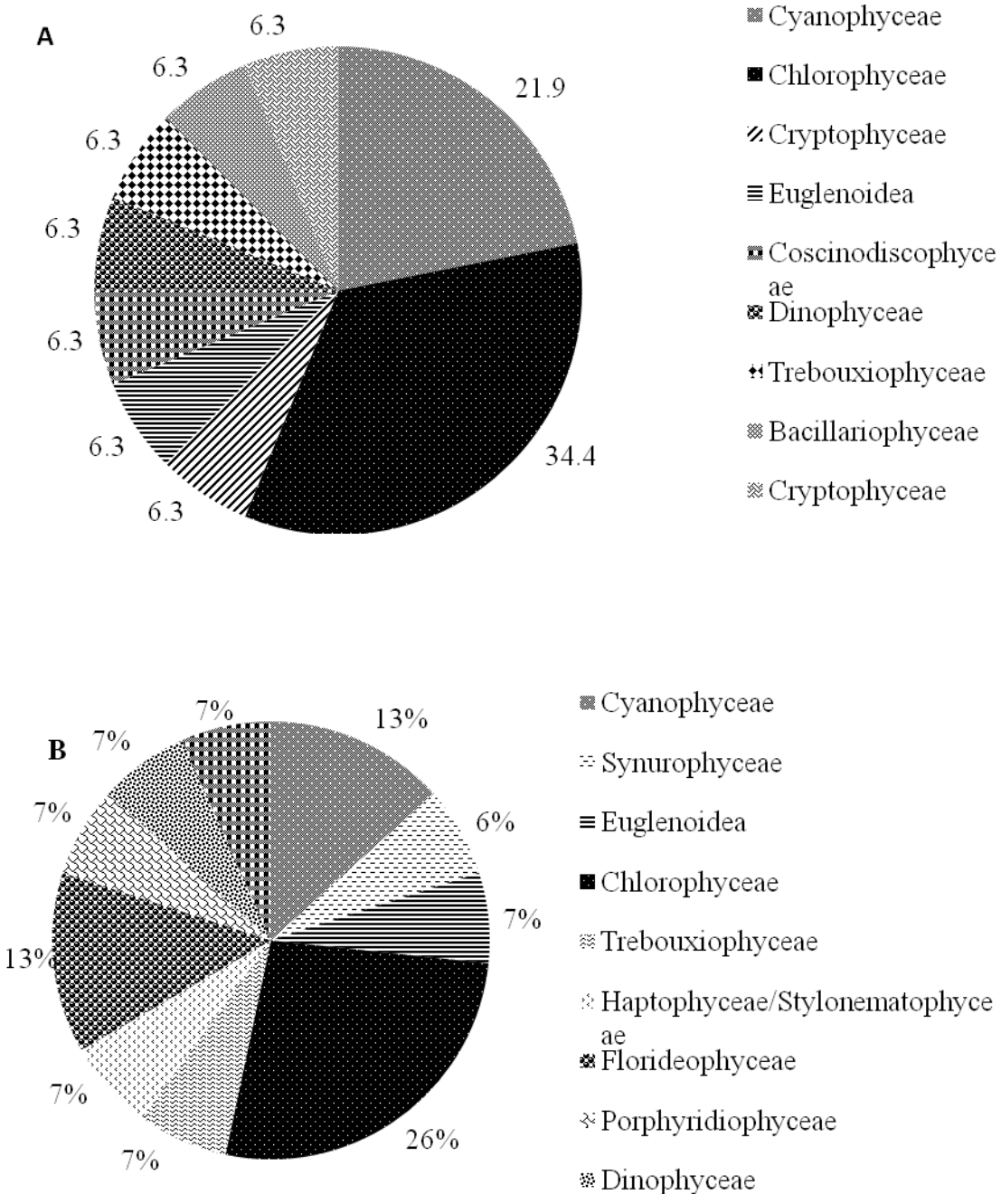

Figure 35: Relative abundance of microalgae classes in Goreangab dam during the (A) dry and (B) wet seasons.

During the dry season at Goreangab dam the genus *Mycrocystis* had the highest relative abundance (70.2 %), followed by *Pediastrum* (35.2 %) and then *Cryptomonas* (4 %) (Figure 36). During the wet season the highest relative abundance was also from the genus *Microcystis* (49.9 %), followed by *Cryptomonas* (4 %) and then *Chlorela* (2.3 %) (Figure 36).

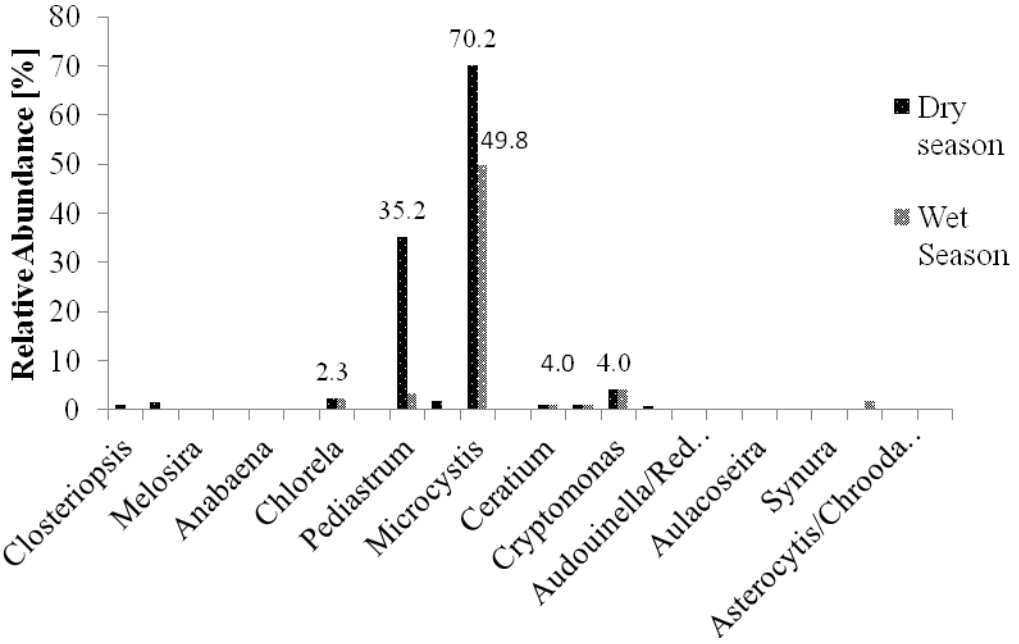

Figure 36: Relative abundance of microalgae genera in Goreangab dam during the dry and wet seasons.

4.1.4 Diversity, Evenness and Richness of microalgae

A high diversity of microalgae genera was identified from all three aquatic systems (Figure 37).

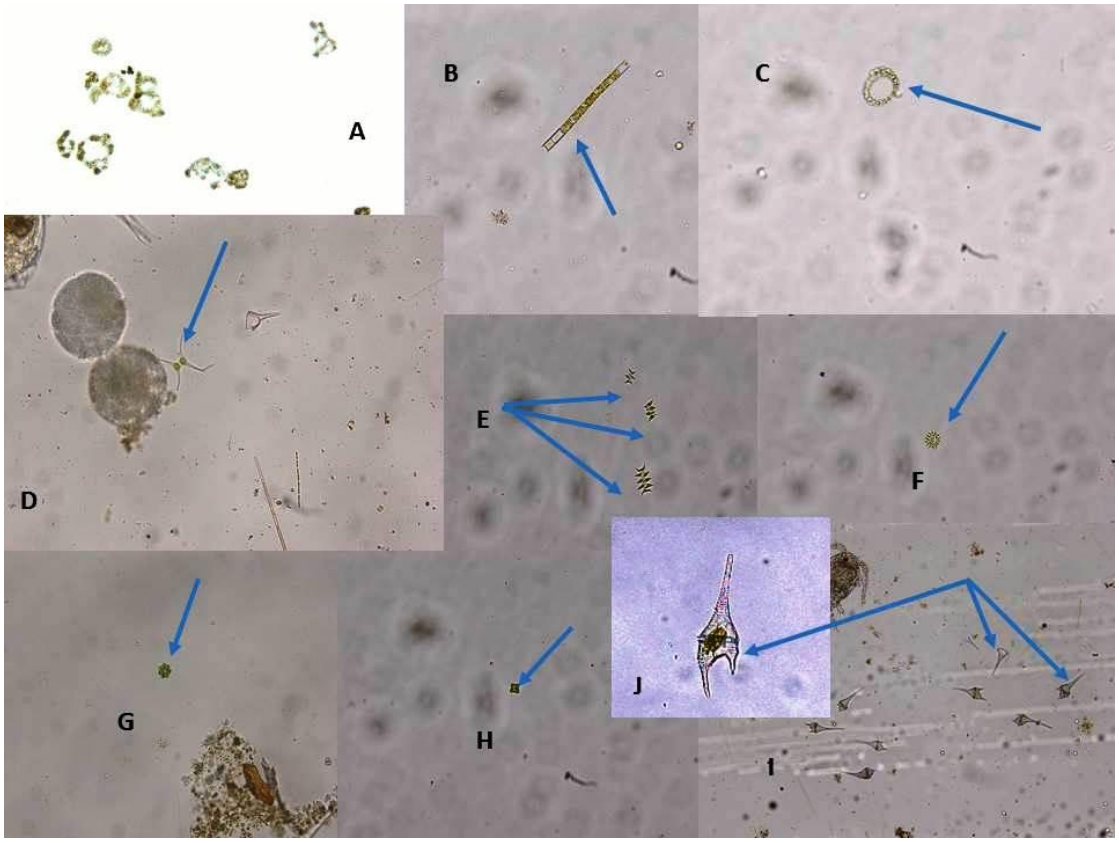

Figure 37: Brightfield photomicrographs at 100x magnification. (A) *Microcystis*, (B) *Melosira*, (C) *Anabaena*, (D) *Staurastrum*, (E) *Scenedesmus*, (F) *Pediastrum*, (G) *Coelestrum*, (H) *Tetraeron*, (I) *Ceratium*, (J) *Ceratium* (400x).

The Shannon-Weiner diversity index (H') for Swakoppoort dam was similar in both the dry and wet seasons at 2.2 and 2.1, respectively. At Goreangab dam diversity indices

within the same range were also found during the dry and wet seasons at 2.7 and 1.9, respectively. The diversity of microalgae was higher in Von Bach dam compared to the other two aquatic systems sampled, especially during the dry season at 4.1 and it was lower during the wet season at 3.1. The overall diagnosis amongst the three dams indicates that Von Bach dam has the highest microalgae diversity (average of 3.2), followed by Goreangab dam at 2.3 and the lowest diversity was in Swakoppoort dam at 2.1 (Figure 38).

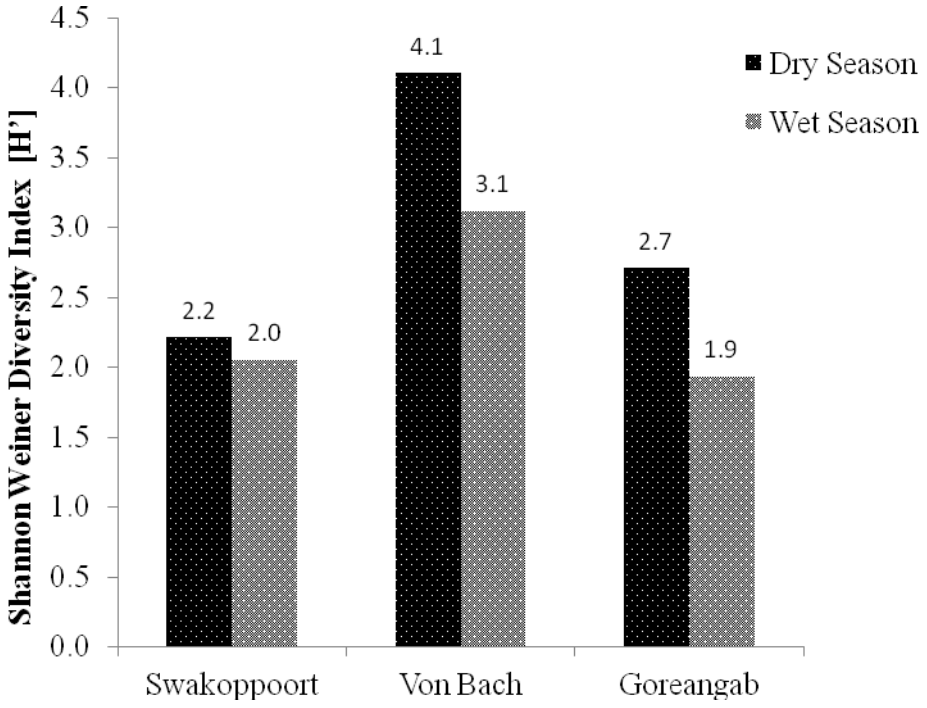

Figure 38: Shannon-Weiner Diversity Index (H') for Swakoppoort, Von Bach and Goreangab dams during the dry and wet seasons.

The Evenness index (J') was the highest during the dry season at Von Bach dam (0.97) and the lowest during the wet season at Goreangab dam (0.46) (Figure 39). Figure 40 shows the Margalef index (d) which was the highest in Swakoppoort dam during the dry season (1.48) and the lowest during the wet season in Goreangab dam (0.98). Furthermore, the Margalef index for all the dams ranged between 1.0 and 1.5.

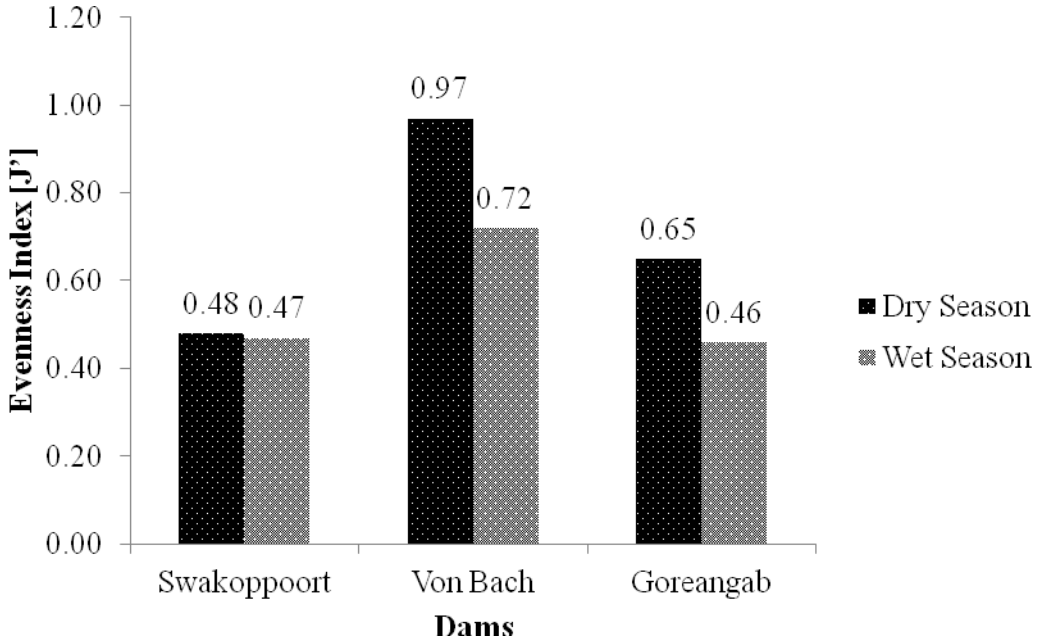

Figure 39: Evenness Index (J') for Swakoppoort, Von Bach and Goreangab dams during the dry and wet seasons.

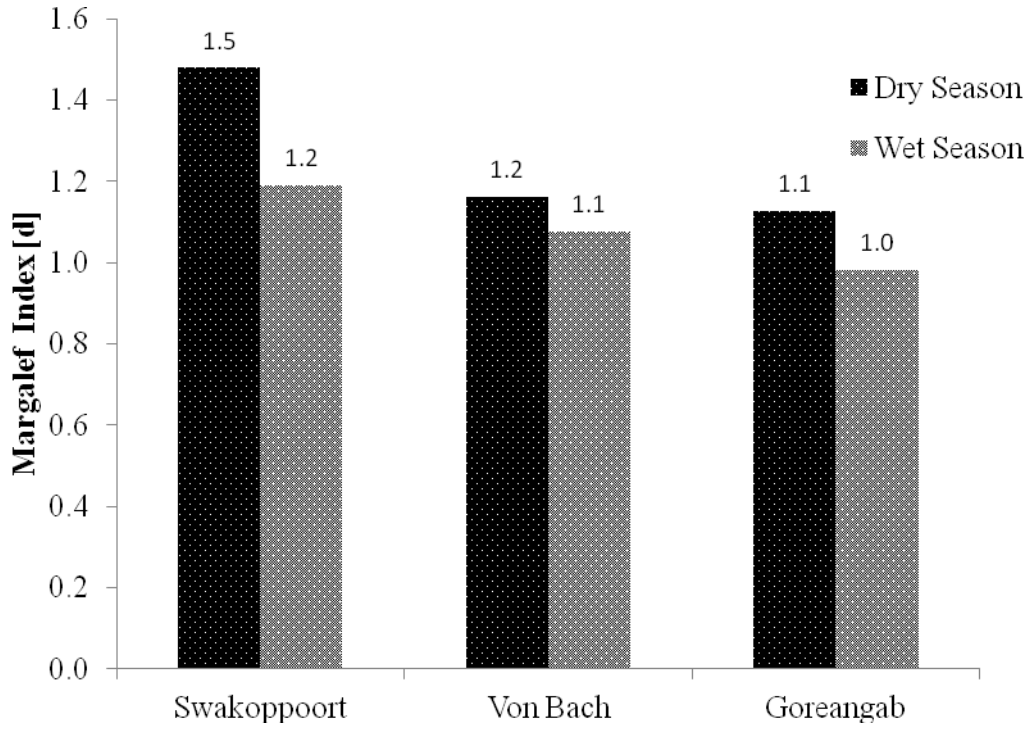

Figure 40: Margalef's Index (d) for Swakoppoort, Von Bach and Goreangab dams during the dry and wet seasons.

4.2 Environmental parameters

Average dam water volume for Swakoppoort dam was 38.01 Mm^3 during the dry season and 29.84 Mm^3 during the wet season (Table 12). The average dam water volume for Von Bach dam was 30.58 Mm^3 during the dry season and 24.69 Mm^3 during the wet season and Goreangab dam had 3.63 Mm^3 during the dry season and 3.63 Mm^3 during the wet season (Table 12).

The mean temperature for Swakoppoort dam in the dry season was 19 ºC ± 1.13 (n = 2)

and in the wet season 25.4 ºC ± 2.26 (*n* = 2), while in Von Bach dam it was 17.95 ºC ± 1.34 (*n* = 2) in the dry season and 25.25 ºC ± 1.63 (*n* = 2) in the wet season and in Goreangab dam it was 16.3 ºC ± 0.99 ºC (*n*=2) in the dry season and 23.4 ºC ± 1.41 ºC (*n* = 2) in the wet season (Table 15).

Turbidity was measured indirectly with a secchi disk and was 32 cm ± 2.83 (n = 2) in Swakoppoort dam during the dry season and 35.5 cm ± 6.36 (*n* = 2) during the wet season (Table 15). In Von Bach dam the secchi disk measurements was 95 cm ± 7.07 (*n* = 2) in the dry season and 89.5 cm ± 27.58 (*n* = 2) during the wet season and at Goreangab dam it was 110 cm ± 14.14 (*n* = 2) during the dry season and 88 cm ± 72.12 (*n* = 2) (Table15).

The mean dissolved oxygen concentration for Swakoppoort dam was 6.07 mg/L ± 4.84 (*n* = 2) during the dry season and 7.3 mg/L ± 1.84 (*n* = 2) during the wet season, while dissolved oxygen levels was 5.53 mg/L ± 3.18 (*n* = 2) in Von Bach dam during the dry season and 5.30 mg/L ± 1.14 (*n* = 2) during the wet season and for Goreangab dam it was 4.17 mg/L ± 4.99 (*n*= 2) during the dry season and 2.86 mg/L ± 0.36 (*n* = 2) during the wet season (Table 15).

The mean pH of the water in Swakoppoort dam during the dry season was 9.32 ± 2 (*n* = 2) and during the wet season 10.27 ± 1.70 (*n* = 2) during the wet season (Table 15). Von Bach dam had a pH of 7.86 ± 1.64 (*n* = 2) during the dry season and 9.22 ± 1.40 (*n* = 2) during the wet season and Goreangab dam had an average pH of 9.66 ± 0.91 (*n* = 2) during the dry season and 10.06 ± 1.96 (*n* = 2) during the wet season (Table 15).

Most of the macronutrients recorded during the wet season were lower than that of the dry season (Table 15). Swakoppoort dam had an average of 0.28 mg/L nitrate ± 0.08 (*n* = 2) during the dry season and 0.02 mg/L ± 0.01 (*n* = 2) during the wet season, while average phosphate concentrations were 0.27 mg/L ± 0.35 (n = 2) during the dry season and 0.07 mg/L ± 0.08 (*n* = 2) during the wet season and average ammonia concentrations were 0.23 mg/L (*n*=2) during the dry season and 0.10 mg/L ± 0.04 (n = 2) during the wet season (Table 15). Von Bach dam had an average nitrate concentration of 0.03 mg/L ± 0.01 mg/L (*n* = 2) during the dry season and 0.04 mg/L ± 0.02 mg/L (*n* = 2) during the wet season. It had an average phosphate concentration of 0.14 mg/L ± 0.15 (*n* = 2) during the dry season and 0.01 mg/L ± 0.01 mg/L (*n* = 2) during the wet season and an average ammonia concentration of 0.35 mg/L ± 0.13 (*n* = 2) during the dry season and 0.06 mg/L (*n* = 2) during the wet season (Table 15). Goreangab dam had an average nitrate concentration of 0.52 mg/L ± 0.06 (*n* = 2) during the dry season and 0.94 mg/L ± 0.37 (*n* = 2) during the wet season, it had an average phosphate concentration of 2.39 mg/L ± 1.49 (*n* = 2) during the dry season and 2.6 mg/L ± 1.27 (*n* = 2) during the wet season; it had average ammonia concentration of 0.30 mg/L ± 0.26 (*n* = 2) during the dry season and 2.44 mg/L ± 1.08 (*n* = 2) during the wet season (Table 15).

According to the statistical analysis performed, all of the environmental parameters measured for all the dams during the dry and wet seasons were not normally distributed (*p* = 0.000). Spearman's correlation tests were conducted for all the dams to consider the monotonic relationship between genus relative abundance and environmental

parameters. Swakoppoort results showed that between genus relative abundance and secchi disc depth, pH and temperature was a weak positive correlation that was not statistically significant (r_s (8) = 0.117, p = 0.450). A weaker positive correlation was observed between genus relative abundance and dissolved oxygen which was also not statistically significant (r_s (8) = 0.057, p = 0.711). The relationships between genus relative abundance and dam water volume, nitrate, phosphate and ammonia were weak, all negatively correlated and not statistically significantly (r_s (8) = -0.117, p = 0.450).

As for Von Bach dam, the relationships between genus relative abundance and secchi disk depth, pH, temperature, dissolved oxygen, dam water volume as well as nitrate, phosphate and ammonia were all strongly and positively correlated but not statistically significant (r_s (8) = 0.000 p = 1).

Goreangab dam results showed that the relationships between genus relative abundance and secchi disk depth, dissolved oxygen as well as dam water volume were very weak positively correlated and not statistically significant (r_s (8) = 0.076 p = 0.658). The relationships between nitrates, phosphates, ammonia, pH and temperature measured were very weak negatively correlated and not statistically significant (r_s (8) = -0.076 p = 0.658).

Table 15: Environmental parameters during the dry and wet months.

	Swakoppoort dam						Von Bach dam						Goreangab dam					
	Dry season			Wet season			Dry season			Wet season			Dry season			Wet season		
Date	14 June 2013	4 June 2014	**Avg.**	14 Nov. 2013	28 Feb. 2014	**Avg.**	14 June 2013	4 June 2014	**Avg.**	14 Nov. 2013	28 Feb 2014	**Avg.**	14 June 2013	4 June 2014	**Avg.**	14 Nov 2013	28 Feb 2014	**Avg.**
Time	11:00	12:56	-	13:46	12:45	-	14:25	16:04	-	15:00	15:13	-	16:50	08:56	-	10:21	09:12	-
Secchi disk (cm)	30	34	32	40	31	35.5	100	90	95	109	70	89.5	120	100	110	139	37	88
Temperature (ºC)	18.2	19.8	19	23.8	27	25.4	18.9	17	17.95	24.1	26.4	25.25	15.6	17	16.3	22.4	24.4	23.4
Dissolved Oxygen (mg/L)	9.49	2.64	6.07	8.6	6	7.3	7.78	3.28	5.53	4.49	6.1	5.30	7.7	0.64	4.17	2.6	3.11	2.86
pH	7.9	10.7	9.3	9.07	11.6	10.34	6.7	9	7.85	8.2	10.2	9.2	10.3	9.02	9.66	8.7	11.4	10.1
NO_3^--N (mg/L)	0.34	0.22	0.28	0.01	0.03	0.02	0.02	0.03	0.03	0.02	0.05	0.04	0.47	0.56	0.52	1.2	0.67	0.94
PO_4^--P (mg/L)	0.02	0.51	0.27	0.01	0.13	0.07	0.03	0.24	0.14	0	0.02	0.01	1.33	3.44	2.39	3.5	1.7	2.6
NH_3-N (mg/L)	0.07	0.39	0.23	0.07	0.13	0.1	0.26	0.44	0.35	0.06	0.06	0.06	0.11	0.48	0.30	3.2	1.67	2.44

4.3 Optimization of neutral intracellular lipid and biomass production in *Nannochloropsis limnetica* SAG 18.99

4.3.1 Pre-cultures

Table 16 shows the initial (as reflected on the BG_{11}^{0} stock solution) and final macronutrient concentrations at the end of the cultivation period. It also shows the bio dry mass (BDM) accumulation during this time.

4.3.1.1 Cell density

Figure 40 shows the cell densities/concentrations of the three Flasks cultivated for 14 days. Flask 1 had an $R^2 = 0.9556$, Flask 2 had an $R^2 = 0.9497$ and Flask 3 had an $R^2 = 0.9653$, indicating a near linear increase in cell number over time. Cell development, concentrations and morphology of the pre-cultures during the cultivation process are illustrated in Figure 42 A-G. Cells showed in Figure 42 F and G were used to inoculate the main-cultures.

Table 16: Final macronutrient concentrations and bio dry mass (BDM) weights for pre-cultures.

Flask	**Initial chloride (mg/L) -** $CaCl_2.2H_2O$	**Final chloride (mg/L) -** $CaCl_2.2H_2O$	**Initial nitrate (mg/L) -** $NaNO_3$	**Final nitrate (mg/L) -**$NaNO_3$	**Initial phosphate (mg/L) -** $K_2HPO_4.3H_2O$	**Final phosphate (mg/L) -** $K_2HPO_4.3H_2O$	**Initial sulfate (mg/L) -** $MgSO_4.7H_2O$	**Final sulfate (mg/L) -** $MgSO_4.7H_2O$	**BDM (mg/L)**
1	36	15.94	1500	840.8	40	-	75	14.0	466.70
2	36	12.47	1500	678.1	40	-	75	11.6	450.0
3	36	12.99	1500	677	40	-	75	10.9	536.70

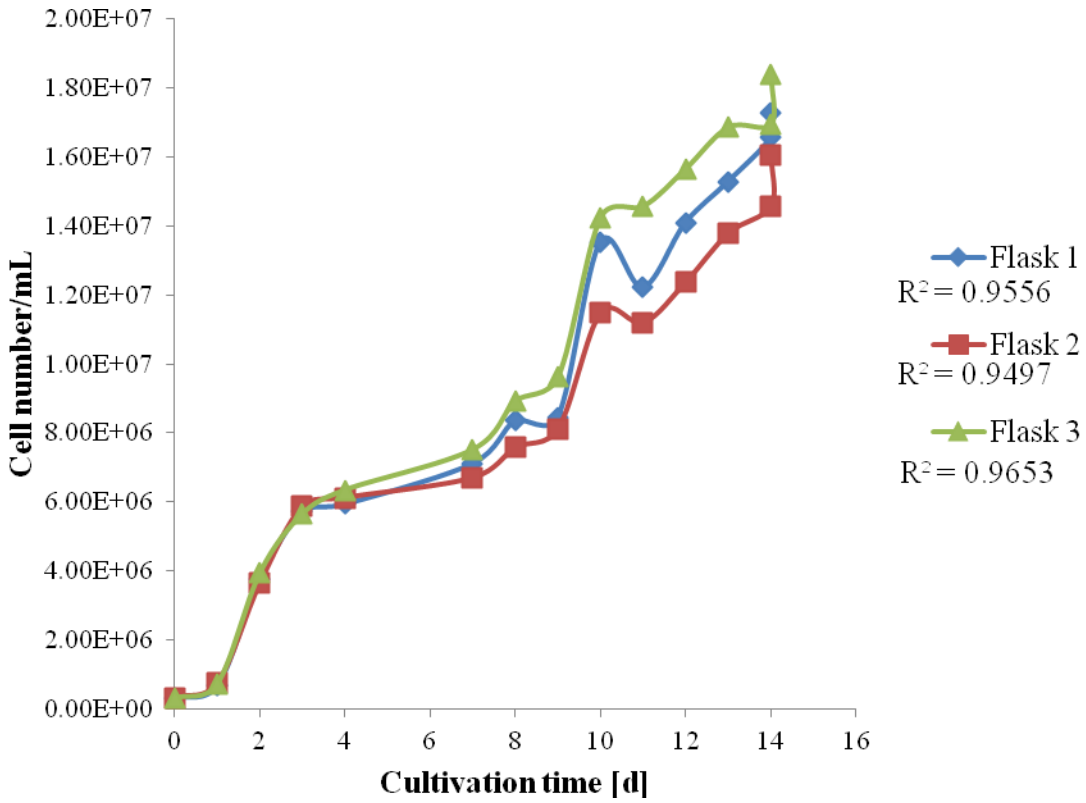

Figure 41: Cell concentrations of the pre-cultures during the 14 days of cultivation.

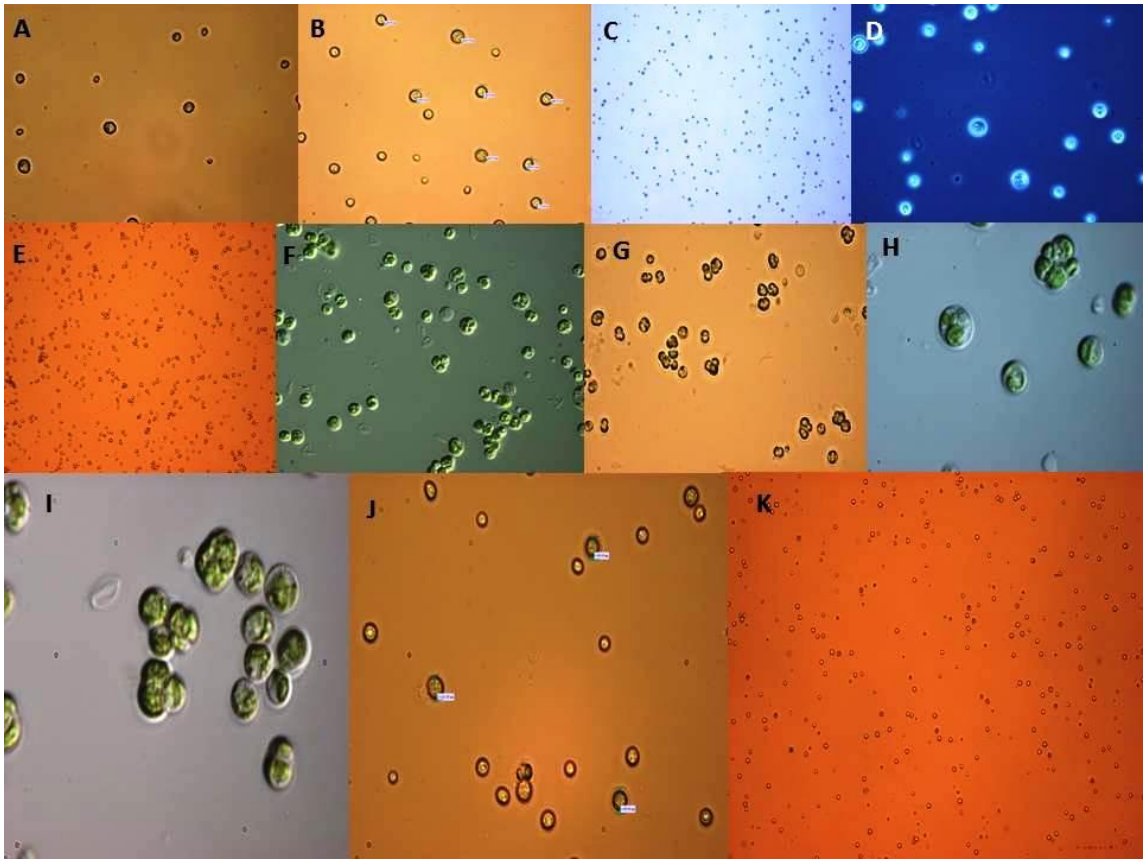

Figure 42: Series of Brightfield, Phase Contrast and DIC Light photomicrographs for pre-cultures and main-cultures. (A) Day 30/07/15 (400x), (B) Day 31/07/15 (400x), (C) Day 06/08/15 (100x), (D) Day 07/08/15 (400x), (E) Day 27/08/15 (100x), (F & G) Day 28/08/15 (400x), (H & I) Day 28/08/15 (1000x) (main-culture), (J) Day 03/09/15 (400x) (main-culture), (K) Day 04/09/15 (400x) (main-culture).

