CARBON DIOXIDE ABSORPTION USING FRESH WATER ALGAE AND IDENTIFYING POTENTIAL USES OF ALGAL BIOMASS

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A dissertation submitted to the Faculty of Engineering and the Built Environment, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Masters of Science in Engineering.

Johannesburg 2011

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in Engineering at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

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Signature of candidate

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ABSTRACT

Across the world, industrialization and the consequent emergence of economies reliant on fossil fuels have inevitably resulted in the adverse condition of atmospheric warming known as the greenhouse effect. Anthropogenic CO_2 has been identified as one of the major causes. This research focuses on investigating the possibility of exploiting the natural photosynthetic process in fresh-water South African microalgae in order to reduce CO_2 emissions, and in this way to create a more benign environment. In order to investigate the conditions that would make the large-scale cultivation of these microalgae possible, we first considered the factors that limit their growth. The research was carried out using batch cultures to study the effects on the microalgae of varying amounts of light intensity, photoperiod, and of concentrations of nitrogen and phosphate ions and CO_2. The results showed that growth was best at a light intensity of 10 000lux for 12 hours a day, or a photoperiod of at least 16 hours per day at 6 000lux. A maximum growth rate of 0.67 per day was obtained with an optimal ammonium nitrate concentration of 300mg/l. The species under investigation showed no tendency to discriminate between different types of nitrogen ions. The most favourable phosphate ion concentration was found to be 500mg/l, with a maximum growth rate of 0.84 per day. Feeding microalgae with CO_2 resulted in substantial growth, while a gas flow rate of 50ml/min of 100% CO_2 yielded a rate of 1.27 per day. However, the maximum growth rate of 2.0 per day was achieved with 5 while in10% CO_2 at 50ml/min.

DEDICATION

To Jehovah the almighty saviour

My wife Tarisai, my mother Gladys and the entire Kativu family

ACKNOWLEDGEMENTS

I would like to thank my supervisors, Professors D Hildebrandt and D Glasser, and my co-supervisors, Dr. T S Matambo and Dr. K Rumbold, for the insightful advice, encouragement, constructive criticism, expertise and guidance they gave me during the course of this work.

Also, I appreciate the assistance I received from technicians at the Centre of Material Process and Synthesis (COMPS), the School of Chemical and Metallurgical Engineering (and its workshop teams), the School of Animal, Plant and Environmental Science, the School of Molecular and Cell Biology, and the School of Chemistry. I would like to thank the University of Stellenbosch's Central analytical Facility for performing the DNA sequencing and analysis.

During the course of this project I received both encouragement and moral support from my fellow post-graduate students at COMPS and the School of Chemical and Metallurgical Engineering, and I wish to express my gratitude.

I am indebted to COMPS for giving me financial assistance for the entire period during which I was working on this dissertation.

Lastly, but not least, I would like to thank my wife and my family for the patience, understanding and unstinting support they gave me throughout my efforts to complete this project.

CONFERENCE PRESENTATIONS

1) E Kativu, T S Matambo, D Hildebrandt , D Glasser and K Rumbold, (2010) The effect of nitrogen ions concentration on the growth of a South African fresh water high CO_2 tolerant microalgae, *American Institute of Chemical Engineers (AIChE) Spring meeting, San Antonio TX USA, 24 March 2010. (Abstract, Oral presentation*)

2) E Kativu, T S Matambo, D Hildebrandt, D Glasser and K Rumbold, (2010) The effect of nitrogen ions concentration on the growth of a South African fresh water high CO_2 tolerant microalgae, *South African Institute of Chemical Engineers (SAIChE), Gauteng postgraduate student symposium, Pretoria, 19 October 2010. (Abstract, Oral presentation)*

3) E Kativu, T S Matambo, D Hildebrandt, and D Glasser (2010), Effects of CO_2 On South African Fresh Water Microalgae Growth, *Wits post graduate Cross faculty symposium, Johannesburg, South Africa 26–29 October 2010. (Abstract, Oral presentation)*

4) E Kativu, T S Matambo, D Hildebrandt, and D Glasser (2010), Effects of CO_2 On South African Fresh Water Microalgae Growth, *American Institute of Chemical Engineering (AIChE) annual meeting, Salt Lake City, UT, USA, 7–12 November 2010.*

AWARDS

Third best oral presentation South African Institute of Chemical Engineers (SAIChE) 2010, Gauteng Postgraduate Student Symposium, 19 October 2010, Pretoria, Johannesburg South Africa, Title: The effect of nitrogen ions concentration on the growth of South African fresh water microalgae tolerant to high CO_2 levels.

PUBLICATIONS

E Kativu, T S Matambo, D Hildebrandt and D Glasser, Effects of CO_2 on South African Fresh Water Microalgae Growth. The article was presented at AIChE 2010 annual meeting oral presentation and it was invited for publication in the *Journal of Environmental Progress and Sustainable energy*. Article is has been reviewed and revised for publication on the journal's December 2011 special issue.

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List of Abbreviations

Carbon Anhydrase	CA
Carbon dioxide	CO_2
Carbon Capture and Storage	CCS
Carbonflourochlorides	CFCs
Coal to Liquid Technology	CLT
Green House Gases	GHG
Intergovernmental Panel on Climate Change	IPCC
Internal Transcriber Spacer	ITS
Monoethanolamine	MEA
National Center of Biotechnology Information	NCBI
Nitric Oxides	NO_x
Nicotinamide Adenine Dinucleotide Phosphate	NADP
Polymerase Chain Reaction	PCR
Phosphogyceric Acid	PGA
Sulphur Oxides	SO_x

CHAPTER ONE

1. Introduction

1.1 Literature review

1.1.1 Global warming

'Global warming' refers to an increase in the average temperature in the lower atmosphere that causes climatic change (IPCC, 2001). Unprecedented anthropogenic emissions of green house gases (GHGs) are probably the cause of the current alteration in climatic patterns across the world (Huntley and Redalje, 2007). Major causes of GHGs are water vapour, carbon dioxide (CO_2), methane (CH_4) sulphur oxides (SO_x), nitrogen oxides (NO_x), chlorofluorocarbons (CFCs) and many other gases created by human activities (Liu, 1994). The steady escalation in anthropogenic activities has led to an increase in the amount of CO_2 and other GHGs in the atmosphere. Emissions of carbon dioxide in particular have grown exponentially from 280ppm before industrialization occurred in the mid-nineteenth century to 367ppm in 1999, 379ppm in 2005 and to current levels of 385ppm, mainly owing to a dependency on fossil fuels to meet energy needs (Chang and Yang, 2003; Le Treut, *et al.,* 2007, Hansen *et al.*, 2008). The prevailing CO_2 levels have surpassed the safety limit of 350ppm which is said to be dangerous to sustain life on earth (Hansen *et al*., 2008).

Water vapour is the major contributor to global warming, but there is little that can be done to reduce it, since it is a natural phenomenon that has been occurring since the

earth came into existence. However, a growing factor in climate change is the dramatic increase in the world's production of CO_2, which (especially from 1990 onwards) has been responsible for an estimated 60% of the rise in global warming, and is continuing its upward trend (Hall and House, 1995). Efforts are being made to reduce the contribution of CO_2 and other manmade gases to the threat posed by the global warming phenomenon. If unmitigated, would eventually overtax the capacity of natural managed and human systems to adapt (IPCC, 2007).

The United Nations promoted the Kyoto Protocol (1997), which has the objective of reducing the GHGs produced by signatory countries by 5.2% (calculated on the basis of the emission statistics in 1990). More than 170 countries have ratified the protocol (Gutierrez *et al*., 2008). This was followed in 2009 by the Copenhagen Accord, which aimed not only to decrease GHGs but to contain climate change by reducing the growth in emissions, first to 2ºC and then 1.5ºC by 2015 (Rogelj *et al.,* 2010).

1.1.2 Carbon capture storage and sequestration

Although various strategies to mitigate CO_2 emissions are being explored, so far not one mitigation technology offers an ideal solution. Carbon dioxide capture and storage (CCS) is a process that consists of separating CO_2 from its industrial and energy-related sources, and transporting it to a storage location for long-term isolation (IPCC, 2007). Carbon dioxide sequestration methods can be divided into three categories: 1) chemical reaction approach to capturing CO_2 (for example by mineral carbonization); 2) storage approach, which involves the injection and entrapment of the CO_2 within pressure or structural boundaries (such as geological formations and deep ocean storage); and 3) Biological approach, by means of

which CO_2 is fixed into biomass by photosynthesis (for instance in natural forests, plantations, food crops and aquatic photosynthetic micro-organisms).

Chemical absorption, which is used in CO_2 capturing, involves cyclical carbonation/de-carbonation reactions in which gaseous CO_2 reacts with solid metal oxide to yield metal carbonate. The metal oxide is regenerated when the metal carbonate is heated beyond its calcination temperature (Gupta and Fan, 2002).

Equation (1.1) illustrates the carbonization and the calcinations reaction.

$$MO + CO_2 \underset{\text{Calcination}}{\overset{\text{Carbonization}}{\rightleftharpoons}} MCO_3 \qquad (1.1).$$

A monoethanolamine (MEA) solvent is widely used as a CO_2 chemical absorption method (Rensik *et al.,* 2004). It involves the desorption of the CO_2 into an aqueous MEA-CO_2 solution, when heated, regenerates the MEA in a reversible reaction. Water vapour in the regenerated CO_2 can be separated from it easily by condensation. The carbon dioxide fixation and desorption reactions are represented by Equation 1.2.

$$CO_2 + 2C_2H_4OH{-}NH_2 \underset{\text{Heating}}{\rightleftharpoons} C_2H_4OH{-}NHCO_2^- + C_2H_4OH{-}NH_3^+ \qquad (1.2).$$

Other processes used in neutralizing CO_2 from flue gas of power plants include membrane separation, cryogenic fractionation, and adsorption (using molecular sieves). However, they are even less energy-efficient and more expensive than the

chemical absorption method because of the very low CO_2 partial pressure in the flue gas (Stewart and Hessami, 2005).

Another GHG mitigation strategy used by power plants involves the storage of CO_2 in geological formations. Carbon dioxide from can be sequestrated into anyone of the following types of site: active oil reservoirs, coal beds, depleted oil and gas reservoirs, deep aquifers, mined salt domes and rock caverns (Figure 1-1) (Herzog *et al.,* 1993). The main problems encountered in using these technologies are uncertainty as to the volumes the chosen site can accommodate and its long-term integrity; and operational storage concerns like the cost of transporting the CO_2 to the storage site (Ormerod, 1994). The question of how securely stored carbon dioxide can be contained is the most important, as there is the risk of the CO_2 escaping and returning to the atmosphere, and causing a public safety hazard such as asphyxiation and consequent liability (Herzog *et al.,* 1997).

Because carbon dioxide is soluble, all the CO_2 emitted naturally finds its way into the oceans, which currently contain around 144 billion tones of CO_2 (Herzorg *et al.,* 1997). However, deep ocean storage involves discharging CO_2 directly into the ocean. This can be done in several ways: 1) Dry ice is released at the surface of the ocean by a ship; 2) liquid CO_2 is injected at a depth of about 1 000 metres through a pipe towed by a moving vessel, which results in a rising droplet plume; 3) liquid CO_2 is injected at a depth of about 1 000 metres by a manifold lying on the ocean bottom,causing a rising droplet plume; 4) a dense CO_2–seawater mixture, which forms a sinking bottom gravity current, is created at a depth of between 500–1 000 metres; and 5) liquid CO_2 is introduced to a depression in the sea floor to create a

stable ‘deep lake’ at a depth of about 4 000 metres (Nakashiki *et al.,* 1991; Haugan and Drange, 1992; Liro *et al.*, 1992; Ozaki *et al.,* 1995; Ohsumi, 2004).

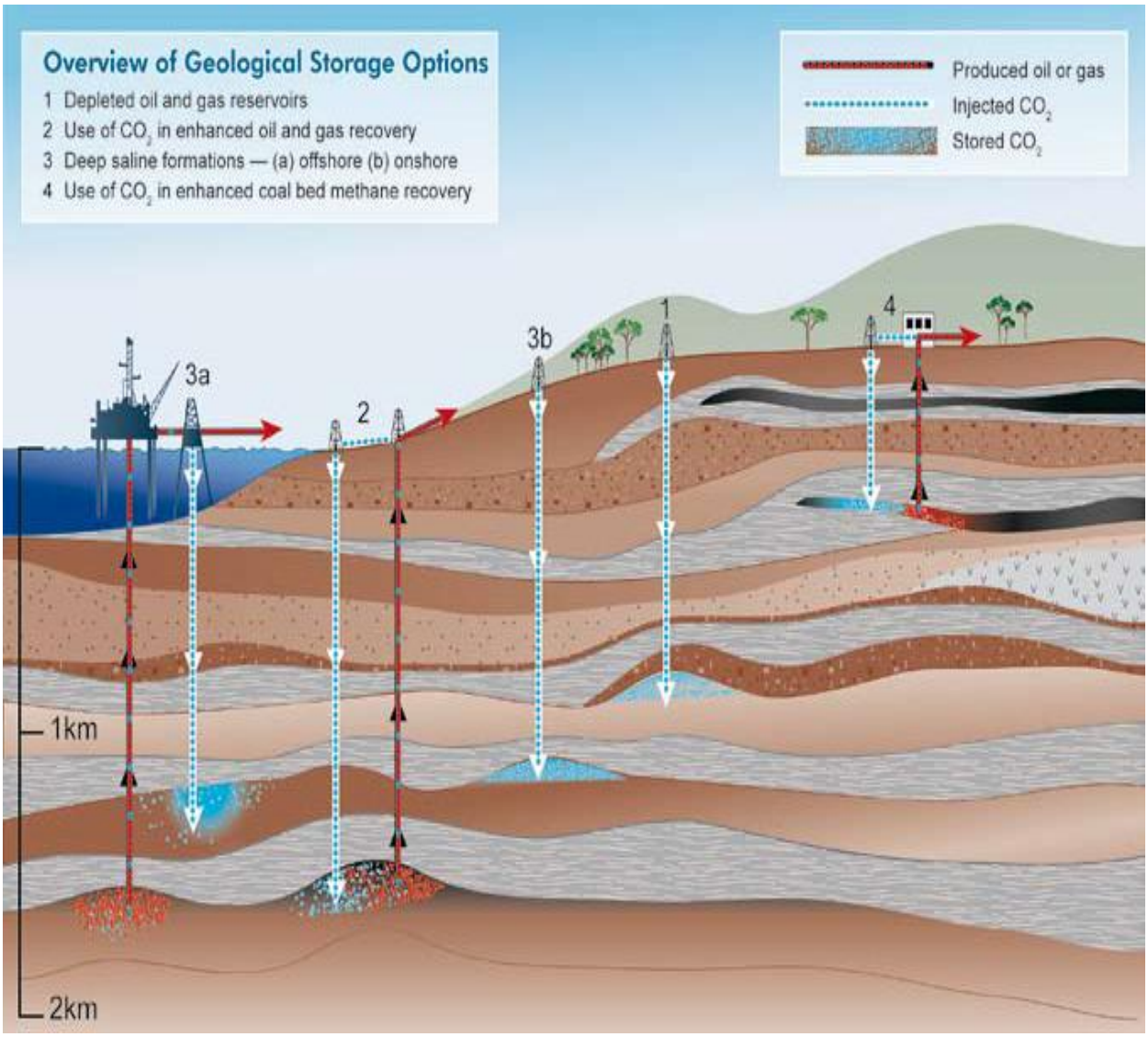


Figure 1-1: Overview of different types of CO_2 storage in geological formations. (taken from IPCC, 2005)

Although very little information is available on the consequences of these forms of intentional injection of CO_2 into the sea, a number of potential hazards have been identified. For example, releasing CO_2 in the deep ocean is likely to disturb the heterotrophic diverse fauna to be found on the sea floor (Ahnert and Schriever,

2001). Contaminants such as sulfides would also displace oxygen, which in turn could lead to depletion in sea animals (IPCC, 2005). Other concerns are the prohibitive cost of transporting the CO_2 and depositing it in the ocean, and the fear that it might eventually escape into the atmosphere.

The CCS technologies described above are not only costly and energy-consuming, but offer marginal mitigation benefits. It is therefore necessary to develop more cost-effective and sustainable alternatives to curb the soaring emission rate (Wang *et al*., 2008). Many scientific studies have been, and are being, made to address the problem. The most common long-term strategy for reducing emissions of CO_2 and other GHGs is to replace fossil fuels with alternative forms of energy obtained from renewable resources (Hildebrandt *et al.,* 2009a). Other important means include optimizing processes, carbon and energy efficiencies (Chakraborty *et al.,* 2008; Hildebrandt *et al.*, 2009b).

1.1.3 Biomitigation

The global biosphere absorbs nearly two billion tonnes of CO_2 annually, which is approximately one-third of the amount of the world's carbon emissions (Socolow *et al.,* 2004). This suggests a biological means of sequestrating CO_2 that has aroused increasing interest in the scientific world. Many researchers believe that the natural process of photosynthesis is the best near to intermediate-term solution to the problem of carbon emissions (Bayless, 2001).

Biological CO_2 sequestration can be grouped into two categories, terrestrial and aquatic ecosystems.

1.1.3.1 Terrestrial ecosystems

The term ‘terrestrial ecosystem’ includes natural and plantation forests; crop- and grassland, food and biomass crops. Globally 500 billion tonnes of CO_2 are fixed annually by terrestrial vegetation. This is 20 times more than the amount of CO_2 released by fossil fuel consumption (Skjanes *et al*., 2007). Europe’s terrestrial biosphere absorbs 7–12% of its anthropogenic CO_2 emissions (Janssens *et al.,* 2003). However, the potential for increased CO_2 capture by plant agriculture has been estimated as capable of contributing only 3–6% towards reducing fossil fuel emissions which is largely because of the slow growth rates of conventional terrestrial plants (Skjanes *et al.,* 2007; Wang, 2008). Other major concerns associated with CO_2 mitigation using terrestrial ecosystems are that the conversion of wetlands and flood plains to forest plantations would hamper ecological functions in that mono-culture plantations would reduce biodiversity, and hence increase the risk of disease and catastrophic failure (Sathaye *et al*., 2007). Forestation on rich agricultural land could diminish food security; and large-scale forests/plantations/crops are vulnerable to fires, ploughing, harvesting and other potential disturbances (Jandl *et al.,* 2007).