Figures 43 to 45 follow the growth rates (in this case optical density set at 750 nm) and the pH changes during the cultivation duration for pre-cultures. There was an increase in pH from 7.5 to 8.7 over the 2 week period as cell density increased for all the pre-

cultures. Growth was not normally distributed (p = 0.032). Kruskal Wallis test indicated that growth rate was the same across all Flasks and was not statistically significant (p = 0.733). pH was normally distributed (p = 0.194). One-way Anova showed that it was not statistically significant (p = 0.580) amongst all the flasks. Spearman's test conducted to assess the relationship between pH and growth rate amongst all the Flasks during the cultivation process showed that it was very strong, positively correlated and statistically significant (r_s (8) = 0.988; p = 0.000).

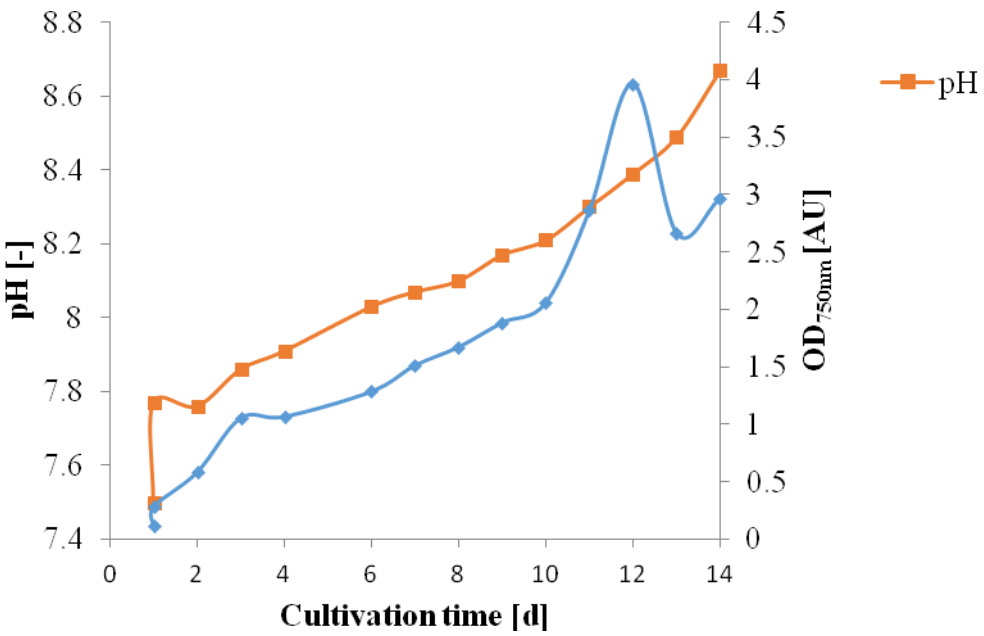

Figure 43: pH and optical densities in Flask 1 during the pre-culture establishment over 2 weeks.

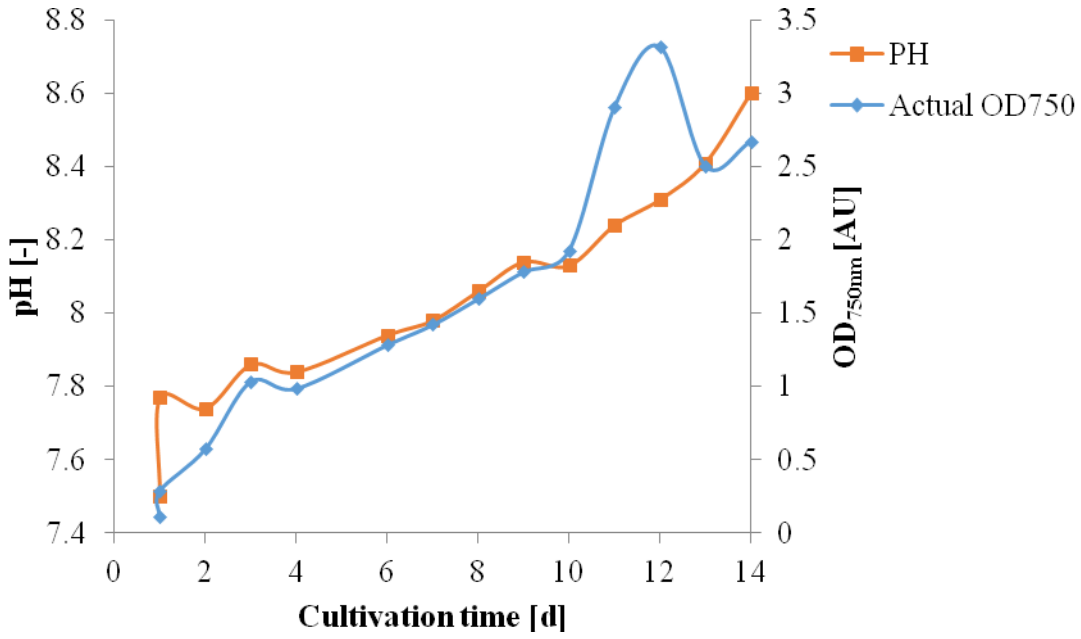

Figure 44: pH and optical densities in Flask 2 during the pre-culture establishment over 2 weeks.

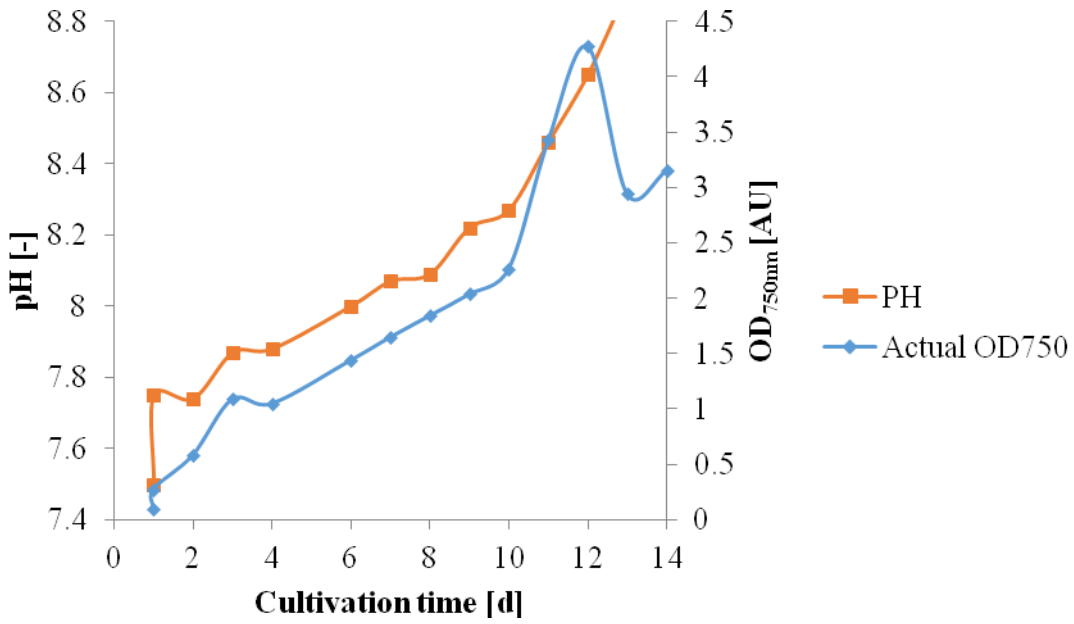

Figure 45: pH and optical densities in Flask 3 during the pre-culture establishment over 2 weeks.

4.3.2 Main-culture

Table 17 shows the initial macronutrient IC results of the BG_{11}^{0} stock solutions prepared for normal nitrate (3.53 M) (A1 & A2), nitrate replete (7.06 M) (B1 & B2), moderate nitrate deplete (1.765 M) (C1 & C2), and high nitrate deplete (0.8825 M) (D1 & D2) cultivated under the same conditions of light, pH, temperature and CO_2 concentration. However, for this section to generate results, Flasks A1, B2, C2 and D1 will be chosen to represent the considered levels of nitrate concentrations since they reflect the closest amounts of the expected concentrations derived from the BG_{11}^{0} stock solution (Rippka

et al., 1979). In reporting results here Flask/s and treatment/s will be used interchangeably as having the same meaning.

Table 17: Macronutrient concentrations before inoculations for main-cultures.

Flask	**Chloride (mg/L)** - $CaCl_2.2H_2O$	**Nitrate (mg/L)** - $NaNO_3$	**Phosphate (mg/L)** - $K_2HPO_4.3H_2O$	**Sulfate (mg/L)** - $MgSO_4.7H_2O$
Normal Nitrate (3.53 M)				
A1	15.15	1351.64	6.15	22.13
A2	14.15	1277.24	6.53	21.06
Replete Nitrate (7.06 M)				
B1	9.14	1416.47	5.87	15.02
B2	13.92	2164.88	6.8	25.33
Moderate Nitrate deplete (1.765 M)				
C1	13.76	603.61	5.96	22.62
C2	14.72	649.2	4.43	21.45
Hight Nitrate deplete (0.8825 M)				
D1	16.30	362.32	4.69	26.53
D2	14.14	618.75	7.28	22.20

4.3.2.1 Cell density

Cell density could not be captured during the main-culture cultivation because of malfunctioning of the Flow cytometer during this time. Figure 42 (H-K) shows the early and intermediary growth stages of *N. limnetica* for Flask A1.

4.3.2.2 Growth rates (Optical density: OD) in relation to pH and macronutrients

Optical cell density (OD) revealed a sigmoidal growth curve in Flasks A1 and B2 and for the linear phase R^2 was 0.785 and 0.8568, respectively (Figures 46 and 47). For Flask C2 a sigmoidal growth curve was obtained during the first 6 days of cultivation with a linear phase of R^2 of only 0.148, but after day 6 OD decreased to zero (Figure 48). As for Flask D1 the sigmoidal growth curve was obtained during the first 6 days at a linear phase of $R^2 = 0.7982$ (Figure 49).

The highest growth rate therefore, for A1 was on 10 day^{-1}, at 15.10 hours at the OD of 17.92. The highest growth rate for Flask B2 was on 8 $days^{-1}$, obtained after 15.17 hours and the OD measured at that time was 16.38. The highest growth rate for Flask C2 was 6 $days^{-1}$ obtained after 11.32 hours at an OD of 16.11. The highest growth rate for D1 was 10 $days^{-1}$, 16.19 hours at 21.68 OD.

Shapiro-Wilk test showed that the growth rates across all of the Flasks during cultivation were not normally distributed ($p = 0.012$). Kruskal Wallis showed that they were statistically significant ($p = 0.001$). Therefore, the null hypothesis was rejected. Similarly, pH and all the macronutrients in all of the Flasks during cultivation were not normally distributed (i.e. pH ($p = 0.000$), chloride ($p = 0.000$), nitrate ($p = 0.010$), phosphate ($p = 0.000$) and sulfate ($p = 0.000$)). Kruskal Wallis showed that pH ($p = 0.671$), chloride ($p = 0.104$), phosphate ($p = 0.180$) and sulfate ($p = 0.319$) were statistically not significant. Nitrate, however was statistically significant ($p = 0.000$).

Correlation analysis using Spearman's between growth rates vs pH was very weak and

positively correlated and not statistically significant at (r_s (8) = 0.041, p = 0.759); between growth and chloride was weak and negatively correlated and not statistically significant at (r_s (8) = -0.014, p = 0.915), between growth rates and nitrate was weak and negatively correlated and not statistically significant at (r_s (8) = -0.164, p = 0.209); between growth rates and phosphate was moderate and negatively and statistically significant at (r_s (8) = -0.520, p = 0.001); between growth rates and sulfate was very strong and negatively correlated and statistically significant (r_s (8) = -0.887, p = 0.000).

4.3.2.3 Bio dry mass (BDM) in relation to pH and macronutrients

During the interpretation of the BDM results for this section the correlation factor was not used to calculate the BDM for the days not gravimetrically sampled. This was because the BDM generated by correlation was not a true and accurate reflection. Therefore, BDM interpretation was only based on the three days gravimetrically sampled.

BDM increased linearly over time for all of the Flasks (A1 (R^2 = 0.9987), B2 (R^2 = 0.987), C2 (R^2 = 0.9868) D1 (R^2 = 0.9611, OD)) (Figures 46-49). The highest BDM for Flask A1 was measured at 4985 mg/L on day 14, 0.374 % neutral intracellular lipid content, 7.4 pH, 16.4 OD at 18.695 mg/L chloride, 664.0 mg/L nitrate, 0.25 phosphate and 0.088 mg/L sulfate. Flask B2 shows the highest BDM captured was 5775 mg/L, on day 14, 19.26 hours, at pH of 7.61, 0.316 % lipid content, 17.113 mg/L chloride, 1902.78 mg/L nitrate, 0.628 mg/L phosphate and 0 mg/L sulfate. The highest BDM for Flask C2 was 7780 mg/L at a growth rate of 14 $days^{-1}$ after 19.47 hours at a pH of 7.1,

lipid content of 0.463 %, OD of 0.044 at 15.583 mg/L chloride, 60.27 mg/L nitrate, 0.182 mg/L phosphate and 0 sulfate. The highest BDM for Flask D1 was 6630 mg/L at 14 $days^{-1}$, 19.52 hours, pH 7.08, neutral intracellular lipid at 0.71 %, and OD at 19.2 at 18.405 mg/L chloride, 3.503 mg/L nitrate and 0 mg/L phosphate and sulfate.

The lowest BDM for Flask A1 was 604 mg/L after 0.4 hours, at a pH of 7.7, neutral intracellular lipid content of 2.45 %, OD of 0.114 at 15.826 mg/L chloride, 1337.31 mg/L nitrate, 7.048 mg/L phosphate and 22.145 mg/L sulfate. The lowest BDM for B2 was 586.7 mg/L at day^{-1} 0, 5.02 hours, at 13.651 mg/L chloride, 2426.456 mg/L nitrate, 6.731 mg/L phosphate and 20.001 mg/L sulfate. Flask C2 had its lowest BDM at 486.67 mg/L, OD of 0.587 at 15.091 mg/L chloride, 693.207 mg/L nitrate, 7.713 mg/L phosphate and 22.716 mg/L sulfate. Flask D1 lowest BDM measured was 253 mg/L at 0 $days^{-1}$, 5.15 hours, pH 7.63 at 8.905 mg/L chloride, 203.126 mg/L nitrate, 4.569 mg/L phosphate and 13.814 mg/L sulfate.

Bio dry mass showed a normal distribution of $p = 0.055$ during the cultivation process across all of the Flasks and statistical significance using the one way Anova showed that it was statistically not significant ($p = 0.939$). Therefore, the null hypothesis was accepted. Since BDM was only sampled three times throughout the cultivation process, its relationship to growth rates, pH, lipids and macronutrients was statistically analyzed during those days. During this time BDM was normally distributed ($p = 0.055$); lipids were normally distributed ($p = 0.081$); pH was normally distributed ($p = 0.151$); growth rates were not normally distributed ($p = 0.019$); nitrate was normally distributed ($p = 0.180$), chloride was not normally distributed ($p = 0.016$), phosphate was not normally

distributed (p = 0.001), sulfate was not normally distributed (p = 0.047). Pearson's correlation test showed that the relationship between BDM and lipids was strong and negatively correlated and statistically significant (r_s (8) = -0.765, p = 0.004); Pearson's correlation test showed that the relationship between BDM and pH was strong and negatively correlated and statistically significant (r_s (8) = -0.673, p = 0.017); Spearman's correlation test showed that the relationship between BDM and growth rates was moderate and positively correlated and statistically not significant (r_s (8) = 0.529, p = 0.077); Pearson's correlation test showed that the relationship between BDM and nitrate was weak, negatively correlated and statistically not significant (r_s (8) = -0.356, p = 0.256); Spearman's correlation test showed that the relationship between BDM and chloride was moderate, positively correlated and statistically not significant (r_s (8) = 0.517, p = 0.085); Spearman's correlation test showed that the relationship between BDM and phosphate was strong, negatively correlated and statistically significant (r_s (8) = -0.616, p = 0.033); Spearman's correlation test showed that the relationship between BDM and sulfate was strong, negatively correlated and statistically significant (r_s (8) = -.0.901, p = 0.000).

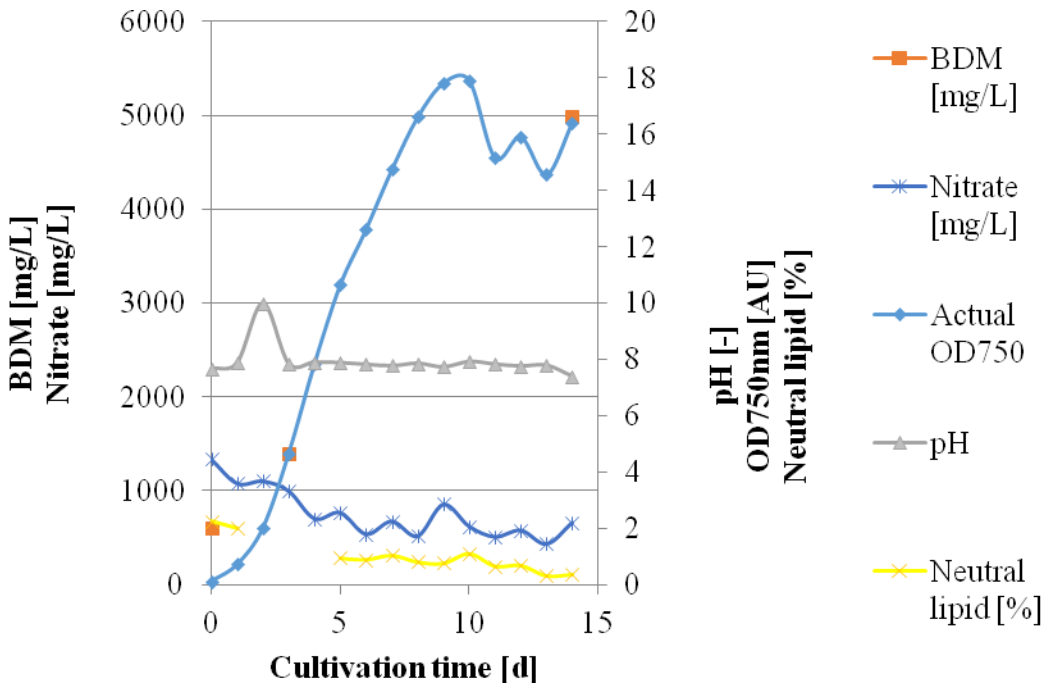

Figure 46: BDM, OD, neutral intracellular lipid, pH and nitrate in Flask A1 during the 2 week main-culture.

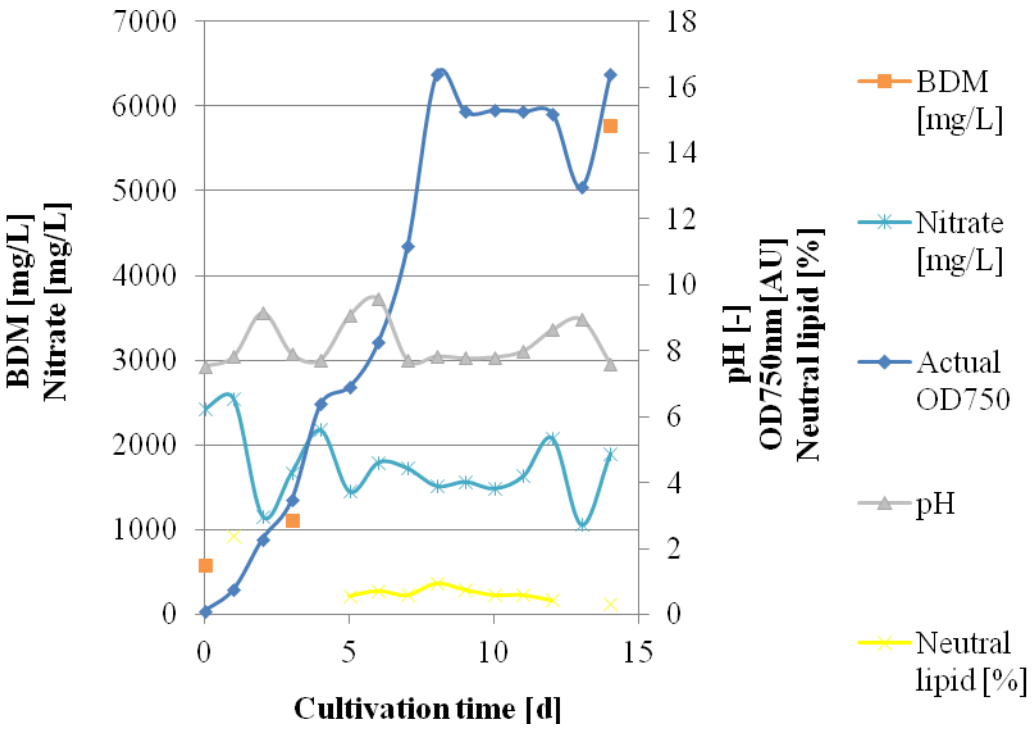

Figure 47: BDM, OD, neutral intracellular lipid, pH and nitrate in Flask B2 during the 2 week main-culture period.

4.3.2.4 Neutral intracellular lipid (%) in relation to pH and macronutrients

The calibration curve of Dillschneider, 2013 was not used to interpret the neutral intracellular lipids content during this study. This was because the relative fluorescence for all the three Flasks (A1, B2, C2, D1) remained low and below the threshold (< 2%) throughout the culture period (Figures 46-49). Therefore, only relative fluorescence was used to interpret the neutral intracellular lipid data. The linear regression for A1, B2, C2, D1 were respectively 0.8344, 0.6066, 0.6842 and 0.6747.

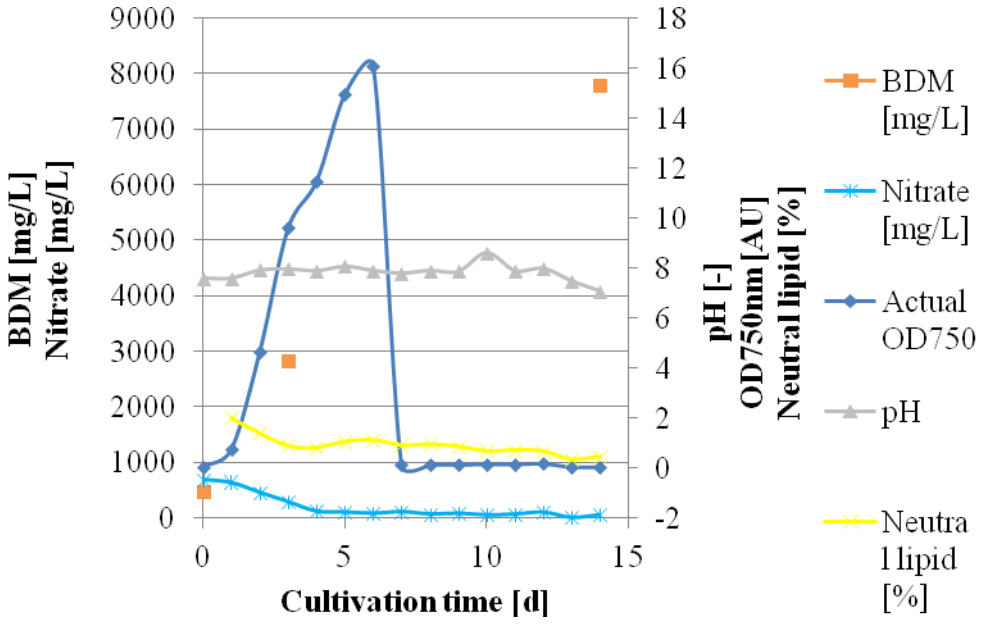

Figure 48: BDM, OD, neutral intracellular lipid, pH and nitrate in Flask C2 during the 2 week main-culture.

The highest neutral intracellular lipid for Flask A1 was 2.245 %, 4.50 hours, day^{-1} 0 at 15.826 mg/L chloride, 1337.31 mg/L nitrate, 7.048 mg/L phosphate and 22.145 mg/L sulfate. The highest neutral intracellular lipid captured for Flask B2 was 2.387 % at 1 day^{-1}, 6.28 hours at 14.716 mg/L chloride, 2550.439 mg/L nitrate, 3.707 mg/L phosphate and 20.433 mg/L sulfate. The highest neutral intracellular lipid captured for Flask C2 was 2.025 % at 0 days^{-1}, 6.45 hours at 15.395 mg/L chloride, 638.972 mg/L nitrate, 4.341 mg/L phosphate and 21.967 mg/L sulfate. The highest neutral intracellular lipid captured for Flask D1 was 1.95 % at 1 days^{-1}, 6.47 hours at 13.388 mg/L chloride, 255.183 mg/L nitrate, 2.532 mg/L phosphate and 18.995 mg/L sulfate.

The lowest neutral intracellular lipid captured for Flask A1 was 0.33 % on day^{-1} 13, 17.09 hours 11.417 mg/L chloride, 433.359 mg/L nitrate, and 0 mg/L phosphate and sulfate. The lowest neutral intracellular lipid captured for Flask B2 was 0.316 % at day^{-1} 14, 19.26 hours at 17.113 mg/L chloride, 1902.78 mg/L nitrate, 0.628 mg/L phosphate and 0 mg/L sulfate. The lowest neutral intracellular lipid captured for C2 was 0.33 % at 13 days^{-1}, 18.10 hours at 22.758 mg/L chloride, 5.902 mg/L nitrate, 0.211 mg/L phosphate and 0 mg/L sulfate. The lowest neutral intracellular lipid captured was 0.426 % at 13 days^{-1}, 18.22 hours at 12.485 mg/L chloride, 112.145 mg/L nitrate and 0 mg/L phosphate and sulfate.

Relative fluorescence data captured for intracellular lipid analysis, showed that during the cultivation process all of the data were not to be normal (p = 0.000) and the Kruskal Wallis test showed it to be statistically significant (p = 0.112). Therefore, the null hypothesis was accepted. Correlation tests were carried out to see whether there was a relationship between lipids, growth rates, pH and macronutrients during the time of BDM sampling. Pearson's correlation test showed that the relationship between lipids and pH was very weak, positively correlated and not statistically significant (r_s (8) = 0.173, p = 0.591); The relationship between lipids and growth rates was moderate, negative correlated and statistically not significant (r_s (8) = -0.501, p = 0.087); The relationship between lipids and nitrate was moderate, positively correlated and statistically not significant (r_s (8) = 0.232, p = 0.469). Spearman's correlation test showed that the relationship between lipids and chloride was moderate, negatively correlated and not statistically significant (r_s (8) = -0.441, p = 0.152); and between lipids

and phosphate was moderate, positively correlated and statistically not significant (r_s (8) = 0.520, p = 0.083).

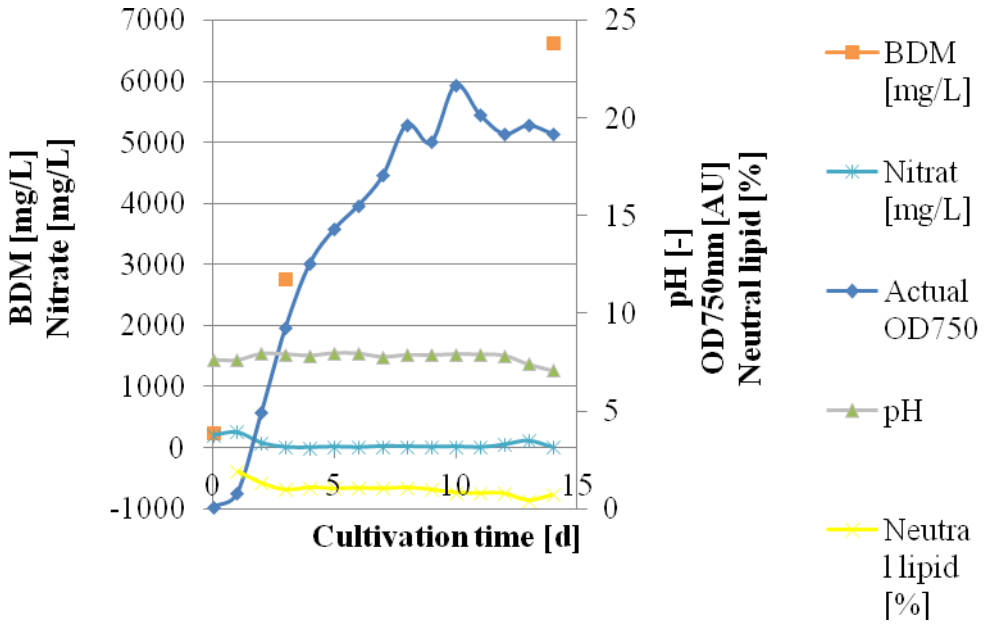

Figure 49: BDM, OD, neutral intracellular lipid, pH and nitrate in Flask D1 during the 2 week main-culture period.

4.3.2.5 pH in relation to macronutrients per treatment

Since pH plays a pivotal role in the ability of the microalgae cells to absorb macronutrients from the medium for biosynthesis, its fluctuations were carefully monitored. The linear regression pH values for A1, B2, C2 and D1 were R^2 = 0.1448, 0.0005, 0.0148 and 0.1389, respectively. The linear regression values for A1

macronutrients were nitrate $R^2 = 0.6486$, phosphate $R^2 = 0.3562$, chloride $R^2 = 0.1143$ and sulfate $R^2 = 0.7138$. The linear regression values for B2 were nitrate $R^2 = 0.151$, phosphate $R^2 = 0.4866$, chloride $R^2 = 0.0477$ and sulfate $R^2 = 0.8547$. The linear regression values for C2 were nitrate $R^2 = 0.6518$, phosphate $R^2 = 0.635$, chloride $R^2 = 0.1568$ and sulfate $R^2 = 0.9417$. The linear regression values for D1 were nitrate $R^2 = 0.2296$, phosphate $R^2 = 0.6647$, chloride $R^2 = 0.2744$ and sulfate $R^2 = 0.7473$.

The highest pH for Flask A1 was 9.98 on day^{-1} 2, 6.51 hours, 2.10 OD, at 19.048 mg/L chloride, 1103.828 mg/L nitrate, 0.591 phosphate and 17.717 mg/L sulfate. The highest pH for Flask B2 was 9.15 on day^{-1} 2, 7.26 hours, 2.29 OD, at 7.191 mg/L chloride, 1162.356 mg/L nitrate, 0 mg/L phosphate and 8.005 mg/L sulfate. The highest pH for Flask C2 was 8.61 on day^{-1} 10, 18.05 hours, 0.153 OD at 10.576 mg/L chloride, 53.719 mg/L nitrate, 0.199 mg/L phosphate and 0 mg/L sulfate. The highest pH measured for Flask D1 was 7.97 on day^{-1} 5, 12.10 hours, 14.31 OD at 19.248 mg/L chloride, 13.198 mg/L nitrate, 0.213 mg/L phosphate and 3.795 mg/L sulfate.