1.1.3.2 Aquatic ecosystems

Those ecosystems defined as aquatic include all photosynthetic organisms that inhabit marine, brackish and fresh-water bodies. Algae comprise the dominant photosynthetic organisms in aquatic ecosystems, and are responsible for approximately 50% of total photosynthetic primary production (Giordano *et al.,* 2005). Microalgae are a group of fast-growing unicellular or simple multi-cellular

microorganisms with the ability to fix CO_2 while capturing solar energy with an efficiency of 10–50 times greater than that of terrestrial plants (Usui and Ikenouchi, 1997; Li *et al.,* 2008). Approximately half their dry weight is carbon, which is derived from carbon dioxide (Sanchaz, 2003). The approximate molecular formula of microalgae dry biomass is $CO_{0.48}H_{1.83}N_{0.11}P_{0.01,}$ (Globbelaar, 2004) which suggests that for every Kg of microalgae formed; approximately 1.88 Kg of carbon dioxide is fixed. This is why, of all the biological methods of directly capturing and utilizing the CO_2 emitted by power-generating plants, microalgae systems offer the best technological option.

Several species of microalgae have been studied and found to be viable for CO_2 sequestration strategies. *Chlorococcum littorale* was able to grow in a 60% CO_2 environment when the stepwise adaptation technique was used (Kodama *et al.,* 1993). Another species with high CO_2 tolerance is *Euglena gracilis*, which is capable of growing under a 5–45% concentration of CO_2. However, its highest growth rate was observed at only 5%, and growth stopped when the concentration was higher than 45% CO_2 (Nakano *et al.,* 1996). Findings by Hirata *et al.,* (1996a; 1996b) reported that *Chlorella* sp*.* UK001 could be cultured successfully under 10% CO_2 conditions, while Hanagata *et al.* (1992) recorded that *Chlorella* sp. could be grown under a 40% concentration. Furthermore, Maeda *et al*., (1995) found a strain of *Chlorella* sp. T-1 that was capable of growth under 100% CO_2 concentration, although the highest growth rate occurred with 10%. *Scenedesmus* sp. was shown to be able to grow under 80% CO_2 conditions, but the maximum cell mass was observed when the CO_2 concentration was 10–20% (Hanagata *et al.,* 1992).

Cyanidium caldarium and some other species of *Cyanidium* are capable of growing in pure CO_2 (Seckbach *et al.,* 1971; Graham and Wilcox, 2000).

Microalgae are of particular interest to researchers seeking methods of carbon mitigation because of their rapid growth rates (up to 10 times that of trees) and their potential for significantly higher-efficiency solar conversion than can be found in land plants (Herzog *et al.,* 1997). These algae usually double their biomass every 24 hours; but during the exponential phase the doubling time can be as short as three-and-a-half hours (Chisti, 2007). Culturing microalgae for CO_2 absorption is quite expensive: but it becomes a more feasible option when the process is coupled with wastewater treatment; biofuel production; novel bioproducts such as bioplastics, nutraceuticals and organic fertilizers; and animal and human feeds (Metting, 1996; Chisti, 2007; Wang, 2008).

1.1.4 Algae

Algae are polyphyletic, non-cohesive assemblages of O_2–evolving photosynthetic organisms that include seaweeds (macroalgae) and a highly diverse group of microscopic single and multi-cellular organisms (microalgae) (Metting, 1996). Algae vary in size and shape, from microalgae of less than 1µm to macroalgae (giant kelps) with fronds 60m in length. They inhabit fresh, marine and brackish water environments, (Valeem and Shameel, 2009) and are also found in almost every other part of the earth. They range from those growing on the snow of some American mountains to others living in lichen associations on bare rocks, desert soils, and hot springs, and algae existing symbiotically with fungi, plants and animals (Darley, 1982; Lee, 2008).

Most algae are capable of using inorganic carbon as a nutrient source. These are referred to as autotrophic (lithotrophic or halophytic). They are also regarded as photoautotrophic or photolithotrophic because of their dependence on light as a source of energy (Megonigal *et al.,* 2004). Microalgae that are capable of oxidizing inorganic compounds for energy are known as chemoautotrophic or chemolithotrophic. Some species (known as heterotrophs) depend on organic compounds for growth, while those of them that depend on light for energy are called photoheterotrophs (photoorganotrophs). Those known for oxidizing organic compounds for food are known as chemoheterotrophs (organoheterophs) (Lee, 2008).

Auxotrophic species require some organic compounds for growth, not to meet their energy needs but for nutrients such as vitamins. Some autotrophic algae are capable of using organic compounds, therefore are called mixotrophic or facultative heterotrophy algae can also be phagocytic or halozoic, osmotrophic, saprophytic, and parasitic species (Sigee, 200; Lee, 2008).

Generally algae are categorized into two kingdoms: prokaryotes and eukaryotes. *Cyanophyta* and *Prochlorophyta* are classified as prokaryotes, while the rest are eukaryotes. The latter can be separated into three groups.

i) These are algae with chloroplasts surrounded by the two membranes of the chloroplast envelope. Examples include *Glaucophyta*, *Rhodophyta* and *Chlorophyta* (Tengs *et al.,* 2000) (Figure 1-2.).

ii) This group of algae is characterized by chloroplasts surrounded by a membrane of chloroplast endoplasmic reticulum. Among this type are *Dinophyta* and *Euglenophyta*.

iii) In this case the algae contain chloroplasts encircled by two membranes of chloroplast endoplasmic reticulum. Instances are *Heterokophyta* and *Cryptophyta* (Figure 1-2).

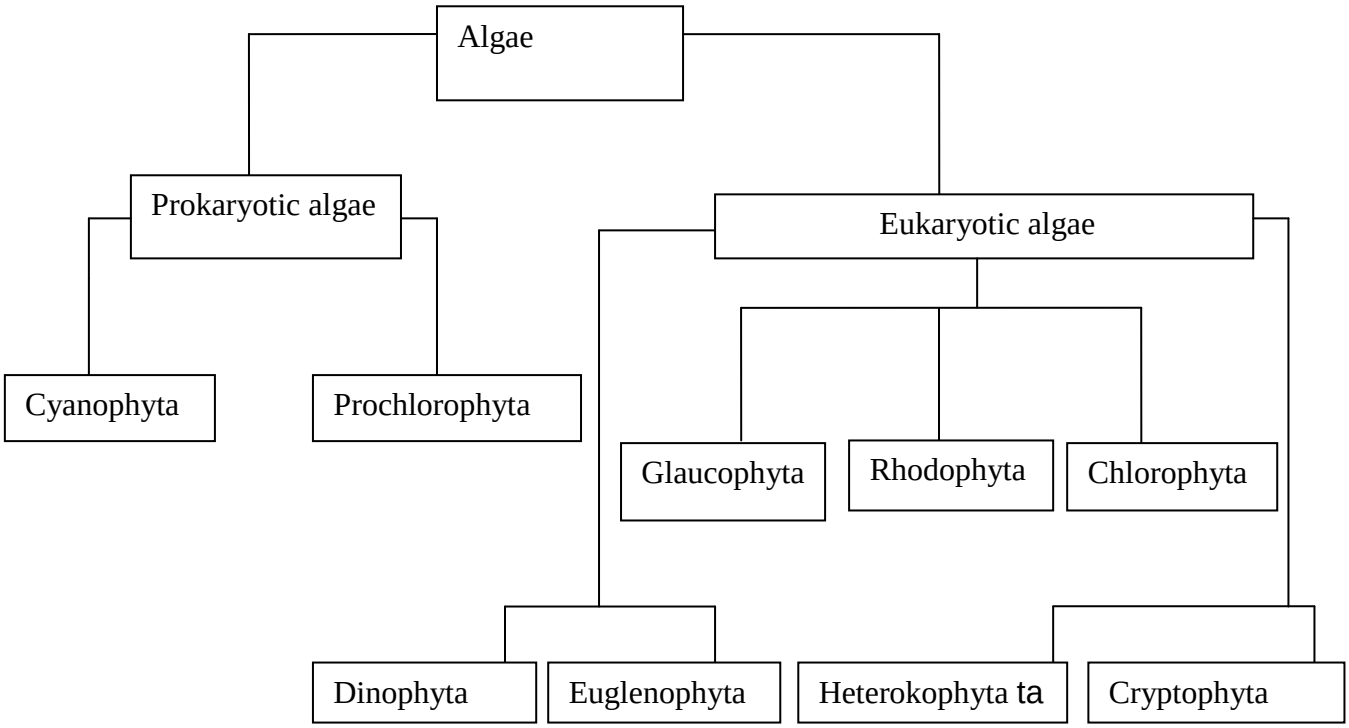


Figure 1-2: The tree diagram shows the different classes of microalgae as described by Lee, 2008

1.1.5 Culturing systems for microalgae

Microalgae culture is one of the biotechnologies that have recently been developed (Milledge, 2010). Mass culturing of microalgae on a commercial scale has been performed successfully on species such as C*hlorella*, S*pirulina*, *Scenedesmus, Dunaliella salina, Haematococcuspluviali* and *Porphyridium cruentum* (Chaumont,

1993 and Borowitzka, 1999). Although these microorganisms are abundant in nature they have not hitherto made the subject of scientific investigation. Another reason is that they have genetic ability to produce a wide range of compounds and chemicals that could be of great commercial value (Grossman, 2005). There are probably well over 30 000 species of microalgae, only a few hundred of which are cultured in laboratories at present. A handful of these have been characterized in detail by researchers, who have also assessed their economic potential (Chaumont, 1993).

Microalgae biomass is produced in specially engineered facilities, the fundamental design and infrastructure of which are dictated by the growth requirements of the microalgae of interest and the value of the final product (Meeting, 1996). In general microalgae can be produced in open ponds or closed systems (such as photobioreactors), and the culturing methods used can be continuous, semi-continuous or batch (Jorquera *et al.,* 2010).

i. Open pond system

Open ponds can be grouped into natural systems (for example lakes, lagoons and ponds) or artificial ponds and containers, which can take various forms. These include shallow unstirred, circular stirred and raceway ponds and tanks (Zittelli *et al.,* 2003, Kunjapung and Eldridge, 2010) (Figure 1-3). Open ponds remain the most commonly-used culturing method because they are relatively simple to construct, and require low-cost operation and maintenance. However, they have numerous limitations. These include poor utilization of light by microalgae cells, high evaporation, low mass transfer, loss or diffusion of CO_2 into the atmosphere, and the large amount of land required (Ugwu, 2008). Furthermore, they are susceptible to

chemical and biological contamination. Culturing of a single species (unialgal) depends on the competitiveness of the microalgae being grown, that is, whether or not they can resist contamination from other microalgae species. It follows that unialgal species can be cultured successfully using open pond systems, but that their specific needs must be recognized. In other words, they must be cultivated in a highly selective environment. For example, *Chlorella* grows well in nutrient-rich media; *Spirulina* requires a high pH and bicarbonate concentration; while *D.salina* grows best under highly saline conditions (Soong, 1980; Belay, 1997; Borowitzka and Borowitzka, 1988).

As a result of the need to control the environment in which microalgae are cultivated, open pond systems are not always suitable for the purpose. They are most commonly used for wastewater treatment, aquaculture, stock and human feeds (Metting, 1996).

ii. Closed systems

Microalgae are also grown in three types of closed system: photoautotrophically, mixotrophically or hetrotrophically. Closed systems are usually chosen when the species being grown is uncompetitive with other, unwanted, species. They are also preferred when reagents of high value (such biopharmaceuticals or isotopically lablled research compounds) are being produced (Wijffels *et al*., 2010). There are various forms of closed systems: these include stirred tank and airlifting reactors, bag culture, and flat plate photo, tubular photo and vertical column photo bioreactors (Figure 1-4).

The advantages and disadvantages of using open and closed systems are set out in Table 1-1.

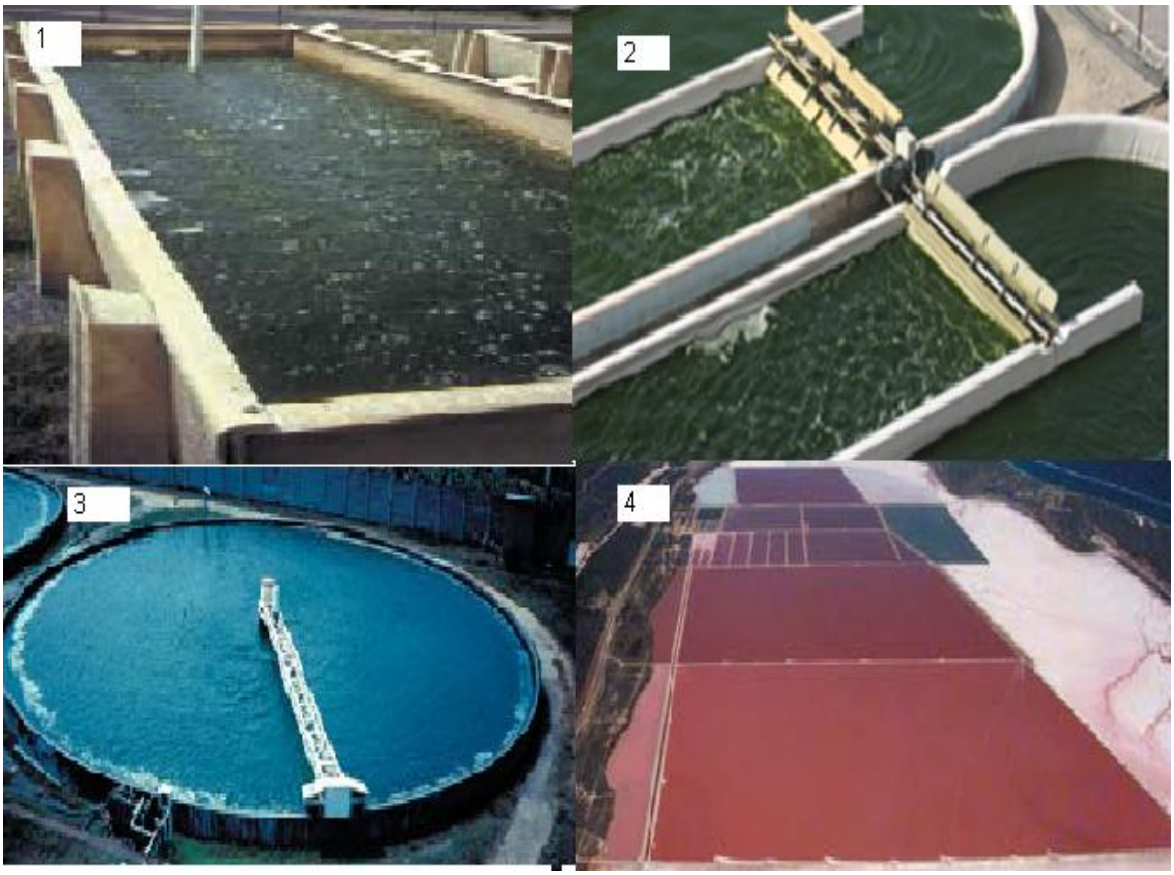


Figure 1-3: Different types of open pond microalgae culturing systems. 1: deep aerated tank, 2: race away pond system, 3: circular centre pivot tank and 4: shallow unstirred tank (Anderson, 2005)

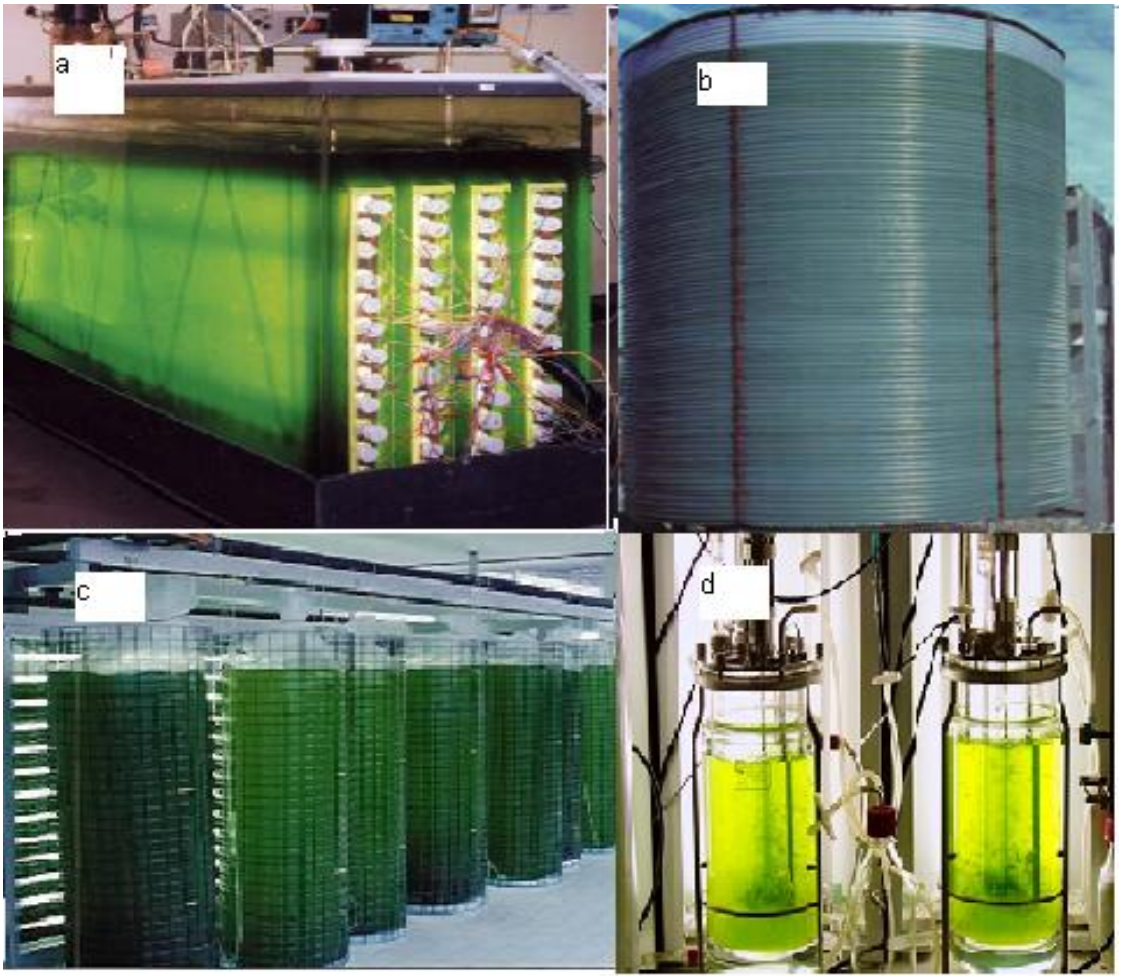


Figure 1-4: Different types of closed system for culturing microalgae: a) flat plat photo bioreactor (Anderson, 2005); b) Biocoil 1000 L tubular photo bioreactor (Anderson, 2005); c) bag culture; and d) air lifted tubular bioreactor.

Table 1-1: *Advantages and disadvantages of open and closed microalgae culturing systems (Mata et al., 2010)*

Culturing systems	Advantages	Disadvantages
Open pond	relatively economical; easy to clean up after cultivation; good mass cultivation algae	difficult to control the culture conditions; difficult to grow algal cultures for short periods; relatively unproductive; wasteful of land; easy to contaminate culturing conditions; only a few algae species can be successfully be grown.
Closed	highly productive; with good mass transfare, mixing; large illumination surface; easily maintained pure pure culture; easily controlled growth parameters	expensive because construction requires sophisticated materials; difficult to scale up; operational intensive; risk because culture may suffer hydrodynamic stress

1.1.6 Factors limiting algal growth

Microalgal growth can be limited by a number of factors. It is therefore vital that the designer of a large-scale culturing system should understand and accommodate them. Growth is influenced by both physical and biotic factors, (Borowitzka, 1999) and the limiting factors for optimal growth conditions for maximal microalgae biomass yield are listed in Table 1.2.

Table 1-2: *Factors limiting microalgal growth (Moheimani, 2005)*

Abiotic factors	light (quality and quantity); temperature; concentration and bioavailability of nutrients, oxygen, carbon, pH ,salinity, presence of toxic chemicals
Biotic factors	the presence of pathogens (bacteria, fungi and viruses); competition from other algae
Operational factors	impediments to growth such as shear produced by mixing; inappropriate, dilution rate, depth, harvest frequency and addition of bicarbonate

Figure 1-5: Photosynthetic conversion of CO_2 into microalgae biomass, adapted from Bayless, *et al.*, 2000

i. Light

Light influences the photosynthetic mechanism of microalgae directly, and is therefore an important factor in creating optimal conditions for culturing (Falkowski *et al*., 1985). Moreover, this factor has also an effect on their enzymatic activities and macromolecule syntheses (Foy and Smith, 1980). The degree to which algae utilize light depends on a number of conditions, for

example the position of the cell in a given instance, the density of the culture, and the pigmentation of the cells. The intensity of light is one of the important determinants of biomass productivity in large-scale microalgae cultures, (De la Noue and De Pauw, 1988) and day length (in other words, the number of hours of light during a 24 hour period) is another, because it influences the circadian rhythm of photosynthesis, respiration, cellular division, and growth rate (Hobson *et al*., 1979; Redalje and Laws, 1983; Piquemal, 1990). A lack or oversupply of light can limit growth even when the nutrients and temperature supplied are near optimal. However light is not a limiting factor in open outdoor culturing systems such as ponds (Richmond and Grobbelaar, 1986).

Plotting the growth curve relating to the response of microalgae to light shows three distinct stages:

a) a light-limited section in which the growth rate intensifies with increasing light intensity
b) a light-saturated area in which the growth rate is independent of the light intensity
c) a photo-inhibited part in which the growth rate decreases with a further increase in photon flux density.

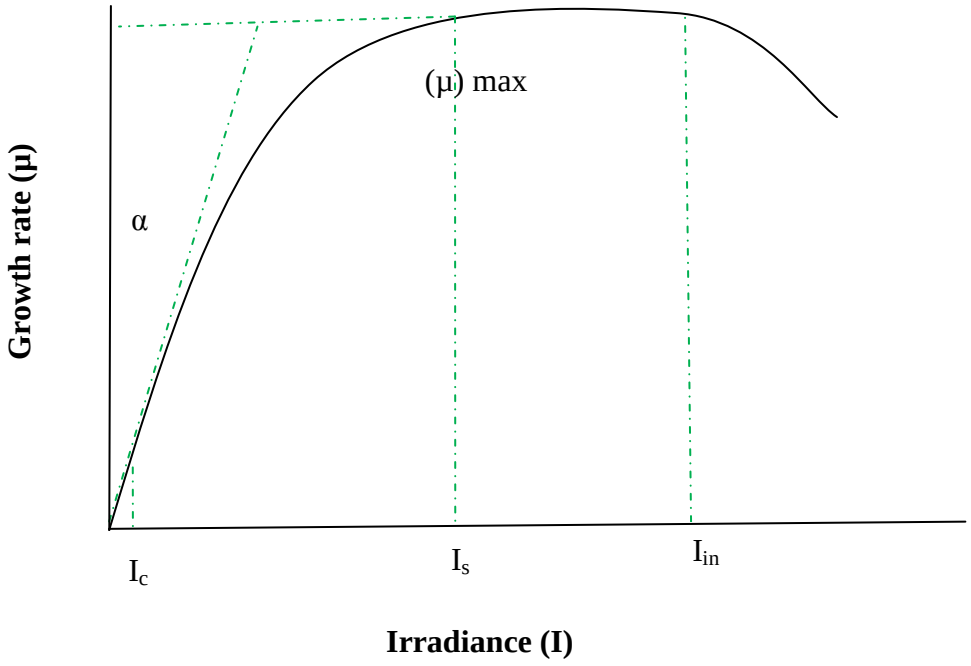


Figure 1-6: Light response growth curve of growth rate (μ/I), I_c = light compensation point, I_s = light saturation irradiance and I_{in} = light irradiance value where photoinhibition occurs, adapted from (Goldman, 1980)

ii. Nutrients

Photoautotrophic microalgal growth is mainly dependent on nutrients such as carbon, nitrogen, phosphorus and micronutrients (Richmond, 1988). It follows that any deficiency or excess in these requirements will limit their growth.

Microalgae require **nitrogen** to grow and build biomass (Barnese and Schelske, 1993). It is widely accepted that nitrogen affects marine and fresh water phytoplankton productivity. Total nitrogen concentrations in water bodies can range

from as low as 100–200µg/l to as high as 10 000µg/l in a heavily-polluted river (Forley and Bobbi, 2003). Nitrogen deficiency in algae results in alterations in growth, physiological responses and chemical composition. For example, there is an increase in lipid production when the algal cells are nitrogen-deprived (Stephenson *et al.,* 2010; Verma *et al.,* 2010). In addition El- Baky, *et al*. (2009) noted that biochemical compounds such as phenolic compounds and chlorophyll are also influenced by amount of nitrogen available.

Algae take up nitrates by means of a common high affinity transport system involving the permease (a transporter) and, to a lesser extent, diffusion (Hu, *et al.,* 2000). Once inside the cell, the nitrates are reduced to nitrite by nitrate reductase, which further reduces it to ammonium (Herrero and Flores, 1997). The ammonium is then incorporated into carbon skeletons, mainly through the operation of the glutamine synthetase-glutamate synthase cycle (Muro-Pastor, 2003).

Microalgae can assimilate inorganic nitrogen forms such as nitrates, ammonia, and inorganic urea, while some species (such as blue-green algae) can fix molecular nitrogen (Fidalgo *et al.,* 1998; Ismal, *et al.,* 2007). Ammonium and nitrate salts are the main sources of nitrogen. Some microalgal species exhibit a preference for inorganic nitrogen sources (Syrrete, 1962). Research by Fidalgo *et al.,* (1998) showed that *I. galbana*, grew better in urea than in nitrates at the exponential growth phase, their findings concluded that there was no significant difference in the dry weight obtained with a low concentration of NH_4Cl, $NaNO_3$ and a similar amount of urea.

However, several research reports have indicated that most microalgae do not discriminate between types of nitrogen source. Increasing the concentration of total nitrogen ions increases both biomass productivity and growth rate (Giordano and Bowes, 1997). However, a very high concentration slightly reduces growth but does not stop it, probably because of nitrate regulation. Introducing more nitrogen ions into the medium raises nitrate reductase activity, and it leads to production and accumulation of NH_4^+ (Giordano and Beardall 2008). The nitrate regulation process, which induces inhibition of nitrate assimilation, probably slows down microalgal growth. In addition, higher nitrate reductase activity can lead to a toxic accumulation of nitrite inside the cells, causing reduced nitrate uptake and inhibition of growth (Jeanfils *et al.,* 1993).

Phosphorus is an element required for microalgal growth, especially for generating and transforming metabolic energy (Eixler *et al.,* 2006; Sun and Wang, 2009). Phosphorus is an essential nutrient that constitutes cells, nucleotides and nucleic acids (Correll, 1999). In natural lakes phosphate concentrations are very dilute and are therefore at levels that limit microalgal growth (Correll, 1999).

Phosphates generally occur in different forms: orthophosphates (which are the salt form of phosphoric acid), polyphosphates and organic phosphates (Powell *et al*., 2009). There are two mechanisms through which phosphates are incorporated into the microalgal biomass. Firstly, the cell actively absorbs phosphates to meet its metabolic needs. Secondly, it engages in a 'luxury' uptake of phosphates which it uses to form polyphosphates that are either acid-soluble or acid-insoluble (Miyachi *et al.,* 1964). Acid-soluble polyphosphates are readily available to assist metabolism,

while acid-insoluble polyphosphates are reserved for conditions when the phosphate levels in the medium are limited (Powell *et al*., 2008).

The concentration of phosphates in the culturing medium affects microalgae growth, as is demonstrated by the more rapid uptake of phosphates in a phosphate-limited environment a medium containing inadquate phosphates (Singh *et al*., 2007). Polyphosphate pools are prevalent in the presence of a high phosphate concentration. Scientists have also reported that phosphate uptake is optimal in alkaline pH, stimulated by Na^+ ions, and inhibited by K^+ ions (Eberius and Yingchol, 1974; Singh *et al*., 2007).

The role of **carbon** as a nutrient for photoautotrophic microalgae is central to the research described in this dissertation. Carbon is the paramount element required for photosynthesis and photosynthesizing organism takes it up as carbon dioxide gas. Carbon is used to build up basic macromolecules of all organisms such as sugars, proteins, lipids etc.

The aquatic environment generally contains insufficient CO_2 for photoautotrophic algae, for two reasons. Water has a limited capacity to hold gaseous CO_2, and the diffusion rate of $CO_{2\ (aq),}$ which is 10^{-4} to that of the atmosphere, is slow (Satoh, *et al.,* 2001). Inorganic carbon (Ci) exists in different forms in water, as demonstrated in the equilibrium equation 1.3 (Yang and Gao ,2003).

$$CO_{2\ (aq)} + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-} \qquad (1.3).$$

As the pH in the medium drops, an increase in CO_2 concentration can be observed. Different species of dissolved inorganic carbon (DIC) occur in various dissociation

fractions that are dependent on pH, as illustrated in the diagram below (Figure 1-7). The solubility of carbon dioxide decreases with increasing temperature and pressure (Zhu *et al*., 2008).

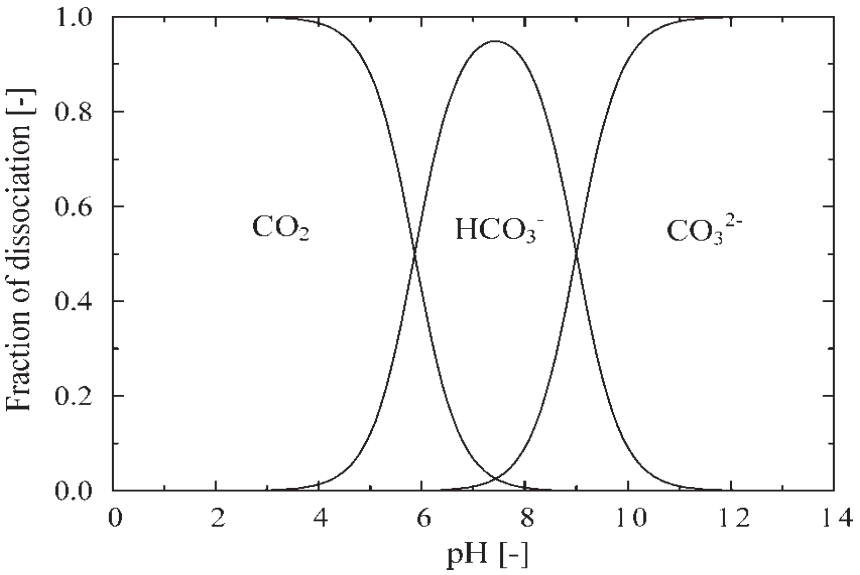


Figure 1-7: Chemical equilibrium of dissolved inorganic carbon species as a function of culture medium pH (Ota et al., 2009).

1.1.7 Mechanisms of CO_2 absorption and fixation in microalgae

The Ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) enzyme is known to fix Ci in the form of CO_2 in the dark (light-independent) reaction of photosynthesis (Calvin cycle) (Jacbs-Lopes and Franco 2010). When pure CO_2 dissolves in an aqueous solution, the pH drops below 7, and this creates an acidic environment. The dominant Ci species in an acidic solution will be CO_2 and HCO_3^- (Ota *et al.,* 2009). Microalgae have developed various means of ensuring that the Rubsico enzyme is well supplied with CO_2 that are referred to as carbon- concentrating mechanisms (CCMs), (Beardall, 2004) by means of which inorganic carbon moves across the plasma membrane. These CCMs include passive diffusion of CO_2 through the

aquaporins, active uptake of HCO_3^-, and facilitated diffusion of HCO_3^- (Smith and Bidwell, 1980). In addition Moroney (2007) reported that most algae contain the enzyme carbonic anhydrase (CA), which is found on the external side of the plasma membrane, in the cytoplasm and inside the chloroplast. Extracellular CA accelerates the equilibrium of CO_2 and HCO_3^- in an alkaline medium, so that CO_2 is formed at a rate sufficient to support photosynthesis (Fridlyand, 1997). In an alkaline environment, microalgal species with active HCO_3^- transport on the plasma membrane. The extracellular CA catalyses the hydration of the CO_2 that escapes from the cell, and this sharply reduces the CO_2 efflux to the surrounding medium. Regardless of the form in which Ci enters the cell, all Ci will be hydrated into HCO_3^- in the cytosol. The HCO_3^- then diffuses into carboxysomes, where it is converted to CO_2 by CA (Figure 1-8). In the carboxysome, the CO_2 concentration builds up to a higher steady state than in the bulk medium, thus strongly favouring the carboxylase over the oxygenase activity (Rubisco) (Giordano, *et al.,* 2005). Rubisco catalyses either carboxylation or oxygenation of Ribulose biphosphate during the Calvin cycle of photosynthesis. There is evidence of C4 carbon metabolism pathway in some microalgae and this improves the efficiency in assimilation and fixation of carbon in low levels of carbon environment (Reinfelder *et al.,* 2000).

CCMs are an evolutionary trait that developed for microalgae to adapt to low levels of carbon dioxide concentrations in aquatic environments. Very high levels of carbon dioxide may cause changes in microalgae physiology and metablosim which includes reduction in stomatal density, Rubsco levels, chlorophyll, and lower photoresipiration (Papazi *et a*l., 2008). Microalgae species that flourishes in high levels of carbon dioxide might still possess the genetic capability to photosynthesize under high CO_2

concentrations and increased illumination (Papazi *et al.,* 2008). Species that could adopt the state of transition in favour of PS1 could grow well in high levels of CO_2 (Miyachi *et al.,* 2003). Other findings shows long-term elevation of CO_2 would down regulate the activity of CCM, which results in reduced or no activity of CA (Sobrino *et al*., 2008).

Microalgae posses other means of fixing inorganic carbon other than the Calvin cycle and this makes microalgae efficient in fixing carbon into biomass at high levels of carbon dioxide. Some of the secondary carbon fixation mechanisms are 3-hydroxyprropionate pathway which uses the acetyl-CoA carboxylase and propionyl-CoA carboxylase to fix CO_2 into glycoxylate and intermideate in carbon metabolism (Milner *et al.,* 2009).

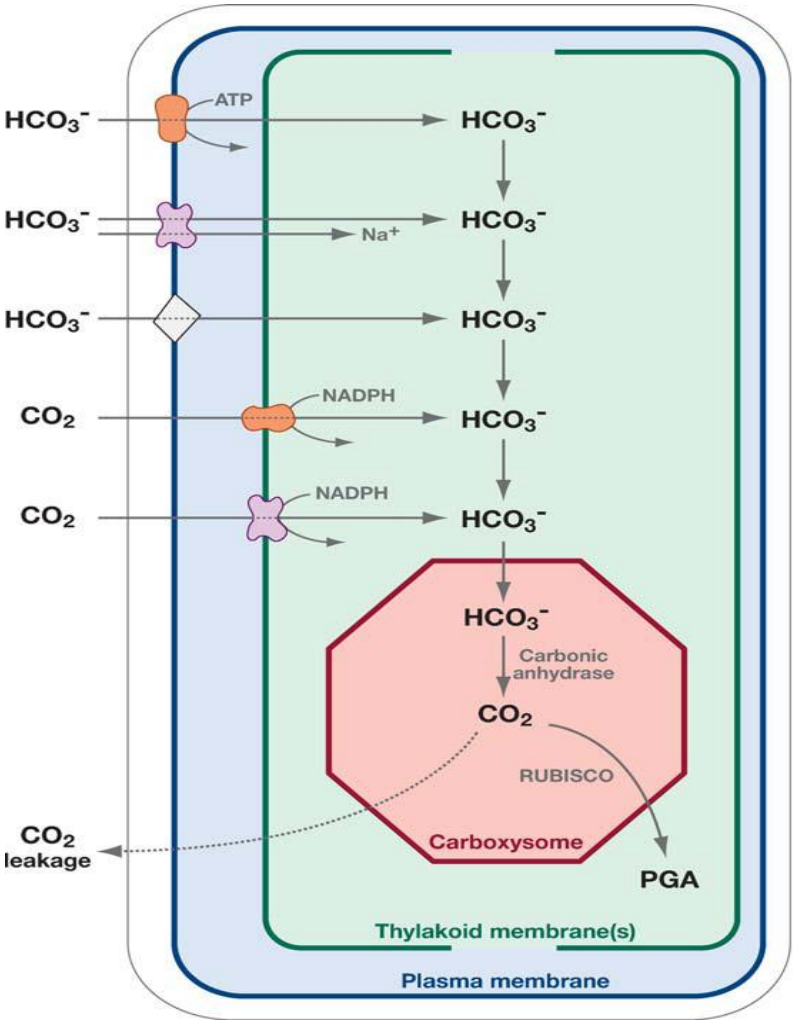


Figure 1-8: A schematic model showing inorganic transport and CO_2 accumulation in a microalgal cell (Giordano *et al.,* 2005).