The lowest pH for Flask A1 was 7.4 and day^{-1} 14, 19.06 hours, 16.4 OD at 18.695 mg/L chloride, 664.021 mg/L nitrate, 0.25 mg/L phosphate and 0.088 mg/L sulfate. The lowest pH for Flask B2 measured was 7.53 on day^{-1} 0, 5.02 hours, 0.105 OD at 13.651 mg/L chloride, 2426.456 mg/L nitrate, 6.731 mg/L phosphate and 20.001 mg/L sulfate. The lowest pH for Flask C2 was 7.07, 14 $days^{-1}$, 19.47 hours, at 15.583 mg/L chloride, 60.27 mg/L nitrate, 0.182 mg/L phosphate and 0 sulfate. The lowest pH value recorded for Flask D1 measured was 7.08 on 14 $days^{-1}$, 19.52 hours at 18.405 mg/L chloride, 3.503 mg/L nitrate and 0 mg/L phosphate and sulfate.

CHAPTER 5

5. DISCUSSION

5.1 Ecological study of microalgae

Acceleration in human population and activity leads to increasing pressure on water quality and demand. This is especially detrimental for hyper arid countries such as Namibia that rely heavily on storage resevoirs and inter-basin transfers to curb water shortage. Short term solutions for water shortage do not answer the need for water pollution prevention and mitigation which leaves most of the countries in the developing world destitute.

In Namibia water quality deterioration in freshwater systems is a result of discharges from surface run-offs of especially rivers and their tributaries in catchment areas. These affluents which mostly occur in the wet season, transport and distribute waste material and especially inorganic nutrients from surrounding urban areas, sewage ponds, agricultural and mining activitiese into water storage reservoirs (Nyenje, Foppen, Uhlenbrook, Kulabako & Muwanga 2010; Sirunda & Mazvimavi, 2014). Lakes, impoundments and other confined water bodies as well as slow moving rivers with low mixing levels and, therefore, aeration accumulate nutrients and are susceptible to microalgal blooms and eutrophication, unlike fast flowing waters with lower retention times (Rast & Thornton 1996; Anderson et al, 2002).

The Namibian sub-topical climate provides a good environment for microalgae blooms of especially blue-green microalgae which are indicators of eutrophic freshwater environments (Gunnarsson & Sanseovic, 2001). This is especially true in man-made dams for potable water. Von Bach, Swakoppoort and Goreangab dams are Namibian freshwater dams that share the same catchment area. Similarities of nutrients from Swakop River and its tributaries have been documented by Shinana (2011) and Lehmann (2010). Sirunda and Mazvimavi (2014) even reported the negative effects of inter-basin transfer on water quality from Swakoppoort dam to Von Bach dam.

Microalgae blooms in freshwater systems act as primary producers and biofilters (Shaharuddin, Chang, Zakaria & AB Ghani, 2015) and can be used as bioindicators for numerous environmental factors such as pH, nitrogen (nitrate, nitrite and ammonia-nitrogen), phosphorus, salinity and oxygen depletion (Shaharuddin et al., 2015). Therefore, their diversity and composition can be used as a way of assessing the level of anthropogenic stress to aquatic systems (Shaharuddin et al., 2015). However, certain strains of these microalgae have pharmaceutical, nutracuetical and lipid biosynthetic abilities and thus can be harvested and applied to produce various valuable products that can offset the cost of water treatment processes associated with microalgae blooms (Kuo, 2010). For example, blue-green microalgae over the years have received great attention in the biotechnology world due to their physiological processes, including light-induced hydrogen evolution by biophotolysis (Skulberg, 1994). Some strains in this class act as food and fodder due to their high protein content, vitamins and other

essential growth factors (Borowitzka & Borowitzka, 1988). Many others are a source of valuable products (Richmond, 1990) that are essential in the pharmaceutical world such as pigments and antibiotics (Falch et al., 1995). Therefore, it is vital before any resource acquisition to identify the strains that are uniquely indigious to utilize to full potential.

To consider and understand the dynamics of microalgae in freshwater systems, they need to be viewed within the context of spatial heterogeneity and seasonal variations (Kirke, 2001; Ochumba & Kibaara, 2008; Reynolds & Walsby, 2008; Kuo, 2010; Sirunda & Mazvimavi, 2014). The narrative of this study was, therefore, to seek whether there is a significant difference in the seasonal relative genera abundance, diversity, richness and evenness of microalgae in Von Bach, Swakoppoort and Goreangab dams in central Namibia. Furthermore, increased levels of nutrients (i.e. phosphate, nitrate and ammonia) change microalgae community composition through induced changes in predation, resource limitation, light availability and biological effects on sediments, therefore, these were also considered (Anderson et al., 2002; Kuo, 2010). Moreover, a balance of many other factors such as light, water column stability, water turbulence, water temperature, pH, conductivity, turbidity, amongst others influence microalgae species composition. Amongst these, turbidity, water temperature, dissolved oxygen, pH and dam water volumes were considered in this study.

Relative genera abundance, diversity, evenness and richness of microalgae

In diversity studies, species richness is the total number of species present in a given area, species diversity takes into account how individuals are distributed amongst those species, whereas evenness describes how equally individuals are distributed amongst the species (Aslam, 2009). Dominant class and genera throughout the seasons in aquatic systems may be as a result of sufficient nutrient availability, good conditions and growth rates (Hossain, Khan, Sarkar & Haque, 2005). Many species of freshwater microalgae and especially from the class Chlorophyceae proliferate intensively in eutrophic waters due to their higher growth rates; however, they do not accumulate to form dense surface scums of high cell density. These blooms are generally caused by blue-green microalgae such as *Microcycstis*, *Anabeana*, *Aphanizomenon*, *and Oscillatoria* amongst others.

Blue-green microalgae can outcompete other phytoplankton in freshwater systems due to their many advantages. First they are mostly picoplankton (0.2 - 2 µm) providing an excellent surface to volume ratio for nutrient assimilation (Chorus & Bartram, 1999). Secondly, they have evolved specialized intracellular gas vesicles that allow them to actively seek water depths with optimal growth conditions (Hossain et al., 2005). Additionally, they secrete slime or carry out surface undulation of cells to enable photomovement (Häder, 1987; Paerl, 1988). Thirdly, they recquire very little energy to maintain cell function and structure when compared to other plankton (Gons, 1977; Van Liere, Mur, Gibson & Herdman, 1979). Therefore, they can maintain a higher growth

rate when compared to other plankton during low light intensities (Chorus & Bartram, 1999).

The current study can support this, since Chlorophyceae was the most dominant class followed by Cyanophycea for both dry and wet seasons. Whilst, the differentiation during genera specifications showed that *Microcystis* was the highest genus for both seasons for all the dams, except for Von Bach dam where *Aulacoseira* was the most dominant, while *Ceratium* was the most dominant during the wet season. Besides high growth rates and advantageous physiological features of blue-green microalgae, the differences in class and genera abundance can also be attributed to many other factors surrounding the dams. This is because blue-green microalgae prefer stable water conditions with low flows, long retention times, light winds and minimal turbulence (Nswgovau, 2016). In this case Goreangab dam was the most favourable for blue-green blooms since it is less hindered in the sense of mechanical extraction of water as well as its constant high influx of macronutrients from its surrounding environments. Nevertheless, there was no statistical significance in relative genera amongst the dams during the dry vs wet seasons. However, there is a need to investigate this further with a greater sampling size and frequency.

The Shannon-Weiner diversity index (H') has a threshold of 1.5 to 3.5. It increases as both the richness and the evenness of the community increase (Magurran, 2013). During this study, Von Bach dam had the highest diversity average followed by Goreangab dam and the lowest diversity was found in Swakoppoort dam. Species

eveness varied between 0.46-0.65 for Swakoppoort and Goreangab dams, but was higher for Von Bach dam (0.72 -0.97). Species richness was around 1 for all the Dams and throughout all the seasons, except for Swakopppoort dam during the dry season where it was 1.5.

Turbidity

Low turbidity is a result of low suspended particles in the water column. It is primarily influenced by surrounding geological environment and slow moving water that allows particulate matter to settle out of the water column (Nswgovau, 2016). During low turbidity, more light is available in the water for chlorophyll containing organisms, thus enables optimal growth conditions for blue-green microalgae. In turbid lakes, blue-green microalgae have a competitive advantage over other plankton due to their dense growths and specialised pigments (i.e. carotenoids) that allow for maximum light absorption and protection of cells from photoinhibition (Cohen-Bazir and Bryant, 1982; Paerl, Tucker & Bland, 1983). Källqvist (1981) studied the different growth rates of phytoplankton in a eutrophic Norwegian lake. The author found that while the growth rate of diatoms (*Asterionella*, *Diatoma* and *Synedra*) and cyanobacterium (*Planktothrix*) was the same at 2 m depth, the previous grew faster at 1 m depth. However, at below 3 m only *Planktothrix* grew (Chorus & Bartram, 1999). Another study by Van Liere et al. (1979) demonstrated competition between cyanobacteria and other phytoplankton. Here, green microalga (*Scenedesmus*

protuberans) grew faster at high light intensities whilst cyanobacterium (*Planktothrix agardhii*) was faster at low light intensities. During conditions of low light intensity, *Planktothrix* out competed *Scenedesmus* and during high light intensities, the biomass of the *Scenedesmus* increased rapidly, resulting in an increase in turbidity and decrease in light availability. This in the end increased the growth rate of the *Planktothrix,* which became dominant after 20 days (Chorus & Bartram, 1999). Therefore, the study concluded that, although cyanobacteria may not reach the maximum growth rates of Chlorophyta, at very low light intensities their overall growth rate is higher and, therefore, they can outcompete Chlorophyta (Chorus & Bartram, 1999). This is especially true in nutrient rich eutrophic waters with high turbidity and poor nutritional conditions (Chorus & Bartram, 1999).

During the current study, the secchi disk results for turbidity were highest at Goreangab dam during both dry and wet seasons. At the same time although not statistically significant genus relative abundance against turbidity was very weak positively correlated. This would be associated with the *Microcystis* blooms during both seasons because these blooms are known to increase water turbidity as they flow with the water in any direction (Sirunda & Mazvimavi, 2014). Swakoppoort dam had the second highest *Microcystis* abundance, lowest average diversity, eveness and highest richness when compared to the other dams. Von Bach dams' turbidity in relation to relative abundance was not statistically significant. Therefore, turbidity did not play a role in the microalgae relative abundance in this study. This demonstrates

that other factors, other than turbidity affect relative abundance of microalgae during these times.

5.1.1 Effect of abiotic factors on relative genera abundance of microalgae

Temperature

Temperature is the one of the most important abiotic factors which control microalgae population and composition in freshwater systems (Tas, 2011). Its increase intensifies the effect of toxic substances in aquatic habitats and as a result effects the growth of microalgae species and communities in aquatic systems (Tas, 2011). Moreover, temperature also affects the accessibility of microalgae to nutrients in the form of thermal stratification. During this time the top layer of the water column becomes warmer and the lower layer remains cooler (Nswgovau, 2016). As the two layers stop mixing, the upper layer becomes more stable and the growth of blue-green microalgae blooms is encouraged (Nswgovau, 2016). This is often the case when anoxic water bodies result in bottom waters during stratification, which may lead to increased nutrient release from the sediments (Nswgovau, 2016).

Robarts and Zohary (1987) and Nswgovau (2016) associated maximum growth rates of blue-green microalgae to develop during the warmer months with intermediary light intensity at optimal temperatures of above 25 °C. These optimum temperatures are higher than those required for green microalgae and diatoms (Chorus & Bartram,

1999). This is evident in tropical regions with higher water temperatures, where these blooms persist throughout the year. However, this is not the case in temperate regions (i.e. coastal regions of Namibia), whereby these blooms do not persist throughout the winter months as the low water temperature is less favorable. Additionally, blue-greens can also diminish when exposed for long periods to high light intensities (Nswgovau, 2016).

To determine the dominance of two *Microcystis* species in eutrophic bodies in Japan, scientists isolated the species and studied the effects of temperature, light intensity and nutrient concentrations on growth rates. They concluded that temperature amongst the other environmental factors was the most important factor determining the dominance of the two species (Imai, Chang & Nakano, 2009). Another study by Nalewajko and Murphy (2001) found the optimal growth temperature for *Microcystis* sp. and *Anabaena* sp. isolated from Lake Biwa to be between 28 and 32 °C. During the current study the water temperature in all of the three dams were optimal for microalgae growth during both seasons. Lower temperatures were however recorded during the dry season when compared to the wet season. Accordingly, higher diversity, richness and eveness were recorded for all the dams during the dry season.

Correlation test for temperature against relative genera abundance was not statistically significant and, therefore had no affect. Even though the temperatures recorded in Von Bach dam were in the range described as optimum for growth for all microalgae species, interestingly enough *Microcystis* was only recorded during the wet season with

a 4 % relative abundance.

Dissolved oxygen

High temperatures increase the metabolism and respiration activity of aquatic organisms of especially microalgae which eventually leads to blooms. The resulting consequences are their degradation leading to decrease in dissolved oxygen concentrations (Tas, 2011). Additionally, dissolved oxygen concentration is affected by numerous other factors such as water movement/mixing, pollution, inflow of freshwater from other sources, production of oxygen by plants and its consumption by animals and bacteria (Tas, 2011). The complete lack of oxygen in aquatic environment is referred to as anoxia at 0 mg/L and partial lack or low oxygen level is referred to as hypoxia (< 2 - 3 mg/L). During a complete lack of oxygen only certain microorganisms can exist (Sucurinet, 2016). During the time of this study all the dams had above optimum oxygen concentration in surface water conducive for the survival of most aquatic organisms. The relative abundance correlation against dissolved oxygen for all the dams was not statistically significantly. This indicated that dissolved oxygen concentrations did not affect relative genera abundance of microalgae, although absolute abundance of the microalgae may affect oxygen concentrations. This will however, vary throughout the day, depending on photosynthetic activity.

This high productivity is usually associated with high abundance and senescence of *Microcystis* blooms that lead to oxygen depletion by bacteria. Swakopoort dam had the

highest dissolved oxygen concentration during both seasons.

pH

pH is another important factor regulating life in aquatic systems. Low pH levels facilitate the solubilization of ammonia, heavy metals and salts whilst high pH levels increase the concentration of carbon dioxide and carbonic acid concentrations (Sirunda & Mazvimavi, 2014). Since all living organisms release carbon dioxide, pH increase and decrease is affected by respiration and photosynthesis rates which determine its net addition or removal. These processes are affected by sunlight, water temperature, biomass of plants, animals and microorganisms in the water and bottom sediments (Tucker, & D'Abramo, 2008). pH is the highest during daylight when microalgae and underwater plants remove carbon dioxide from the water through photosynthesis which exceeds respiration and it is low as the sun begins to set and eventually stops as respiring organisms add carbon dioxide to the water. Day time photosynthesis is equal to respiration and pH remains within an optimum range tolerated by most aquatic organisms. However, when plants or microalgae multiply rapidly, more carbon dioxide is removed each day by photosynthesis than is added during the night by respiration. This is very common in lakes and water bodies experiencing a high influx of nutrients from the surrounding environments as well as waterbodies with low total hardness and moderate to high total alkalinity (Tucker & D'Abramo, 2008).

In freshwater systems, good development by organisms is at 6.5 - 8.5 pH range (Tas,

2011). Fatal effects of pH on aquatic organisms are when the pH is below 4.5 and when it is above 9.5 (Sirunda & Mazvimavi, 2014). However, several studies have observed some green microalgae and *Microcystis* to grow above these thresholds. Goldman, Azov, Riley and Dennett (1982) showed that it is difficult to separate metabolic from chemical factors that influence the pH tolerance limits of the individual species. The study demonstrated that two freshwater species namely *Scenedesmus obliquus* (Turp.) Kutz. and *Chlorella vulgaris* (Beij.) grew up to a pH of 10.6, although *C. vulgaris* was more adversely affected by the alkaline pH than was *Scenedesmus obliquus*. They further found that the lower pH limits were controlled by the production of alkalinity concomitant with NO_3^- uptake, whereby the upper pH limits in the case of *Scendesmus obliquus* by metabolic control. Therefore, concluding that the availability of inorganic carbon was not an influencing factor in setting the maximum attained pH (Goldman, Azov, Riley & Dennett, 1982). Another study by McLachlan and Gorham (1962) showed that *Microcystis aeruginosa* Kütz. (strain NRC-1) grew well throughout the pH range 6.5 to 10 when provided with suitable media.

Sampling during the current study was carried out during daytime where pH levels were expected to be of optimum equilibrium for most microalgae species. However, the high levels of pH during both seasons can be attributed to the highetened nutrient composition and, therefore, primary production. For example, Goreangab dam had the highest average recorded alkaline pH levels during these times. This is usually associated with the natural water properties and influx of various nutrients into the dam. Goreangab dams' genus relative abundance against pH was very weak negatively

correlated and not statistically significant. Swakoppoort had the second highest pH averages during both seasons and Von Bach dam had the lowest pH that were a direct reflection of the low nutrient concentrations measured during the time. Correlation tests for both Swakoppooort and Von Bach dam of genus relative abundance against pH showed no statistically significance. Therefore, pH did not have an influence on the relative genera abundance of microalgae in this study.

Dam water volumes

The dam water volumes have a direct effect on nutrient availability, saturation, dilution and, therefore, microalgae abundance. High water volumes increase the amounts of nutrients and sediments into impounded water bodies due to runoff and increased erosion respectively (Dresen & Korth, 1994). Additionally, when groundwater volumes are high, older septic systems that are located near freshwater impoundments can flood (Dresen & Korth, 1994). Low water volumes increase the amount of aquatic plants and can be harmful environments for fish at the same time. This is because low water volumes can implicate water body stratification. During stratification, the thermocline prohibits nutrients (especially phosphorus) from circulating throughout the water body, eventually trapping it near the bed of the water body in the deeper water areas (Dresen & Korth, 1994). If water volumes decrease and water bodies do not stratify, nutrients can circulate freely throughout the water column creating microalgae blooms (Dresen & Korth, 1994). Low water volumes can therefore, contribute to microalgae populations. This is because microalgae and especially blue-green microalgae can take advantage of

nutrients normally contained in deeper water released from the sediments due to wave action (Dresen & Korth, 1994). On the other hand when water volumes are not optimum or high and stratification breaks, macronutrients that are released are usually oxidized in the hypolimnion layer below the thermocline where there are no microalgae available to utilize the total phosphorus (Sirunda & Mazvimavi, 2014). Herein it can be concluded that internal phosphorus contributes less to the eutrophication process of the water and that the most effective way to control eutrophication in water impoundments is to reduce external nutrients (Sirunda & Mazvimavi, 2014).

Dam water volumes can fluctuate naturally due to precipitation which varies widely from season to season and year to year. In some cases some natural lakes with stream inflows show the effect of rainfall almost immediately; however others may not reflect changes in precipitation for months (Dresen & Korth, 1994). For instance, heavy rainfall leads water volumes to rise when rain enters freshwater impoundments as groundwater. In the same accord, longer retention times range from several days for some small impoundments to many years for large seepage lakes with no surface outlets (Dresen & Korth, 1994). During this study, differences were found in dam water volumes during the two seasons. Goreangab dam water volumes were positively correlated but not statistically significant. Therefore, it did not have an effect on the relative abundance of microalgae. Swakoppoort dam water volume was higher during the dry season when compared to the wet season. The differences could be attributed to run offs/discharges from nearby tributaries in the Swakop

catchment as well as retention times. The same goes for Von Bach dam which had a higher water volume during the dry season.

Macronutrients

Nitrogenous compounds such as nitrate (NO_3^--N), nitrite (NO_2^--N), ammonium nitrogen (NO_4^+-N) and organic nitrogen (Org-N) are important determinants of water quality and, therefore, microalgae growth and abundance (Tas, 2011). This is especially true for ammonium compounds which are increased by pollution from discharges containing decaying organic material that may be detrimental to aquatic life (Tas, 2011). Nitrate is a common macronutrient form of nitrogen in oxygen-rich waters, hence readily available for microalgae growth (Tas, 2011). On the other hand, phosphorus in the form of orthophosphate (PO_4^{3-}) is the most important limiting macronutrient affecting the productivity of natural waters and, therefore, eutrophication (Tas, 2011). Total phosphorus concentration in natural waters is affected by numerous factors such as morphometer of basin, the chemical content of the region's geological structure, organic matter flow into water, domestic waste and organic metabolism in the water (Tas, 2011). Since phosphate is the primary limiting factor in freshwater aquatic systems for microalgae, it is depleted by plants and microalgae before any other nutrients (Lakeaccessorg, 2016). After depletion, it is recycled back into water through the microbial loop. Microorganisms in the sediment degrade dead organic matter and phoshpates are released as soluble phosphate into the bottom water through diffusion

processes. These then enter the surface water during de-stratification of the dam when bottom waters are upwelled to the surface (Nswgovau, 2016). This release occurs when the water is anoxic and the sediments freely release it into the water column which promotes microalgae growth (Nswgovau, 2016). During these times when microalgae growth is high and in turn forms thick layers of scum in eutrophic systems, light penetration is halted and nutrients below that depth are not assimilated (Lakeaccessorg, 2016). Despite this, in some freshwater systems such as the tropics and sub-tropics nitrogen is the limiting nutrient due to the excessive input of phosphorous and long growing seasons (Yang et al., 2008). Lin et al. (2008), reported such an incident at the Ten Mile Creek of Indian River Lagoon where total phosphorous was > 0.2 mg/L and chlorophyll a and turbidity increased with addition of available nitrogen (0.2 ~ 6.0 mg/L), however not affected by addition of reactive phosphorous (Yang et al., 2008).

Several studies have documented the relationships of microalgae blooms and especially blue-greens to macronutrients. Yang et al. (2008) reported in their literature that red tides occur when the nitrogen concentration in the water reaches 0.3 mg/L and phosphorous concentration reaches 0.02 mg/L. Richardson et al. (2007) showed a ecological variation in algal, macrophyte, macro invertebrate and swamp community structures in the Everglades areas when total phosphorous exceeded the surface water mean threshold concentration of 0.015 mg/L (Yang et al., 2008). Therefore, a threshold zone of 0.012 ~ 0.015 mg/L of total phosphorous is considered protective for all trophic levels (Yang et al., 2008).

Blue-green microalgae blooms can occur when the concentration of nutrients is low but

more frequently when the concentrations of nutrients is high (Nswgovau, 2016). Sirunda and Mazvimavi (2014) indicated that *Microcystis* and *Anabaena* cannot be controlled by nutrient deprivation as they are able to fix nitrogen from the atmosphere and only require about 0.01 mg/L phosphate to cause a bloom. Nitrogen and phosphate concentrations in municipal wastewaters are ussually 30 - 40 mg/L and 5 - 10 mg/L respectively (Williams & Laurens, 2010). Ahrens and Sander (2010) found that *Chlorella* and *Scenedesmus* grow in a wide range of wastewaters, with sub-optimal ratios of nitrogen and phosphate, with complete decomposition of nitrogen and phosphate in 10 days.

To compare the optimum N:P ratio for growth between eukaryotic microalgae and cyanobacteria, Schreurs, (1992) found 16-23 molecules N:1 molecule of P and 10-16 molecules N:1 molecule P respectively. As a result demonstrating that cyanobacteria can proliferate in low nutrient levels and, therefore, can outcompete most plankton. Another advantage of blue-green microalgae over other plankton as regards to nutrients is their storage of essential nutrients and metabolites within their cytoplasm (Chorus & Bartram, 1999). Here surplus nutrients are stored when in excess and broken down during the times of limitation (Chorus & Bartram, 1999). For instance, during a lack of nitrogen, the primary products of photosynthesis are channelled towards the synthesis and accumulation of glycogen and lipids (Chorus & Bartram, 1999). During phosphorus limitations, cyanobacteria contain up to 2 - 4 cell divisions storage that is equivalent to a 4 - 32 fold increase in biomass (Chorus & Bartram, 1999). Furthermore, during high total phosphate concentrations and consecutive increase in phytoplankton density,

cyanobacteria are still at an advantage (Chorus & Bartram, 1999). This is because cyanobacteria thrive in high turbidity and low light availability due to their specialized features (Chorus & Bartram, 1999).

However and on the contrary to the preceding studies, other studies tend to differ stating that cyanobacteria (esp. *Microcystis*) need high concentrations of phosphorous and to proliferation. For example, a study by Nalewajko and Murphy (2001) showed that *Microcystis* does not bloom due to low availability of phosphorus and nitrogen and that *Anabaena* has an efficient phosphorus-uptake and storage system that allows them to grow during low phosphorus levels. In the same study, *Microcystis* growth in a minus-phosphorus medium ceased in 7 - 9 days, compared with 12 - 13 days for *Anabaena*, suggesting that *Microcystis* will not grow well in phosphorus limited waters. Moreover, a study by Davis, Berry, Boyer and Gobler (2009) observed the highest growth rates of toxic *Microcystis* cells in concurrent increases of temperature and phosphorus concentrations. There authors suggested that the future implications of eutrophication and climate warming would be to enhance the growth of toxic, rather than non-toxic populations of *Microcystis*.

Macronutrients (phosphate, ammonia and nitrate) recorded during the times of sampling for all the dams for both seasons were above optimal range for microalgae growth. At the same time lower concentrations of nutrients were observed during the dry season. These anomalies may have been attributed to several factors: (i) very low dissolved

oxygen concentrations recorded during the sampling time. This since dissolved oxygen directly oxydises pure elements from sediments in forms that can be synthesized by phytoplankton (Tas, 2011); (ii) At the time of sampling the macronutrients may have been exhausted by uptake during microalgae blooms. Since rates of nutrient uptake were not measured, the once-off nutrient concentration is only a reflection of the difference in production and input of nutrients and the uptake of nutrients. Low nutrient concentrations are therefore, an indication that uptake exceeds production and input.

Nevertheless, Goreangab dam had the highest recorded concentrations of macronutrients. This was mainly due to its close proximity and susceptibility to many industrial and domestic discharges. Thus, anthropogenic inputs are significant. Additionally, the most abundant microalgae in the dam were blue-greens, particularly *Microcystis* and *Anabaena*, which are known to be effective nutrient utilisers and are not affected by nutrient deprivation (Sirunda & Mazvimavi, 2014). The relationships between genus relative abundance against nitrates, phosphates, ammonia was very weak negatively correlated and not statistically significant. Swakoppoort dam had the second highest macronutrient concentrations, however not statistically significant.

5.2 Optimization of neutral intracellular lipid and biomass production in *Nannochloropsis limnetica* SAG 18.99

Microalgae are quickly becoming a prominent source of production for various commodities ranging from fertilizers for livestock, cosmetics, pigments, metabolites to

biodiesel production. This is mainly due to their advantages over terrestrial plants in that they can be grown in arid areas unsuitable for conventional agriculture (i.e. desert areas) in large reservoirs of saline and wastewater (Tabah, 2011). During processing, water used is far less than that of traditional crops; they do not displace food crops; their production is not seasonal, and biomass can be harvested daily (Gouveia & Oliveria, 2009; Tabah, 2011).

Microalgae growth is affected by various abiotic and biotic elements (Moheimani, 2005; Kumar et al., 2010; Mata et al., 2010). Therefore, their biochemical properties can be altered by controlling growth conditions and nutrient availability to influence the lipid content needed to produce biofuels (Sforza et al., 2010). For example, light intensity and temperature are frequently used as physical stimuli to increase or decrease biomass production (Hu et al., 2008). Equally certain levels of carbon dioxide supplementation and decrease in nutrients can increase lipid content, especially TAG fraction (Gordillo et al., 1998b; Huntley & Redalje, 2007; Francisco et al., 2010; Tang et al., 2011). The effects of especially nitrogen deficiency in microalgae culture showed enhanced biosynthesis and lipid accumulation (Juneja et al., 2013). Therefore, attempts to increase lipid concentration through nitrogen limitation are carefully evaluated to ensure high lipid productivity (Metting, 1996; Juneja et al., 2013). In the current study *Nannochloropsis limnetica* was cultured in BG_{11}^{0} medium in highly controlled conditions in lab-scaled bioreactors as an attempt to manipulate and increase the neutral intracellular lipid content and biomass productivity. Nile red (9-(Diethylamino) -5H

benzo [α] phenoxazin- 5-one, staining method was employed in this study to identify, reveal and confirm the formation of cytoplasmic lipid and or intracellular lipid droplets in this microalgae (Abdo et al., n.d).

5.2.1 Pre-cultures

During the pre-culture cultivation microalgae proliferated in a characteristic pattern consisting of lag, exponential, stationary and declining phases. Growth rates were close to identical during cultivation although their biomass generation varied. pH remained constant and had no influence on the growth rate and, therefore, biomass productivity. Macronutrients during the cultivation process were consumed as expected, whilst phosphate as the primary element was entirely depleted at the end of the cultivation process, indicating phosphate as the limiting nutrient for growth in this microalga.

5.2.2 Main-cultures

5.2.2.1 Growth rates (OD)

Since the main-cultures were inoculated with exponentially growing pre-cultures there was a small lag phase that lasted only two days (Fogg & Thake, 1987). The exponential growth phase or log phase, showed the microalgae to be optimally adapted to the cultivation conditions hence maximum growth was observed. Immediately thereafter, the cells started to decelerate which indicates limitations of the surrounding conditions

in the substrate like for instance nutrients, light, pH, and CO_2 needed for cell metabolism (Fogg & Thake, 1987).

Several studies indicated that additional nitrogen concentration leads to higher growth rates and biomass yields in microalgae. For instance a study by Becerra-Dórame, López-Elías and Martínez-Córdova (2010) reported a positive correlation between nutrient concentration and cell density, with a high cell density with richer medium for *Dunaliella* sp. Rocha et al. (2003) reported similar results obtained with marine *Nannochloropsis gaditana* where additional concentrations of nitrogen sources to F/2 medium led to the increment of the growth rate and Converti et al. (2009) observed a gradual increase in the growth rate of *Nannochloropsis oculata* as a result of increase of $NaNO_3$ concentration from 0.075 to 0.150 to 0.300 g/L. However, this cannot be said for this study since the highest growth rate was recorded in Flask C2 (6 $days^{-1}$) which represented moderate nitrate deplete and the lowest growth rate was in Flask D1 (10 $days^{-1}$) which represented the highest nitrate deplete.

5.2.2.2 Bio dry mass (BDM)

During strain characterization, the success of a microalga strain to yield lipids is not the only determining factor and should not be considered without simultaneously considering biomass productivity (Rattanapoltee & Kaewkannetra, 2013). During nitrogen depletion in microalgae culture, despite the reduced growth rates, carbon is diverted to either lipids or carbohydrates. Carbohydrates in this case may reach above 70 % of dry mass without reduction in productivity and, therefore, lipid accumulation is

often associated with a reduction in biomass productivity (Rodolfi et al., 2009). This is most likely because lipid content during nitrogen deficiency is obtained at the expense of other components such as proteins and carbohydrates (Rodolfi et al., 2009). Nevertheless, in some green microalgae, the mass of cellular lipid accumulation derived from newly fixed carbon during nitrogen deficiency may be higher than the total biomass present at the onset of stress (Fogg, 1966; Shifrin & Chisholm, 1981).

In the current study, BDM increased linearly and exponentially over time for all of the Flasks starting lowest in the beginning (0 $days^{-1}$) of cultivation and highest at the end (14 $days^{-1}$) expressing the relative ecological success of the strain adapting to the variant nitrate concentrations as was shown by Fogg and Thake (1987). BDM and growth rates was moderate and positively correlated and statistically significant and between BDM and lipids was strong and negatively correlated and statistically significant. Flasks C2 representing moderate nitrate depelete achieved the highest BDM, hence agreeing to the notion that biomass can be generated on the expense of lipids.