1.1.8 Microalgae toleration to gases other than CO_2

Carbon dioxide, together with other flue gases such as nitric oxides (NO_x) and sulphur oxides (SO_x), which are said to be toxic to biological systems, is released from factories that burn fossil fuels (Allam and Spilsbury, 1992; Verma *et al.,* 2010). The SO_x are oxidized to sulphates that accumulate, causing a decrease in pH and growth inhibition in microalgal culturing conditions (Stepan *et al.,* 2002). Although some researchers have suggested that these toxic gases should be removed

because they hinder the CO_2 mitigation for which the microalgae are being grown. Removal of these substances is costly, and would make the technology of the microalgae systems unaffordable. Algal tolerance to NO_x and SO_x varies from species to species. Acidophilic microalgae strains are not inhibited by SO_x and NO_x (Yoshihara *et al.,* 1996). Lee and co-researchers induced tolerance of 250ppm of SO_x and 300ppm of NO_x was by using very high inoculum concentrations of microalgae; and adjusting both the gas flow rate and the pH during the first 8 hours of inoculation (Lee *et al.,* 2000; Lee and Lee, 2003). Regardless of the flow rate, *Monoraphium minutum* was found to be able to tolerate up to 200ppm SO_x and 150ppm of NO_x (Zeiler *et al.,* 1995).

1.1.9 Potential uses of the microalgae biomass

The culturing of microalgae for CO_2 absorption is an expensive process. However, it can be made economically viable if the biomass produced is used both for carbon mitigation and for other purposes.

Algae biomass could play an important role in supplying food security for both animals and humans. Microalgae have high nutritional value because of the wide range of essential nutrients such as provitamins, minerals and polyunsaturated fatty acids they provide (Pandey and Tiwari, 2010). *Chlorella, Spirulina, Scenedesmus* and *Caelastrum* have a protein content of 60%, while 18 of the 22 known amino acids are found in these microalgae (Brown, 1991). They contain essential amino acids, including those containing sulphur, which are usually missing from most human diets (Kolb *et al.,* 2004). They are also a rich source of polyunsaturated fatty acids, which include omega 3 fatty acids, hexadecatrienoic acid methyl ester,

octadecatetraenoic acid methyl ester, eicosapentaenoic acid methyl ester, docosahexaenoic acid methyl ester, arachidonic acid and γ-linolenic acid (Spolaore *et al.,* 2006). Microalgae are regarded as a good source of protein for humans, animals and fish grown in aquaculture hatcheries. Nevertheless, microalgae grown in the effluent from wastewater treatment plants should not be used for consumption by humans, animals or fish, as they may contain a variety of contaminants, ranging from heavy metals and pesticide residues to pathogenic bacteria and viruses (De La Noue and De Pauw, 1988).

Microalgae also contain bioactive compounds that are effective medicinally (Gustafson, 2004). Several of these, such as lipid anti-oxidants, β-carotene, sterols and toxins, make algae a good source of nutraceuticals (Rasmussen *et al.,* 2007). The therapeutic action of treatments based on microalgae on diseases associated with oxidative stress has attracted a great deal of attention (Chen *et al.,* 2009). Microalgae can be used to treat a range of medical conditions because of their properties. For example, they are anti-bacterials, bronchodilators, polysynaptic blockers and analgesics, and prevent odema, convulsions and inflammation (De La Noue and De Pauw, 1988).

The chemicals found in algal biomass host that can be exploited industrially to generate revenue. Microalgae also produce sugars, polysaccharides, dyes, bioflocculants, pigments and oils (De La Noue and De Pauw, 1988, Pereira *et al.,* 2009). Other valuable chemicals include phycobiliproteins, which are fluorescent dyes used in certain immunoassay and cell separations (Curtin, 1985) and asthaxanthin, lutein, zeaxanthin, lycopen and bixin, which are useful components of food colourants, cosmetics and pharmaceutical products (Chen *et al*., 2009). In

addition, microalgae produce a number of osmoregulatory substances such as sorbitol, mannitol, cyclohexanetiol and bio-emulsifiers (De La Noue and De Pauw, 1988).

Paleotobatanical evidence suggests that microalgae are responsible for the major sources of hydrocarbon that have been found in a variety of oil-rich deposits that date from the Ordovician period (about 490 million years ago) to the present. (Borrego *et al.,* 1996). Microalgae generally have lipids in their biomass, and most of these fatty acids are suitable for biodiesel production. Again, *Botrococcus braunii* and many other species are being tested for the production of long-chain hydrocarbons of C_{30-36} (Tsukahara and Sawayama, 2005). Algae contain substantial amounts of starch and other polysaccharides, which in theory can be fermented to produce ethanol and other high-energy chemicals (Chen *et al.,* 2009).

Many algal species can also induce hydrogen production by biophotolysis (Huesemann *et al.,* 2010). Other findings by De La Noue and De Pauw (1988) proved that marine blue-green non-hetrocystous algae (*Oscillataria*) grown in a growth chamber of 20 x 3 square feet could produce enough hydrogen to yield 1 000 Kilowatt-hours (KWh) of electricity per month.

Whole dry algal cells or extraction residues are usable as feed stocks for thermochemical conversion processes such as gasification, pyrolysis, and hydrothermal liquefaction, which produce syngas, bio-oil, biopolyols and biochar. Bio-syngas ($CO + H_2$) when combusted can generate heat or electricity, and bio-oil can be used as heating oil or processed further to make liquid transportation fuels. Biopolyols serve as chemical stocks for material synthesis (Chen *et al.,* 2009).

Microalgae biomass or extract residues can be used as organic fertilizers and soil conditioners. Biochar is known to make one of the best land restoration feeds available. De La Noue and De Pauw (1988) reported that 1 kg of microalgae could replace 60kg of conventional fertilizer.

Wastewater from agricultural processing plants contains a high level of nitrates and phosphates, which causes eutrophication (House *et al.*, 1994). Microalgae are capable of utilizing these waste materials by incorporating both inorganic and organic forms of nitrogen and phosphates into their biomass (Hameed and Ebrahim, 2007). The processes involved in the removal of these nutrients are precipitation, stripping and luxury uptake by the algal biomass (Shahalam, 2009). Efficient bioremediation of wastewater by this means depends on whether the substance to be removed is limiting to the microalgae; whether there is adequate mixing of water and algae and whether there is continual removal of the algal biomass (Noue and Pauw, 1988, Martin *et al.,* 1985).

Microalgae can bioremediate most of the biologically sequestered trace metals to be found in aquatic environments. Their ability to absorb and metabolize trace metals is associated with their extensive surface, volume ratios, the presence of high-affinity, metal-binding groups on their cell surfaces, and their efficient metal uptake and storage systems (Saxema and Misra, 2010). Microalgae may adsorb up to 10% of their biomass as metals. (Rajamani *et al.,* 2007) These comprise the essential trace metals required for metabolism, but also include toxic heavy metals, which often compete with the trace metals for adsorption and absorption (Aquino and Stuckey, 2007). Microalgae can sequester the toxic heavy metals efficiently.

1.2: Background and motivation

South Africa and the world at large are in dire need of abatement strategies to counter the unprecedentedly high CO_2 levels that are believed to be causing the unstable weather patterns the world is currently experiencing. This phenomenon is generally referred to as climate change. Africa's industrial powerhouse, South Africa, obtains over 80% of its energy needs from fossil fuels. The most recent research indicates that South Africa emits over 222 million tonnes of carbon dioxide into the atmosphere every year. Matimba power station, which is located in Limpopo province, is ranked 19th in the list of the world's highest CO_2 emitters (Laura, 2007).

Although efforts are being made to find alternative and sustainable sources of energy, the world cannot afford to forgo the use of fossil fuels because of the need to meet its energy requirements. However, through the Kyoto protocol and similar international initiatives, countries around the world are seeking sustainable means of curbing CO_2 emissions. In 2005 the United Nations Intergovernmental Panel for Climate Change (IPCC) suggested that natural sequestration of CO_2 was pivotal to creating a more benign environment.

One of the methods of reducing CO_2 emissions that is attracting attention from scientists is to convert the CO_2 into microalgal biomass, which is also useful in many other applications, such as biofuels, organic fertilizers, stock feeds, nutraceuticals, other high-energy chemicals and pharmaceutical products. Microalgae now represent the world's best hope of offsetting the detrimental effects of greenhouse gases. These algae are phytoplankton organisms that account for at least 50% of global photosynthetic productivity (Giordano *et al.,* 2005). They generally have high growth

rates and are easily grown in aquatic habitats on unproductive land. They can be cultivated in systems designed to produce biomass, take up anthropogenic CO_2 and remove certain water pollutants simultaneously (Clarens *et al.,* 2010).

Among the several factors that affecting microalgal growth are the supply of nitrogen, phosphate, carbon, light, temperature and micronutrients. A thorough understanding of the conditions that limit microalgal growth is therefore important to enable engineers to design culturing systems that operate at optimal efficiency.

1.3 Research problem

Scientists the world over are making concerted efforts to find sustainable means of sequestrating CO_2 and minimizing the emission of not only carbon dioxide but other greenhouse gases. Microalgae can play a pivotal role in providing carbon sinks and reducing atmospheric carbon dioxide, which in turn will reduce effects of global warming. Understanding the growth-limiting factors that could affect the large-scale cultivation of indigenous microalgae is vital to a clear determination of the conditions necessary for maximum biomass yield. The research described in this dissertation seeks to establish a clear picture of what algae will and will not tolerate, in order to define optimal microalgae growth conditions (such as light, nutrients, pH and carbon dioxide concentrations) to obtain higher growth rates and biomass yield.

1.4 Justification

Because South Africa is a coal-based economy, which makes heavy use of processes such as coal to liquid (CTL), thermal electricity generation and various forms of chemical manufacture, it produces a great deal of CO_2. As both a

developing country and Africa's industrial and economic hub, South Africa has an obligation to lead continental efforts to reduce CO_2 and other greenhouse gas emissions. One of the technologies that can assist this endeavor is the culturing of microalgae. Investigating the factors that limit the growth of South Africa's native microalgae is a necessary first step towards making informed recommendations for designing microalgae CO_2 sequestration systems in developing countries like South Africa. We are not aware of any other research into the use of natural native fresh water microalgae for CO_2 sequestration systems that has been carried out in South Africa.

1.5 Hypothesis

Microalgal growth is limited by environmental factors such as light intensity and nutrients like nitrogen and phosphates as well as carbon dioxide. When there is an appropriate supply of light, nutrients and carbon, a maximum biomass yield and growth rate can be achieved in a large-scale microalgal culturing system.

1.6 Objectives

Growth-limiting factors' refer to those elements required by microalgae in specific amounts, which, if not supplied, will inhibit their growth. The intention of the research was to determine the most favourable conditions to secure maximum growth rate and dry weight yield as a function of:

1) Identify the microalgae species that tolerate high CO_2
2) Light intensity
3) Photoperiod
4) Nitrogen ion species
5) Nitrogen ion concentration
6) Confirm the nitrogen limitation by adding nitrogen ions to a nitrogen ion-depleted medium.
7) Effect of total phosphate ion concentrations
8) pH
9) Measure the effect of adding CO_2 to microalgae
10) Effect of CO_2 flow rates and concentration
11) Establish ways to enhance CO_2 tolerance at high flow rates

CHAPTER TWO

2. Materials and Methods

2.1 Reagents

All reagents used in this study were of analytical grade, and sourced from Merck, South Africa (unless stated otherwise). These were used as obtained, without further purification.

Utra-high-purity (UHP) grade N_2 and CO_2 gases were supplied by AFROX South Africa.

2.2 Equipment

The light used for plant life support (photosynthesis) was provided by F36/Gro-T8 fluorescent tubes which emit a high level of blue and red radiation (Sylvania Gro lux, Germany), alternated with Osram F36/33-640 cool white fluorescent tubes (Sylvania Gro lux, Germany). The light intensity (lux) was measured using a Major Tech MT940 light meter (MAJOR TECH, Germany). One lux unit meter reading is equivalent to $0.0079 Wm^{-2}$. The cultures were kept in constant motion by magnetic stirrers, and the change in absorbance in the culture was measured using a Unico® 4802 UV/VIS double beam spectrophotometer (Unico® Instruments, Co Ltd, Shangai). An Olympus BH2 microscope with a Nikon DXM1200 camera was used to observe the morphological features of the microalgae being studied, while individual cells were examined for identification purposes using means of an Olympus Cell® system. The pH of the culture was monitored by a Crison pH meter Basic 20^+ (Lasec, South Africa). Brooks Smart mass flow controllers (Brooks Instrument B.V, Holland) were used to mix gases, CO_2 and N_2 (v/v).

2.3 Microalgal growth

2.3.1 Microalgae Collection

Fresh-water green microalgae samples that were used in the experiments were collected from Johannesburg Zoo Lake, South Africa using 250ml sample collecting bottles. Johannesburg Zoo Lake is a recreational manmade lake that is managed by Johannesburg City Parks. Two culturing approaches were used which were Zoo lake water supplemented with Beijerinck medium and distilled water supplemented with Beijerinick medium. Zoo Lake water was collected in two liters bottles. Microalgae samples from Zoo Lake were filtered using a vacuum filter pump with a nitrocellulose (0.45µm) filter paper. Microalgae cells remained on the filter paper and 50ml of distilled water were used to wash the microalgae from the filter paper into the culturing medium.

Water for culturing collected from the Zoo Lake was filtered using a vacuum filter pump fitted with a nitrocellulose filter paper (0.45µl). The filtrate was autoclaved for 20 min at 121ºC and cooled then used for culturing microalgae. Unused samples were kept at 4°C.

All the glassware and equipment relating to the experiments were autoclaved before use, and aseptic conditions were maintained throughout.

Fifty milimeters of water containing the collected microalgae from Zoo Lake were grown in Beijerinick medium (Appendix A1). An experiment was set up to investigate the comparative effect on the culture growth of microalgae of filtered Zoo Lake water as against distilled water.

Figure 2-1: Microalgae collection from the Johannesburg Zoo Lake

2.3.2 Microalgal culturing conditions

Culturing was done at fixed room temperature (25ºC) with a humidty of 70% and light intensity of 6 000lux for 24 hours per day, unless otherwise stated. Ambient air was supplied using an aquarium DARO air pump, at a flow rate maintained at 50ml/min in all experiments except those relating to pH and CO_2. All experiments except those using higher concentrations of CO_2 were carried out in Phytotron growth chambers. Beijerinick medium was used as the standard media throughout. Constant mixing was ensured by the use of a magnetic stirrer. The apparatus in the phytotron was set up as illustrated in Figure 2-2.

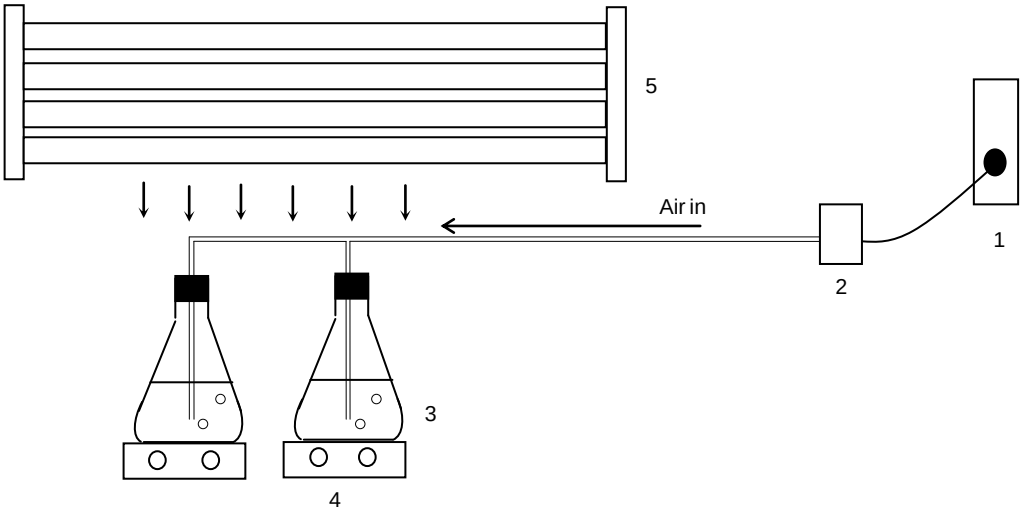


Figure 2-2: Diagrammatic representation of the experimental set-up in the phytotron growth chamber 1) power mains, 2) aquarium air pumps, 3) culturing flasks, 4) magnetic stirrers, 5) gro lux fluorescent tubes

In order to select species that tolerate high CO_2 levels, pure CO_2 was bubbled through the medium for 12 hours a day at a flow of 10ml/min. Microalgae that were tolerant to high CO_2 levels were used for further experiments. Sterile conditions were maintained throughout the experiments.

Contaminates were monitored using an Olympus BH2 microscope during culturing period and any changes in morphological features were noted.

2.3.3 Identification of the microalgae investigated

High CO_2 tolerant microalgae obtained from Johannesburg Zoo Lake were unknown; therefore molecular methods were used to identify the exact species. A sample of 250ml of medium containing cultured microalgae were collected and sent to Stellenbosch University for identification using DNA extraction and sequencing (As described in Appendix A5 and A6). Extracted DNA was amplified using polymerase chain reaction (PCR) technique. This was achieved using the Internal Transcriber region (ITS2) which was amplified using primer pair ITS03F-800 and ITS05R-700

universal primers (Table 2-1). The ITS 2 is a conserved and universal genE in all photosynthesizing microalgae and is used in identifying unknown microalgae.

***Table* 2-1**: *Primers used for microalgal identification*.

Primer Name	Region	Primer Sequence	Reference
ITS 03F-800	ITS	5' CGATGAAGAACGYAGCGA3'	Hoef-Emden, 2007
ITS 05R-700	ITS	5' TACTTGTTCGCTATCGGTCTCT 3'	

The PCR reagents were prepared as follows: 1x Epicenter Failsafe Premix A (*Separations*); 0.5*μ*M of each primer; 0.25 U Failsafe Enzyme and 20ng DNA. PCR was performed using a Geneamp® PCR system 9700 (*Applied Biosystems*) with the following conditions: 95°C for 5 minutes, followed by 40 cycles at 95°C for 30 seconds, 45°C at 50 minutes, and 72°C for 60 seconds, with a final extension of 72°C for 10 minutes. PCR fragments were prepared for sequencing. Sequencing was carried out in a 3730xl DNA analyzer (Applied Biosystems). The resulting sequence were edited with SequenceScanner V1 (Applied Biosystems) and Chromas V233 programmes. The sequences data were run through the National Center for Biotechnology Information (NCBI) data base to compare with other algal, internal transcriber spacer 2 sequences for identification (Schultz *et al.*, 2006). Morphology of the unknown algal samples were viewed under a light microscope (Olympus Cell® system) attached to an IX-81 inverted fluorescence imaging and an F-view-II cooled CCD camera (Soft Imaging Systems). Morphological features were compared to internet images of similar algae.

2.3.4 Measurement of microalgal growth

An absorption spectrum of the algae was carried out and the highest absorbance was used for monitoring growth (Appendix A2). Cell growth was monitored, using a Unico® 4802 UV/VIS double beam spectrophotometer. Absorption was correlated to cell dry mass by filtering 50ml of sample through a 0.45µm nitrocellulose filter, and subsequently drying the filter overnight in an oven at 100ºC. The dry mass was then cooled in a desiccator. All experiments were done in triplicates.

Cell growth rates were measured according to equation (2.1) (Hu et al., 2000).

$$\mu = (\ln X2 - \ln X1)/\Delta t, \qquad (2.1).$$

where

µ is the specific growth rate

X1 is the biomass at time 1

X2 is the biomass at time 2

Δt is the difference in time between X1 and X2.

2.3.5 Effect of light intensity and photoperiod

Light provide energy for the process of photosynthesis. Accordingly, an experiment was set up to investigate light intensity and photoperiod (number of hours of exposure to light) requirements inorder to achieve optimal specific growth rate and biomass yield. Light intensity was measured using a light meter (Major Tech MT940). Light intensity was varied by adjusting the distance between the microalgae-culture flasks and the light source. Light intensity ranged from 0, 3 000, 6 000, 8 000 and 10 000lux. The photoperiod was measured by exposing the algae to a source of light

for specific duration of time per day. Photoperiod was controlled by covering the microalgae cultures with aluminum foil to create darkness when required. Exposure times were as follows 0, 8, 12, 16, 20 and 24 hours per day. Photo period experiments were carried out at a light intensity of 6000lux.

The total light intensity was calculated by multiplying the light intensity measured with the light exposure duration (hours). Total light intensity is a calculated value not the actual reading from the light meter.

2.3.6 Effect of nitrogen ions

The effects of nitrogen ions on the growth rate and biomass yield were investigated. Nitrogen ions (NH_4NO_3) concentrations within the media were varied as follows: 0, 18.75, 37.5, 75, 150, 300, 750 and 1500mg/l. Cultures were allowed to grow for 12 days during which the growth rates and biomass yield were measured.

In order to determine the importance of nitrogen ions on microalgal growth, samples containing 0, 18.75 and 37.5mg/l nitrogen ions were selected. These cultures were grown for 12 days to ensure nitrogen depletion in cultures. On day 12 the standard Beijerenick medium containing 150mg/l of nitrogen ions were added to the culture flasks containing 18.75 and 37.5mg/l of nitrogen ions. While Beijerinick nutrients except nitrogen were added to the third flask, which initially contained no nitrogen ions, on the same day. Atmospheric air containing ambient CO_2 concentrations was supplied at a flow rate of 500ml/min.

A theoretical estimation of the expected dry biomass yield per given amount of nitrogen ions in the medium was determined. An estimation of percentage of the nitrogen ions in the medium that was used was carried out. The nitrogen ions species

used was NH_4NO_3 of which 35.443% is nitrogen. Assuming that all the nitrogen is converted to algal biomass ($CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$), it means 6.5925% of the algal biomass will be nitrogen. Therefore expected algal biomass was calculated as follows 5.38 times the amount nitrogen ions in the medium and then a constant will be added. The constant added will be the amount of algae biomass obtained when culturing is done without adding any nitrogen ions.

Upon obtaining an optimal nitrogen ion concentration, investigation on the effects of different nitrogen ion species (ammonium nitrate, ammonium chloride and potassium nitrate) was determined on microalgal cultures.

2.3.7 Effect of phosphate ions

To investigate the degree to which phosphate influences microalgal growth, six flasks containing varied concentrations of phosphate ions (0, 250, 500, 1 000, 2 000 and 4 000mg/l) were prepared. Effects of total phosphates were determined under culture growth conditions of the best nitrogen ions concentrations from section 2.3.6 experiments. Ambient air flow rate was set at 500ml/min and all other parameters were as described in section 2.3.2.

2.3.8 Effect of pH

The influence of pH on microalgal growth was also examined. Four different pH values of 5.5, 6.0, 6.5 and 7, were set up by adjusting using 1M HCl, 1M NaOH and 50mM of HEPES buffer. A control culture was also set up in which the pH was not buffered or adjusted. Pure CO_2 was bubbled through the cultures at 10ml/min, and standard culture conditions were maintained throughout the experiment.

2.3.9 Effect of adding CO_2 gas

In order to confirm the importance of CO_2 on the growth of microalgae, two experiments were set up as follows, one was supplied with ambient air while the other with pure CO_2. The gas flow rate for both experiments were at 50ml/min. optimum nitrogen and phosphate ions, light intensity and photoperiod obtained above were used (hereafter referred to as optimal culturing conditions) were selected. The pH was controlled using 50mM of HEPES buffer.

2.3.10 Effect of CO_2 flow rate

The higher the flow rate the more CO_2 is supplied to microalgae. An experiment to find out the effect of adding pure CO_2 to the culture at different flow rates was conducted. Carbon dioxide was bubbled continuously into five different flasks containing optimal culturing conditions at flow rates of 10, 20, 50, 100 and 250ml/min.

2.3.11 Effect of elevated CO_2 concentrations

Raising the concentration of CO_2 is believed to encourage a high specific growth rate and increase maximum biomass yield. An investigation on the effect of higher CO_2 concentrations on microalgal growth by bubbling different concentrations of various CO_2 (5, 10, 25, 50 and 100%) was determined using gas flow rates of 20, 50 and 100 ml/min on each of the CO_2 concentration. CO_2 concentrations of 5, 10, 25, and 50% were made up to 100% by mixing nitrogen gas via a Brooks Smart mass flow controller (Brooks Instrument B.V, Holland). Optimum growth conditions were maintained.

2.3.12 Enhancement of high CO_2 tolerance at high flow rates

A further experiment was carried out to find means of raising the tolerance of microalgae to high levels of CO_2 by bubbling pure CO_2 through the cultures for 3, 6, 9, 12, 15 and 24 hours per day at 250ml/min. All other culturing conditions were kept optimal.

CHAPTER THREE

3 Results

3.1 Preculturing experiments

3.1.1 Determining the most effective culturing conditions

This research aimed to investigate and determine the sensitivity of the growth rates and biomass yields of microalgae collected from Johannesburg Zoo Lake to environmental factors. The results were intended to provide information vital to establishing the best growth conditions for native microalgae when designing culturing systems (Appendix A2). A microalgae absorption spectrum was carried out and microalgae understudy absorbed most at 680nm and this was then used to monitor microalgae growth in all experiments. The initial series of experiments we set up aimed to identify which water source which was ideal for growing fresh water algae from Zoo Lake. Water from Zoo Lake was used and compared to that of distilled water. Both water sources were supplemented with Beijerinick nutrient medium. The results showed that microalgae grew best in nutrient supplemented distilled water. There was a steady increase in biomass yield and a specific growth rate of about 0.10 per day when nutrient supplemented distilled water was used. Microalgae cultivated in nutrient supplemented Zoo Lake water had a high growth rate on the first day of the experiment, but from day two of culturing growth ceased, resulting in a five times lower growth rate of 0.02 per day (Figure 3-1).

Because nutrient supplemented distilled water provided the best culturing conditions, it was used in all the experiments that followed.

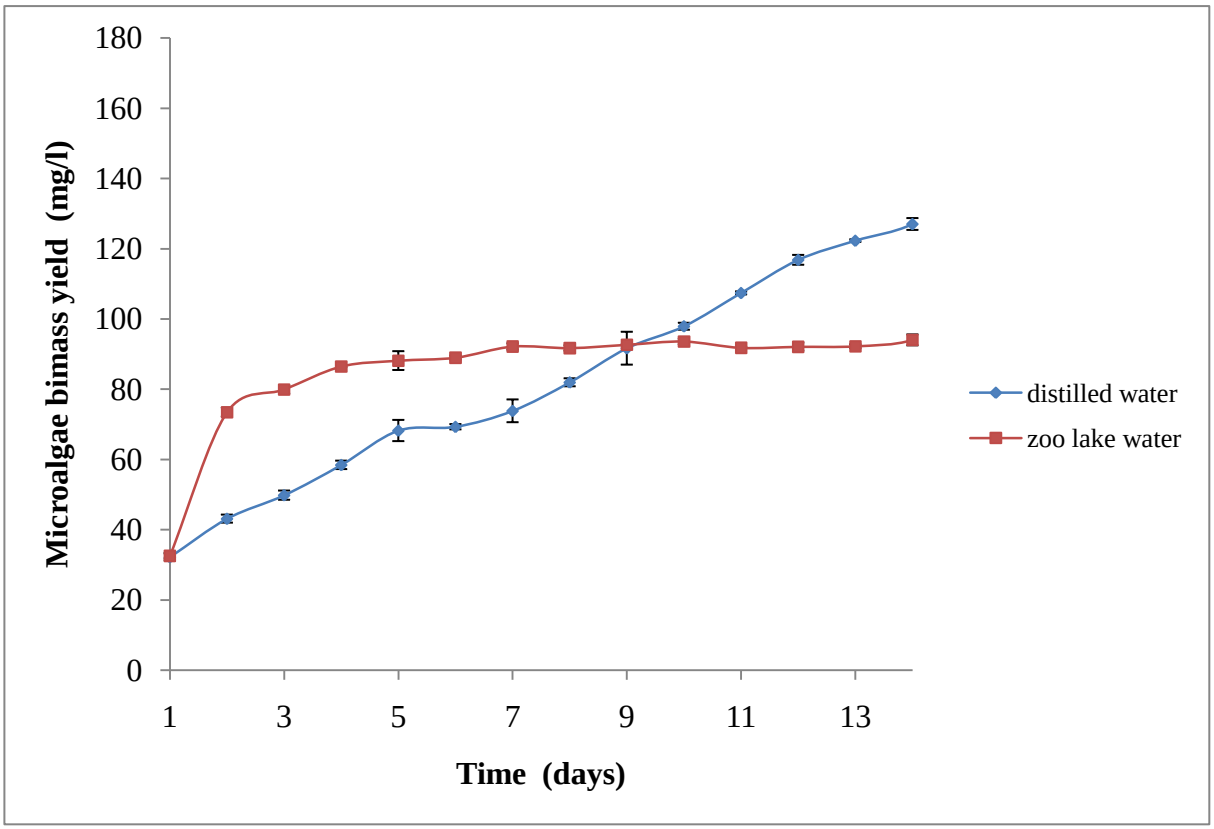


Figure 3-1: Graphs representing the most effective culturing water for the Zoo Lake microalgae, under 6 000lux for 24 hours, 1 000mg/l phosphate and 150mg/l nitrogen ions.

3.1.2 Establishing a culture that is high in CO_2 tolerance

When the microalgae from Zoo Lake were collected, it was not known whether they could tolerate high CO_2 levels. In determining this ambient was bubbled into a set of culturing flasks while pure CO_2 gas was bubbled into another set of culturing flasks. Visual observations under a light microscope revealed that cultures bubbled with ambient air contained a number of microalgal species that differed morphologically. However, in cultures that were bubbled with pure CO_2, only a single dominant microalgae species was obtained. This dominant microalgal species was used in the subsequent experiments. It should be noted that there were no attempts made to isolate a monoalgal culture but CO_2 was used to selectively grow this high CO_2

tolerant species. Due to technical problems with the camera attached to the microscope, pictures showing the morphological differences between the species in ambient and CO_2 bubbled cultures could not be developed.

3.2 Identification of the microalgae

High CO_2 tolerant microalgae were unknown, and morphological features observed could not give conclusive identification. Molecular methods were employed to conclusively identify the Zoo lake microalgae that flourish in elevated CO_2 level. The Central Analytical Facility at Stellenbosch University was engaged to identify the microalgae by means of DNA analysis. Polymerase Chain Reaction (PCR) amplification was performed on the Internal Transcriber Spacer region 2 (ITS), produced a single DNA band (Figure 3-2).

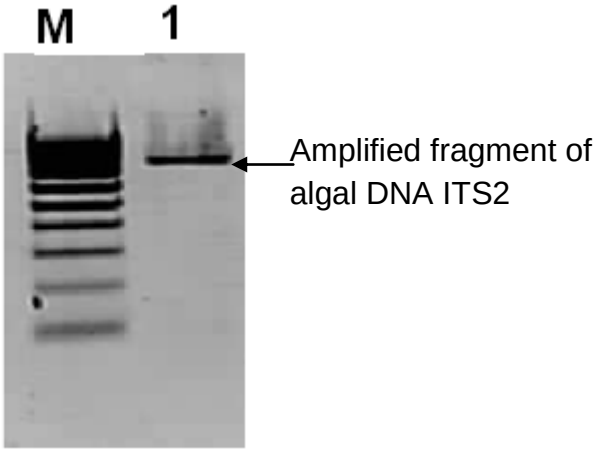
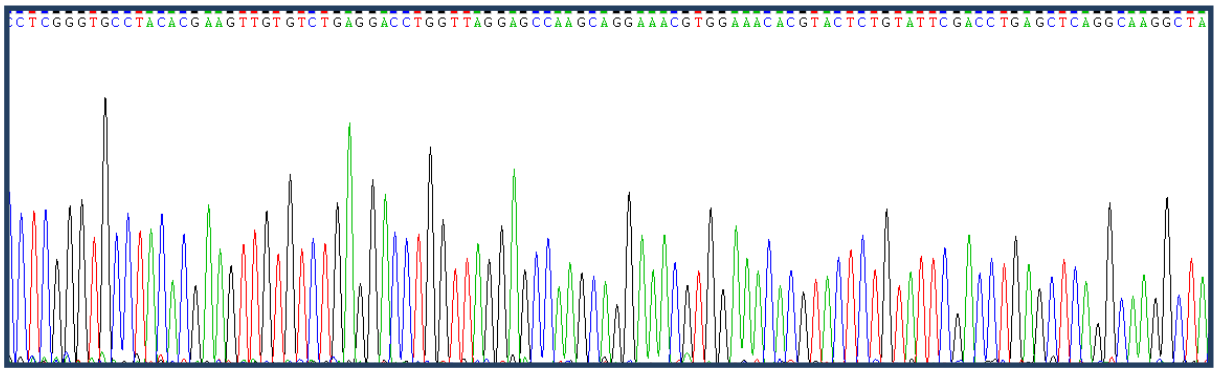


Figure 3-2: Agarose gel showing amplified algae DNA fragment. Lane M: Molecular marker; Lane 1: DNA fragment of algae collected from Johannesburg Zoo Lake.

Sequenced DNA were viewed on chroma V2.33, which showed the presence of a single species (Figure 3-3)

A



B

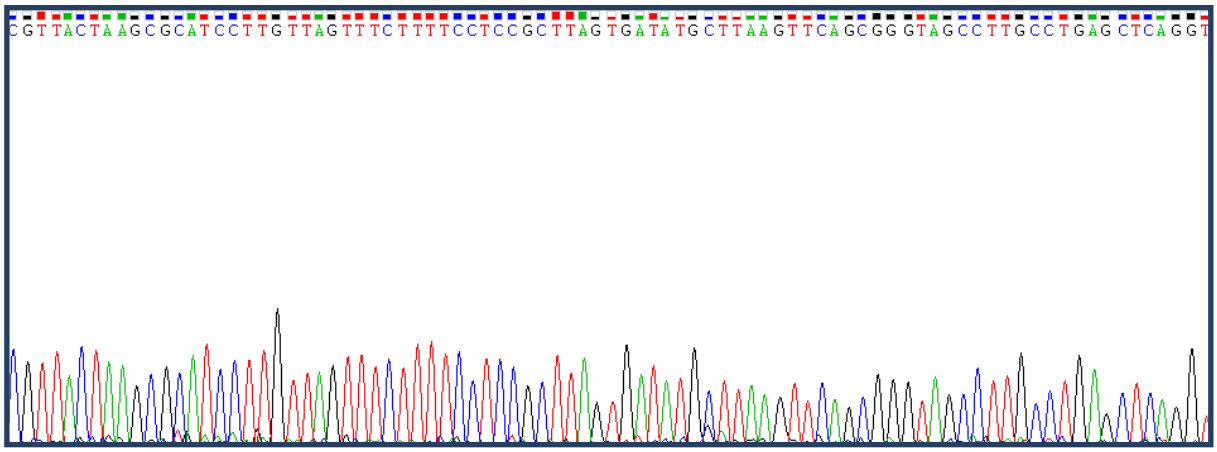


Figure 3-3: Chromatograms of DNA sequence of algae from Johannesburg Zoo Lake. A: DNA Sequence analysis of the ITS2 region of algae isolated from Johannesburg Zoo Lake using forward primer. B: DNA Sequence analysis of the ITS2 region using reverse primer.Viewed using chromas v2.33