5.2.2.3 Neutral intracellular lipid productivity

Generally, the production and accumulation of lipids inside microalgae cells occur under nutrient stress where the growth rates are reduced. The production of these intracellular lipids is to act as protection mechanisms for cells against stressful conditions (Rattanapoltee & Kaewkannetra, 2013). This occurs especially at the end of

the growth stage upon nutrient limitation and depletion (Mohammady & Fathy, 2007). In some microalgae, despite the higher lipid content, the actual lipid yield can be lower under nutrient stress than in nutrient replete conditions due to a much lower growth rate (Sheehan et al., 1998; Liu & Benning, 2013). During this study, relative fluorescence data recorded for all the Flask was the highest only on the first couple of days during the lag phase and decelerated until at the end of the cultivation. Therefore, there was no lipid accumulation. Correlation tests carried out between lipids and growth rates was moderate, negative correlated and statistically not significant and between lipids and nitrate was moderate, positively correlated however statistically not significant.

The differences in the percentage fluorescence at this time may be attributed to the physiological response of these microalgae to adapt which include metabolism to growth and, therefore, increase in the levels of enzymes and metabolites involved in cell division and carbon fixation. Since all the cultivation variables were identical except for nitrate, the adaptation response was due to the nitrate variations.

Similar responses to nitrogen variability have been studied by several scientists over the years. Nitrogen limitation is used to increase the lipid content in microalgae, but not at the expense of other nutrients such as phosphate that help in the formation of lipids. Nitrogen is important for protein and cell growth and during its deficiency excess carbon from photosynthesis is morphed into storage molecules like for instance starch and TAG (Rattanapoltee & Kaewkannetra, 2013). However, this cannot be said for all microalgae. Borowitzka and Borowitzka (1988) reported a variety of responses by green

microalgae from several fold increases from log phase values (i.e. *Chlorella pyrenoidosa*), to no change or even a slight reduction (i.e. *Dunaliella* spp. and *Tetraselmis suecica*). Additionally, some *Chlorella* sp. also from the same genera has been found to accumulate starch under nitrogen starvation, whereas others predominantly accumulate neutral intracellular lipids (Hu, 2004). Benemann and Oswald (1996) along with Shifrin and Chisholm (1981) showed that diatoms, which usually exhibit a high log phase do not increase their lipid content during nitrogen starvation.

Other studies demonstrated that not only nitrogen variability/deficiency and or limitation but also other macronutrients alternate cellular lipids of microalgae. For example a study by Krienitz and Wirth (2006) showed that the highest concentrations of PUFA in *N. limnetica* were obtained in non-aerated suspension cultures, with a high content of phosphate (40 mg/L K_2HPO_4) in the culture medium. In the current study to the contrary, the microalgae experienced a phosphate deficiency in the medium, whilst nitrate remained until the end of the cultivation process. Therefore, lipid accumulation may not have been synthesized.

Another factor which may have negatively contributed to the results of the neutral intracellular lipid analysis could have been the use of the Nile red method. During staining, neutral intracellular lipids in microalgal cells observed using a fluorescence microscope have a yellow fluorescence and without staining the cells have the red fluorescence (Rattanapoltee & Kaewkannetra, 2013). However, this is not the same in

many microalgae species and several factors need to be considered towards the success of this method. These include: (1) the polarity of solvent to dissolve Nile red, (2) the difference in measuring conditions which may affect the combination of Nile red to lipid component in the cells and (3) the fluorescence intensity (Rattanapoltee & Kaewkannetra, 2013).

Abdo et al. (n.d) demonstrated false results of this method in their experiment. During their research, some blue-green isolates (i.e. *Microcystis aeruginosa* and *Phormidium rimosum*) were successfully stained and, therefore, reflected yellow parts on the contrary to other blue-greens (*Oscillatoria limnetica* and *Oscillatoria limosa)* that were omitting yellow fluorescence colour without being stained and green isolates (*Chlamydomonas variabills, Chlorella vulgaris, Haematococcus pluvialis, Scenedesmus obliquus* and *Scenedesmus quadriquada*) which were not clear since their whole cells were stained in red.

Other problems associated with using the Nile red staining is linked to microalgae species containing thick, rigid cell walls that are impenetrable to the dye. Some marine *Nannochloropsis* spp. (i.e. esp. *Nannochloropsis gadinata*) contain cell walls whose recalcitrance presents a significant barrier to biocommodity extraction (Scholz et al., 2014). The species in this genus are difficult to stain and not only with Nile Red (Sheehan et al., 1998) but also with an array of other dyes including PicoGreen and SYTOX Green (Veldhuis et al., 1997). This is mostly due to the small size of intracellular lipid droplets and natural low cell wall permeability (Doan & Obbard,

2011). For example in marine *Nannochloropsis oculata* the cellulose anomeric carbon C1 in the cell wall only appeared in the rigid (CP) spectrum whilst remaining carbons showed up in the spectra of both rigid and mobile zones (Nannochloropsisorg, 2016).

Doan and Obbard (2011) during the screening of local microalgae strains from Singapore coastal waters for biodiesel neutral intracellular lipid feedstock confirmed the contrast of the *Nannochloropsis* spp. cell wall. They found that the Nile red flouresence method for staining *Nannochloropsis* was inadequate, and, therefore, a more efficient Nile red staining method needed to be proposed. Doan and Obbard (2011) in their study proposed a modified, novel staining procedure that can be applied for high-throughput screening and cell sorting of *Nannochloropsis* sp. with elevated intracellular lipid. The study advised glycerol-Nile red method in conjunction with flow cytometry since glycerol unlike DMSO does not inhibit subsequent growth of sorted cells.

CHAPTER 6

6. CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

6.1.1 Relative genera abundance of microalgae

Microalgae as primary producers play a substantial role in the health of any aquatic system. Their ability to assimilate various nutrients can be either a con or a pro. The former leads to microalgae blooms occuring in stagnant waters and, therefore, eutrophication. These processes are problematic for water purification and in extreme conditions lead to loss of life for other aquatic organisms, livestock and even humans. The latter provides an array of opportunities through harvesting naturally occurring microalgae blooms and or isolating and culturing them for various products such as food and feedstock for human and animal life respectively, biofuel production and valuable metabolites (Kuo, 2010).

The current study identified and quantified the various microalgae species present in three eutrophic systems of Namibia during both dry and wet seasons to genus level. Additionally, since understanding microalgae bloom spatial dynamics is a critical factor in predicting their occurrence and determining and developing their preventative and migratory measures, capturing environmental factors was critical (Lopez et al., 2008). The study successfully addressed the two hypotheses which were firstly to statistically

analyze whether there exists seasonal differences in genera abundances of the microalgae per dam and secondly whether there exists a correlation of relative genera abundance against environmental parameters measured at the said time. It was found that relative genera abundance was not statistically significant ($p > 0.05$) and, therefore, the null hypothesis was accepted. Secondly, the correlations tests carried out showed that environmental parameters were not statistically significant ($p > 0.05$) and, therefore although playing a role had no statistically significant influence on the relative genera abundance during this time. The similarity observed in classes and genera throughout the seasons in all of the dams is most likely to sharing the same catchment Swakop catchment. Nevertheless, deviations were captured amongst classes and genera per season which could relate to mechanical extractions, topography, nutrient enrichment and or any other environmental factors not assessed during the present study.

The class Chlorophycea was the most abundant in all the dams throughout both seasons, however the genus *Microcystis* was dominant due to their physiological advantage over other microalgae. This was especially the case in Goreangab dam which is characterized by optimum combination of environmental factors such as high macronutrients (esp. high phosphates and nitrates), temperature and pH levels that fed to this abundance. Furthermore, to add to this anomaly, although dissolved oxygen concentrations in 100 % saturated fresh water ranges between 7.56 mg/L at 30 °C to 14.62 mg/L at 0 °C, colder water carries twice as much dissolved oxygen than warm water (Wetzel, 2001), with variations ranging from seasonal to time of day or night (Statemnus, 2016). Moreover, water with high concentrations of dissolved

minerals similar to those measured in Goreangab dam are believed to have a lower dissolved oxygen concentration when compared to other fresh water bodies with the same temperature (Statemnus, 2016). The study therefore concludes that numerous amounts of microalgae species identified during the current research have the potential to be cultivated for various outputs as described in the preceding chapters, especially from the group Chlorophyceae which was the most abundant class in both seasons across all the dams. Furthermore, since environmental parameters may have played a role in relative genera abundance, however not statistically significant, cultivation parameters can be diversified without relying too much on external influences.The microalgae can thus be either harvested directly from these systems as they act as natural incubators or isolates can be cultivated in the best chosen production systems.

6.1.2 Optimization of neutral intracellular lipid and biomass production in *Nannochloropsis limnetica* SAG 18.99

The main objective and, therefore, hypothesis of this part of the current research was to optimize the neutral intracellular lipid and biomass production in *Nannochloropsis limnetica*. Thhe initial part of this study was focused on the characterization of the strains by use of pre-cultures to understand their growth physiology. The second part which was referred to as the main-cultivation was to apply environmental stress and in

this case nitrogen variations to evaluate its effect on neutral intracellular lipid and biomass production.

During strain characterization although there was a slight biomass deviation at the end of the cultivation process across all the Flasks, growth rates were not statistically significant ($p = 0.733$). pH remained constant and, therefore, had no influence on the growth rate and biomass productivity. Macronutrients during the cultivation process were consumed as expected, whilst phosphate as the primary element was entirely consumed.

During the main-cultivation growth rates were statistically significant ($p = 0.001$). Therefore, the null hypothesis was rejected. Bio dry mass was statistically not significant ($p = 0.939$). Therefore, the null hypothesis was accepted. Biomass increase was an exponential success in all of the 4 Flasks. Relative fluorescence data captured for lipid analysis, statistically significant ($p = 0.112$). Therefore, the null hypothesis was rejected. pH was not statistically significant ($p > 0.05$) therefore, remained constant throughout the cultivation process.

Studies by Illman, Scragg and Shales (2000) and Bhola et al. (2011) demonstrated reductions in microalgae yield under nutrient stress condition that promote high lipid content. On the contrary to this common belief the highest growth rates and bio dry mass were recorded in the “moderate nitrate deplete” concentration (Flask C2) in the current study.

Similarly, lipids increased under nutrient depletion. In this study there was no lipid accumulation throughout the cultivation process for all of the Flasks. In fact the highest relative fluorescence which equates the percentage of stained lipid content was recorded was under "replete" nitrogen concentration (Flask B2) during lag phase on 1 day^{-1}. This is a condition only experienced in non-oleaginous species which either cease growth or divert assimilated carbon into storage polysaccharides (Rodolfi et al., 2009). Under prolonged nutrient deficiency growth ultimately ceases to exist. However, under nutrient limitation, the cell culture adapts to the insufficient nutrient concentrations, whereby, the intracellular level of the limiting nutrient determines the growth rate and biomass composition (Rodolfi et al., 2009), since microalgae are generally expected to utilize endogenous reserves to compensate for the lack (Rodolfi et al., 2009). At the same time, productivity is reduced in comparison with nutrient-sufficient growth (Rodolfi et al., 2009). The study therefore concludes that variations in nitrogen did not optimize neutral intracellular lipids as expected and that the biomass composition needs to be characterized.

6.2 RECOMMENDATIONS

6.2.1 Relative genera abundance of microalgae

This study was successful in addressing the two hypotheses. However, it was limited to two samplings for both wet and dry seasons and, therefore, should be regarded as an initial step towards understanding the dynamics of these blooms in these three systems during these times. More so, since irregularity in rainfall made it difficult to distinguish between the two seasons, numerous other factors played a vital role in microalgae growth and need to be considered.

First, to have a clear and true representation of microalgae species compositions, quantities and influence of environmental factors on them, monthly monitoring spanning over a year and sampling at least every second week should be carried out. Secondly, microalgae vary from a few (2 - 3) pico/nanometers to more than 100 μm (Ren, 2014), therefore, various mesh sizes need to be considered. Thirdly, microalgae growth is affected by numerous other nutrients besides the ones captured in this study, such as manganese (Mn), silicon (Si) and iron (Fe). These nutrients can effectively influence the outcome of species composition, dominance, structure and abundance under cultural eutrophication (Anderson et al., 2002). This would help understand the cumulative effects of these environmental parameters on microalgae compositions. The increase in sample size would therefore allow more elaborative statistical analysis.

Furthermore, chlorophyll a and inorganic carbon should be monitored since the former is a direct indicator of plant, microalgae and cyanobacteria biomass and these contain 1 to 2 % chlorophyll a and the high abundance of the latter lowers dissolved oxygen

concentration (Sirunda & Mazvimavi, 2014). Moreover, sampling should be carried out at the same time for each sampling since productivities of systems change throughout the duration of the day as described in the preceding chapters.

Freshwater reservoirs are complex systems in terms of mixing and flushing dynamics. Therefore, abiotic turbidity from episodic sediment loading and the availability of phosphate in the epilimnion layer is appreciable (Anderson et al., 2002; Sirunda & Mazvimavi, 2014). Thus, the presence of phosphate in aquatic systems may not be used to successfully predict the occurrence and extent of late summer cyanobacteria blooms and that light can be the primary resource limiting microalgae growth (Anderson et al., 2002).

6.2.2 Optimization of neutral intracellular lipid and biomass production in *Nannochloropsis limnetica* SAG 18.99

The study proposes the Nile red method be used in conjunction with other conventional gravimetric methods for lipid quantification and validation. Secondly other macronutrients such as phosphate concentration along with variables such as light intensity and CO_2 should also be manipulated for lipid and biomass accumulation for future studies.

REFERENCES

Abdo, S. M., Ahmed, E., El-Enin, S. A., El Din, R. S., & Ali12, G. E. D. G, (n.d.). Qualitative and quantitative determination of lipid content in microalgae for biofuel production.

Abed, R. M. M., Dobretsov, S., & Sudesh, K. (2009). Applications of cyanobacteria in biotechnology. *Journal of Applied Microbiology*, *106*(1), 1-12.

Achten, W. M. J., Verchot, L., Franken, Y. J., Mathijs, E., Singh, V. P., Aerts, R., & Muys, B. (2008). *Jatropha* bio-diesel production and use. *Biomass and Bioenergy*, *32*(12), 1063-1084.

Agarwal, A. K. (1998). Vegetable oils versus diesel fuel: development and use of biodiesel in a compression ignition engine. *TIDE*, *8*(3), 191-204.

Ahmad, A. L., Yasin, N. M., Derek, C. J. C., & Lim, J. K. (2011). Microalgae as a sustainable energy source for biodiesel production: a review. *Renewable and Sustainable Energy Reviews*, *15*(1), 584-593.

Ahrens, T., & Sander, H. (2010). Microalgae in wastewater treatment: green gold from sludge. *Bioforum Eur*, *14*(3), 16-18.

Akintayo, E. T. (2004). Characteristics and composition of *Parkia biglobbossa* and *Jatropha curcas* oils and cakes. *Bioresource technology*, *92*(3), 307-310.

Akkerman, I., Janssen, M., Rocha, J., & Wijffels, R. H. (2002). Photobiological hydrogen production: photochemical efficiency and bioreactor design. *International Journal of Hydrogen Energy*, *27*(11), 1195-1208.

Al-Qasmi, M., Raut, N., Talebi, S., Al-Rajhi, S., & Al-Barwani, T. (2012, July). A review of effect of light on microalgae growth. *In Proceedings of the World Congress on Engineering*, (Vol. 1, pp. 4-6).

Amin, S. (2009). Review on biofuel oil and gas production processes from microalgae. *Energy Conversion and Management*, *50*(7), 1834-1840.

Andersen, J. H., Conley, D. J., & Hedal, S. (2004). Palaeoecology, reference conditions and classification of ecological status: the EU Water Framework Directive in practice. *Marine Pollution*

Bulletin, *49*(4), 283-290.

Andersen, R. A. (2005). Algal culture techniques. *Physiological Society of America, Elsevier Academic Press, California, USA*.

Andersen, R. A., Brett, R. W., Potter, D., & Sexton, J. P. (1998). Phylogeny of the Eustigmatophyceae based upon 18S rDNA, with emphasis on *Nannochloropsis*. *Protist*, *149*(1), 61-74.

Anderson, D. M., Andersen, P., Bricelj, V. M., Cullen, J. J., & Rensel, J. J. (2001). *Monitoring and management strategies for harmful algal blooms in coastal waters* (pp. 201-203). Unesco.

Anderson, D. M. (2004). Prevention, control, and mitigation of harmful algal blooms: multiple approaches to HAB management. *Harmful Algae Management and Mitigation*, 123-130.

Anderson, D. M. (2009). Approaches to monitoring, control and management of harmful algal blooms (HABs). *Ocean & Coastal Management*, *52*(7), 342-347.

Anderson, D. M., Glibert, P. M., & Burkholder, J. M. (2002). Harmful algal blooms and eutrophication: nutrient sources, composition, and consequences. *Estuaries*, *25*(4), 704-726.

Anderson, D. M., & Morel, F. M. (1978). Copper sensitivity of *Gonyaulax tamarensis*. *Limnology Oceanography*, *23*(2), 283-295.

Annadotter, H., Cronberg, G., & Jönsson, L. (2000). The toxicity of blue-green algae (cyanobacteria) in Lake Finjasjön, Sweden in relation to Environmental Factors–A five-year study. *In Proceedings from the 5th International Conference on the Conservation and Management of Lakes," Strategies for lake ecosystems beyond*.

Antal, T. K., & Lindblad, P. (2005). Production of H_2 by sulphur-deprived cells of the unicellular cyanobacteria *Gloeocapsa alpicola* and *Synechocystis* sp. PCC 6803 during dark incubation with methane or at various extracellular pH. *Journal of Applied Microbiology*, *98*(1), 114-120.

Apt, K. E., & Behrens, P. W. (1999). Commercial developments in microalgal biotechnology. *Journal of Phycology*, *35*(2), 215-226.

Ariño, X., Ortega-Calvo, J. J., Hernandez-Marine, M., & Saiz-Jimenez, C. (1995). Effect of sulfur starvation on the morphology and ultrastructure of the cyanobacterium *Gloeothece* sp. PCC 6909. *Archives of Microbiology*, *163*(6), 447-453.

Ashton, P. (2000). Potential environmental impacts associated with the proposed abstraction of water from the Okavango River in Namibia. *Southern African Journal of Aquatic Sciences*, *25*(1), 175-182.

Aslam, M. (2009). Diversity, species richness and evenness of moth fauna of Peshawar. *Pakistan Entomology*, *2*, 99-102.

Azov, Y. (1982). Effect of pH on inorganic carbon uptake in algal cultures. *Applied and Environmental Microbiology*, *43*(6), 1300-1306.

Baek S. H., Shimode S., Han M. S., & Kikuchi T. (2008). Growth of dinoflagellates, *Ceratium furca* and *Ceratium fusus* in Sagami Bay, Japan: the role of nutrients. *Harmful Algae*, 7, 729–739.

Baker, N. R. (2008). Chlorophyll fluorescence: a probe of photosynthesis in vivo. *Annual Review of Plant Biology, 59*, 89-113.

Banerjee, A., Sharma, R., Chisti, Y., & Banerjee, U. C. (2002). *Botryococcus braunii*: a renewable source of hydrocarbons and other chemicals. *Critical Reviews in Biotechnology*, *22*(3), 245-279.

Barbosa, M. J. G. V. (2003). Microalgal photobioreactors: Scale-up and optimization. *The Netherlands: Wageningen University*, *168*.

Barnwal, B. K., & Sharma, M. P. (2005). Prospects of biodiesel production from vegetable oils in India. *Renewable and Sustainable Energy Reviews*, *9*(4), 363-378.

Barsanti, L., & Gualtieri, P. (2014). *Algae: anatomy, biochemistry, and biotechnology*. CRC press.

Bartram, J., & Ballance, R. (Eds.). (1996). *Water quality monitoring: a practical guide to the design and implementation of freshwater quality studies and monitoring programmes*. CRC Press.

Basha, S. D., & Sujatha, M. (2007). Inter and intra-population variability of *Jatropha curcas* (L.) characterized by RAPD and ISSR markers and development of population-specific SCAR markers. *Euphytica*, *156*(3), 375-386.

Basili, M., & Fontini, F. (2012). Biofuel from *Jatropha curcas*: environmental sustainability and option value. *Ecological Economics*, *78*, 1-8.

Beardall, J., & Raven, J. A. (2013). Limits to phototrophic growth in dense culture: CO_2 supply and light. *In Algae for Biofuels and Energy* (pp. 91-97). Springer Netherlands.

Becerra-Dórame, M., López-Elías, J. A., & Martínez-Córdova, L. R. (2010). An alternative outdoor production system for the microalgae *Chaetoceros muelleri* and *Dunaliella* sp. during winter and spring in Northwest Mexico. *Aquacultural Engineering*, *43*(1), 24-28.

Becker, E. W. (2007). Microalgae as a source of protein. *Biotechnology Advances*, 25, 207-210.

Becker, E. W. (2008). *Microalgae biotechnology and microbiology*, v. VII. Cambridge University Press.

Belcher, H., & Swale, E. (1976). *A beginner's guide to freshwater algae*. HMSO.

Bellinger, E. G., & Sigee, D. C. (2010). Introduction to freshwater algae. *Freshwater Algae: Identification and Use as Bioindicators*, 1-40.

Beltrami, E., & Carroll, T. O. (1994). Modeling the role of viral disease in recurrent phytoplankton blooms. *Journal of Mathematical Biology*, *32*(8), 857-863.

Ben-Amotz, A., & Avron, M. (1990). The biotechnology of cultivating the halo tolerant alga *Dunaliella*. *Trends in Biotechnology*, *8*, 121-126.

Ben-Amotz, A., Tornabene, T. G., & Thomas, W. H. (1985). Chemical profile of selected species of microalgae with emphasis on lipids. *Journal of Phycology*, *21*(1), 72-81.

Benemann, J. R., & Oswald, W. J. (1996). *Systems and economic analysis of microalgae ponds for conversion of CO {sub 2} to biomass. Final report* (No. DOE/PC/93204--T5). California Univ., Berkeley, CA (United States). Dept. of Civil Engineering.

Bergh, Ø., Børsheim, K. Y., Bratbak, G., & Heldal, M. (1989). High abundance of viruses found in aquatic environments. *Nature*, *340*(6233), 467-468.

Berman-Frank, I., & Dubinsky, Z. (1999). Balanced growth in aquatic plants: Myth or reality? Phytoplankton use the imbalance between carbon assimilation and biomass production to their strategic advantage. *Bioscience*, *49*(1), 29-37.

Bernard, O., & Rémond, B. (2012). Validation of a simple model accounting for light and temperature effect on microalgal growth. *Bioresource technology*, *123*, 520-527.

Bhola, V., Desikan, R., Santosh, S. K., Subburamu, K., Sanniyasi, E., & Bux, F. (2011). Effects of parameters affecting biomass yield and thermal behaviour of *Chlorella vulgaris*. *Journal of Bioscience and Bioengineering*, *111*(3), 377-382.

Bibeau, E. (2009). Microalgae Technologies & Processes for Biofuels/Bioenergy Production in British Columbia.

Biggs, B. J. (2000). Eutrophication of streams and rivers: dissolved nutrient-chlorophyll relationships for benthic algae. *Journal of the North American Benthological Society*, *19*(1), 17-31.

Blanco, A. M., Moreno, J., Del Campo, J. A., Rivas, J., & Guerrero, M. G. (2007). Outdoor cultivation of lutein-rich cells of *Muriellopsis* sp. in open ponds. *Applied microbiology and Biotechnology*, *73*(6), 1259-1266.

Borowitzka, M. A. (1997). Microalgae for aquaculture: opportunities and constraints. *Journal of Applied Phycology*, *9*(5), 393-401.

Borowitzka, M. A. (1999). Commercial production of microalgae: ponds, tanks, tubes and fermenters. *Journal of Biotechnology*, *70*(1), 313-321.

Borowitzka, M. A. (2016). Algal Physiology and Large-Scale Outdoor Cultures of Microalgae. *In The Physiology of Microalgae* (pp. 601-652). Springer International Publishing.

Borowitzka, M. A., & Borowitzka, L. J. (1988). *Micro-algal biotechnology*. Cambridge University Press.

Borowitzka, M. A., Huisman, J. M., & Osborn, A. (1991). Culture of the astaxanthin-producing green alga *Haematococcus pluvialis*. Effects of nutrients on growth and cell type. *Journal of Applied Phycology*, *3*(4), 295-304.

Bosma, R., Van Spronsen, W. A., Tramper, J., & Wijffels, R. H. (2003). Ultrasound, a new separation technique to harvest microalgae. *Journal of Applied Phycology*, *15*(2-3), 143-153.

Botana, L. M. (2014). *Seafood and Freshwater toxins: pharmacology, physiology, and detection*. CRC Press.

Bowles, D. (2007). Micro-and macro-algae: utility for industrial applications.*Outputs from the EPOBIO project. University of York: CNAP*.

Boyce, R. L. (2005). Life under your feet: Measuring soil invertebrate diversity. *Teaching Issues and Experiments in Ecology*, *3*, 4-5.

Brányiková, I., Maršálková, B., Doucha, J., Brányik, T., Bišová, K., Zachleder, V., & Vítová, M. (2011). Microalgae—novel highly efficient starch producers. *Biotechnology and Bioengineering*, *108*(4), 766-776.

Bräutigam, K., Dietzel, L., Kleine, T., Ströher, E., Wormuth, D., Dietz, K. J., & Nunes-Nesi, A. (2009). Dynamic plastid redox signals integrate gene expression and metabolism to induce distinct metabolic states in photosynthetic acclimation in Arabidopsis. *The Plant Cell*, 21(9), 2715-2732.

Brown, M. R., Dunstan, G. A., Norwood, S., & Miller, K. A. (1996). Effects of harvest stage and light on the biochemical composition of the diatom *Thalassiosira pseudonana*. *Journal of Phycology*, *32*(1), 64-73.

Butterwick, C., Heaney, S. I., & Talling, J. F. (2005). Diversity in the influence of temperature on the growth rates of freshwater algae, and its ecological relevance. *Freshwater Biology*, *50*(2), 291-300.

Campanella, L., Cubadda, F., Sammartino, M. P., & Saoncella, A. (2001). An algal biosensor for the monitoring of water toxicity in estuarine environments. *Water Research*, *35*(1), 69-76.

Cao, L., Guisen, D., Bingbin, H., Qingyi, M., Huimin, L., Zijian, W., & Fu, S. (2007). Biodiversity and water quality variations in constructed wetland of Yongding River system. *Acta Ecologica Sinica*, *27*(9), 3670-3677.

Carmichael, W. (2008). A world overview—One-hundred-twenty-seven years of research on toxic cyanobacteria—Where do we go from here? *In Cyanobacterial harmful algal blooms: State of the science and research needs* (pp. 105-125). Springer New York.

Carpenter, S. R., Caraco, N. F., Correll, D. L., Howarth, R. W., Sharpley, A. N., & Smith, V. H. (1998). Nonpoint pollution of surface waters with phosphorus and nitrogen. *Ecological Applications*, *8*(3), 559-568.

Carrieri, D., Ananyev, G., Costas, A. M. G., Bryant, D. A., & Dismukes, G. C. (2008). Renewable hydrogen production by cyanobacteria: nickel requirements for optimal hydrogenase activity. *International Journal of Hydrogen Energy*, *33*(8), 2014-2022.

Carvalho, A. P., Meireles, L. A., & Malcata, F. X. (2006). Microalgal reactors: a review of enclosed system designs and performances. *Biotechnology Progress*, *22*(6), 1490-1506.

Cashman, D., Foster, C., McCluskey, K., & Zhang, Y. (2014). Identifying Opportunities to Reduce Water Pollution and Encourage Voluntary Compliance in Windhoek, Namibia. *Undergraduate Interactive Qualifying Project No. E-project-050814-051919). Retrieved from Worcester Polytechnic Institute Electronic Projects Collection: http://www. wpi. edu/Pubs/E-project/Available/E-project-050814-051919/unrestricted/City_-_Final_IQP_Report. pdf.*

Cenciani, K., Bittencourt-Oliveira, M. C., Feigl, B. J., & Cerri, C. C. (2011). Sustainable production of biodiesel by microalgae and its application in agriculture. *African Journal of Microbiology Research*, *5*(26), 4638-4645.

Change, I. P. O. C. (2007). Climate change 2007: The physical science basis. *Agenda*, *6*(07), 333.

Chaumont, D. (1993). Biotechnology of algal biomass production: a review of systems for outdoor mass culture. *Journal of Applied Phycology*, *5*(6), 593-604.

Cheirsilp, B., & Torpee, S. (2012). Enhanced growth and lipid production of microalgae under mixotrophicculture condition: effect of light intensity, glucose concentration and fed-batch cultivation. *Bioresource Technology*, *110*, 510-516.

Chen, B., Landsman-Roos, N., Naughton, R., & Olenyik, K. (2008). *Jatropha curcas L.*: Biodiesel Solution or All Hype? *A scientific approach, economic and political analysis of the future energy crop. Chicago Univ., Energy and Energy Policy, Spring*, 1001-1005.

Chen, D. (2011). *Liquid transportation fuels from algal oils*. The University of Tulsa.

Chen, G. Q., & Chen, F. (2006).Growing phototrophic cells without light. *Biotechnology Letters*, *28*(9), 607-616.

Chen, W., Zhang, C., Song, L., Sommerfeld, M., & Hu, Q. (2009). A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. *Journal of Microbiological Methods*, *77*(1), 41-47.

Chen, P. Y., Lee, P. F., Ko, C. J., Ko, C. H., Chou, T. C., & Teng, C. J. (2011). Associations between water quality parameters and planktonic communities in three constructed wetlands, Taipei. *Wetlands*, *31*(6), 1241-1248.

Chen, Y. H., & Walker, T. H. (2011). Biomass and lipid production of heterotrophic microalgae *Chlorella protothecoides* by using biodiesel-derived crude glycerol. *Biotechnology Letters*, *33*(10), 1973-1983.

Cheng, D., & He, Q. (2014). Assessment of environmental stresses for enhanced microalgal biofuel production—an overview. *Frontiers in Energy Research*, *2*, 26.

Chenl, Y., & Celia, D. G. (1994). Effects of pH on the growth and carbon uptake of marine phytoplankton. *Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire*, *3755*, 83-94.

Chisti, Y. (2007). Biodiesel from microalgae. *Biotechnology Advances*, *25*(3), 294-306.

Chisti, Y. (2008). Response to Reijnders: do biofuels from microalgae beat biofuels from terrestrial plants. *Trends in Biotechnology*, *26*(7).

Choi, S. L., Suh, I. S., & Lee, C. G. (2003). Lumostatic operation of bubble column photobioreactors for *Haematococcus pluvialis* cultures using a specific light uptake rate as a control parameter. *Enzyme and Microbial Technology*, *33*(4), 403-409.

Chojnacka, K., & Marquez-Rocha, F. J. (2004). Kinetic and stoichiometric relationships of the energy and carbon metabolism in the culture of microalgae. *Biotechnology*, *3*(1), 21-34.

Chorus, I., & Bartram, J. (1999). *Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management*. E & F Spon, London. (p.416).

Choudhary, M., Jetley, U. K., Khan, M. A., Zutshi, S., & Fatma, T. (2007). Effect of heavy metal stress on proline, malondialdehyde, and superoxide dismutase activity in the cyanobacterium *Spirulina platensis*-S5. *Ecotoxicology and Environmental Safety*, *66*(2), 204-209.

Cisneros, B. J., & Rose, J. B. (Eds.). (2009). *Urban Water Security: Managing Risks: UNESCO-IHP* (Vol. 5). CRC Press.

Clarens, A. F., Resurreccion, E. P., White, M. A., & Colosi, L. M. (2010). Environmental life cycle comparison of algae to other bioenergy feedstocks. *Environmental Science & Technology*, *44*(5), 1813-1819.

Cohen, Z., Norman H. A., Heimer, Y. M. (1995). Microalgae as a source of omega 3 fatty acids. *World Review of Nutrition and Dietetics*, 77, 1-31.

Cohen-Bazire, G., & Bryant, D. A. (1982). Phycobilisomes: composition and structure [Algae, cyanobacteria]. *Botanical Monographs (USA)*.

Cole, J. J. (1982). Interactions between bacteria and algae in aquatic ecosystems. *Annual Review of Ecology and Systematics*, *13*, 291-314.

Coleman, J. R., & Colman, B. (1981). Inorganic carbon accumulation and photosynthesis in a blue-green alga as a function of external pH. *Plant Physiology*, *67*(5), 917-921.