DNA alignment of the ITS2 region of the Zoo Lake *Desmodesmus sp* (Genebank accession numbers EUB502836.1) revealed a 99% identity as shown in Figure 3-4

```
Desmodesmus      GATACGTAGTGTGAATTGCAGAATTCCGTGAACCATCGAATCTTTGAACGCATATTGCGC 300
Zoo              --------------ATTGCAGA-TTCCGTGA-CCATCGAATCTTTGAACGCATATTGCGC 44
                             ******** ******** **************************

Desmodesmus      TCGACCCCTCGGGGAAGAGCATGTCTGCCTCAGCGTCGGTTTACACCCTCACCCCACTTC 360
Zoo              TCGACCCCTCGGGGAAGAGCATGTCTGCCTCAGCGTCGGTTTACACCCTCACCCCACTTC 104
                 ************************************************************

Desmodesmus      CCTCACAGGAAGCGCTTGCTGCGTCGTTTGACCAGCAACTGGGATGGATCTGGCCCTCCC 420
Zoo              CCTCACAGGAAGCGCTTGCTGCGTCGTTTGACCAGCAACTGGGATGGATCTGGCCCTCCC 164
                 ************************************************************

Desmodesmus      AATCGAAGCAATTCGATTGGGTTGGCTGAAGCACAGAGGCTTAAACTGGGACCCGTACCG 480
Zoo              AATCGAAGCAATTCGATTGGGTTGGCTGAAGCACAGAGGCTTAAACTGGGACCCGTACCG 224
                 ************************************************************

Desmodesmus      GGCTCAACTGGATAGGTAGCAACACCCTCGGGTGCCTACACGAAGTTGTGTCTGAGGACC 540
Zoo              GGCTCAACTGGATAGGTAGCAACACCCTCGGGTGCCTACACGAAGTTGTGTCTGAGGACC 284
                 ************************************************************

Desmodesmus      TGGTTAGGAGCCAAGCAGGAAACGTGGAAACACGTACTCTGTATTCGACCTGAGCTCAGG 600
Zoo              TGGTTAGGAGCCAAGCAGGAAACGTGGAAACACGTACTCTGTATTCGACCTGAGCTCAGG 344
                 ************************************************************

Desmodesmus      CAAGGCTACCCGCTGAACTTAAGCAT---------------------------------- 626
Zoo              CAAGGCTACCCGCTGAACTTAAGCATATCACTAAGCGGAGGAAAAGAAACTAACAAGGAT 404

                 *************************
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Figure 3-4: Multiple sequence alignment of the ITS2 region of algae isolated from Johannesburg Zoo Lake (Zoo) and *Desmodesmus sp* (Accession number EU502836.1). The star (*) represents regions that are identical. Percentage identity was 99%. Alignment was performed using ClustalW.

Morphological identification using a light microscope showed that microalgae was similar to that of *Desmodesmus sp* (Figure 3-5) (image obtained from botany.natur.cuni.cz/algo/caup-list.html).

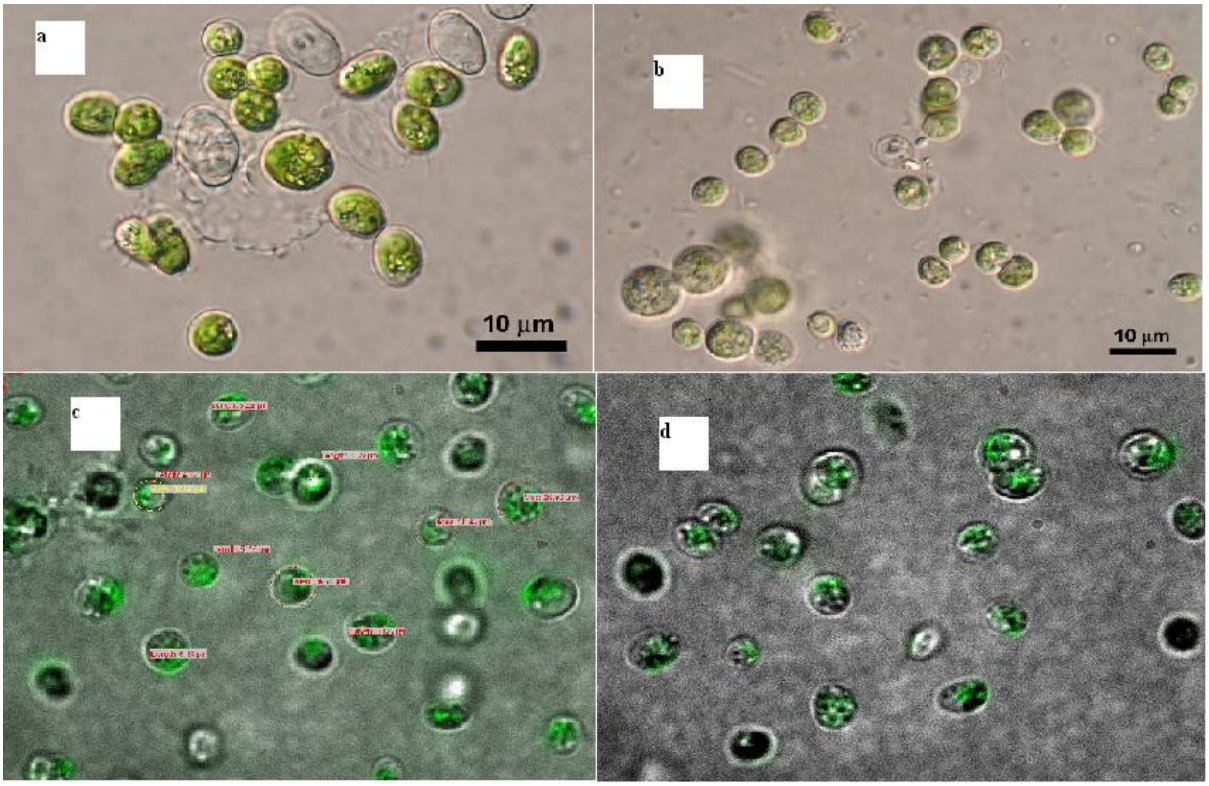

Figure 3-5: (a) and (b) are photographs of *Desmodesmus sp*. taken from botany.natur.cuni.cz/algo/caup-list.html and (c) and (d) are photographs of the microalgae under investigation (taken by Dr. Ben Loos, CAF, Stellenbosch University).

3.3 Effect of light intensity and photoperiod on microalgae growth

3.3.1 Effects of light intensity

Effect of light intensity on the growth of *Desmondesmus sp was* investigated. Cultures that were not exposed to any light showed a very slow growth rate, averaging 0.04 per day, and a total microalgal dry biomass yield of 31.99mg/l on day 12 of culturing. When microalgae were exposed to a light intensity of 3 000lux, a growth rate of 0.28 per day was observed, with a maximum dry biomass of 418.32mg/l on day 12. However, the growth rate improved to 0.45 per day using 6 000lux, although the dry weight yield of 374.33mg/l on day 12 was slightly lower than

that of the microalgae exposed to 3 000lux. Results in terms of total dry weight of algae grown under 8 000lux and 10 000lux were 654.65mg/l and 690.00mg/l on day 12 (Figure 3-6), and their specific growth rates were 0.50 per day and 0.56 per day respectively (Figure 3-9).

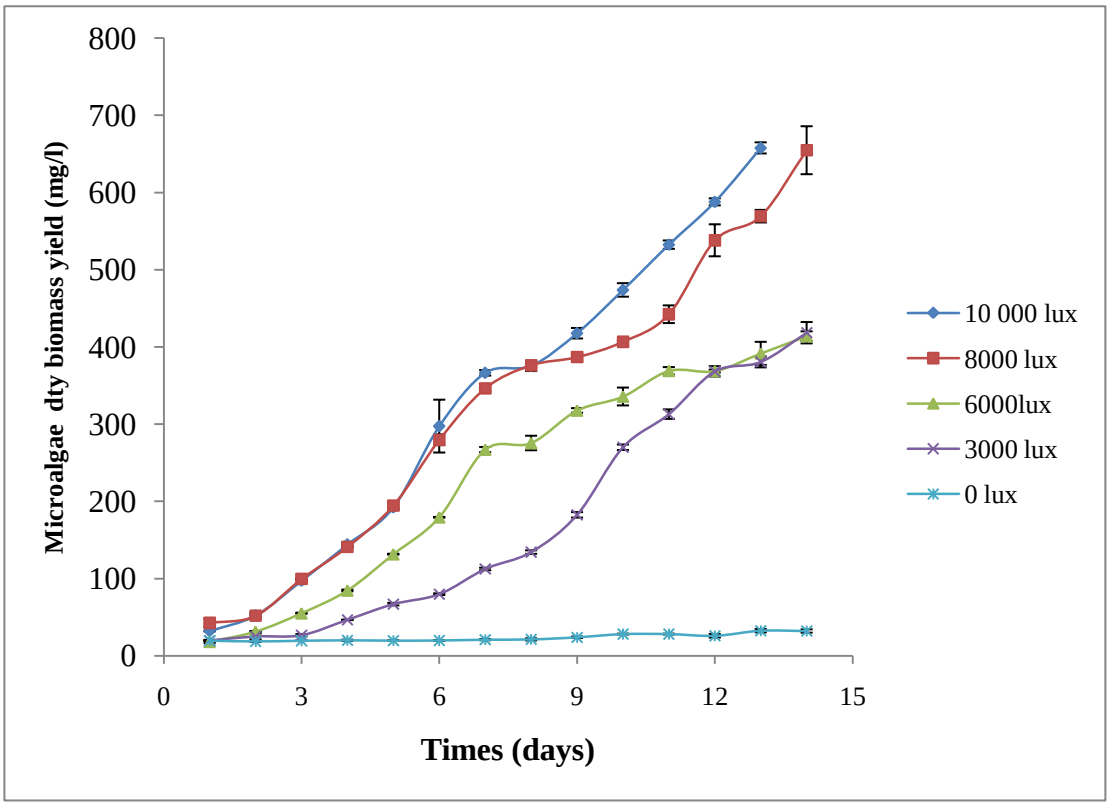

Figure 3-6: Effect of light intensity on microalgal growth. The light intensity was varied from 0 to10 000lux over a 12-hour photoperiod. Atmospheric air was bubbled at 500ml/min. Microalgae were grown in standard Beijerinick medium.

3.3.2 Effects of photoperiod

The duration of light exposure to which microalgae may influence their circadian rhythm and affect their growth rate overall. As a result an experiment was set up to determine the influence of length of daylight on the growth rate and biomass yield of the microalgae.

When the duration of the exposure to light was varied from 0 to 24 hours per day at 6 000lux, the growth rate and dry weight yield increased proportionally with the hours. When no light was provided to the algae, a growth rate of 0.04 per day and a total of 31.99mg/l of biomass were achieved. Exposing microalgae for 8 hours a day at 6 000 lux resulted in a total microalgal biomass of 272.72mg/l on day 12, and a growth rate of 0.35 per day. When the light period was increased to 12 hours a day, the growth rate increased to 0.47 per day, with a dry biomass yield of 370.67mg/l on day 12 of culturing. Exposure of the microalgae to light for 20 and 24 hours a day showed a similar growth rate, 0.68 per day, but their dry biomass yields on day 12 were 559.06 and 665.43mg/l respectively (Figure 3-7).

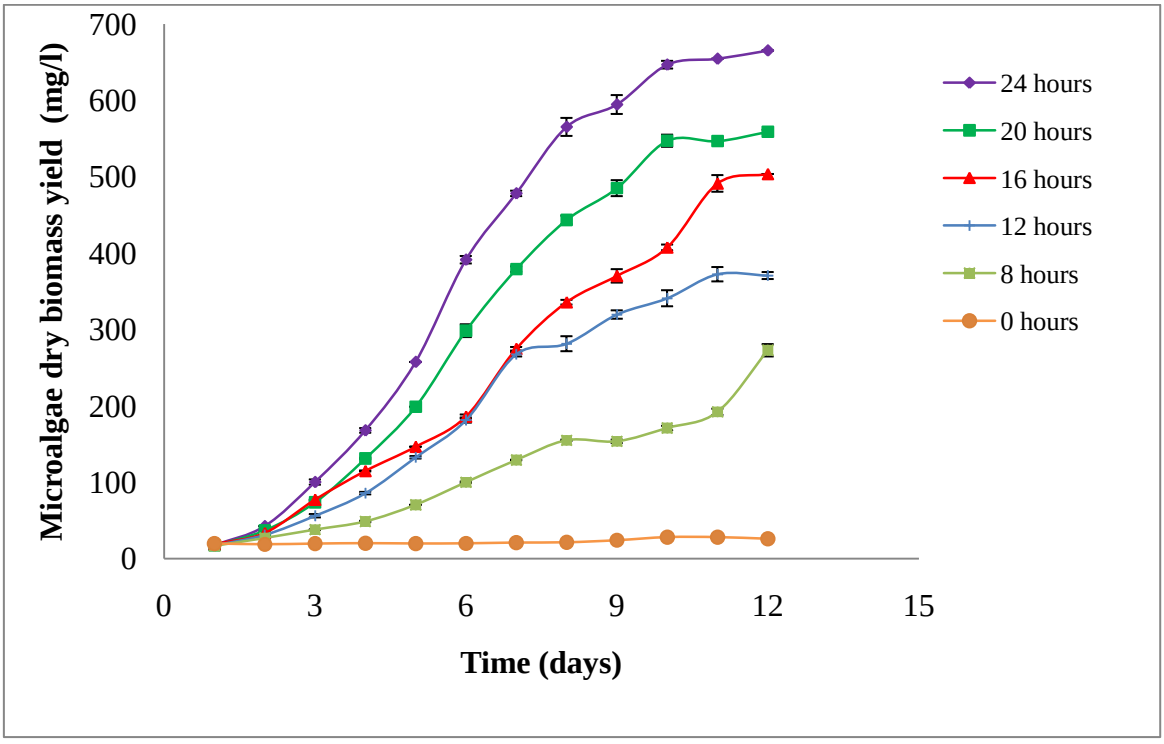

Figure 3-7: Effect of varying photoperiods on microalgal growth at 6 000lux. Atmospheric air was bubbled at 500ml/min. The photoperiod was varied from 0 to 24 hours a day. In total darkness the biomass yield was 31.99mg/l, while 24 hours of light achieved the highest amount of biomass with 665.43mg/l.

3.3.3 Effects of total light intensity

To test the conclusions made on light intensity and photoperiod experiments, an analysis of the relationship between the total light intensity and growth rate were carried out. Total light intensity is the product of the measured light intensity and the number of hours during which the microalgae are exposed to the source of light. The growth rates used were taken from the results of the light intensity and photoperiod experiments (Figures 3-6 and 3-7). From zero lux total light intensity to 96 000lux total light intensity, the growth rate was seen to increase in a linear fashion, but at 120 000lux the growth rate appeared to have reached its maximum point. Any further increase in total light intensity did not alter the growth rate. Total light intensity is a calculated value and not the light intensity read from the light meter.

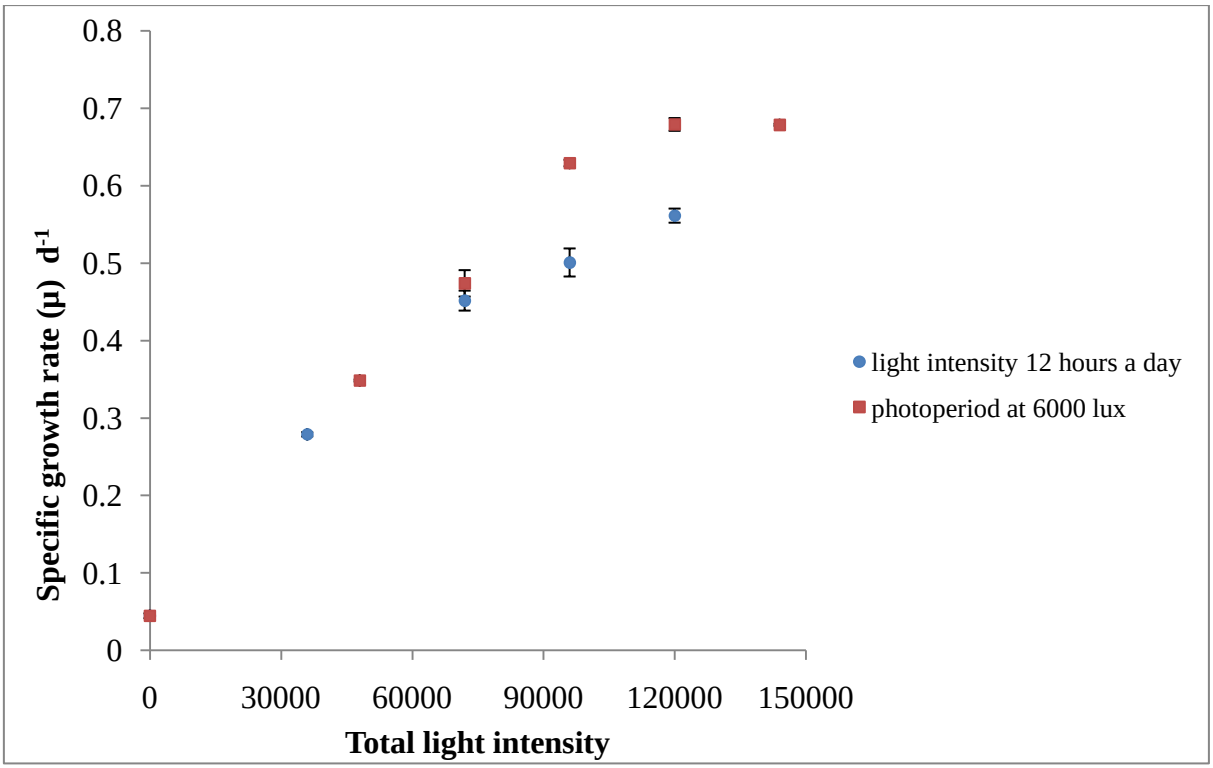

Figure 3-8: Effect of total light intensity on the microalgal growth rate. The total light intensity was varied from 0 to 144 000lux per day. A total light intensity of 0lux per day resulted in a low growth rate of 0.04 per day. The highest growth rate was achieved at 0.68 per day at 120 000lux per day. Atmospheric air was bubbled at 500ml/min.

3.4 Effects of nutrients on microalgal growth

3.4.1 Effect of nitrogen ion concentration

Once the optimal light intensity for the culturing of the *Desmodesmus sp* microalgae had been determined, experiments were carried out to investigate the importance of nitrogen ions on their growth and biomass yield. Apart from the nitrogen, the concentration of which was varied in this experiment, the growth medium contained all the other nutrients of Beijerinick medium (Appendix A1). The culturing flasks to which no nitrogen ions had been added yielded an average growth rate of 0.08 per day and a maximum biomass of 69.74mg/l on day 12 (Figure 3-9). At nitrogen ion concentrations of 18.75mg/l and 37.5mg/l, rapid growth was observed for the first four days of culturing. Thereafter the increase in growth appeared to level off gradually (Figure 3-9). The standard Beijerinick medium contained 150mg/l of nitrogen ions, and the microalgae cultured at this concentration had a growth rate of 0.53 per day and an average biomass yield of 361.32mg/l after the 12 days of culturing. When 300mg/l of nitrogen concentration was added to the medium, the growth rate increased to 0.67 per day, and by day 12 the total dry biomass was 628.34 mg/l. A further increase in the concentration of nitrogen ions to above 300mg/l resulted in a slight decrease in both growth rate and dry biomass. Using concentrations of 375, 750 and 1 500mg/l of nitrogen ions yielded lower total dry biomass at 511.91, 480.85 and 463.82mg/l, while their growth rate also decreased to 0.65, 0.48 and 0.46 per day respectively (Figure 3-11).

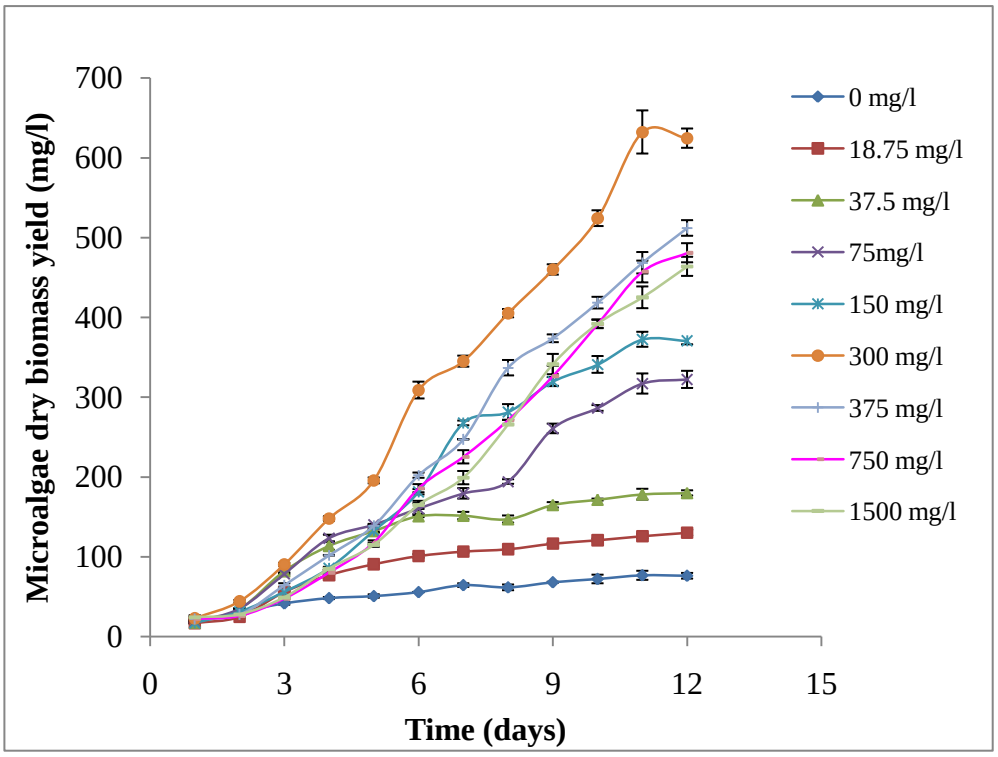

Figure 3-9: Effect of nitrogen ion concentration on microalgal growth. The concentration of nitrogen ions was varied from 0 to 1 500mg/l under 6 000lux for 24 hours a day. A dry biomass of 76.05mg/l was obtained using 0mg/l of nitrogen ions and a high dry biomass of 624.46mg/l was obtained using 300mg/l of nitrogen ion concentration.

To determine whether nitrogen ions can limit microalgal growth, three algal culturing flasks containing 0, 18.75 and 37.5mg/l nitrogen ions were allowed to grow for additional six days. The three different cultures reached a stationary phase on day 12, 150mg/l of nitrogen ions were added to the cultures that had originally contained 18.75 and 37.5mg/l nitrogen ions. The growth rate suddenly increased two fold (0.13 to 0.26) in the first culture (initial nitrogen ion concentration of 18.75mg/l), while that of the second culture (initially containing 37.5mg/l nitrogen ions concentrations) was even higher, at 2.5 times higher than the earlier growth rate (0.14 to 0.35 per day). The overall dry biomass yield for the two cultures rose from 118.26 and 165.56mg/l respectively on day 12 to a maximum of 356.22 (3 fold increase) and 597.243mg/l (3.6 fold increase) respectively on day 18, as shown in Figure 3-10. A control was

included that contained no nitrogen ions added to the culture, and no growth increase was observed after day 18 (Figure 3-10).

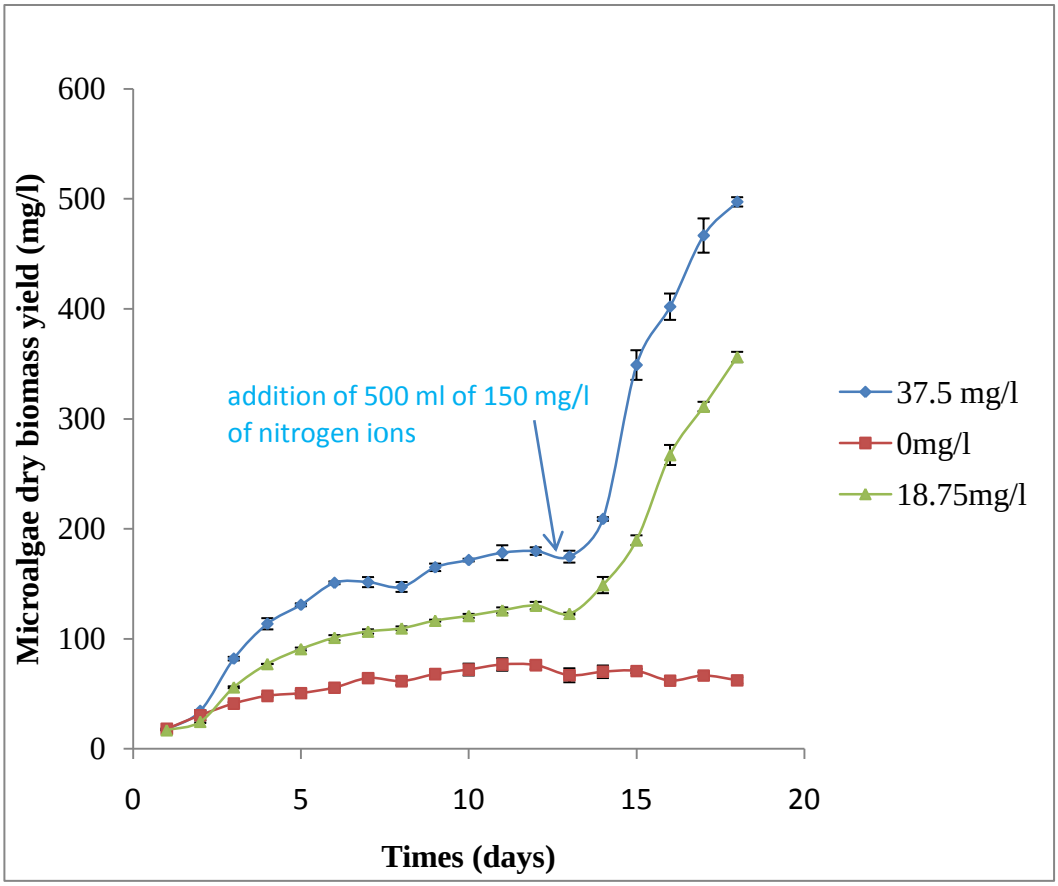

Figure 3-10: Effect of adding nitrogen ions to a nitrogen ion-depleted medium. Cultures containing 0, 18.75 and 37.5mg/l nitrogen ions with a light intensity of 6 000lux and a photoperiod of 24 hours a day were grown until microalgal growth leveled off. Once growth ceased, 500ml of 150mg/l nitrogen ions were added to cultures originally containing 18.75 and 37.5mg/l of nitrogen ions. A secondary growth curve was achieved with growth rates of 0.26 and 0.35 per day for 18.75 and 37.5mg/l respectively**.** Atmospheric air was bubbled at 500ml/min.

3.4.2 Effect of nitrogen ions on growth rate and biomass yield

A theoretical analysis of the microalgal biomass yield obtained at different nitrogen ion concentrations as described in section 2.3.6 were undertaken. An approximate formula ($CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$) suggested by Grobelaar (2004) was used to make an expected estimate of the dry biomass yield. At low concentrations of nitrogen ions,

most of the nitrogen in the medium was converted into microalgal biomass. At a concentration of 18.75mg/l nitrogen ions, approximately 69.34% of the nitrogen in the medium was used to make biomass. However, the conversion percentage to biomass decreased with an increase in concentration of the nitrogen ions. Although microalgae grown with 300mg/l nitrogen ions recorded the highest growth rate and dry weight yield of 0.67 per day and 628.34mg/l, only 37.34% was converted into biomass after 12 days of culturing. Table 3-1 illustrates the nitrogen ion conversion percentages.

Table 3-1: *Showing the obtained biomass and the expected biomass yield in relation the nitrogen ion concentration in the medium*

Nitrogen ion concentration (mg/l)	Microalgae biomass obtained (mg/l)	Expected microalgae biomass yield (mg/l)	% conversion of nitrogen ions to microalgae biomass (mg/l)
0	69.74	69.74	
18.75	118.26	170.545	69.34
37.5	165.56	271.35	61.01
75	302.49	472.96	63.96
150	361.32	876.1801	41.24
300	628.34	1682.62	37.34
375	511.91	2085.84	24.54
750	480.85	4101.94	11.72
1500	463.82	8134.141	5.70

The growth rate increased with a higher concentration of nitrogen ions. A slight reduction in growth rate was observed in nitrogen ion concentrations above 300mg/l. At concentrations of 750 and 1 500mg/l the growth rate appeared to stabilize (Figure 3-11).

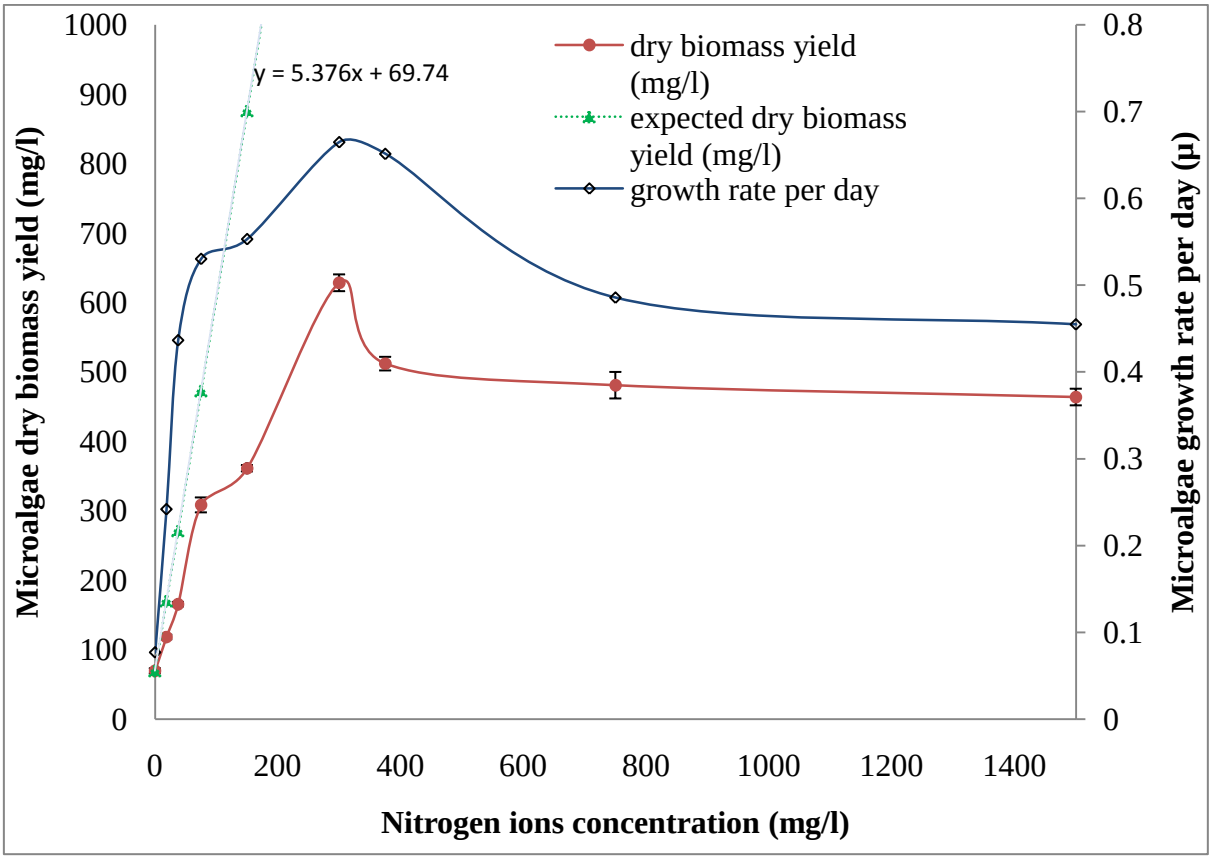

Figure 3-11: Dry biomass yield of microalgae cultured under different nitrogen ion concentrations after 12 days of culturing. Nitrogen ion concentrations were varied from 0 to 1 500mg/l. The growth rate increased from 0.08 per day when using 0mg/l nitrogen ions to as high as 0.67 per day when using 300mg/l nitrogen ions. Theoretical analysis produced a line of expected dry biomass per given amount of nitrogen ions in the medium.

3.4.3 Effect of different types of nitrogen ion

The experiments in this section were conducted to determine the sensitivity of *Desmodesmus sp* microalgae to different forms of nitrogen ion. Three types of inorganic nitrogen were selected: ammonium nitrate, potassium nitrate and ammonium chloride. Microalgae cultured with potassium nitrate and ammonium chloride recorded a growth rate of 0.59 per day for both, while ammonium nitrate achieved 0.62 per day. The total dry weight obtained on day 12 for potassium nitrate

was 654.65mg/l, ammonium chloride 676.23mg/l and ammonium nitrate 665.43mg/l (Figure 3-12).

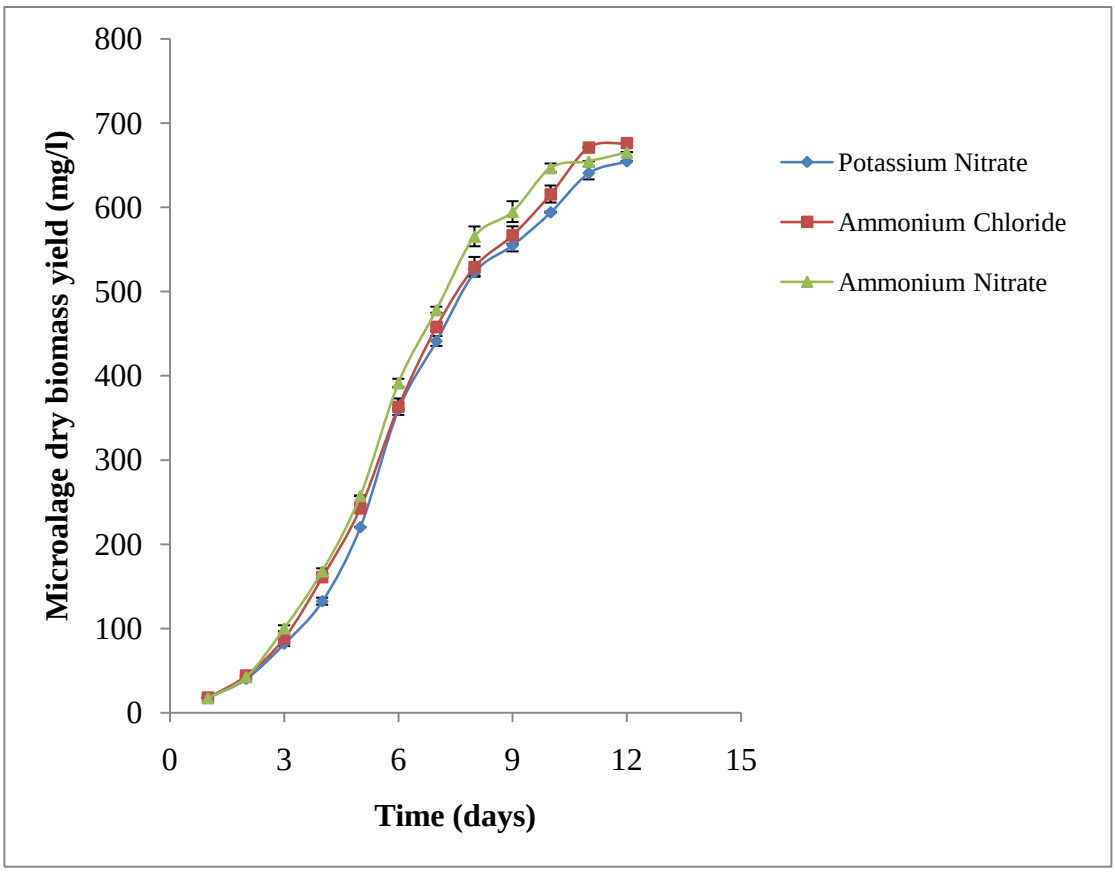

Figure 3-12: Effect of different nitrogen ion species on microalgal growth. Three nitrogen ion sources, ammonium nitrate, ammonium chloride and potassium nitrate, were used to investigate the effect of different nitrogen ion species on the growth of microalgae. A nitrogen ion concentration of 300mg/l was used. Light intensity of 6 000lux and a photoperiod of 24 hours a day and an atmospheric air flow rate of 500ml/min were used.

3.4.4 Effect of total phosphate ions