Collier, J. L., & Grossman, A. R. (1992). Chlorosis induced by nutrient deprivation in *Synechococcus* sp. strain PCC 7942: not all bleaching is the same. *Journal of Bacteriology*, *174*(14), 4718-4726.

Colman, B., Huertas, I. E., Bhatti, S., & Dason, J. S. (2002). The diversity of inorganic carbon acquisition mechanisms in eukaryotic microalgae. *Functional Plant Biology*, *29*(3), 261-270.

Converti, A., Casazza, A. A., Ortiz, E. Y., Perego, P., & Del Borghi, M. (2009). Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. *Chemical Engineering and Processing: Process Intensification*, *48*(6), 1146-1151.

Conway, D., Kelly, P. M., Subak, S., & Downing, T. E. (1995). *The impacts of climate change on Africa* (No. 12). CSERGE, School of Environmental Sciences, University of East Anglia.

Conway, D., Persechino, A., Ardoin-Bardin, S., Hamandawana, H., Dieulin, C., & Mahé, G. (2009). Rainfall and water resources variability in sub-Saharan Africa during the twentieth century. *Journal of Hydrometeorology*, 10(1), 41-59.

Costa, J. A. V., Colla, L. M., & Filho, P. D. (2003). *Spirulina platensis* growth in open raceway ponds using fresh water supplemented with carbon, nitrogen and metal ions. *Zeitschrift fur naturforshung C*, *58*(1/2), 76-80.

Costa, J. A. V., & De Morais, M. G. (2011). The role of biochemical engineering in the production of biofuels from microalgae. *Bioresource Technology*, *102*(1), 2-9.

Craigie, J. S., & Hellebust, J. A. (Eds.). (1978). *Handbook of phycological methods: physiological and biochemical methods*. Cambridge University Press.

Csordas, A., & Wang, J. K. (2004). An integrated photobioreactor and foam fractionation unit for the growth and harvest of *Chaetoceros* spp. in open systems. *Aquacultural Engineering*, *30*(1), 15-30.

Cunningham, F. X., Dennenberg, R. J., Jursinic, P. A., & Gantt, E. (1990). Growth under red light enhances photosystem II relative to photosystem I and phycobilisomes in the red alga *Porphyridium cruentum*. *Plant Physiology*, *93*(3), 888-895.

Curtis, T. P., Mara, D. D., & Silva, S. A. (1992). The effect of sunlight on faecal coliforms in ponds: implications for research and design. *Water Science and Technology*, *26*(7-8), 1729-1738.

Dallas, H. F., & Day, J. A. (2004). *The effect of water quality variables on aquatic ecosystems: a review*. Pretoria: Water Research Commission.

Damiani, M. C., Popovich, C. A., Constenla, D., & Leonardi, P. I. (2010). Lipid analysis in Haematococcuspluvialis to assess its potential use as a biodiesel feedstock. *Bioresource Technology*, *101*(11), 3801-3807.

Darley, W. M., & Darley, W. M. (1982). *Algal biology: a physiological approach* (No. 589.31 D37).

Das, D., & Veziroğlu, T. N. (2001). Hydrogen production by biological processes: a survey of literature. *International Journal of Hydrogen Energy*, *26*(1), 13-28.

Das, P., Lei, W., Aziz, S. S., & Obbard, J. P. (2011). Enhanced algae growth in both phototrophic and mixotrophic culture under blue light. *Bioresource Technology*, *102*(4), 3883-3887.

Davis, T. W., Berry, D. L., Boyer, G. L., & Gobler, C. J. (2009). The effects of temperature and nutrients on the growth and dynamics of toxic and non-toxic strains of *Microcystis* during cyanobacteria blooms. *Harmful Algae*, *8*(5), 715-725.

De la Pena, M. R. (2007). Cell growth and nutritive value of the tropical benthic diatom, *Amphora* sp., at varying levels of nutrients and light intensity, and different culture locations. *Journal of Applied Phycology*, *19*(6), 647-655.

Del Campo, J. A., García-González, M., & Guerrero, M. G. (2007). Outdoor cultivation of microalgae for carotenoid production: current state and perspectives. *Applied Microbiology and Biotechnology*, *74*(6), 1163-1174.

Delia, A. S., Caruso, G., Melcarne, L., Caruso, G., Parisi, S., & Laganà, P. (2015). Biological Toxins from Marine and Freshwater Microalgae. *In Microbial Toxins and Related Contamination in the Food Industry* (pp. 13-55). Springer International Publishing.

Demirbas, A. (2008). Production of biodiesel from algae oils. *Energy Sources, Part A: Recovery, Utilization, and Environmental Effects*, *31*(2), 163-168.

Demirbas, A. (2010). Use of algae as biofuel sources. *Energy Conversion and Management*, *51*(12), 2738-2749.

Demirbas, A. H., & Demirbas, I. (2007). Importance of rural bioenergy for developing countries. *Energy Conversion and Management*, *48*(8), 2386-2398.

Demirbas, A., & Demirbas, M. F. (2011). Importance of algae oil as a source of biodiesel. *Energy Conversion and Management*, *52*(1), 163-170.

De Pauw, N., Morales, J., & Persoone, G. (1984). Mass culture of microalgae in aquaculture systems: progress and constraints. *Hydrobiologia*, *116*(1), 121-134.

Department of Water Affairs, (1988). Evaporation map for Namibia. Report No: 11/1/8/1/H1, October 1988, p.11.

De Pauw N, Persoone G (1988). Microalgae for aquaculture. In Borowitzka MA, Borowitzka LJ (eds), *Microalgal Biotechnology*, Cambridge University Press, New York, pp. 197-221.

Dermoun, D., Chaumont, D., Thebault, J. M., & Dauta, A. (1992). Modelling of growth of Porphyridium cruentum in connection with two interdependent factors: light and temperature. *Bioresource Technology*, *42*(2), 113-117.

De Vita, G., Endresen, K., & Hunt, L. C. (2006). An empirical analysis of energy demand in Namibia. *Energy Policy*, *34*(18), 3447-3463.

Dillschneider, R., Steinweg, C., Rosello-Sastre, R., & Posten, C. (2013). Biofuels from microalgae: photoconversion efficiency during lipid accumulation. *Bioresource Technology*, *142*, 647-654.

Divakara, B. N., Upadhyaya, H. D., Wani, S. P., & Gowda, C. L. (2010). Biology and genetic improvement of *Jatropha curcas L.*: a review. *Applied Energy*, *87*(3), 732-742.

Doan, T. T. Y., & Obbard, J. P. (2011). Improved Nile red staining of *Nannochloropsis* sp. *Journal of Applied Phycology*, *23*(5), 895-901.

Dolan, J. M., Podnar, G., Stancliff, S., Lin, E., Hosler, J., Ames, T., & Elfes, A. (2007). Harmful algal bloom characterization via the telesupervised adaptive ocean sensor fleet. *Robotics Institute*, 187.

Donato-Rondón, J. C., Morales-Duarte, S. J., & Castro-Rebolledo, M. I. (2010). Effects of eutrophication on the interaction between algae and grazers in an Andean stream. *Hydrobiologia*, *657*(1), 159-166.

Dortch, Q. (1990). The interaction between ammonium and nitrate uptake in phytoplankton. *Marine ecology progress series. Oldendorf*, *61*(1), 183-201.

Dortch, Q., Rabalais, N. N., Turner, R. E., & Qureshi, N. A. (2001). Impacts of changing Si/N ratios and phytoplankton species composition. *Coastal Hypoxia: Consequences for Living Resources and Ecosystems*, 37-48.

Dragone, G., Fernandes, B. D., Vicente, A. A., & Teixeira, J. A. (2010). Third generation biofuels from microalgae. *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*, *2*, 1355-1366.

Dresen, M. D., & Korth, R. M. (1994). Life on the edge: owning waterfront property.

Dunahay, T., Benemann, J., & Roessler, P. (1998). *A look back at the US Department of Energy's Aquatic Species Program: Biodiesel from algae* (Vol. 328). Golden: National Renewable Energy Laboratory.

Dunahay, T. G., Jarvis, E. E., Dais, S. S., & Roessler, P. G. (1996). Manipulation of microalgal lipid production using genetic engineering. *In Seventeenth Symposium on Biotechnology for Fuels and Chemicals* (pp. 223-231). Humana Press.

Du Pisani, P. L. (2006). Direct reclamation of potable water at Windhoek's Goreangab reclamation plant. *Desalination*, *188*(1), 79-88.

Dutta, D., De, D., Chaudhuri, S., & Bhattacharya, S. K. (2005). Hydrogen production by Cyanobacteria. *Microbial Cell Factories*, *4*(1), 1.

Eberhard, S., Finazzi, G., & Wollman, F. A. (2008). The dynamics of photosynthesis. *Annual Review of Genetics*, *42*, 463-515.

Edward, G. B., & David, C. S. (2010). Freshwater Algae Identification and Use as Bioindicators. *A John Wiley & Sons, Ltd*, 101.

Ekholm, P., & Krogeru, K. (2003). Determining algal-available phosphorus of differing origin: routine phosphorus analyses versus algal assays. *Hydrobiologia*, 492, 29–42.

Elsey, D., Jameson, D., Raleigh, B., & Cooney, M. J. (2007). Fluorescent measurement of microalgal neutral lipids. *Journal of Microbiological Methods*, *68*(3), 639-642.

Eppley, R. W. (1972). Temperature and phytoplankton growth in the sea. *Fisheries Bulletin*, *70*(4), 1063-1085.

Eppley, R. W., & Sloan, P. R. (1966). Growth rates of marine phytoplankton: correlation with light absorption by cell chlorophyll a. *Physiologia Plantarum*, *19*(1), 47-59.

Eriksen, N. T. (2008). The technology of microalgal culturing. *Biotechnology Letters*, *30*(9), 1525-1536.

Esquível, M. G., Amaro, H. M., Pinto, T. S., Fevereiro, P. S., & Malcata, F. X. (2011). Efficient H_2 production via *Chlamydomonas reinhardtii*. *Trends in Biotechnology*, *29*(12), 595-600.

Ettl, H., & Gaertner G (Eds.). (1988). Süsswasserflora von Mitteleuropa. Gustav Fischer Verlag, Stuttgart.

Evangelista, V., Barsanti, L., Frassanito, A. M., Passarelli, V., & Gualtieri, P. (Eds.). (2008). *Algal toxins: Nature, occurrence, effect and detection*. Springer Science & Business Media.

Fábregas, J., Abalde, J., Herrero, C., Cabezas, B., & Veiga, M. (1984). Growth of the marine microalga *Tetraselmis suecica* in batch cultures with different salinities and nutrient concentrations. *Aquaculture*, *42*(3-4), 207-215.

Fábregas, J., Maseda, A., Domínguez, A., & Otero, A. (2004). The cell composition of *Nannochloropsis* sp. changes under different irradiances in semicontinuous culture. *World Journal of Microbiology and Biotechnology*, *20*(1), 31-35.

Fábregas, J., Vázquez, V., Cabezas, B., & Otero, A. (1993). Tris not only controls the pH in microalgal cultures, but also feeds bacteria. *Journal of Applied Phycology*, *5*(5), 543-545.

FACT (Food, Agriculture, Conservation and Trade Act). (2007). Annual Progress Report Jatropha oil for local development in Mozambique, Technical report, FACT, 29.

Falch, B. S., König, G. M., Wright, A. D., Sticher, O., Angerhofer, C. K., Pezzuto, J. M., & Bachmann, H. (1995). Biological activities of cyanobacteria: Evaluation of extracts and pure compounds. *Planta Medica*, *61*(04), 321-328.

Falconer, I. R. (2008). Health effects associated with controlled exposures to cyanobacterial toxins. In *Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs* (pp. 607-612). Springer New York.

Falkowski, P. G., Dubinsky, Z., & Wyman, K. (1985). Growth-irradiance relationships in phytoplankton. *Limnology and Oceanography*, *30*(2), 311-321.

Fang, Y. Y. (2004). Water eutrophiocation in Li-Yang Reservoir and its ecological remediation countermeasures. *Journal of Soil and Water Conservation*, *18*(6), 183-186.

Fawley, K. P., & Fawley, M. W. (2004). Diversity and ecology of freshwater *Nannochloropsis* (Eustigmatophyceae). Abstract 29. In *58th Annual meeting of PSA, Williamsburg, VA, USA./http://www. psaalgae. org/meeting/PSA2004-Program-all. pdf S.*

Fawley, K. P., & Fawley, M. W. (2007). Observations on the diversity and ecology of freshwater *Nannochloropsis* (Eustigmatophyceae), with descriptions of new taxa. *Protist*, *158*(3), 325-336.

Fawley, M. W. (1984). Effects of Light Intensity and Temperature interactions on Growth characteristics of *Phaedactylum tricornutum* (Bacillariophyceae) 1. *Journal of Phycology*, *20*(1), 67-72.

Fawley, M. W., Fawley, K. P., Bailey, C., Fakhr, M., Hoffman, M., Dimmer, S., & Johnson, J. (2004). The microalgae of Itasca State Park, Minnesota, lakes.

Fawley, M. W., Fawley, K. P., & Owen, H. A. (2005). Diversity and ecology of small coccoid green algae from Lake Itasca, Minnesota, USA, including *Meyerella planktonica*, gen. et sp. nov. *Phycologia*, *44*(1), 35-48.

Fernández, F. A., Hall, D. O., Guerrero, E. C., Rao, K. K., & Grima, E. M. (2003). Outdoor production of *Phaeodactylum tricornutum* biomass in a helical reactor. *Journal of Biotechnology*, *103*(2), 137-152.

Fernández, F. G., Pérez, J. A., Sevilla, J. M., Camacho, F. G., & Grima, E. M. (2000). Modeling of eicosapentaenoic acid (EPA) production from *Phaeodactylum tricornutum* cultures in tubular photobioreactors. Effects of dilution rate, tube diameter, and solar irradiance. *Biotechnology and Bioengineering*, *68*(2), 173-183.

Fietz, S., Bleiss, W., Hepperle, D., Koppitz, H., Krienitz, L., & Nicklisch, A. (2005). First record of *Nannochloropsis limnetica* (Eustigmatophyceae) in the autotrophic picoplankton from Lake Baikal. *Journal of Phycology*, 41, 780–790.

Fisher, T., Berner, T., Iluz, D., & Dubinsky, Z. (1998). The kinetics of the photoacclimation response of *Nannochloropsis* sp. (Eustigmatophyceae): a study of changes in ultrastructure and PSU density. *Journal of Phycology*, *34*(5), 818-824.

Fogg, G. E. (1966). The extracellular products of algae. *Oceanography and Marine Biology: An Annual Review*, *4*(19), 212.

Fogg, G. E., & Thake, B. (1987). *Algal cultures and phytoplankton ecology*. University of Wisconsin Press.

Foster, K. W., & Smyth, R. D. (1980). Light Antennas in phototactic algae. *Microbiological Reviews*, *44*(4), 572.

Francisco, E. C., Neves, D. B., Jacob-Lopes, E., & Franco, T. T. (2010). Microalgae as feedstock for biodiesel production: carbon dioxide sequestration, lipid production and biofuel quality. *Journal of Chemical Technology and Biotechnology*, *85*(3), 395-403.

Freire, I., Cortina-Burgueño, A., Grille, P., Arizcun, M. A., Abellán, E., Segura, M., ... & Otero, A. (2016). Nannochloropsis limnetica: A freshwater microalga for marine aquaculture. *Aquaculture*, *459*, 124-130.

Fristachi, A., Sinclair, J. L., Hall, S., Berkman, J. A. H., Boyer, G., Burkholder, J., & Morton, S. L. (2008). Occurrence of cyanobacterial harmful algal blooms workgroup report. *In Cyanobacterial harmful algal blooms: state of the science and research needs* (pp. 45-103). Springer New York.

Fuentes, M. R., Sánchez, J. G., Sevilla, J. F., Fernández, F. A., Pérez, J. S., & Grima, E. M. (1999). Outdoor continuous culture of Porphyridium cruentum in a tubular photobioreactor: quantitative analysis of the daily cyclic variation of culture parameters. *Journal of Biotechnology*, *70*(1), 271-288.

Fuggi, A., Pinto, G., Pollio, A., & Taddei, R. (1988). The role of glycerol in osmoregulation of the acidophilic alga *Dunaliella acidophila* (Volvocales, Chlorophyta): effect of solute stress on photosynthesis, respiration and glycerol synthesis. *Phycologia*, *27*(4), 439-446.

Fuhrman, J. A., & Suttle, C. A. (1993). Viruses in marine planktonic systems. *Oceanography*, *6*(2), 51-63.

Fujiki, H., Suganuma, M., Suguri, H., Yoshizawa, S., Takagi, K., Nakayasu, M., & Sugimura, T. (1990). New tumor promoters from marine natural products. *In ACS symposium series* (Vol. 418, pp. 232-240). Oxford University Press.

Gallagher, E. (2008). *The Gallagher review of the indirect effects of biofuels production*. London: Renewable Fuels Agency.

Gast, R. J., Sanders, R. W., & Caron, D. A. (2009). Ecological strategies of protists and their symbiotic relationships with prokaryotic microbes. *Trends in Microbiology*, *17*(12), 563-569.

Gavrilescu, M., & Chisti, Y. (2005). Biotechnology—a sustainable alternative for chemical industry. *Biotechnology Advances*, *23*(7), 471-499.

Gehl, K. A., & Colman, B. (1985). Effect of external pH on the internal pH of *Chlorella saccharophila*. *Plant Physiology*, *77*(4), 917-921.

Gensemer, R. W., Smith, R. E., & Duthie, H. C. (1993). Comparative Effects of pH and Aluminium on Silica-limited growth and nutrient uptake in *Asterionella Ralfsii var. Americana* (Bacillariophyceae) 1. *Journal of Phycology*, *29*(1), 36-44.

Goel, P. K. (2006). *Water pollution: causes, effects and control*. New Age International.

Goldman, J. C., Azov, Y., Riley, C. B., & Dennett, M. R. (1982). The effect of pH in intensive microalgal cultures. I. Biomass regulation. *Journal of Experimental Marine Biology and Ecology*, *57*(1), 1-13.

Gondwana collection, namibia. (2016). *Webspaceconfigde*. Retrieved 19 September, 2016, fromhttp://typo3.p232710.webspaceconfig.de/home/namibia/stampsstories/stamps-13-05-21-pelicans/.

Gons, H. J. (1977). *On the light-limited growth of Scenedesmus protuberans Fritsch: ecological and physiological aspects of the energy requirements for growth and maintenance of a planktonic green alga* (Doctoral dissertation, Universiteit van Amsterdam).

Good, N. E., Winget, G. D., Winter, W., Connolly, T. N., Izawa, S., & Singh, R. M. (1966). Hydrogen ion buffers for biological research. *Biochemistry*, *5*(2), 467-477.

Gordillo, F. J., Goutx, M., Figueroa, F. L., & Niell, F. X. (1998a). Effects of light intensity, CO_2 and nitrogen supply on lipid class composition of *Dunaliella viridis*. *Journal of Applied Phycology*, *10*(2), 135-144.

Gordillo, F. J., Jiménez, C., Figueroa, F. L., & Niell, F. X. (1998b). Effects of increased atmospheric CO2 and N supply on photosynthesis, growth and cell composition of the cyanobacterium *Spirulina platensis* (Arthrospira). *Journal of Applied Phycology*, *10*(5), 461-469.

Gouveia, L., & Oliveira, A. C. (2009). Microalgae as a raw material for biofuels production. *Journal of Industrial Microbiology & Biotechnology*, *36*(2), 269-274.

Greenberg, A. E., Clesceri, L. S., & Eaton, A. D. (1992). Standard methods for the examination of water and wastewater. American Public Health Association.

Greene, R. M., Geider, R. J., Kolber, Z., & Falkowski, P. G. (1992). Iron-induced changes in light harvesting and photochemical energy conversion processes in eukaryotic marine algae. *Plant Physiology*, *100*(2), 565-575.

Greenspan, P., Mayer, E. P., & Fowler, S. D. (1985). Nile red: a selective fluorescent stain for intracellular lipid droplets. *The Journal of Cell Biology*, *100*(3), 965-973.

Grewe, C., & Griehl, C. (2008). Time-and media-dependent secondary carotenoid accumulation in *Haematococcus pluvialis*. *Biotechnology Journal*, *3*(9-10), 1232-1244.

Griffiths, M. J., & Harrison, S. T. (2009). Lipid productivity as a key characteristic for choosing algal species for biodiesel production. *Journal of Applied Phycology*, *21*(5), 493-507.

Grima, E. M., Belarbi, E. H., Fernández, F. A., Medina, A. R., & Chisti, Y. (2003). Recovery of microalgal biomass and metabolites: process options and economics. *Biotechnology Advances*, *20*(7), 491-515.

Grima, E. M., Pérez, J. S., Camacho, F. G., Sevilla, J. F., & Fernandez, F. A. (1994). Effect of growth rate on the eicosapentaenoic acid and docosahexaenoic acid content of *Isochrysis galbana* in chemostat culture. *Applied Microbiology and Biotechnology*, *41*(1), 23-27.

Grobbelaar, J. U. (1994). Turbulence in mass algal cultures and the role of light/dark fluctuations. *Journal of Applied Phycology*, *6*(3), 331-335.

Gross, M. (2013). *Development and optimization of algal cultivation systems* (M.S. Thesis, Iowa State

University, Ames, USA)

Guckert, J. B., & Cooksey, K. E. (1990). Triacylglycerol Accumulation and Fatty Acid Profile Changes In *Chlorella* (Chlorophyta) during high ph-induced cell cycle inhibition1. *Journal of Phycology*, *26*(1), 72-79.

Gunnarsson, H., & Sanseovic, A. M. (2001). Possible linkages between algae toxins in drinking water and related illnesses in Windhoek, Namibia.

Guschina, I. A., & Harwood, J. L. (2006). Lipids and lipid metabolism in eukaryotic algae. *Progress in Lipid Research*, *45*(2), 160-186.

Haag, A. L. (2007, May 31). Algae Bloom Again. *Nature*, (447), 520-521.

Häder, D.P. (1987). Photomovement. In: P. Fay and C. Van Baalen [Eds] The Cyanobacteria. Elsevier, Amsterdam, 325-345.

Hameed, M. A. (2007). Effect of algal density in bead, bead size and bead concentrations on wastewater nutrient removal. *African Journal of Biotechnology*, *6*(10).

Han, W., Li, C., Miao, X., & Yu, G. (2012). A novel miniature culture system to screen CO2-sequestering microalgae. *Energies*, *5*(11), 4372-4389.

Hankamer, B., Lehr, F., Rupprecht, J., Mussgnug, J. H., Posten, C., & Kruse, O. (2007). Photosynthetic biomass and H2 production by green algae: from bioengineering to bioreactor scale-up. *Physiologia Plantarum*, *131*(1), 10-21.

Hannon, M., Gimpel, J., Tran, M., Rasala, B., & Mayfield, S. (2010). Biofuels from algae: challenges and potential. *Biofuels*, *1*(5), 763-784.

Hansen, P. J. (2002). Effect of high pH on the growth and survival of marine phytoplankton: implications for species succession. *Aquatic Microbial Ecology*, *28*(3), 279-288.

Hardie, L. P., Balkwill, D. L., & Stevens, S. E. (1983). Effects of iron starvation on the physiology of the cyanobacterium *Agmenellum quadruplicatum*. *Applied and Environmental Microbiology*, *45*(3), 999-

1006.

Hargreaves, J. W., & Whitton, B. A. (1976). Effect of pH on growth of acid stream algae. *British Phycological Journal*, *11*(3), 215-223.

Harker, M., Tsavalos, A. J., & Young, A. J. (1996). Factors responsible for astaxanthin formation in the chlorophyte *Haematococcus pluvialis*. *Bioresource Technology*, *55*(3), 207-214.

Harrness. (2005). Harmful Algal Research and Response: A National Environmental Science Strategy 2005-2015. Ramsdell, J.S., Anderson, D.M., and Glibert, P.M. (eds.) Ecological Society of America, Washington, D.C., 96pp.

Harris, E. H. (2009). *Vol. 1: Introduction to Chlamydomonas and its laboratory use*. Amsterdam [etc.]: Elsevier [etc.].

Hart, R. (2006). Food web (bio-) manipulation of South African reservoirs-viable eutrophication management prospect or illusory pipe dream? A reflective commentary and position paper. *Water SA*, 35(4), 567-575.

Harvey, P. J., Psycha, M., Kokossis, A., Abubakar, A. L., Trivedi, V., Swamy, R., ... & Gartner, S. (2012, June). Glycerol production by halophytic microalgae: Strategy for producing industrial quantities in saline water. In *Proceedings of the 20th European Biomass Conference and Exhibition, Milan, Italy* (pp. 18-22).

Havlik, I., Lindner, P., Scheper, T., & Reardon, K. F. (2013). On-line monitoring of large cultivations of microalgae and cyanobacteria. *Trends in Biotechnology*, *31*(7), 406-414.

Healey, F. P. (1982). Phosphate. *The Biology of Cyanobacteria*, (19), 105-124.

Healey, F. P., & Hendzel, L. L. (1979). Indicators of phosphorus and nitrogen deficiency in five algae in culture. *Journal of the Fisheries Board of Canada*, *36*(11), 1364-1369.

Heasman, M., Diemar, J., O'connor, W., Sushames, T., & Foulkes, L. (2000). Development of extended shelf-life microalgae concentrate diets harvested by centrifugation for bivalve molluscs–a summary. *Aquaculture Research*, *31*(8-9), 637-659.

Held, P. (2011). Monitoring Algal Cell Growth Using Absorbance and Fluorescence. BioTek Application Note, www.biotek.com.

Heraud, P., Wood, B. R., Tobin, M. J., Beardall, J., & McNaughton, D. (2005). Mapping of nutrient-induced biochemical changes in living algal cells using synchrotron infrared microspectroscopy. *FEMS Microbiology Letters*, *249*(2), 219-225.

Hernandez, J. P., de-Bashan, L. E., Rodriguez, D. J., Rodriguez, Y., & Bashan, Y. (2009). Growth promotion of the freshwater microalga Chlorella vulgaris by the nitrogen-fixing, plant growth-promoting bacterium *Bacillus pumilus* from arid zone soils. *European Journal of Soil Biology*, *45*(1), 88-93.

Hepperle, D., & Krienitz, L. (2001). Systematics and ecology of chlorophyte picoplankton in German inland waters along a nutrient gradient. *International Review of Hydrobiology*, *86*(3), 269-284.

Heyns, P, Montgomery, S, Pallett, J & Seely, M K. (Eds). (1997). Namibia's water: A decision makers' guide. Windhoek: Ministry of Agriculture, Water and Rural Development/Desert Research Foundation of Namibia.

Hibberd, D. J. (1981). Notes on the taxonomy and nomenclature of the algal classes Eustigmatophyceae and Tribophyceae (synonym Xanthophyceae). *Botanical Journal of the Linnean Society*, *82*(2), 93-119.

Ho, S. H., Chen, C. Y., & Chang, J. S. (2012). Effect of light intensity and nitrogen starvation on CO_2 fixation and lipid/carbohydrate production of an indigenous microalga *Scenedesmus obliquus* CNW-N. *Bioresource Technology*, (*113)*, 244-252.

Holdsworth, E. S. (1985). Effect of growth factors and light quality on the growth, pigmentation and photosynthesis of two diatoms, *Thalassiosira gravida* and *Phaeodactylum tricornutum*. *Marine Biology*, *86*(3), 253-262.

Holm-Hansen, O., Nishida, K., Moses, V., & Calvin, M. (1959). Effects of mineral salts on short-term incorporation of carbon dioxide in *Chlorella*. *Journal of Experimental Botany*, *10*(1), 109-124.

Horner, D. S., Heil, B., Happe, T., & Embley, T. M. (2002). Iron hydrogenases–ancient enzymes in modern eukaryotes. *Trends in Biochemical Sciences*, *27*(3), 148-153.

Hossain, M. M., Khan, S., Sarkar, M. R. U., & Haque, M. M. (2005). Microalgal community structure in experimental carp-pangasiid catfish polyculture ponds. *Bangladesh Journal of Fisheries Research*, *9*(2), 121-132.

Hossain, A. B. M. S., Salleh, A., Boyce, A. N., Chowdhury, P., & Naqiuddin, M. (2008). Biodiesel fuel production from algae as renewable energy. *American Journal of Biochemistry and Biotechnology*, *4*(3), 250-254.

Hu, H., & Gao, K. (2003). Optimization of growth and fatty acid composition of a unicellular marine picoplankton, *Nannochloropsis* sp., with enriched carbon sources. *Biotechnology Letters*, *25*(5), 421-425.

Hu, H., & Gao, K. (2006). Response of growth and fatty acid compositions of *Nannochloropsis* sp. to environmental factors under elevated CO 2 concentration. *Biotechnology Letters*, *28*(13), 987-992.

Hu, Q. (2004). 5 Environmental Effects on Cell Composition. *Handbook of microalgal Culture: Biotechnology and Applied Phycology*, 83.

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., & Darzins, A. (2008). Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *The Plant Journal*, *54*(4), 621-639.

Hu, Q., Guterman, H., & Richmond, A. (1996). A flat inclined modular photobioreactor for outdoor mass cultivation of photoautotrophs. *Biotechnology and Bioengineering*, *51*(1), 51-60.

Hudnell, H. K. (Ed.). (2008). *Cyanobacterial harmful algal blooms: state of the science and research needs* (Vol. 619). Springer Science & Business Media.

Huntley, M. E., & Redalje, D. G. (2007). CO_2 mitigation and renewable oil from photosynthetic microbes: a new appraisal. *Mitigation and Adaptation Strategies for Global Change*, *12*(4), 573-608.

Huynh, M., & Serediak, N. (2006). Algae identification field guide. *Agriculture and Agri-Food Canada. 40 pp. P-9. Online: http://publications. gc. ca/collections/collection_2011/agr/A125-8-2-2011-eng. pdf 77 Health Canada, 2013. Enviornmental and Workplace Health, Blue-Green Algae (Cyanobacteria) and their Toxins. Online: http://www. hc-sc. gc. ca/ewh-semt/pubs/water-eau/cyanobacter-eng. php*.

Illman, A. M., Scragg, A. H., & Shales, S. W. (2000). Increase in *Chlorella* strains calorific values when grown in low nitrogen medium. *Enzyme and Microbial Technology*, *27*(8), 631-635.

Imai, H., Chang, K. H., & Nakano, S. I. (2009). Growth responses of harmful algal species *Microcystis* (Cyanophyceae) under various environmental conditions. *Interdisciplinary Studies on Environmental Chemistry—Environmental Research in Asia*, 269-275.

Info-namibiacom. (2016). *Info-namibiacom*. Retrieved 19 September, 2016, from http://www.info-namibia.com/info/weather.

Inglesi, R. (2010). Aggregate electricity demand in South Africa: conditional forecasts to 2030. *Applied Energy*, *87*(1), 197-204.

Iwkume, T. N., & Rasuno, M. (1985). Photosynthesis and primary production of *Microcystis aeruginosa* in Lake Kasumigaura. *Journal of Plankton Research*, (*7)*, 303-312.

Izaguirre, G. (2008). Harmful algal blooms and cyanotoxins in Metropolitan Water District's Reservoirs. *Advances in Experimental Medicine and Biology*, (*619)*, 182-184.

Jacobsen, M., Webster, M., & Vairavamoorthy, K. (Eds.). (2012). *The future of water in African cities: Why waste water?* World Bank Publications.

Janssen, M. (2002). *Cultivation of microalgae: effect of light/dark cycles on biomass yield* (p. 184). publisher not identified.

Janssen, M., Tramper, J., Mur, L. R., & Wijffels, R. H. (2003). Enclosed outdoor photobioreactors: Light regime, photosynthetic efficiency, scale-up, and future prospects. *Biotechnology and Bioengineering*, *81*(2), 193-210.

Ji, C. F., Yu, X. J., Chen, Z. A., Xue, S., Legrand, J., & Zhang, W. (2011). Effects of nutrient deprivation on biochemical compositions and photo-hydrogen production of *Tetraselmis subcordiformis*. *International Journal of Hydrogen Energy*, *36*(10), 5817-5821.

Jin, E. S., & Melis, A. (2003). Microalgal biotechnology: Carotenoid production by the green algae *Dunaliella salina*. *Biotechnology and Bioprocess Engineering*, *8*(6), 331-337.

Jongschaap, R. E. E., Corré, W. J., Bindraban, P. S., & Brandenburg, W. A. (2007). *Claims and facts on Jatropha curcas L.: global Jatropha curcas evaluation. breeding and propagation programme* (No. 158). Plant Research International.

Julies, E., Naomab, E. (2011). *Effect of rain water input and run-off on phosphate and nitrate concentrations in selected surface waters sources in and around Windhoek* (Bachelor of Science Research Thesis, University of Nambia).

Juneja, A., Ceballos, R. M., & Murthy, G. S. (2013). Effects of environmental factors and nutrient availability on the biochemical composition of algae for biofuels production: a review. *Energies*, *6*(9), 4607-4638.

Kalacheva, G. S., Zhila, N. O., Volova, T. G., & Gladyshev, M. I. (2002). The effect of temperature on the lipid composition of the green alga *Botryococcus*. *Microbiology*, *71*(3), 286-293.

Kalin, M., Wheeler, W. N., & Meinrath, G. (2004). The removal of uranium from mining wastewater using algal/microbial biomass. *Journal of Environmental Radioactivity*, *78*(2), 151-177.

Källqvist, T. (1981). Hydroecological field experiment. Incubation of Natural Phytoplankton in Lake Gjersjøen. *Norwegian Institute for Water Research, F-80402, Oslo*, 21.

Kan, Y., & Pan, J. (2010). A One-Shot solution to bacterial and fungal contamination in the green alga

Chlamydomonas reinhardtii culture by using an antibiotic cocktail1. *Journal of Phycology*, *46*(6), 1356-1358.

Kanazawa, K. K., & Gordon, J. G. (1985). The oscillation frequency of a quartz resonator in contact with liquid. *Analytica Chimica Acta*, (*175*) 99-105.

Karatay, S. E., & Dönmez, G. (2011). Microbial oil production from thermophile cyanobacteria for biodiesel production. *Applied Energy*, *88*(11), 3632-3635.

Kemka, N., Njiné, T., Togouet, S. H. Z., Menbohan, S. F., Nola, M., Monkiedje, A., & Compère, P. (2006). Eutrophication of lakes in urbanized areas: the case of Yaounde Municipal Lake in Cameroon, Central Africa. *Lakes & Reservoirs: Research & Management*, *11*(1), 47-55.

Kennish, M. J. (1992). *Ecology of Estuaries. Anthropogenic effects*. CRC. Press. *Inc., Boca Raton F*, *1*.

Kheira, A. A. A., & Atta, N. M. (2009). Response of *Jatropha curcas L*. to water deficits: Yield, water use efficiency and oilseed characteristics. *Biomass and Bioenergy*, *33*(10), 1343-1350.

Khoeyi, Z. A., Seyfabadi, J., & Ramezanpour, Z. (2012). Effect of light intensity and photoperiod on biomass and fatty acid composition of the microalgae, *Chlorella vulgaris*. *Aquaculture International*, *20*(1), 41-49.

Kilham, S., Kreeger, D., Goulden, C., & Lynn, S. (1997). Effects of nutrient limitation on biochemical constituents of *Ankistrodesmus falcatus*. *Freshwater Biology*, *38*(3), 591-596.

Kim, H. S., Hwang, S. J., Shin, J. K., An, K. G., & Yoon, C. G. (2007). Effects of limiting nutrients and N: P ratios on the phytoplankton growth in a shallow hypertrophic reservoir. *Hydrobiologia*, *581*(1), 255-267.

Kirke, B. K. (2001). Pumping downwards to prevent algal blooms. *In Poster presentation, IWA 2nd World Water Congress, Berlin, Germany*.

Kishimoto, M., Okakura, T., Nagashima, H., Minowa, T., Yokoyama, S. Y., & Yamaberi, K. (1994). CO2 fixation and oil production using micro-algae. *Journal of Fermentation and Bioengineering*, *78*(6), 479-482.

Knuckey, R. M., Brown, M. R., Robert, R., & Frampton, D. M. (2006). Production of microalgal concentrates by flocculation and their assessment as aquaculture feeds. *Aquacultural Engineering*, *35*(3), 300-313.

Kobayashi, M., Kakizono, T., & Nagai, S. (1993). Enhanced carotenoid biosynthesis by oxidative stress in acetate-induced cyst cells of a green unicellular alga, *Haematococcus pluvialis*. *Applied and Environmental Microbiology*, *59*(3), 867-873.

Koch, F. (2004). Mendelsohn, J., Jarvis, A., Roberts, C. & Robertson, T. (2002): Atlas of Namibia. A Portrait of the Land and its People.-Cape Town (David Philip Publishers; New Africa Books (Pty) Ltd).-200 S., zahlr. Farb-und s/w-Abb. sowie Karten, Diagramme und Tab. ISBN 0-86486-516-3. SAR 250,-. *Zoosystematics and Evolution*, *80*(1), 129-129.

Koh, M. Y., & Ghazi, T. I. M. (2011). A review of biodiesel production from *Jatropha curcas L*. oil. *Renewable and Sustainable Energy Reviews*, *15*(5), 2240-2251.

Konopka, A., & Brock, T. D. (1978). Effect of temperature on blue-green algae (cyanobacteria) in Lake Mendota. *Applied and Environmental Microbiology*, *36*(4), 572-576.

Krienitz, L., Hepperle, D., Stich, H. B., & Weiler, W. (2000). *Nannochloropsis limnetica* (Eustigmatophyceae), a new species of picoplankton from freshwater. *Phycologia*, *39*(3), 219-227.

Krienitz, L., & Wirth, M. (2006). The high content of polyunsaturated fatty acids in *Nannochloropsis limnetica* (Eustigmatophyceae) and its implication for food web interactions, freshwater aquaculture and biotechnology. *Limnologica-Ecology and Management of Inland Waters*, *36*(3), 204-210.

Kromkamp, J., & Limbeek, M. (1993). Effect of short-term variation in irradiance on light harvesting and photosynthesis of the marine diatom *Skeletonema costatum*: a laboratory study simulating vertical mixing. *Microbiology*, *139*(9), 2277-2284.

Kudo, I., Miyamoto, M., Noiri, Y., & Maita, Y. (2000). Combined effects of temperature and iron on the growth and physiology of the marine diatom *Phaeodactylum tricornutum* (Bacillariophyceae). *Journal of Phycology*, *36*(6), 1096-1102.

Kumar, S., Chaube, A., & Jain, S. K. (2012). Sustainability issues for promotion of *Jatropha* biodiesel in Indian scenario: a review. *Renewable and Sustainable Energy Reviews*, *16*(2), 1089-1098.

Kumar, A., Ergas, S., Yuan, X., Sahu, A., Zhang, Q., Dewulf, J., & Van Langenhove, H. (2010). Enhanced CO_2 fixation and biofuel production via microalgae: recent developments and future directions. *Trends in Biotechnology*, *28*(7), 371-380.

Kumar, M., & Sharma, M. P. (2014). Potential assessment of microalgal oils for biodiesel production: A review. *Journal of Material and Environmental Science*, *3*, 757-766.

Kuo, C. T. (2010). *Harvesting natural algal blooms for concurrent biofuel production and hypoxia mitigation* (Doctoral dissertation, University of Illinois at Urbana-Champaign).

Kyle, D. J., Gladue, R. M. (1991). Eicosapentaenoic acids and methods for their production. International Patent Application, Patent Cooperation Treaty Publication WO 91/14427, October 3, 1991.

Lahnsteiner, J., & Lempert, G. (2007). Water management in Windhoek, Namibia. *Water Science & Technology*, *55*(1-2), 441-448.

Lakeaccessorg. (2016). *Lakeaccessorg*. Retrieved 13 September, 2016, from http://www.lakeaccess.org/ecology/lakeecologyprim18.html.

Lakesteilacoomcom. (2016). *Lakesteilacoomcom*. Retrieved 19 September, 2016, from http://lakesteilacoom.com/about/solar-bees/.

Lane, A. E., & Burris, J. E. (1981). Effects of environmental pH on the internal pH of *Chlorella pyrenoidosa*, *Scenedesmus quadricauda*, and *Euglena mutabilis*. *Plant Physiology*, *68*(2), 439-442.

Lardon, L., Helias, A., Sialve, B., Steyer, J. P., & Bernard, O. (2009). Life-cycle assessment of biodiesel production from microalgae. *Environmental Science & Technology*, *43*(17), 6475-6481.

Laurinavichene, T. V., Fedorov, A. S., Ghirardi, M. L., Seibert, M., & Tsygankov, A. A. (2006). Demonstration of sustained hydrogen photoproduction by immobilized, sulfur-deprived *Chlamydomonas reinhardtii* cells. *International Journal of Hydrogen Energy*, *31*(5), 659-667.

Lavens, P., & Sorgeloos, P. (1996). *Manual on the production and use of live food for aquaculture* (No. 361). Food and Agriculture Organization (FAO).

Lee, R.E (1980) “Phycology,” Cambridge University Press, New York.

Lee, Y. K. (2001). Microalgal mass culture systems and methods: their limitation and potential. *Journal of Applied Phycology*, *13*(4), 307-315.

Le Fol, Y. O. A. N. N. (2012). *Renewable Energy Transition for A Sustainable Future in Namibia* (Doctoral dissertation, Master's Thesis. Department of Development and Planning, Aalborg University. http://projekter. aau. dk/projekter/files/63642504/Mast er_Thesis_Yoann_Le_Fol. pdf).

Lehmann, F. L. (2010). *Water quality of the Swakoppoort dam, Namibia* (Masters' Thesis, Albert-Ludwig University, Freiberg. Germany).

Lehr, F., & Posten, C. (2009). Closed photo-bioreactors as tools for biofuel production. *Current Opinion in Biotechnology*, *20*(3), 280-285.

Lembi, C. A., & Waaland, J. R. (1988). *Algae and human affairs*. Cambridge University Press.

Letcher, P. M., Lopez, S., Schmieder, R., Lee, P. A., Behnke, C., Powell, M. J., & McBride, R. C. (2013). Characterization of *Amoeboaphelidium protococcarum*, an algal parasite new to the Cryptomycota isolated from an outdoor algal pond used for the production of biofuel. *PLoS One*, *8*(2), e56232.

Levasseur, M., Thompson, P. A., & Harrison, P. J. (1993). Physiological Acclimation of marine phytoplankton to different nitrogen sources. *Journal of Phycology*, *29*(5), 587-595.

Lcecomna. (2016). *Lcecomna*. Retrieved 6 September, 2016, from http://lce.com.na/portfolio/water-augmentation-study/.

Li, Y., Horsman, M., Wang, B., Wu, N., & Lan, C. Q. (2008a). Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans*. *Applied Microbiology and Biotechnology*, *81*(4), 629-636.

Li, Y., Horsman, M., Wu, N., Lan, C. Q., & Dubois-Calero, N. (2008b). Biofuels from microalgae. *Biotechnology Progress*, *24*(4), 815-820.

Li, X., Xu, H., & Wu, Q. (2007). Large-scale biodiesel production from microalga *Chlorella protothecoides* through heterotrophic cultivation in bioreactors. *Biotechnology and Bioengineering*, *98*(4), 764-771.

Liang, Y., Sarkany, N., & Cui, Y. (2009). Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions. *Biotechnology Letters*, *31*(7), 1043-1049.

Lin, Y., He, Z., Yang, Y., Stoffella, P. J., Phlips, E. J., & Powell, C. A. (2008). Nitrogen versus

phosphorus limitation of phytoplankton growth in Ten Mile Creek, Florida, USA. *Hydrobiologia*, *605*(1), 247-258.

Lin, C. Y., & Lay, C. H. (2005). A nutrient formulation for fermentative hydrogen production using anaerobic sewage sludge microflora. *International Journal of Hydrogen Energy*, *30*(3), 285-292.

Linderholm, K., Tillman, A. M., & Mattsson, J. E. (2012). Life cycle assessment of phosphorus alternatives for Swedish agriculture. *Resources, Conservation and Recycling*, *66*, 27-39.

Liu, B., & Benning, C. (2013). Lipid metabolism in microalgae distinguishes itself. *Current Opinion in Biotechnology*, *24*(2), 300-309.

Liu, B. H., & Lee, Y. K. (2000). Secondary carotenoids formation by the green alga *Chlorococcum* sp. *Journal of Applied Phycology*, *12*(3-5), 301-307.

Liu, C. G., Jin, X. C., Sun, L., Zhong, Y., Dai, S. G., & Zhuang, Y. Y. (2005). Effects of pH on growth and species changes of algae in freshwater. *Journal of Agro-Environment Science*, *24*(2), 294-298.

Liu, X. J., Jiang, Y., & Chen, F. (2005). Fatty acid profile of the edible filamentous cyanobacterium *Nostoc flagelliforme* at different temperatures and developmental stages in liquid suspension culture. *Process Biochemistry*, *40*(1), 371-377.

Liu, Y., Li, L., & Jia, R. (2011). The Optimum Resource Ratio (N: P) for the Growth of *Microcystis Aeruginosa* with Abundant Nutrients. *Procedia Environmental Sciences*, *10*, 2134-2140.

Liu, Z. Y., Wang, G. C., & Zhou, B. C. (2008). Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*. *Bioresource Technology*, *99*(11), 4717-4722.

Lopez, C. B., Jewett, E. B., Dortch, Q. T. W. B., Walton, B. T., & Hudnell, H. K. (2008). Scientific assessment of freshwater harmful algal blooms. Interagency Working Group on Harmful Algal Blooms, Hypoxia, and Human Health of the Joint Subcommittee on Ocean Science and Technology. Available from http://www.cop.noaa.gov/stressors/extremeevents/hab/ habhrca/FreshwaterReport_final_2008.pdf.

Lynn, S. G., Kilham, S. S., Kreeger, D. A., & Interlandi, S. J. (2000). Effect of nutrient availability on the biochemical and elemental Stoichiometry in the freshwater diatom *Stephanodiscus Minutulus* (Bacillariophyceae). *Journal of Phycology*, *36*(3), 510-522.

Madigan, M. T., Martink, J. M., & Parker, J. (1996). Brock. Biology of Microorganisms. Prentice Hall, 8. Auflage Kapitel 14.3 Enrichment and Isolation Methods.

Magnusson, T.S. (2005). Urban water security – Local conditions and regional context: A case study of attitudes and water use behavior in Windhoek. Linkoping University, Department of water and environment studies. Linkoping, Sweden: Linkoping University.

Magurran, A. E. (2013). *Measuring biological diversity*. John Wiley & Sons.

Makkar, H. P., Kumar, V., Oyeleye, O. O., Akinleye, A. O., Angulo-Escalante, M. A., & Becker, K. (2011). *Jatropha platyphylla*, a new non-toxic *Jatropha* species: Physical properties and chemical constituents including toxic and antinutritional factors of seeds. *Food Chemistry*, *125*(1), 63-71.

Malisawa, M. S., & Rautenbach, C. D. W. (2012). Evaluating water scarcity in the Southern African Development Community (SADC) region by using a climate moisture index (CMI) indicator. *Water Science and Technology: Water Supply*, *12*(1), 45-55.

Mallick, N. (2002). Biotechnological potential of immobilized algae for wastewater N, P and metal removal: a review. *Biometals*, *15*(4), 377-390.

Markou, G., Chatzipavlidis, I., & Georgakakis, D. (2012). Carbohydrates production and bio-flocculation characteristics in cultures of *Arthrospira* (Spirulina) *platensis*: improvements through phosphorus limitation process. *BioEnergy Research*, *5*(4), 915-925.

Malapascua, J. R., Chou, H. N., Lu, W. J., & Lan, J. C. W. (2012). Development of an indirect method of microalgal lipid quantification using a lysochrome dye, Nile red. *African Journal of Biotechnology*, *11*(70), 13518-13527.

Mata, T. M., Martins, A. A., & Caetano, N. S. (2010). Microalgae for biodiesel production and other applications: a review. *Renewable and Sustainable Energy Reviews*, *14*(1), 217-232.

Matthew, T., Zhou, W., Rupprecht, J., Lim, L., Thomas-Hall, S. R., Doebbe, A., & Schenk, P. M. (2009). The metabolome of *Chlamydomonas reinhardtii* following induction of anaerobic H_2 production by sulfur depletion. *Journal of Biological Chemistry*, *284*(35), 23415-23425.

McKendrick, J., & Williams, R. K. (1968). The effects of urban drainage on Lake McIlwaine, Rhodesia, and subsequent water purification difficulties. In *Conference of the Institute of Water Pollution Control, East London*.

McLachlan, J., & Gorham, P. R. (1962). Effects of pH and Nitrogen Sources on Growth of *Microcystis aeruginosa* Kütz. *Canadian Journal of Microbiology*, *8*(1), 1-11.

Meding, A. (2016, February 25). Land of Thirst. *Informante*. Retrieved from. http://www.informante.web.na/land-thirst.17369.

Menge, J. (2010). Treatment of wastewater for re-use in the drinking water system of Windhoek. *City of Windhoek, Namibia (http://www. wastewater. co. za/)*.

Metcalf, E., Asano, T., Burton, F., Leverenz, H., Tsuchihashi, R., & Tchobanoglous, G. (2007). Water Reuse: Issues, Technologies, and Applications.

Metting Jr, F. B. (1996). Biodiversity and application of microalgae. *Journal of Industrial Microbiology*, *17*(5-6), 477-489.

Metzger, P., & Largeau, C. (2005). *Botryococcus braunii*: a rich source for hydrocarbons and related ether lipids. *Applied Microbiology and Biotechnology*, *66*(5), 486-496.

Miao, X., & Wu, Q. (2004). High yield bio-oil production from fast pyrolysis by metabolic controlling of *Chlorella protothecoides*. *Journal of Biotechnology, 110*(1), 85-93.

Miao, X., & Wu, Q. (2006). Biodiesel production from heterotrophic microalgal oil. *Bioresource Technology, 97*(6), 841-846.

Miao, X., Wu, Q., & Yang, C. (2004). Fast pyrolysis of microalgae to produce renewable fuels. *Journal of Analytical and Applied Pyrolysis, 71*(2), 855-863.

Miyachi, S., Miyachi, S., & Kamiya, A. (1978). Wavelength effects on photosynthetic carbon metabolism in *Chlorella*. *Plant and Cell Physiology, 19*(2), 277-288.

Moazami-Goudarzi, M., & Colman, B. (2012). Changes in carbon uptake mechanisms in two green marine algae by reduced seawater pH. *Journal of Experimental Marine Biology and Ecology, 413*, 94-99.

Moazami, N., Ranjbar, R., Ashori, A., Tangestani, M., & Nejad, A. S. (2011). Biomass and lipid productivities of marine microalgae isolated from the Persian Gulf and the Qeshm Island. *Biomass and*

Bioenergy, *35*(5), 1935-1939.

Mofijur, M., Masjuki, H. H., Kalam, M. A., Hazrat, M. A., Liaquat, A. M., Shahabuddin, M., & Varman, M. (2012). Prospects of biodiesel from *Jatropha* in Malaysia. *Renewable and Sustainable Energy Reviews*, *16*(7), 5007-5020.

Mohammady, N. G. E., & Fathy, A. A. (2007). Hurnic Acid lVIitigates Viability Reduction, Lipids and Fatty Acids of *Dunaliella salina* and *Nannochloropsis salina* Grown under Nickel Stress. *International Journal of Botany*, *3*(1), 64-70.

Moheimani, N. R. (2005). *The culture of coccolithophorid algae for carbon dioxide bioremediation* (Doctoral dissertation, Murdoch University).

Morgado, F., Antunes, C., & Pastorinho, R. (2003). Distribution and patterns of emergence of suprabenthic and pelagic crustaceans in a shallow temperate estuary (Ria de Aveiro, Portugal). *Acta Oecologica*, *24*, S205-S217.

Moroney, J. V., & Tolbert, N. E. (1985). Inorganic carbon uptake by *Chlamydomonas reinhardtii*. *Plant*

Physiology, *77*(2), 253-258.

Morris, I., Glover, H. E., & Yentsch, C. S. (1974). Products of photosynthesis by marine phytoplankton: the effect of environmental factors on the relative rates of protein synthesis. *Marine Biology*, *27*(1), 1-9.

Morweiser, M., Kruse, O., Hankamer, B., & Posten, C. (2010). Developments and perspectives of photobioreactors for biofuel production. *Applied Microbiology and Biotechnology*, *87*(4), 1291-1301.

Mukherjee, B., Nivedita, M., & Mukherjee, D. (2010). Plankton diversity and dynamics in a polluted eutrophic lake, Ranchi. *Journal of Environmental Biology*, 31, 827-839.

Munoz, R., & Guieysse, B. (2006). Algal–bacterial processes for the treatment of hazardous contaminants: a review. *Water Research, 40*(15), 2799-2815.

Muradyan, E. A., Klyachko-Gurvich, G. L., Tsoglin, L. N., Sergeyenko, T. V., & Pronina, N. A. (2004). Changes in lipid metabolism during adaptation of the *Dunaliella salina* photosynthetic apparatus to high CO_2 concentration. *Russian Journal of Plant Physiology, 51*(1), 53-62.

Musheko, J. (2015, March 9). Goreangab residents drink from the dam. *The Villager*. Retrieved from http://www.thevillager.com.na/articles/8153/Goreangab-residents-drink-from-the-dam/.

Nakamura, Y., & Imamura, M. (1983). Change in properties of starch when photosynthesized at different temperatures in *Chlorella vulgaris*. *Plant Science Letters*, *31*(2), 123-131.

Nakamura, Y., & Miyachi, S. (1982). Effect of temperature on starch degradation in *Chlorella vulgaris* 11h cells. *Plant and Cell Physiology*, *23*(2), 333-341.

Namwatercom. (2016). *Namwatercom*. Retrieved 6 September, 2016, from http://www.namwater.com.

Nannochloropsisorg. (2016). *Nannochloropsisorg*. Retrieved 20 September, 2016, from http://www.nannochloropsis.org/blog/2014/05/pictures/.

Nalewajko, C., & Murphy, T. P. (2001). Effects of temperature, and availability of nitrogen and phosphorus on the abundance of *Anabaena* and *Microcystis* in Lake Biwa, Japan: an experimental approach. *Limnology*, *2*(1), 45-48.

Negoro, M., Shioji, N., Miyamoto, K., & Micira, Y. (1991). Growth of microalgae in high CO_2 gas and effects of SOx and NOx. *Applied Biochemistry and Biotechnology*, *28*(1), 877-886.

Newenergyandfuelcom. (2016). *Newenergyandfuelcom*. Retrieved 19 September, 2016, from http://newenergyandfuel.com/wp-content/uploads/2009/03/algaeventure-harvester-block-diagram.jpg.

Neweracomna. (2016a). *Neweracomna*. Retrieved 19 September, 2016, from https://www.newera.com.na/wp-content/uploads/Swakoppoort-dam-.jpg.

Neweracomna. (2016b). *Neweracomna*. Retrieved 19 September, 2016, from https://www.newera.com.na/wp-content/uploads/City-2.jpg.

New era publication corporation. (2016). *New Era Newspaper Namibia*. Retrieved 19 September, 2016, from https://www.newera.com.na/2016/02/23/saving-water-absolutely-crucial-namwater-dams-run-dry/.

Nhapi, I., & Tirivarombo, S. (2004). Sewage discharges and nutrient levels in Marimba River, Zimbabwe. *Water SA*, *30*(1), 107-113.

Nielsen, D. C. (2008, January). Oilseed productivity under varying water availability. *In Proceedings of 20th annual central plains irrigation conference and exposition* (pp. 30-33).

Nitsan, Z., Mokady, S., & Sukenik, A. (1999). Enrichment of poultry products with ω3 fatty acids by dietary supplementation with the alga *Nannochloropsis* and mantur oil. *Journal of Agricultural and Food Chemistry*, *47*(12), 5127-5132.

Nolen, S. L. (1989, February). Limnology of Tenkiller Ferry Lake, Oklahoma, 1985-86. In *Proceedings of the Oklahoma Academy of Science* (Vol. 69, pp. 45-56).

NRC (Namibian Resource Consultant). (2000) "City of Windhoek: Gorengab dam Environmental Impact Assessment and Environmental Management Planning." Consultancy Report.

Nswgovau, (2016). *Nswgovau*. Retrieved 13 September, 2016, from http://www.water.nsw.gov.au/water-management/water-quality/algal-information/what-causes-algal-blooms.

Nyenje, P. M., Foppen, J. W., Uhlenbrook, S., Kulabako, R., & Muwanga, A. (2010). Eutrophication and nutrient release in urban areas of sub-Saharan Africa—A review. *Science of the Total Environment*, *408*(3), 447-455.

Oberholster, P. J., & Ashton, P. J. (2008). State of the nation report: An overview of the current status of water quality and eutrophication in South African rivers and reservoirs. *Parliamentary Grant Deliverable. Pretoria: Council for Scientific and Industrial Research (CSIR).*

Ochumba, P. B. O., & Kibaara, D. I. (2008). Observations on blue-green algal blooms in the open water of Lake Victoria, Kenya. *African Journal of Ecology*, 27(1), 23–24.

Ogbonda, K. H., Aminigo, R. E., & Abu, G. O. (2007). Influence of temperature and pH on biomass production and protein biosynthesis in a putative *Spirulina* sp. *Bioresource Technology*, *98*(11), 2207-2211.

Olguín, E. J., Galicia, S., Mercado, G., & Pérez, T. (2003). Annual productivity of *Spirulina* (*arthrospira*) and nutrient removal in a pig wastewater recycling process under tropical conditions. *Journal of Applied Phycology*, *15*(2-3), 249-257.

Ogunmokun, A. A., Mwandemele, O. D., & Dima, S. J. (2000, November). Use of recycled wastewater from biogas digesters for vegetable production in the Goreangab dam Area of Windhoek Municipality. In *1st WARFSA/WATERNET Symposium, Maputo Mozambique* (pp. 1-2).

Olivier, H., & Olivier, H. (1976). Great dams in southern Africa. Cape Town: Punell (p. 130).

O'neil, J. M., Davis, T. W., Burford, M. A., & Gobler, C. J. (2012). The rise of harmful cyanobacteria blooms: the potential roles of eutrophication and climate change. *Harmful Algae*, *14*, 313-334.

Ono, E., & Cuello, J. L. (2007). Carbon dioxide mitigation using thermophilic

cyanobacteria. *Biosystems Engineering*, *96*(1), 129-134.

Openshaw, K. (2000). A review of *Jatropha curcas*: an oil plant of unfulfilled promise. *Biomass and Bioenergy*, *19*(1), 1-15.

Oshima, Y. A. S. U. K. A. T. S. U., Minami, H. A. R. U. T. A. K. A., Takano, Y. U. T. A. K. A., & Yasumoto, T. (1989). Ichthyotoxins in a freshwater dinoflagellate *Peridinium polonicum*.

Otu, M. K., Ramlal, P., Wilkinson, P., Hall, R. I., & Hecky, R. E. (2011). Paleolimnological evidence of the effects of recent cultural eutrophication during the last 200 years in Lake Malawi, East Africa. *Journal of Great Lakes Research*, *37*, 61-74.

Paerl, H. W. (1988). Growth and reproductive strategies of freshwater blue-green algae (cyanobacteria). *Growth and reproductive strategies of freshwater phytoplankton. Cambridge University Press, Cambridge*, *261315*.

Paerl, H. W., & Fulton III, R. S. (2006). Ecology of harmful cyanobacteria. In *Ecology of harmful algae* (pp. 95-109). Springer Berlin Heidelberg.

Paerl, H. W., Fulton, R. S., Moisander, P. H., & Dyble, J. (2001). Harmful freshwater algal blooms, with an emphasis on cyanobacteria. *The Scientific World Journal*, *1*, 76-113.

Paerl, H. W., Tucker, J., & Bland, P. T. (1983). Carotenoid enhancement and its role in maintaining blue-green algal (*Microcystis aeruginosa*) surface blooms.

Pal, D., Khozin-Goldberg, I., Cohen, Z., & Boussiba, S. (2011). The effect of light, salinity, and nitrogen availability on lipid production by *Nannochloropsis* sp. *Applied Microbiology and Biotechnology*, *90*(4), 1429-1441.

Parent, L., Twiss, M. R., & Campbell, P. G. (1996). Influences of natural dissolved organic matter on the interaction of aluminum with the microalga *Chlorella*: a test of the free-ion model of trace metal toxicity. *Environmental Science & Technology*, *30*(5), 1713-1720.

Parry, M. L. (Ed.). (2007). *Climate change 2007-impacts, adaptation and vulnerability: Working group II contribution to the fourth assessment report of the IPCC* (Vol. 4). Cambridge University Press. Patil, V.

Patil, V., Källqvist, T., Olsen, E., Vogt, G., & Gislerød, H. R. (2007). Fatty acid composition of 12 microalgae for possible use in aquaculture feed. *Aquaculture International*, *15*(1), 1-9.

Pedersen, M. F., & Hansen, P. J. (2003). Effects of high pH on the growth and survival of six marine heterotrophic protists. *Marine Ecology Progress Series*, *260*, 33-41.

Peng, P., & Zhou, W. (2014). The next generation feedstock of biofuel: *Jatropha* or *Chlorella* as assessed by their life-cycle inventories. *Agriculture*, *4*(3), 217-230.

Perales-Vela, H. V., Pena-Castro, J. M., & Canizares-Villanueva, R. O. (2006). Heavy metal detoxification in eukaryotic microalgae. *Chemosphere*, *64*(1), 1-10.

Peterson, H. G., Healey, F. P., & Wagemann, R. (1984). Metal toxicity to algae: a highly pH dependent phenomenon. *Canadian Journal of Fisheries and Aquatic Sciences*, *41*(6), 974-979.

Pigott, M. J., Pigott, P. S., Pigott, B. L., & Pigott, S. F. (1989). *U.S. Patent No. 4,843,976*. Washington, DC: U.S. Patent and Trademark Office.

Poelman, E., De Pauw, N., & Jeurissen, B. (1997). Potential of electrolytic flocculation for recovery of micro-algae. *Resources, Conservation and Recycling*, *19*(1), 1-10.

Poerschmann, J., Spijkerman, E., & Langer, U. (2004). Fatty acid patterns in *Chlamydomonas* sp. as a marker for nutritional regimes and temperature under extremely acidic conditions. *Microbial Ecology*, *48*(1), 78-89.

Posten, C. (2009). Design principles of photo-bioreactors for cultivation of microalgae. *Engineering in Life Sciences*, *9*(3), 165-177.

Powerorganicscom. (2016). *Powerorganicscom*. Retrieved 19 September, 2016, from http://powerorganics.com/images/harvestintake.jpg.

Pruder, G. D., & Bolton, E. T. (1979). The role of CO_2 enrichment of aerating gas in the growth of an estuarine diatom. *Aquaculture*, *17*(1), 1-15.

Pulz, O. (2001). Photobioreactors: production systems for phototrophic microorganisms. *Applied Microbiology and Biotechnology*, *57*(3), 287-293.

Pulz, O., & Gross, W. (2004). Valuable products from biotechnology of microalgae. *Applied Microbiology and Biotechnology*, *65*(6), 635-648.

Qiang, H., & Richmond, A. (1996). Productivity and photosynthetic efficiency of *Spirulina platensis* as affected by light intensity, algal density and rate of mixing in a flat plate photobioreactor. *Journal of Applied Phycology*, *8*(2), 139-145.

Rabalais, N. N., Turner, R. E., Justic, D., Dortch, Q., & Wiseman Jr, W. J. (1999). Characterization of hypoxia: topic I report for the integrated assessment on hypoxia in the Gulf of Mexico.

Radakovits, R., Jinkerson, R. E., Darzins, A., & Posewitz, M. C. (2010). Genetic engineering of algae for enhanced biofuel production. *Eukaryotic Cell*, *9*(4), 486-501.

Radakovits, R., Jinkerson, R. E., Fuerstenberg, S. I., Tae, H., Settlage, R. E., Boore, J. L., & Posewitz, M. C. (2012). Draft genome sequence and genetic transformation of the oleaginous alga *Nannochloropsis gaditana*. *Nature Communications*, *3*, 686.

Ragauskas, A. J., Williams, C. K., Davison, B. H., Britovsek, G., Cairney, J., Eckert C. A., Frederick Jr, W. J., Hallett, J. P., Leak, D. J., Liotta, C. L., Mielenz, J. R., Murphy, R., Templer, R., Tschaplinski, T. (2006). The path forward for biofuels and biomaterials. *Science*, 31, 484-489.

Rai, L. C., Mallick, N., Singh, J. B., & Kumar, H. D. (1991). Physiological and biochemical characteristics of a copper tolerant and a wild type strain of *Anabaena doliolum* under copper stress. *Journal of Plant Physiology*, *138*(1), 68-74.

Rajvanshi, S., & Sharma, M. P. (2012). Microalgae: a potential source of biodiesel. *Journal of Sustainable Bioenergy Systems*, *2*(03), 49.

Ralph M., JI-Dong G.U. (2010). Environmental microbiology (2^{nd} ed.), Wiley and Blackwell, United States of America.

Ramanan, R., Kim, B. H., Cho, D. H., Oh, H. M., & Kim, H. S. (2016). Algae–bacteria interactions: Evolution, ecology and emerging applications. *Biotechnology Advances*, *34*(1), 14-29.

Ramanan, R., Vinayagamoorthy, N., Sivanesan, S. D., Kannan, K., & Chakrabarti, T. (2012). Influence of CO2 concentration on carbon concentrating mechanisms in cyanobacteria and green algae: a proteomic approach. *Algae*, *27*(4), 295.

Rao, A. R., Dayananda, C., Sarada, R., Shamala, T. R., & Ravishankar, G. A. (2007). Effect of salinity on

growth of green alga *Botryococcus braunii* and its constituents. *Bioresource Technology, 98*(3), 560-564.

Ras, M., Steyer, J. P., & Bernard, O. (2013). Temperature effect on microalgae: a crucial factor for outdoor production. *Reviews in Environmental Science and Bio/Technology*, *12*(2), 153-164.

Rasmussen, L. V., Rasmussen, K., & Bruun, T. B. (2012). Impacts of *Jatropha*-based biodiesel production on above and below-ground carbon stocks: A case study from Mozambique. *Energy Policy, 51*, 728-736.

Rast, W., & Thornton, A. J. (1996). Trends in eutrophication research and control. *Hydrological Processes*, 10, 295–313.

Rattanapoltee, P., & Kaewkannetra, P. (2013). Nile red, an alternative fluorescence method for quantification of neutral lipids in microalgae. *World Academy of Science, Engineering and Technology, International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering*, *7*(9), 889-893.

Raven, J. A., & Geider, R. J. (1988). Temperature and algal growth. *New Phytologist, 110*(4), 441-461.

Rawson, D. S. (1956). Algal indicators of trophic lake types. *Limnology and Oceanography, 1*(1), 18-25.

Redfield, A. C. (1963). The influence of organisms on the composition of sea-water. *The Sea*, 26-77.

Reid, H. (2007). *The economic impact of climate change in Namibia: how climate change will affect the contribution of Namibia's natural resources to its economy* (Vol. 7, No. 2). IIED.

Reifel, K. M., McCoy, M. P., Rocke, T. E., Tiffany, M. A., Hurlbert, S. H., & Faulkner, D. J. (2002). Possible importance of algal toxins in the Salton Sea, California. *In The Salton Sea* (pp. 275-292). Springer Netherlands.

Reijnders, L., & Huijbregts, M. A. J. (2008). Biogenic greenhouse gas emissions linked to the life cycles of biodiesel derived from European rapeseed and Brazilian soybeans. *Journal of Cleaner Production*, *16*(18), 1943-1948.

Reitan, K. I., Rainuzzo, J. R., & Olsen, Y. (1994). Effect of nutrient limitation on fatty acid and lipid content of marine microalgae1. *Journal of Phycology*, *30*(6), 972-979.

Rellán, S., Osswald, J., Saker, M., Gago-Martinez, A., & Vasconcelos, V. (2009). First detection of anatoxin-a in human and animal dietary supplements containing cyanobacteria. *Food and Chemical Toxicology*, *47*(9), 2189-2195.

Ren, T. (2014). *Primary factors affecting growth of microalgae optimal light exposure duration and frequency* (Doctoral dissertation, Iowa State University).

Renaud, S. M., & Parry, D. L. (1994). Microalgae for use in tropical aquaculture II: Effect of salinity on growth, gross chemical composition and fatty acid composition of three species of marine microalgae. *Journal of Applied Phycology*, *6*(3), 347-356.

Renaud, S. M., Thinh, L. V., Lambrinidis, G., & Parry, D. L. (2002). Effect of temperature on growth, chemical composition and fatty acid composition of tropical Australian microalgae grown in batch cultures. *Aquaculture*, *211*(1), 195-214.

Rether, C. (2011). Untersuchung des Potenzials Antennen-reduzierter Mutanten der Mikroalge *Chlamydomonas reinhardtii* zur Verbesserung des Lichteintrags in Photobioreaktoren,KIT, Karlsruhe.

Reynolds, C. S., & Walsby, A. E. (2008). Water blooms. *Biological Reviews*, 50(4), 437–481.

Rhee, G. Y. (1982). Effects of environmental factors and their interactions on phytoplankton growth. *In Advances in Microbial Ecology* (pp. 33-74). Springer US.

Richardson, C. J., King, R. S., Qian, S. S., Vaithiyanathan, P., Qualls, R. G., & Stow, C. A. (2007). Estimating ecological thresholds for phosphorus in the Everglades. *Environmental Science & technology*, *41*(23), 8084-8091.

Richmond, A. (2004). Biological principles of mass cultivation. *Handbook of microalgal culture: Biotechnology and applied phycology*, 125-177.

Richmond, A. (1990). Large scale microalgal culture and applications. *Progress in Phycological Research*, *7*, 269-330.

Richmond, A. (Ed.). (2008). *Handbook of microalgal culture: biotechnology and applied phycology*. John Wiley & Sons.

Richmond, A., & Vonshak, A. (1978). Spirulina culture in Israel. *Archiv für Hydrobiologie–BeiheftErgebnisse der Limnologie*, *11*, 274-280.

Richardson, K., Beardall, J., & Raven, J. A. (1983). Adaptation of unicellular algae to irradiance: an analysis of strategies. *New Phytologist*, *93*(2), 157-191.

Riebesell, U., Revill, A. T., Holdsworth, D. G., & Volkman, J. K. (2000). The effects of varying CO_2 concentration on lipid composition and carbon isotope fractionation in *Emiliania huxleyi*. *Geochimica et Cosmochimica Acta*, *64*(24), 4179-4192.

Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M., & Stanier, R. Y. (1979). Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *Microbiology*, *111*(1), 1-61.

Robarts, R. D., & Zohary, T. (1987). Temperature effects on photosynthetic capacity, respiration, and growth rates of bloom-forming cyanobacteria. *New Zealand Journal of Marine and Freshwater Research*, *21*(3), 391-399.

Rocha, G., Trotta, R., Martins, C. J. A. P., Melchiorri, A., Avelino, P. P., & Viana, P. T. P. (2003). New constraints on varying α. *New Astronomy Reviews*, *47*(8), 863-869.

Rodolfi, L., Chini Zittelli, G., Bassi, N., Padovani, G., Biondi, N., Bonini, G., & Tredici, M. R. (2009). Microalgae for oil: Strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. *Biotechnology and Bioengineering*, *102*(1), 100-112.

Rodriguez, C., Van Buynder, P., Lugg, R., Blair, P., Devine, B., Cook, A., & Weinstein, P. (2009). Indirect potable reuse: a sustainable water supply alternative. *International Journal of Environmental*

Research and Public Health, *6*(3), 1174-1203.

Roset, J., Gibello, A., Aguayo, S., Domínguez, L., Álvarez, M., Fernández-Garayzabal, J. F., & Muñoz, M. J. (2002). Mortality of rainbow trout [*Oncorynchus mykiss* (Walbaum)] associated with freshwater dinoflagellate bloom [*Peridinium polonicum* (Woloszynska)] in a fish farm. *Aquaculture Research*, *33*(2), 141-145.

Rossignol, N., Lebeau, T., Jaouen, P., & Robert, J. M. (2000). Comparison of two membrane–photobioreactors, with free or immobilized cells, for the production of pigments by a marine diatom. *Bioprocess Engineering*, *23*(5), 495-501.

Rossini, G. P. (2014). Biological Assays for Toxin Detection: Characteristics, Potential, Critical Issues. *Toxins and Biologically Active Compounds from Microalgae*, *1*, 449.

Rotatore, C., & Colman, B. (1991). The acquisition and accumulation of inorganic carbon by the unicellular green alga *Chlorella ellipsoidea*. *Plant, Cell & Environment*, *14*(4), 377-382.

Round, F. E. (1984). The ecology of algae, Cambridge, Cambridge University Press.

Saka, S., & Kusdiana, D. (2001). Biodiesel fuel from rapeseed oil as prepared in supercritical methanol. *Fuel*, *80*(2), 225-231.

Sandnes, J. M., Källqvist, T., Wenner, D., & Gislerød, H. R. (2005). Combined influence of light and temperature on growth rates of *Nannochloropsis oceanica*: linking cellular responses to large-scale biomass production. *Journal of Applied Phycology*, *17*(6), 515-525.

Sánchez, J. F., Fernández-Sevilla, J. M., Acién, F. G., Cerón, M. C., Pérez-Parra, J., & Molina-Grima, E. (2008). Biomass and lutein productivity of *Scenedesmus almeriensis*: influence of irradiance, dilution rate and temperature. *Applied Microbiology and Biotechnology*, *79*(5), 719-729.

Sato, N., Hagio, M., Wada, H., & Tsuzuki, M. (2000). Environmental effects on acidic lipids of thylakoid membranes. *Biochemical Society Transactions*, *28*(6), 912-914.

Sawayama, S., Inoue, S., Dote, Y., & Yokoyama, S. Y. (1995). CO_2 fixation and oil production through microalga. *Energy Conversion and Management*, *36*(6), 729-731.

Schindler, D. W., Hecky, R. E., Findlay, D. L., Stainton, M. P., Parker, B. R., Paterson, M. J., & Kasian, S. E. M. (2008). Eutrophication of lakes cannot be controlled by reducing nitrogen input: results of a 37-year whole-ecosystem experiment. *Proceedings of the National Academy of Sciences*, *105*(32), 11254-11258.

Schlagermann, P., Göttlicher, G., Dillschneider, R., Rosello-Sastre, R., & Posten, C. (2012). Composition of algal oil and its potential as biofuel. *Journal of Combustion*, *2012*.

Schmidt, M. (Ed.). (2009). *Planning Power: Review of Electricity Policy in Namibia*. Windhoek. Retrieved from http://www.ippr.org.na/sites/default/files/Review of Electricity Policy in Namibia.pdf.

Schneider, D. (2006) Grow Your Own? *American Scientist*, 656(9): Science Observer.

Schreurs, H. (1992). *Cyanobacterial dominance. Relations to eutrophication and lake morphology* (Unpublished doctoral dissertation, University of Amsterdam, Amsterdam*)*.

Scholz, M. J., Weiss, T. L., Jinkerson, R. E., Jing, J., Roth, R., Goodenough, U., ... & Gerken, H. G. (2014). Ultrastructure and composition of the *Nannochloropsis gaditana* cell wall. *Eukaryotic Cell*, *13*(11), 1450-1464.

Sforza, E., Bertucco, A., Morosinotto, T., & Giacometti, G. M. (2010). Vegetal oil from microalgae: species selection and optimization of growth parameters. *Chemical Engineering Transactions, 20,*199-204.

Shaharuddin, S., Chang, C. K., Zakaria, N. A., & AB Ghani, A. M. I. N. U. D. D. I. N. (2015). Effect of nitrogen and phosphorus concentration in the water of stormwater constructed wetland to freshwater phytoplankton distribution (10 years establishment): Case study in Universiti Sains Malaysia.

Shannon, C. W., & Weaver, W. (1948). The mathematical Theory of Communication. *Press UoI, editor*.

Shaw, G.R., Moore, D.P. & Garnett, C. (2003). Eutrophication and algal blooms. In: Sabljic, A.(ed) Encyclopedia of Life Support Systems (EOLSS). Developed under the Auspices of the UNESCO, Eolss Publishers, Oxford (pp. 22).

Sheehan, J., Dunahay, T., Benemann, J., & Roessler, P. (1998). A look back at the US Department of Energy's aquatic species program: biodiesel from algae. *National Renewable Energy Laboratory*, *328*.

Shi, J., Podola, B., & Melkonian, M. (2007). Removal of nitrogen and phosphorus from wastewater using microalgae immobilized on twin layers: an experimental study. *Journal of Applied Phycology*, *19*(5), 417-423.

Shifrin, N. S., & Chisholm, S. W. (1981). Phytoplankton Lipids: Interspecific differences and effects of

nitrate, silicate and light-dark cycles1. *Journal of Phycology*, *17*(4), 374-384.

ShihHsin, H., ChunYen, C., & JoShu, C. (2012). Effect of light intensity and nitrogen starvation on CO_2 fixation and lipid/carbohydrate production of an indigenous microalga *Scenedesmus obliquus CNW-N*. *Bioresource Technology*, *113*, 244-252.

Shinana, A.K. (2011). *Identification of nutrient sources causing algal blooms in Swakoppoort dam, Namibia* (Bachelor of Science in Geology Research Thesis, University of Nambia).

Silberbauer, M. J., & Moolman, J. (1993). Changes in urban residential land in the Rietspruit Catchment, Southern Transvaal. *Southern African Journal of Aquatic Science*, *19*(1-2), 89-94.

Silva, C.S.P., DaRos, P.C.M., Silva-Stenico, M. E., Fiore, M. F., Castro, H.F. (2011). XVIII Simpósio Nacional de Bioprocessos, Caxias do Sul, RS, Brazil.

Singh, S., Kate, B. N., & Banerjee, U. C. (2005). Bioactive compounds from cyanobacteria and microalgae: an overview. *Critical Reviews in Biotechnology*, *25*(3), 73-95.

Singh, A., Mehta, S. K., & Gaur, J. P. (2007). Removal of heavy metals from aqueous solution by common freshwater filamentous algae. *World Journal of Microbiology and Biotechnology, 23*(8), 1115-1120.

Singh, A., Pant, D., Olsen, S. I., & Nigam, P. S. (2012). Key issues to consider in microalgae based biodiesel production. *Energy Education Science and Technology Part A: Energy Science and Research, 29*(1), 563-576.

Singh, S. P., & Singh, P. (2015). Effect of temperature and light on the growth of algae species: A review. *Renewable and Sustainable Energy Reviews, 50*, 431-444.

Singh, R. N., Vyas, D. K., Srivastava, N. S. L., & Narra, M. (2008). SPRERI experience on holistic approach to utilize all parts of *Jatropha curcas* fruit for energy. *Renewable Energy, 33*(8), 1868-1873.

Sirunda, J. J., & Mazvimavi, D. (2014). The Effects of Water Transfer from Swakoppoort and Omatako dams on the Water Quality of Von Bach dam, Namibia. *In Combating Water Scarcity in Southern Africa* (pp. 79-118). Springer Netherlands.

Skjånes, K., Knutsen, G., Källqvist, T., & Lindblad, P. (2008). H_2 production from marine and freshwater species of green algae during sulfur deprivation and considerations for bioreactor design. *International Journal of Hydrogen Energy, 33*(2), 511-521.

Skulberg, O. M. (1994). Oscillatorialean cyanoprokaryotes and their application for algal culture technology. *Algological Studies/Archiv für Hydrobiologie, Supplement Volumes*, 265-278.

Smith, V. H. (1983). Low nitrogen to phosphorus ratios favor dominance by blue-green algae in lake phytoplankton. *Science, 221*(4611), 669-671.

Solomon, S. (Ed.). (2007). *Climate change 2007-The physical science basis: Working group I contribution to the fourth assessment report of the IPCC* (Vol. 4). Cambridge University Press.

Solovchenko, A., Khozin-Goldberg, I., Recht, L., & Boussiba, S. (2011). Stress-induced changes in optical properties, pigment and fatty acid content of *Nannochloropsis* sp.: implications for non-destructive assay of total fatty acids. *Marine Biotechnology*, *13*(3), 527-535.

Sorokin, C., & Krauss, R. W. (1962). Effects of temperature & illuminance on *Chlorella* growth uncoupled from cell division. *Plant Physiology*, *37*(1), 37.

Sousa, I., Gouveia, L., Batista, A. P., Raymundo, A., & Bandarra, N. M. (2008). Microalgae in novel food products. *Food Chemistry Research Developments*, 75-112.

Spencer, C. P. (1954). Studies on the culture of a marine diatom. *Journal of the Marine Biological Association of the United Kingdom*, *33*(01), 265-290.

Spiritofleadershipinfo. (2016). *Spiritofleadershipinfo*. Retrieved 19 September, 2016, from http://www.spiritofleadership.info/wp-content/uploads/2012/06/sky_harvester1.jpg.

Spolaore, P., Joannis-Cassan, C., Duran, E., & Isambert, A. (2006). Commercial applications of microalgae. *Journal of Bioscience and Bioengineering*, *101*(2), 87-96.

Srivastava, A., & Prasad, R. (2000). Triacylglycerols-based diesel fuels.*Renewable and Sustainable Energy Reviews*, *4*(2), 111-133.

SSERG. (2001). Reconnaissance of the biological limnology of the Salton Sea: final report. Salton Sea Ecosystem Research Group, Department of Biology and Center for Inland Waters, San Diego State University, San Diego, California (pp. 1100).

Staley, J. T., Bryant, M.P., & Holt, J. G. (1989). Oxygenic photosynthetic bacteria. In: Hensyl WR (ed.) Bergey's Manual of Systematic Bacteriology (Vol 3), Williams & Wilkins Co., Baltimore Md. 1711-1746.

Statemnus. (2016). *Statemnus*. Retrieved 13 September, 2016, from https://www.pca.state.mn.us/sites/default/files/wq-iw3-24.pd.

Statistics Agency, N. (2011). Namibia 2011 Population and Housing Main Report. Windhoek: National Planning Commission.

Stednick, J. D. (1991). *Wildland water quality sampling and analysis*. Elsevier.

Stein, J. R., Hellebust, J. A., & Craigie, J. S. (1973). *Handbook of Phycological Methods: Physiological And Biochemical Methods* (Vol. 2). Cambridge University Press.

Steinberg, C. W., & Hartmann, H. M. (1988). Planktonic bloom-forming cyanobacteria and the eutrophication of lakes and rivers. *Freshwater Biology*, 20(2), 279–287.

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

Stockner, J. G. (1991). Autotrophic picoplankton in freshwater ecosystems: the view from the summit. *Internationale Revue der gesamten Hydrobiologie und Hydrographie*, *76*(4), 483-492.

Sucurinet. (2016). *Esaorg*. Retrieved 9 September, 2016, from http://www.esa.org/esa/wp-content/uploads/2012/12/hypoxia.pdf.

Sucurinet. (2016). *Making-biodiesel-bookscom*. Retrieved 19 September, 2016, from http://making-biodiesel-books.com/wp-content/uploads/2012/02/about-algae1.jpg.

Sun, X., Cao, Y., Xu, H., Liu, Y., Sun, J., Qiao, D., & Cao, Y. (2014). Effect of nitrogen-starvation, light intensity and iron on triacylglyceride/carbohydrate production and fatty acid profile of *Neochloris oleoabundans* HK-129 by a two-stage process. *Bioresource Technology*, *155*, 204-212.

Sun, A., Davis, R., Starbuck, M., Ben-Amotz, A., Pate, R., & Pienkos, P. T. (2011). Comparative cost analysis of algal oil production for biofuels. *Energy*, *36*(8), 5169-5179.

Sun, L., Jin, X. C., Zhong, Y., Zhang, D. M., Zhu, L., Dai, S. G., & Zhuang, Y. Y. (2006). [Changes of algal communities in water body with different proportions of nitrogen and phosphorus]. *Ying yong sheng tai xue bao=The journal of applied ecology/Zhongguo sheng tai xue xue hui, Zhongguo ke xue yuan Shenyang ying yong sheng tai yan jiu suo zhu ban*, *17*(7), 1218-1223.

Sunda, W. (1975). *The relationship between cupric ion activity and the toxicity of copper to phytoplankton* (Doctoral dissertation, Massachusetts Institute of Technology and Woods Hole Oceanographic Institution).

Sukenik, A. (1999). Production of eicosapentaenoic acid by the marine eustigmatophyte *Nannochloropsis*. *Chemicals from Microalgae*, 41-56.

Sukenik, A., Carmeli, Y., & Berner, T. (1989). Regulation of fatty acid composition by irradiance level in the eustigmatophyte *Nannochloropsis* sp. *Journal of Phycology*, *25*(4), 686-692.

Suresh, B., & Ravishankar, G. A. (2004). Phytoremediation—A novel and promising approach for environmental clean-up. *Critical Reviews in biotechnology*, *24*(2-3), 97-124.

Tabah, B. (2011). *Optimization of Lipid Production in Marine Microalga Nannochloropsis sp. (Eustigmatophyceae)* (Doctoral dissertation, Ben-Gurion University of the Negev).

Taher, H., Al-Zuhair, S., Al-Marzouqi, A. H., Haik, Y., & Farid, M. M. (2011). A review of enzymatic transesterification of microalgal oil-based biodiesel using supercritical technology. *Enzyme research*.

Takagi, M., Watanabe, K., Yamaberi, K., & Yoshida, T. (2000). Limited feeding of potassium nitrate for intracellular lipid and triacylglycerol accumulation of *Nannochloris* sp. UTEX LB1999. *Applied Microbiology and Biotechnology*, *54*(1), 112-117.

Takagi, M., & Yoshida, T. (2006). Effect of salt concentration on intracellular accumulation of lipids and triacylglyceride in marine microalgae *Dunaliella* cells. *Journal of Bioscience and Bioengineering*, *101*(3), 223-226.

Tambunan, A. H., Situmorang, J. P., Silip, J. J., Joelianingsih, A., & Araki, T. (2012). Yield and physicochemical properties of mechanically extracted crude *Jatropha curcas L* oil. *Biomass and Bioenergy*, *43*, 12-17.

Tan, C. K., & Johns, M. R. (1996). Screening of diatoms for heterotrophic eicosapentaenoic acid production. *Journal of Applied Phycology*, *8*(1), 59-64.

Tang, D., Han, W., Li, P., Miao, X., & Zhong, J. (2011). CO_2 biofixation and fatty acid composition of *Scenedesmus obliquus* and *Chlorella pyrenoidosa* in response to different CO_2 levels. *Bioresource Technology*, *102*(3), 3071-3076.

Tarr, P. (2007). Epupa dam case study. *Water Resources Development*, *23*(3), 473-484.

Tas, B. (2011). Bloom and eutrophication of Hydrodictyon reticulatum (Chlorophyceae) at Civil and Kacalı Stream, Ordu, Turkey. *Energy Education Science and Technology Part A: Energy Science and Research*, *28*(1), 319-330.

Tatsuzawa, H., Takizawa, E., Wada, M., & Yamamoto, Y. (1996). Fatty acid and lipid composition of the acidophilic green alga *Chlamydomonas* sp. *Journal of Phycology*, *32*(4), 598-601.

Theodorou, M. E., Elrifi, I. R., Turpin, D. H., & Plaxton, W. C. (1991). Effects of phosphorus limitation on respiratory metabolism in the green alga *Selenastrum minutum*. *Plant Physiology*, *95*(4), 1089-1095.

Thomas, E. A. (1969). The process of eutrophication in Central European lakes. Eutrophication: Causes, Consequences and Correctives (pp. 29-49). Proceedings of an International Symposium on Eutrophication. National Academy of Sciences, Washington (pp. 661).

Tilman, D. (1982). *Resource competition and community structure*. Princeton University Press.

Tixikcom sro. (2017). *TIXIKcom*. Retrieved 3 March, 2017, from http://www.tixik.com/swakoppoort-dam-3805482.htm.

Tjahjono, A. E., Hayama, Y., Kakizono, T., Terada, Y., Nishio, N., & Nagai, S. (1994). Hyper-accumulation of astaxanthin in a green alga *Haematococcus pluvialis* at elevated temperatures. *Biotechnology Letters*, *16*(2), 133-138.

Tredici, M. R. (2010). Photobiology of microalgae mass cultures: understanding the tools for the next green revolution. *Biofuels*, *1*(1), 143-162.

Tripathi, U., Sarada, R., & Ravishankar, G. A. (2002). Effect of culture conditions on growth of green alga—*Haematococcus pluvialis* and astaxanthin production. *Acta Physiologiae Plantarum*, *24*(3), 323-329.

Tsukahara, K., & Sawayama, S. (2005). Liquid fuel production using microalgae. *Journal of the Japan Petroleum Institute*, *48*(5), 251.

Tsuzuki, M., Ohnuma, E., Sato, N., Takaku, T., & Kawaguchi, A. (1990). Effects of CO_2 concentration during growth on fatty acid composition in microalgae. *Plant Physiology*, *93*(3), 851-856.

Tucker, C. S., & D'Abramo, L. R. (2008). *Managing high pH in freshwater ponds*. Southern Regional Aquaculture Center. South Regional Aquaculture Center. Publication No. 4604.

Tungenicom. (2016). *Tungenicom*. Retrieved 6 September, 2016, from http://www.tungeni.com.

Uellendahl, H., Wang, G., Møller, H., Jørgensen, U., Skiadas, I. V., Gavala, H. N., & Ahring, B. K. (2008). Energy balance and cost-benefit analysis of biogas production from perennial energy crops pretreated by wet oxidation. *Water Science and Technology*, *58*(9), 1841-1847.

Ukeles, R. (1961). The effect of temperature on the growth and survival of several marine algal species. *Biological Bulletin*, *120*(2), 255-264.

Usher, P. K., Ross, A. B., Camargo-Valero, M. A., Tomlin, A. S., & Gale, W. F. (2014). An overview of the potential environmental impacts of large-scale microalgae cultivation. *Biofuels*, *5*(3), 331-349.

Van der Merwe, B. (2000). Integrated water resource management in Windhoek, Namibia. *Water Supply*, *18*(1), 376-381.

Van der Merwe, B., & Menge, J. (1996). Water Reclamation for Potable Reuse in Windhoek, Namibia. *Department of the City Engineer, City of Windhoek, Namibia*.

Van Ginkel, C. E. (2004). A national survey of the incidence of cyanobacterial blooms and toxin production in major impoundments. *Published by: Directorate: Resource Quality Services, Department of water Affairs and Forestry. South Africa*.

Van Zyl, B. (2011). Proposes construction of the New Industrial Wastewater Treatment Plant and Associated Structures. Windhoek: Directorate of Environmental Affairs.

Vaishampayan, A., Sinha, R. P., Hader, D. P., Dey, T., Gupta, A. K., Bhan, U., & Rao, A. L. (2001). Cyanobacterial biofertilizers in rice agriculture. *The Botanical Review*, *67*(4), 453-516.

Van Leeuwe, M. A., & Stefels, J. (1998). Effects of iron and light stress on the biochemical composition of Antarctic *Phaeocystis* sp. (Prymnesiophyceae). II. Pigment composition. *Journal of Phycology*, *34*(3), 496-503.

Valenzuela-Espinoza, E., Millán-Núñez, R., & Núñez-Cebrero, F. (2002). Protein, carbohydrate, lipid and chlorophyll a content in *Isochrysis aff. galbana* (clone T-Iso) cultured with a low cost alternative to the f/2 medium. *Aquacultural Engineering*, *25*(4), 207-216.

Van Liere, L., & Mur, L. R. (1980). Occurrence of *Oscillatoria agardhii* and some related species, a survey. *In Hypertrophic ecosystems* (pp. 67-77). Springer Netherlands.

Van Liere, L., Mur, L.R., Gibson, C.E. & Herdman, M. (1979). Growth and physiology of *Oscillatoria agardhii* and some related species. *A Survey: Developments in Hydrobiology*, 2, 67-77.

Van Oijen, T., Van Leeuwe, M. A., Gieskes, W. W., & de Baar, H. J. (2004). Effects of iron limitation on photosynthesis and carbohydrate metabolism in the Antarctic diatom *Chaetoceros brevis* (Bacillariophyceae). *European Journal of Phycology*, *39*(2), 161-171.

Vasudevan, P. T., & Briggs, M. (2008). Biodiesel production—Current state of the art and challenges. *Journal of Industrial Microbiology & Biotechnology*, *35*(5), 421-430.

Vazquez-Duhalt, R., & Arredondo-Vega, B. O. (1991). Haloadaptation of the green alga *Botryococcus braunii* (race A). *Phytochemistry*, *30*(9), 2919-2925.

Veldhuis, M. J., Cucci, T. L., & Sieracki, M. E. (1997). Cellular DNA Content of Marine Phytoplankton Using Two New Fluorochromes: Taxonomic and Ecological Implications1. *Journal of Phycology*, *33*(3), 527-541.

Verma, N. M., Mehrotra, S., Shukla, A., & Mishra, B. N. (2010). Prospective of biodiesel production utilizing microalgae as the cell factories: A comprehensive discussion. *African Journal of Biotechnology*, *9*(10), 1402-1411.

Vezjak, M., Savsek, T., & Stuhler, E. A. (1998). System dynamics of euthrophication processes in lakes. *European Journal of Operational Research*, *109*(2), 442-451.

Visviki, I., & Palladino, J. (2001). Growth and cytology of *Chlamydomonas acidophila* under acidic stress. *Bulletin of Environmental contamination and Toxicology*, *66*(5), 623-630.

Visviki, I., & Santikul, D. (2000). The pH tolerance of *Chlamydomonas applanata* (Volvocales, Chlorophyta). *Archives of Environmental Contamination and Toxicology*, *38*(2), 147-151.

Volkman, J. K., Jeffrey, S. W., Nichols, P. D., Rogers, G. I., & Garland, C. D. (1989). Fatty acid and lipid composition of 10 species of microalgae used in mariculture. *Journal of Experimental Marine Biology and Ecology*, *128*(3), 219-240.

Von Oertzen, D. (2012). *Namibia's Energy Future: A Case for Renewables*. Konrad Adenauer Stiftung,

Windhoek, Namibia.

Wan, S. P., Sreedevi, T. K., Rao, A. K., & Dixin, Y. (2007). Biofuels: a strategy for enhanced water use efficiency, improved livelihoods and protecting environment in the semi-arid tropics. *Linkages between Energy and Water Management for Agriculture in Developing Countries*, *29*, 31.

Wang, H., Wang, T., Zhang, B., Li, F., Toure, B., Omosa, I. B., & Pradhan, M. (2014). Water and wastewater treatment in Africa–Current practices and challenges. *CLEAN–Soil, Air, Water*, *42*(8), 1029-1035.

Wang, L. (2006). Research on the Relevant Factors of the Algal Growth in Hydrodynamic Condition. *Faculty of Urban Construction & Environment Engineering. Chongqing University, Chongqing*.

Wang, Z. T., Ullrich, N., Joo, S., Waffenschmidt, S., & Goodenough, U. (2009). Algal lipid bodies: stress induction, purification, and biochemical characterization in wild-type and starchless *Chlamydomonas reinhardtii*. *Eukaryotic Cell*, *8*(12), 1856-1868.

Ward, O. P., & Singh, A. (2005). Omega-3/6 fatty acids: alternative sources of production. *Process Biochemistry*, *40*(12), 3627-3652.

Watanabe, T., Kitajima, C., & Fujita, S. (1983). Nutritional values of live organisms used in Japan for mass propagation of fish: a review. *Aquaculture*, *34*(1), 115-143.

Weissman, J. C., & Goebel, R. P. (1987). *Design and analysis of microalgal open pond systems for the purpose of producing fuels: a subcontract report* (No. SERI/STR-231-2840). Solar Energy Research Inst., Golden, CO (USA).

Wetzel, R. G. (2001). *Limnology: lake and river ecosystems*. Gulf Professional Publishing.

Wijffels, R. H., & Barbosa, M. J. (2010). An outlook on microalgal biofuels. *Science (Washington)*, *329*(5993), 796-799.

Williams, P. J. L. B., & Laurens, L. M. (2010). Microalgae as biodiesel & biomass feedstocks: review & analysis of the biochemistry, energetics & economics. *Energy & Environmental Science*, *3*(5), 554-590.

Wojciechowski, J., & Padial, A. A. (2015). Monitoring studies should consider temporal variability to

reveal relations between cyanobacterial abundance and environmental variables. *Anais da Academia Brasileira de Ciências*, *87*(3), 1717-1726.

Wong, P. T. S., Burnison, G., & Chau, Y. K. (1979). Cadmium toxicity to freshwater algae. *Bulletin of Environmental Contamination and Toxicology*, *23*(1), 487-490.

Wong, P. T. S., & Chau, Y. K. (1990). Zinc toxicity to freshwater algae.*Toxicity Assessment*, *5*(2), 167-177.

Wood, J. (2014). Global Potable Reuse Case Study 4: Windhdoek, Naimia. Australian Water Recycling Centre of Excellence. Sydney, Australia: University of New South Wales & Public Utilities Board, Singapore.

World Bank. (2008). *Data and statistics*. Internet document. Available at: go.worldbank.org/WVEGH5U9W0.

World Commission on Environment and Development (WCED). 1987. *Our common future*. Oxford, UK, Oxford University Press.

World Health Organization. (2010). Namibia: Analytical summary. From: http://www.aho.afro.who.int/profiles_information/index.php/Namibia:Analytical_summary_-_The_physical_environment.

Xiao, T., FanXiang, K., Yang, Y., XiaoLi, S., & Min, Z. (2009). Effects of enhanced temperature on algae recruitment and phytoplankton community succession. *China Environmental Science*, *29*(6), 578-582.

Xin, L., Hong-ying, H., Ke, G., & Ying-xue, S. (2010). Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga *Scenedesmus* sp. *Bioresource Technology*, *101*(14), 5494-5500.

Xin, L., Hong-Ying, H., & Yu-Ping, Z. (2011). Growth and lipid accumulation properties of a freshwater microalga *Scenedesmus* sp. under different cultivation temperature. *Bioresource Technology*, *102*(3), 3098-3102.

Xiong, W., Gao, C., Yan, D., Wu, C., & Wu, Q. (2010). Double CO_2 fixation in photosynthesis–fermentation model enhances algal lipid synthesis for biodiesel production. *Bioresource Technology*, *101*(7), 2287-2293.

Xu, H., Miao, X., & Wu, Q. (2006). High quality biodiesel production from a microalga *Chlorella protothecoides* by heterotrophic growth in fermenters. *Journal of Biotechnology*, *126*(4), 499-507.

Xu, X. Q., & Beardall, J. (1997). Effect of salinity on fatty acid composition of a green microalga from an antarctic hypersaline lake. *Phytochemistry*, *45*(4), 655-658.

Yang, X. E., Wu, X., Hao, H. L., & He, Z. L. (2008). Mechanisms and assessment of water eutrophication. *Journal of Zhejiang University Science B*, *9*(3), 197-209.

Yen H.W., Hu I.C., Chen C.Y., Ho S.H., Lee D.J., Chang J.S., Microalgae-based biorefinery – From biofuels to natural products. *Bioresource Technology, 135* (2013) 166.

Yildiz, F. H., Davies, J. P., & Grossman, A. R. (1994). Characterization of sulfate transport in *Chlamydomonas reinhardtii* during sulfur-limited and sulfur-sufficient growth. *Plant physiology*, *104*(3), 981-987.

Yongmanitchai, W. A. R. D., & Ward, O. P. (1991). Growth of and omega-3 fatty acid production by *Phaeodactylum tricornutum* under different culture conditions. *Applied and Environmental Microbiology*, *57*(2), 419-425.

Zang, C., Huang, S., Wu, M., Du, S., Scholz, M., Gao, F., & Dong, Y. (2011). Comparison of relationships between pH, dissolved oxygen and chlorophyll a for aquaculture and non-aquaculture waters. *Water, Air, & Soil Pollution*, *219*(1-4), 157-174.

Zhang, Y. (2010). Hydrothermal liquefaction to convert biomass into crude oil. *Biofuels from Agricultural Wastes and Byproducts*, *201*.

Zhekisheva, M., Boussiba, S., Khozin-Goldberg, I., Zarka, A., & Cohen, Z. (2002). Accumulation of Oleic acid in *Haematococcus pluvialis* (Chlorophyceae) under nitrogen starvation or high light is Correlated with that of astaxanthin esters. *Journal of Phycology*, *38*(2), 325-331.

Zhila, N. O., Kalacheva, G. S., & Volova, T. G. (2011). Effect of salinity on the biochemical composition of the alga *Botryococcus braunii* Kütz IPPAS H-252. *Journal of Applied Phycology*, *23*(1), 47-52.

Zhu, X. G., Long, S. P., & Ort, D. R. (2008). What is the maximum efficiency with which photosynthesis can convert solar energy into biomass? *Current Opinion in Biotechnology*, *19*(2), 153-159.

Zimba, P. V., Rowan, M., & Triemer, R. (2004). Identification of euglenoid algae that produce. *Journal of Fish Diseases*, *27*, 115-117.

Zittelli, G. C., Rodolfi, L., Biondi, N., & Tredici, M. R. (2006). Productivity and photosynthetic efficiency of outdoor cultures of *Tetraselmis suecica* in annular columns. *Aquaculture*, *261*(3), 932-943.

APPENDICES

APPENDIX A

Significance tests for abundance for all three dams

Case Processing Summary

		Cases					
		Valid		Missing		Total	
	DAM	N	Percent	N	Percent	N	Percent
ABUNDANCE	SWD	44	100.0%	0	0.0%	44	100.0%
	VBD	39	100.0%	0	0.0%	39	100.0%
	GD	36	100.0%	0	0.0%	36	100.0%

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	DAM	Statistic	df	Sig.	Statistic	df	Sig.
ABUNDANCE	SWD	.407	44	.000	.336	44	.000
	VBD	.449	39	.000	.268	39	.000
	GD	.403	36	.000	.269	36	.000

a. Lilliefors Significance Correction

Ranks

	SEASON	N	Mean Rank	Sum of Ranks
ABUNDANCE	DRY	60	58.37	3502.00
	WET	58	60.67	3519.00
	Total	118		

Test Statistics[a]

	ABUNDANCE
Mann-Whitney U	1672.000
Wilcoxon W	3502.000
Z	-.367
Asymp. Sig. (2-tailed)	.713

a. Grouping Variable: SEASON

Swakoppoort dam correlation analysis

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
ABUNDANCE	44	95.7%	2	4.3%	46	100.0%
EUPHOTIC	44	95.7%	2	4.3%	46	100.0%
RAINFALL	44	95.7%	2	4.3%	46	100.0%
PH	44	95.7%	2	4.3%	46	100.0%
TEMP	44	95.7%	2	4.3%	46	100.0%
DO	44	95.7%	2	4.3%	46	100.0%
VOLUME	44	95.7%	2	4.3%	46	100.0%
nitrate	44	95.7%	2	4.3%	46	100.0%
phosphate	44	95.7%	2	4.3%	46	100.0%
ammonia	44	95.7%	2	4.3%	46	100.0%
SEASON	44	95.7%	2	4.3%	46	100.0%

Tests of Normality [b]

	Kolmogorov-Smirnov [a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
ABUNDANCE	.407	44	.000	.336	44	.000
EUPHOTIC	.350	44	.000	.636	44	.000
PH	.350	44	.000	.636	44	.000
TEMP	.350	44	.000	.636	44	.000
DO	.342	44	.000	.645	44	.000
VOLUME	.350	44	.000	.636	44	.000
nitrate	.350	44	.000	.636	44	.000
phosphate	.350	44	.000	.636	44	.000
ammonia	.350	44	.000	.636	44	.000
SEASON	.350	44	.000	.636	44	.000

a. Lilliefors Significance Correction

b. RAINFALL is constant. It has been omitted.

Correlations

			ABUNDANCE	EUPHOTIC
Spearman's rho	ABUNDANCE	Correlation Coefficient	1.000	.117
		Sig. (2-tailed)	.	.450
		N	44	44
	EUPHOTIC	Correlation Coefficient	.117	1.000
		Sig. (2-tailed)	.450	.
		N	44	44

Correlations

			ABUNDANCE	RAINFALL
Spearman's rho	ABUNDANCE	Correlation Coefficient	1.000	.
		Sig. (2-tailed)	.	.
		N	44	44
	RAINFALL	Correlation Coefficient	.	.
		Sig. (2-tailed)	.	.
		N	44	44

Correlations

			ABUNDANCE	PH
Spearman's rho	ABUNDANCE	Correlation Coefficient	1.000	.117
		Sig. (2-tailed)	.	.450
		N	44	44
	PH	Correlation Coefficient	.117	1.000
		Sig. (2-tailed)	.450	.
		N	44	44

Correlations

			ABUNDANCE	TEMP
Spearman's rho	ABUNDANCE	Correlation Coefficient	1.000	.117
		Sig. (2-tailed)	.	.450
		N	44	44
	TEMP	Correlation Coefficient	.117	1.000
		Sig. (2-tailed)	.450	.
		N	44	44

Correlations

			ABUNDANCE	DO
Spearman's rho	ABUNDANCE	Correlation Coefficient	1.000	.057
		Sig. (2-tailed)	.	.711
		N	44	44
	DO	Correlation Coefficient	.057	1.000
		Sig. (2-tailed)	.711	.
		N	44	44

Correlations

			ABUNDANCE	VOLUME
Spearman's rho	ABUNDANCE	Correlation Coefficient	1.000	-.117
		Sig. (2-tailed)	.	.450
		N	44	44
	VOLUME	Correlation Coefficient	-.117	1.000
		Sig. (2-tailed)	.450	.
		N	44	44

Correlations

			ABUNDANCE	nitrate
Spearman's rho	ABUNDANCE	Correlation Coefficient	1.000	-.117
		Sig. (2-tailed)	.	.450
		N	44	44
	nitrate	Correlation Coefficient	-.117	1.000
		Sig. (2-tailed)	.450	.
		N	44	44

Correlations

			ABUNDANCE	phosphate
Spearman's rho	ABUNDANCE	Correlation Coefficient	1.000	-.117
		Sig. (2-tailed)	.	.450
		N	44	44
	phosphate	Correlation Coefficient	-.117	1.000
		Sig. (2-tailed)	.450	.
		N	44	44

Correlations

			ABUNDANCE	ammonia
Spearman's rho	ABUNDANCE	Correlation Coefficient	1.000	-.117
		Sig. (2-tailed)	.	.450
		N	44	44
	ammonia	Correlation Coefficient	-.117	1.000
		Sig. (2-tailed)	.450	.
		N	44	44

Ranks

	SEASON	N	Mean Rank	Sum of Ranks
ABUNDANCE	DRY	23	21.09	485.00
	WET	21	24.05	505.00
	Total	44		

Test Statistics [a]

	ABUNDANCE
Mann-Whitney U	209.000
Wilcoxon W	485.000
Z	-.766
Asymp. Sig. (2-tailed)	.444

a. Grouping Variable: SEASON

Von Bach dam correlation analysis

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
ABUNDANCE	39	100.0%	0	0.0%	39	100.0%
EUPHOTIC	39	100.0%	0	0.0%	39	100.0%
RAINFALL	39	100.0%	0	0.0%	39	100.0%
PH	39	100.0%	0	0.0%	39	100.0%
TEMP	39	100.0%	0	0.0%	39	100.0%
DO	39	100.0%	0	0.0%	39	100.0%
VOLUME	39	100.0%	0	0.0%	39	100.0%
nitrate	39	100.0%	0	0.0%	39	100.0%
phosphate	39	100.0%	0	0.0%	39	100.0%
ammonia	39	100.0%	0	0.0%	39	100.0%

Tests of Normality

	Kolmogorov-Smirnov [a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
ABUNDANCE	.449	39	.000	.268	39	.000
EUPHOTIC	.345	39	.000	.637	39	.000
RAINFALL	.345	39	.000	.637	39	.000
PH	.345	39	.000	.637	39	.000
TEMP	.345	39	.000	.637	39	.000
DO	.345	39	.000	.637	39	.000
VOLUME	.345	39	.000	.637	39	.000
nitrate	.345	39	.000	.637	39	.000
phosphate	.345	39	.000	.637	39	.000
ammonia	.345	39	.000	.637	39	.000

a. Lilliefors Significance Correction

Correlations

			ABUNDANCE	EUPHOTIC
Spearman's rho	ABUNDANCE	Correlation Coefficient	1.000	.000
		Sig. (2-tailed)	.	1.000
		N	39	39
	EUPHOTIC	Correlation Coefficient	.000	1.000
		Sig. (2-tailed)	1.000	.
		N	39	39

Correlations

			ABUNDANCE	RAINFALL
Spearman's rho	ABUNDANCE	Correlation Coefficient	1.000	.000
		Sig. (2-tailed)	.	1.000
		N	39	39
	RAINFALL	Correlation Coefficient	.000	1.000
		Sig. (2-tailed)	1.000	.
		N	39	39

Correlations

			ABUNDANCE	PH
Spearman's rho	ABUNDANCE	Correlation Coefficient	1.000	.000
		Sig. (2-tailed)	.	1.000
		N	39	39
	PH	Correlation Coefficient	.000	1.000
		Sig. (2-tailed)	1.000	.
		N	39	39

Correlations

			ABUNDANCE	TEMP
Spearman's rho	ABUNDANCE	Correlation Coefficient	1.000	.000
		Sig. (2-tailed)	.	1.000
		N	39	39
	TEMP	Correlation Coefficient	.000	1.000
		Sig. (2-tailed)	1.000	.
		N	39	39

Correlations

			ABUNDANCE	DO
Spearman's rho	ABUNDANCE	Correlation Coefficient	1.000	.000
		Sig. (2-tailed)	.	1.000
		N	39	39
	DO	Correlation Coefficient	.000	1.000
		Sig. (2-tailed)	1.000	.
		N	39	39

Correlations

			ABUNDANCE	VOLUME
Spearman's rho	ABUNDANCE	Correlation Coefficient	1.000	.000
		Sig. (2-tailed)	.	1.000
		N	39	39
	VOLUME	Correlation Coefficient	.000	1.000
		Sig. (2-tailed)	1.000	.
		N	39	39

Correlations

			ABUNDANCE	nitrate
Spearman's rho	ABUNDANCE	Correlation Coefficient	1.000	.000
		Sig. (2-tailed)	.	1.000
		N	39	39
	nitrate	Correlation Coefficient	.000	1.000
		Sig. (2-tailed)	1.000	.
		N	39	39

Correlations

			ABUNDANCE	phosphate
Spearman's rho	ABUNDANCE	Correlation Coefficient	1.000	.000
		Sig. (2-tailed)	.	1.000
		N	39	39
	phosphate	Correlation Coefficient	.000	1.000
		Sig. (2-tailed)	1.000	.
		N	39	39

Correlations

			ABUNDANCE	ammonia
Spearman's rho	ABUNDANCE	Correlation Coefficient	1.000	.000
		Sig. (2-tailed)	.	1.000
		N	39	39
	ammonia	Correlation Coefficient	.000	1.000
		Sig. (2-tailed)	1.000	.
		N	39	39

Ranks

	SEASON	N	Mean Rank	Sum of Ranks
ABUNDANCE	DRY	19	20.00	380.00
	WET	20	20.00	400.00
	Total	39		

Test Statistics [a]

	ABUNDANCE
Mann-Whitney U	190.000
Wilcoxon W	400.000
Z	.000
Asymp. Sig. (2-tailed)	1.000
Exact Sig. [2*(1-tailed Sig.)]	1.000 [b]

a. Grouping Variable: SEASON

b. Not corrected for ties.

Goreangab dam correlation analysis

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
ABUNDANCE	36	100.0%	0	0.0%	36	100.0%
EUPHOTIC	36	100.0%	0	0.0%	36	100.0%
RAINFALL	36	100.0%	0	0.0%	36	100.0%
PH	36	100.0%	0	0.0%	36	100.0%
TEMP	36	100.0%	0	0.0%	36	100.0%
DO	36	100.0%	0	0.0%	36	100.0%
VOLUME	36	100.0%	0	0.0%	36	100.0%
nitrate	36	100.0%	0	0.0%	36	100.0%
phosphate	36	100.0%	0	0.0%	36	100.0%
ammonia	36	100.0%	0	0.0%	36	100.0%
SEASON	36	100.0%	0	0.0%	36	100.0%

Tests of Normality

	Kolmogorov-Smirnov [a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
ABUNDANCE	.403	36	.000	.269	36	.000
EUPHOTIC	.338	36	.000	.638	36	.000
RAINFALL	.338	36	.000	.638	36	.000
PH	.338	36	.000	.638	36	.000
TEMP	.338	36	.000	.638	36	.000
DO	.338	36	.000	.638	36	.000
VOLUME	.338	36	.000	.638	36	.000
nitrate	.338	36	.000	.638	36	.000
phosphate	.338	36	.000	.638	36	.000
ammonia	.338	36	.000	.638	36	.000
SEASON	.338	36	.000	.638	36	.000

a. Lilliefors Significance Correction

Correlations

			ABUNDANCE	EUPHOTIC
Spearman's rho	ABUNDANCE	Correlation Coefficient	1.000	.076
		Sig. (2-tailed)	.	.658
		N	36	36
	EUPHOTIC	Correlation Coefficient	.076	1.000
		Sig. (2-tailed)	.658	.
		N	36	36

Correlations

			ABUNDANCE	RAINFALL
Spearman's rho	ABUNDANCE	Correlation Coefficient	1.000	-.076
		Sig. (2-tailed)	.	.658
		N	36	36
	RAINFALL	Correlation Coefficient	-.076	1.000
		Sig. (2-tailed)	.658	.
		N	36	36

Correlations

			ABUNDANCE	PH
Spearman's rho	ABUNDANCE	Correlation Coefficient	1.000	-.076
		Sig. (2-tailed)	.	.658
		N	36	36
	PH	Correlation Coefficient	-.076	1.000
		Sig. (2-tailed)	.658	.
		N	36	36

Correlations

			ABUNDANCE	TEMP
Spearman's rho	ABUNDANCE	Correlation Coefficient	1.000	-.076
		Sig. (2-tailed)	.	.658
		N	36	36
	TEMP	Correlation Coefficient	-.076	1.000
		Sig. (2-tailed)	.658	.
		N	36	36

Correlations

			ABUNDANCE	DO
Spearman's rho	ABUNDANCE	Correlation Coefficient	1.000	.076
		Sig. (2-tailed)	.	.658
		N	36	36
	DO	Correlation Coefficient	.076	1.000
		Sig. (2-tailed)	.658	.
		N	36	36

Correlations

			ABUNDANCE	VOLUME
Spearman's rho	ABUNDANCE	Correlation Coefficient	1.000	.076
		Sig. (2-tailed)	.	.658
		N	36	36
	VOLUME	Correlation Coefficient	.076	1.000
		Sig. (2-tailed)	.658	.
		N	36	36

Correlations

			ABUNDANCE	nitrate
Spearman's rho	ABUNDANCE	Correlation Coefficient	1.000	-.076
		Sig. (2-tailed)	.	.658
		N	36	36
	nitrate	Correlation Coefficient	-.076	1.000
		Sig. (2-tailed)	.658	.
		N	36	36

Correlations

			ABUNDANCE	phosphate
Spearman's rho	ABUNDANCE	Correlation Coefficient	1.000	-.076
		Sig. (2-tailed)	.	.658
		N	36	36
	phosphate	Correlation Coefficient	-.076	1.000
		Sig. (2-tailed)	.658	.
		N	36	36

Correlations

			ABUNDANCE	ammonia
Spearman's rho	ABUNDANCE	Correlation Coefficient	1.000	-.076
		Sig. (2-tailed)	.	.658
		N	36	36
	ammonia	Correlation Coefficient	-.076	1.000
		Sig. (2-tailed)	.658	.
		N	36	36

Ranks

	SEASON	N	Mean Rank	Sum of Ranks
ABUNDANCE	DRY	18	19.28	347.00
	WET	18	17.72	319.00
	Total	36		

Test Statistics [a]

	ABUNDANCE
Mann-Whitney U	148.000
Wilcoxon W	319.000
Z	-.452
Asymp. Sig. (2-tailed)	.652
Exact Sig. [2*(1-tailed Sig.)]	.673 [b]

a. Grouping Variable: SEASON

b. Not corrected for ties.

APPENDIX B

All pre-cultures statistical analysis growth rates and pH

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
GROWTH	42	100.0%	0	0.0%	42	100.0%
pH	42	100.0%	0	0.0%	42	100.0%

Tests of Normality

	Kolmogorov-Smirnov [a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
GROWTH	.114	42	.200 *	.942	42	.032
pH	.088	42	.200 *	.963	42	.194

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of GROWTH is the same across categories of FLASK.	Independent-Samples Kruskal-Wallis Test	.733	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

ANOVA

pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.148	2	.074	.553	.580
Within Groups	5.237	39	.134		
Total	5.385	41			

Correlations

			GROWTH	pH
Spearman's rho	GROWTH	Correlation Coefficient	1.000	.988**
		Sig. (2-tailed)	.	.000
		N	42	42
	pH	Correlation Coefficient	.988**	1.000
		Sig. (2-tailed)	.000	.
		N	42	42

**. Correlation is significant at the 0.01 level (2-tailed).

Pre-Flask 1

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
GROWTH	14	100.0%	0	0.0%	14	100.0%
pH	14	100.0%	0	0.0%	14	100.0%

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
GROWTH	.131	14	.200*	.943	14	.451
pH	.078	14	.200*	.993	14	1.000

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Correlations

		GROWTH	pH
GROWTH	Pearson Correlation	1	.965**
	Sig. (2-tailed)		.000
	N	14	14
pH	Pearson Correlation	.965**	1
	Sig. (2-tailed)	.000	
	N	14	14

**. Correlation is significant at the 0.01 level (2-tailed).

Pre-Flask 2

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
GROWTH	14	100.0%	0	0.0%	14	100.0%
pH	14	100.0%	0	0.0%	14	100.0%

Tests of Normality

	Kolmogorov-Smirnov [a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
GROWTH	.136	14	.200 *	.958	14	.693
pH	.085	14	.200 *	.992	14	1.000

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Correlations

		GROWTH	pH
GROWTH	Pearson Correlation	1	.968 **
	Sig. (2-tailed)		.000
	N	14	14
pH	Pearson Correlation	.968 **	1
	Sig. (2-tailed)	.000	
	N	14	14

**. Correlation is significant at the 0.01 level (2-tailed).

Pre-Flask 3

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
GROWTH	14	100.0%	0	0.0%	14	100.0%
pH	14	100.0%	0	0.0%	14	100.0%

Tests of Normality

	Kolmogorov-Smirnov [a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
GROWTH	.182	14	.200 *	.927	14	.280
pH	.149	14	.200 *	.950	14	.562

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Correlations

		GROWTH	pH
GROWTH	Pearson Correlation	1	.930 **
	Sig. (2-tailed)		.000
	N	14	14
pH	Pearson Correlation	.930 **	1
	Sig. (2-tailed)	.000	
	N	14	14

**. Correlation is significant at the 0.01 level (2-tailed).

APPENDIX C

Main-culture: All Flasks growth rate vs variables statistical analysis

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
GR	27	45.0%	33	55.0%	60	100.0%
pH	27	45.0%	33	55.0%	60	100.0%
CHLORIDE	27	45.0%	33	55.0%	60	100.0%
NITRATE	27	45.0%	33	55.0%	60	100.0%
PHOSPHAT	27	45.0%	33	55.0%	60	100.0%
SULPHATE	27	45.0%	33	55.0%	60	100.0%

Tests of Normality

	Kolmogorov-Smirnov [a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
GR	.167	27	.052	.898	27	.012
pH	.389	27	.000	.615	27	.000
CHLORIDE	.271	27	.000	.693	27	.000
NITRATE	.166	27	.056	.894	27	.010
PHOSPHAT	.368	27	.000	.679	27	.000
SULPHATE	.253	27	.000	.781	27	.000

a. Lilliefors Significance Correction

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of GR is the same across categories of NITCONC.	Independent-Samples Kruskal-Wallis Test	.001	Reject the null hypothesis.
2	The distribution of pH is the same across categories of NITCONC.	Independent-Samples Kruskal-Wallis Test	.671	Retain the null hypothesis.
3	The distribution of CHLORIDE is the same across categories of NITCONC.	Independent-Samples Kruskal-Wallis Test	.104	Retain the null hypothesis.
4	The distribution of NITRATE is the same across categories of NITCONC.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.
5	The distribution of PHOSPHAT is the same across categories of NITCONC.	Independent-Samples Kruskal-Wallis Test	.180	Retain the null hypothesis.
6	The distribution of SULPHATE is the same across categories of NITCONC.	Independent-Samples Kruskal-Wallis Test	.319	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Correlations

			GR	pH
Spearman's rho	GR	Correlation Coefficient	1.000	.041
		Sig. (2-tailed)	.	.759
		N	60	60
	pH	Correlation Coefficient	.041	1.000
		Sig. (2-tailed)	.759	.
		N	60	60

Correlations

			GR	CHLORIDE
Spearman's rho	GR	Correlation Coefficient	1.000	-.014
		Sig. (2-tailed)	.	.915
		N	60	60
	CHLORIDE	Correlation Coefficient	-.014	1.000
		Sig. (2-tailed)	.915	.
		N	60	60

Correlations

			GR	NITRATE
Spearman's rho	GR	Correlation Coefficient	1.000	-.164
		Sig. (2-tailed)	.	.209
		N	60	60
	NITRATE	Correlation Coefficient	-.164	1.000
		Sig. (2-tailed)	.209	.
		N	60	60

Correlations

			GR	PHOSPHAT
Spearman's rho	GR	Correlation Coefficient	1.000	-.520**
		Sig. (2-tailed)	.	.001
		N	60	37
	PHOSPHAT	Correlation Coefficient	-.520**	1.000
		Sig. (2-tailed)	.001	.
		N	37	37

**. Correlation is significant at the 0.01 level (2-tailed).

Correlations

			GR	SULPHATE
Spearman's rho	GR	Correlation Coefficient	1.000	-.887**
		Sig. (2-tailed)	.	.000
		N	60	37
	SULPHATE	Correlation Coefficient	-.887**	1.000
		Sig. (2-tailed)	.000	.
		N	37	37

**. Correlation is significant at the 0.01 level (2-tailed).

BDM productivity statistical analysis for all the Main-cultures during cultivation process

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
BDM	12	100.0%	0	0.0%	12	100.0%

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	Df	Sig.	Statistic	df	Sig.
BDM	.217	12	.123	.864	12	.055

a. Lilliefors Significance Correction

ANOVA

BDM

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3701528.982	3	1233842.994	.131	.939
Within Groups	75601656.586	8	9450207.073		
Total	79303185.567	11			

Lipid productivity statistical analysis for all the Flasks during cultivation

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
LIPIDS	50	100.0%	0	0.0%	50	100.0%

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	Df	Sig.	Statistic	df	Sig.
LIPIDS	.203	50	.000	.844	50	.000

a. Lilliefors Significance Correction

Ranks

	NITROCONC	N	Mean Rank
LIPIDS	A1	12	25.58
	B2	10	16.50
	C2	14	26.14
	D1	14	31.21
	Total	50	

	LIPIDS
Chi-Square	5.991
df	3
Asymp. Sig.	.112

a. Kruskal Wallis Test

b. Grouping Variable:

NITROCONC

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of LIPIDS is the same across categories of NITROCONC.	Independent-Samples Kruskal-Wallis Test	.112	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Correlation analysis of lipis vs BDM vs macronutrients vs pH vs growth rates for days sampled for BDM

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
LIPIDS	12	100.0%	0	0.0%	12	100.0%
BDM	12	100.0%	0	0.0%	12	100.0%
NITRATE	12	100.0%	0	0.0%	12	100.0%
CHLORIDE	12	100.0%	0	0.0%	12	100.0%
PHOSPHATE	12	100.0%	0	0.0%	12	100.0%
SULPHATE	12	100.0%	0	0.0%	12	100.0%
PH	12	100.0%	0	0.0%	12	100.0%
GR	12	100.0%	0	0.0%	12	100.0%

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
LIPIDS	.217	12	.125	.877	12	.081
BDM	.217	12	.123	.864	12	.055
NITRATE	.170	12	.200*	.904	12	.180
CHLORIDE	.243	12	.048	.821	12	.016
PHOSPHATE	.364	12	.000	.703	12	.001
SULPHATE	.182	12	.200*	.859	12	.047
PH	.155	12	.200*	.898	12	.151
GR	.229	12	.083	.826	12	.019

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Correlations

		LIPIDS	BDM
LIPIDS	Pearson Correlation	1	-.765**
	Sig. (2-tailed)		.004
	N	12	12
BDM	Pearson Correlation	-.765**	1
	Sig. (2-tailed)	.004	
	N	12	12

**. Correlation is significant at the 0.01 level (2-tailed).

		LIPIDS	NITRATE
LIPIDS	Pearson Correlation	1	.232
	Sig. (2-tailed)		.469
	N	12	12
NITRATE	Pearson Correlation	.232	1
	Sig. (2-tailed)	.469	
	N	12	12

			LIPIDS	CHLORIDE
Spearman's rho	LIPIDS	Correlation Coefficient	1.000	-.441
		Sig. (2-tailed)	.	.152
		N	12	12
	CHLORIDE	Correlation Coefficient	-.441	1.000
		Sig. (2-tailed)	.152	.
		N	12	12

			LIPIDS	PHOSPHATE
Spearman's rho	LIPIDS	Correlation Coefficient	1.000	.520
		Sig. (2-tailed)	.	.083
		N	12	12
	PHOSPHATE	Correlation Coefficient	.520	1.000
		Sig. (2-tailed)	.083	.
		N	12	12

			LIPIDS	SULPHATE
Spearman's rho	LIPIDS	Correlation Coefficient	1.000	.824**
		Sig. (2-tailed)	.	.001
		N	12	12
	SULPHATE	Correlation Coefficient	.824**	1.000
		Sig. (2-tailed)	.001	.
		N	12	12

**. Correlation is significant at the 0.01 level (2-tailed).

		LIPIDS	PH
LIPIDS	Pearson Correlation	1	.173
	Sig. (2-tailed)		.591
	N	12	12
PH	Pearson Correlation	.173	1
	Sig. (2-tailed)	.591	
	N	12	12

			LIPIDS	GR
Spearman's rho	LIPIDS	Correlation Coefficient	1.000	-.501
		Sig. (2-tailed)	.	.097
		N	12	12
	GR	Correlation Coefficient	-.501	1.000
		Sig. (2-tailed)	.097	.
		N	12	12

		BDM	NITRATE
BDM	Pearson Correlation	1	-.356
	Sig. (2-tailed)		.256
	N	12	12
NITRATE	Pearson Correlation	-.356	1
	Sig. (2-tailed)	.256	
	N	12	12

			BDM	CHLORIDE
Spearman's rho	BDM	Correlation Coefficient	1.000	.517
		Sig. (2-tailed)	.	.085
		N	12	12
	CHLORIDE	Correlation Coefficient	.517	1.000
		Sig. (2-tailed)	.085	.
		N	12	12

			BDM	PHOSPHATE
Spearman's rho	BDM	Correlation Coefficient	1.000	-.616*
		Sig. (2-tailed)	.	.033
		N	12	12
	PHOSPHATE	Correlation Coefficient	-.616*	1.000
		Sig. (2-tailed)	.033	.
		N	12	12

*. Correlation is significant at the 0.05 level (2-tailed).

			BDM	SULPHATE
Spearman's rho	BDM	Correlation Coefficient	1.000	-.901**
		Sig. (2-tailed)	.	.000
		N	12	12
	SULPHATE	Correlation Coefficient	-.901**	1.000
		Sig. (2-tailed)	.000	.
		N	12	12

**. Correlation is significant at the 0.01 level (2-tailed).

			BDM	GR
Spearman's rho	BDM	Correlation Coefficient	1.000	.529
		Sig. (2-tailed)	.	.077
		N	12	12
	GR	Correlation Coefficient	.529	1.000
		Sig. (2-tailed)	.077	.
		N	12	12

		BDM	PH
BDM	Pearson Correlation	1	-.673*
	Sig. (2-tailed)		.017
	N	12	12
PH	Pearson Correlation	-.673*	1
	Sig. (2-tailed)	.017	
	N	12	12

*. Correlation is significant at the 0.05 level (2-tailed).