Phosphates play a pivotal role in the culturing of microalgae because they aid the metabolic process and make up the nucleic acids. The experimental investigation undertaken sought to clarify the role of the concentration of total phosphate ions in promoting growth rate and dry biomass yield. Culture containing the standard

Beijerinick nutrients, with the exception of varied phosphate concentration ions between 0–4 000mg/l in seven different culturing flasks were used. Generally the maximum dry biomass yield and growth rate increased linearly at total phosphate ion concentration of 0 – 500mg/l, thereafter a slight decrease was recorded. Figure 3-13 shows the effect of phosphate ions on microalgae biomass yield, while Figure 3.14 demonstrates how the growth rate is affected by the concentration of phosphate ions. When no phosphate was added to the medium, a total dry weight of 396.33mg/l was obtained at an average growth rate of 0.20 per day. The maximum growth rate of 0.63 per day and total dry weight yield of 840.96mg/l was achieved with a total phosphate ion concentration of 500mg/l. At higher phosphate ion concentrations of 1 000, 2 000 and 4 000mg/l the recorded dry weights were 704.05, 695.19 and 601.82mg/l with growth rates of 0.54, 0.511 and 0.48 per day respectively (Figures 3-13 and 3-14).

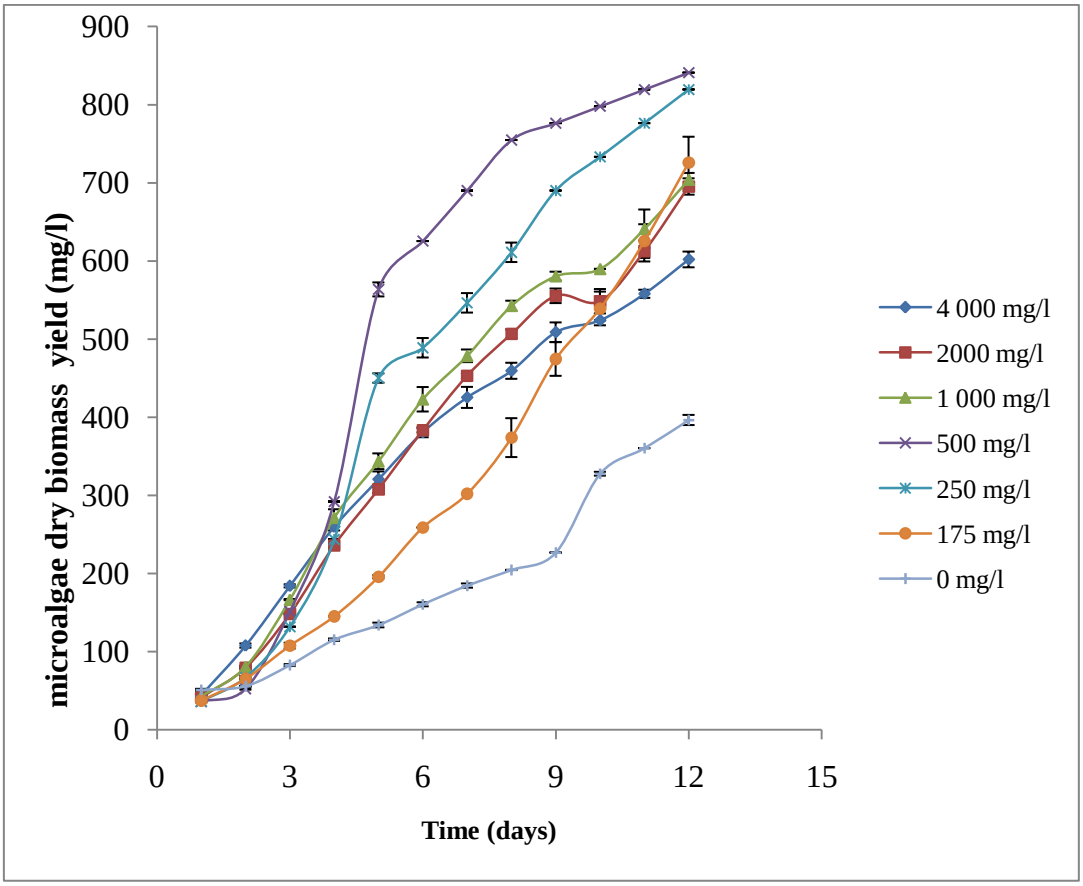

Figure 3-13: Effect of total phosphate ion concentrations on microalgal growth. The total phosphate ion concentration was varied from 0 to 4 000mg/l. The highest dry biomass of 840.96mg/l after 12 days was achieved in a culture containing 500mg/l of total phosphate ions concentration. The culture was exposed to a light intensity of 6 000lux for 24 hours a day and 300mg/l of nitrogen ions concentration. Atmospheric air was bubbled at 500ml/min.

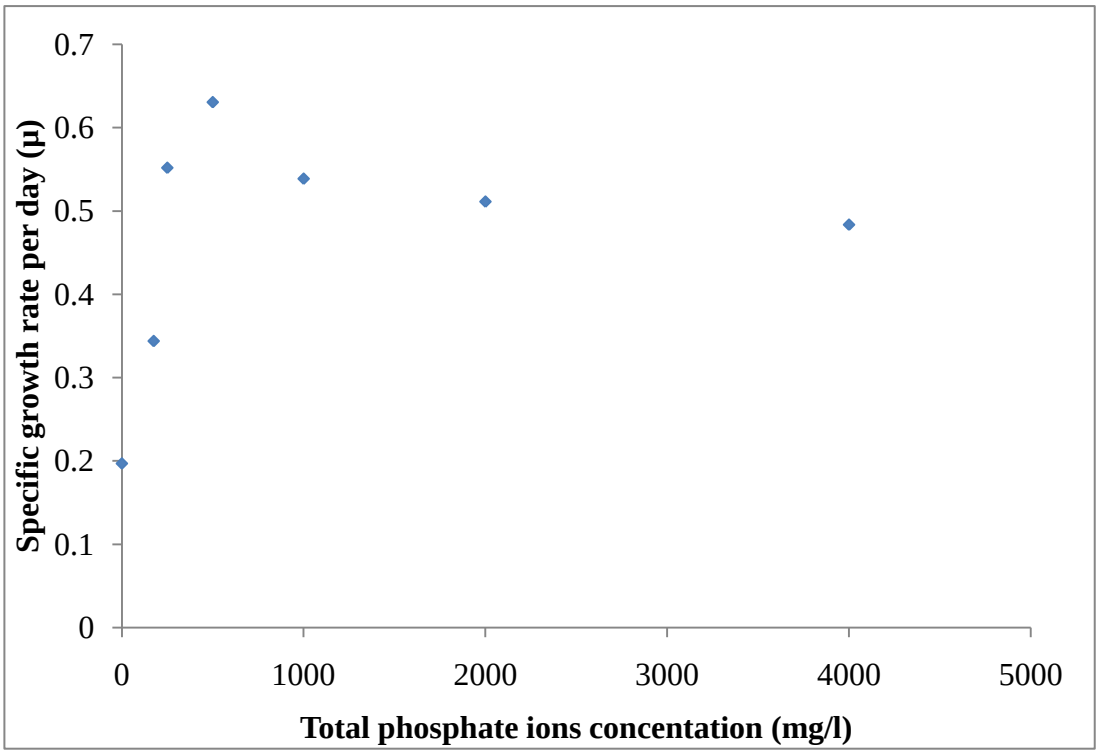

Figure 3-14: Effect of total phosphate ions on microalgal growth rate. The total phosphate concentration was varied from 0 to 4 000mg/l. The highest growth rate of 0.84 per day was achieved using a total phosphate concentration of 500mg/l. Growth conditions were; light intensity of 6000lux for 24 hours a day and 300mg/l of nitrogen ions concentration. Atmospheric air was bubbled at 500ml/min.

3.5 Effect of CO_2

3.5.1 Effect of pH on microalgal growth

The availability of CO_2 species in the medium is influenced by pH, which therefore affects microalgal growth considerably. Experiments were carried out to find the pH most suitable for maximal growth. Pure CO_2 gas was bubbled through the culturing flasks at 10ml/min; the pH was stabilized using HEPES buffer, nitrogen ion concentration of 300mg/l, and 500mg of total phosphate ion concentration were used. The initial pH of unbuffered Beijerinick culturing medium was 6.9. Bubbling pure CO_2 at 10ml/min resulted in a lag phase for the initial 3 days; thereafter microalgal growth proceeded into the exponential phase. A maximum growth rate of

0.61 per day and a total dry biomass yield of 388.78mg/l were obtained when the pH of the medium had been adjusted to 6.5. When the adjusted pH was to 7 and 6, a total dry biomass yield of 344.36mg/l and 346.30mg/l were achieved during the culturing period while

Growth rates of 0.50 and 0.54 per day respectively were observed during the exponential growth phase. As the pH decreased below 6.5, the growth rate and the overall dry biomass declined. A total dry biomass of 164.10mg/l was obtained at a growth of 0.34 per day in a medium buffered at pH 5.5. When the cultures were not buffered, the growth rate was almost similar to that of a pH of 6.5, and a maximum dry biomass yield of 357.73mg/l was obtained (Figure 3.15).

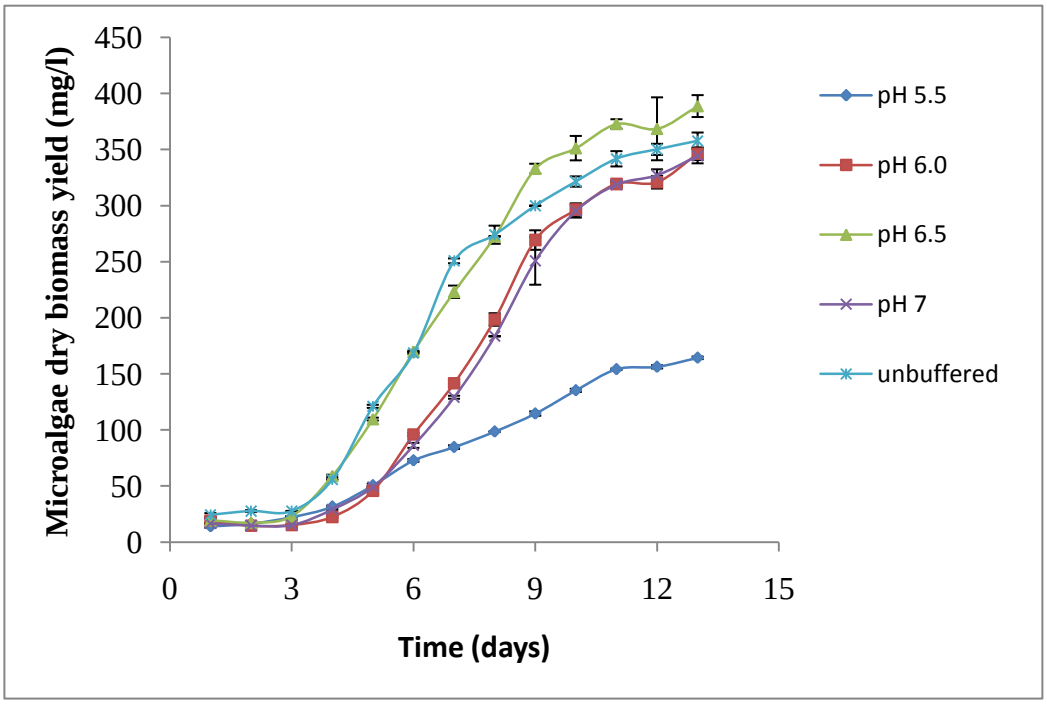

Figure 3-15: Effect of pH on microalgal growth under light intensity of 10 000lux for 24 hours a day in a nutrient medium containing 300mg/l nitrogen ions and 500mg/l of total phosphate ions. Pure CO_2 was bubbled continuously at 10ml/min.

3.5.2 Effect of adding CO_2

Carbon dioxide availability plays a pivotal role in the dark reaction of photosynthesis, which fixes the CO_2 into the biomass building-block, glucose. The next step in this study was to test the effect of adding pure CO_2 gas to the cultures of *Desmodesmus sp* microalgae. A nitrogen ion concentration of 300mg/l and a total phosphate ion concentration of 500mg/l were used in this experiment. In order to determine the effects of CO_2, an experiment was set up as well as the control that had only ambient air bubbled at a flow rate of 50ml/min, from which on day 12 a total dry biomass of 86.76mg/l was obtained. However, when pure CO_2 was bubbled at the same rate as the atmospheric air, the growth rate increased from 0.11 to 0.63 per day, showing a fivefold increase. The total dry biomass yield also increased by a factor of five from 86.76 to 496.81mg/l after 12 days (Figure 3-16).

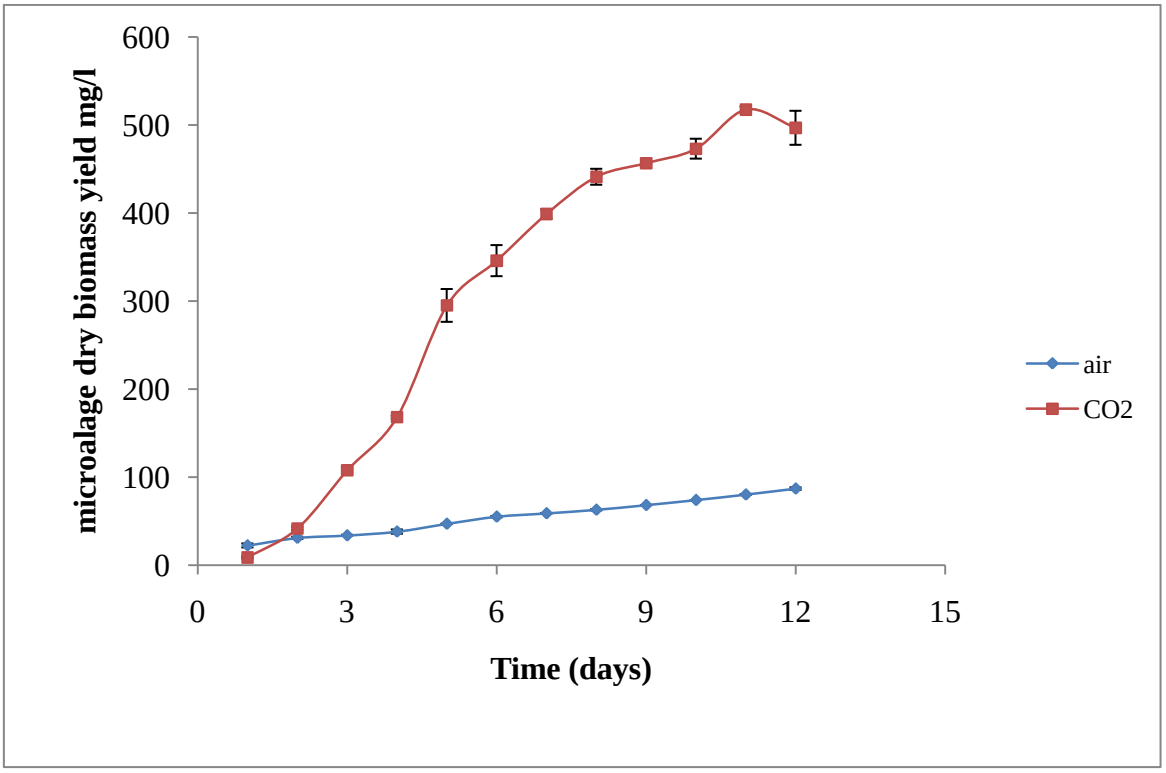

Figure 3-16: Effect on microalgal growth of adding CO_2 at 50ml/min under a light intensity of 10 000lux for 24 hours a day in a nutrient medium containing 300mg/l nitrogen ions and 500mg/l of total phosphate ions.

3.5.3 Effect of flow rate

After finding that the addition of CO_2 gas to the microalgal cultures resulted in a significant increase in both specific growth rate and dry biomass yield, subsequently test were carried out to find out the most efficient flow rate for bubbling CO_2 gas into the culturing flasks. The higher the flow rate, the more CO_2 is fed to algae in a given time. Flow rates of 10, 20, 50, 250ml/min of pure CO_2 gas were bubbled through the flasks to observe their effects on growth rate and dry biomass yield. The investigations indicated that very high flow rates resulted in reduced growth rates and biomass yields after 12 days of culturing. At a flow rate of 250ml/min the specific growth rate was at 0.28 per day and the yield a maximum of 70.01mg/l of dry

biomass. When pure CO_2 gas was bubbled at 100ml/min the growth rate was 0.30 per day, and the resultant biomass was 279.49mg/l. The most effective flow rate was 50 ml/min, which achieved an overall growth rate of 1.26 per day and the highest dry biomass of 496.81mg/l. At 20ml/min the growth rate was 0.70 per day and the biomass yield 366.21mg/l. The lowest flow rate used in this experiment was 10ml/min, which resulted in a growth rate of 0.61 per day and a dry biomass of 357.75mg/l (Figure 3-17).

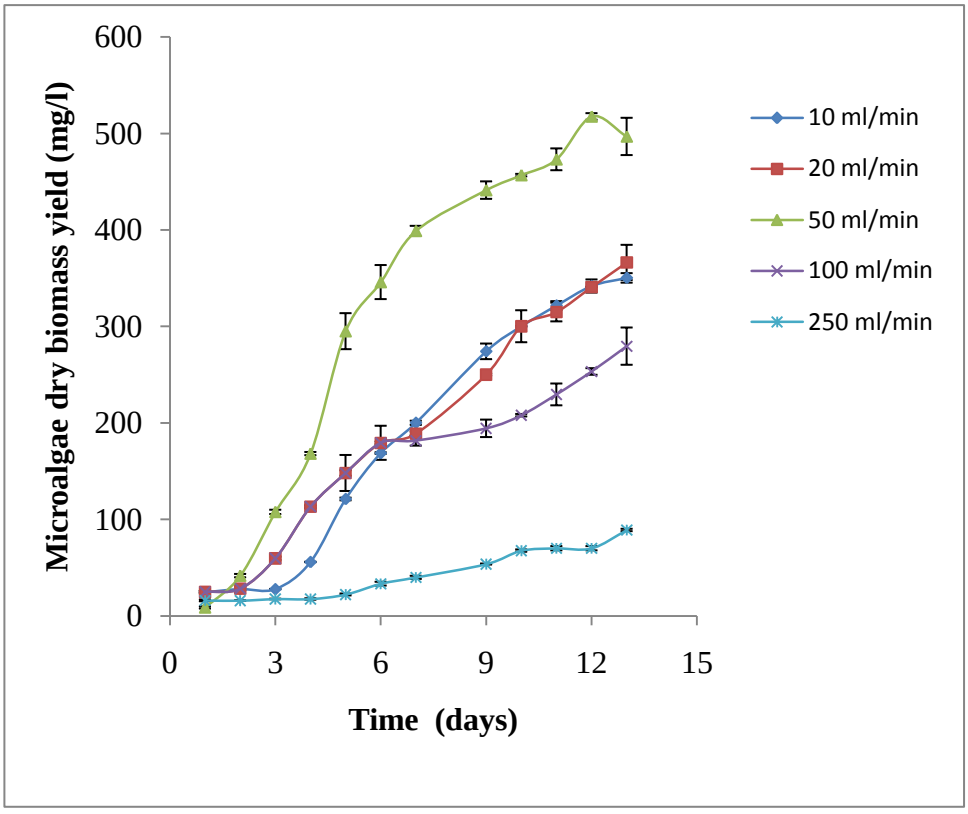

Figure 3-17: Effect of bubbling pure CO_2 at different flow rates on the growth of microalgae under a light intensity of 10 000lux for 24 hours a day in a nutrient medium containing 300mg/l nitrogen ions and 500mg/l of total phosphate ions.

3.5.4 Effect of combined flow rate and concentration of CO_2

Because both the flow rate and concentration of carbon dioxide affected the growth rate and dry biomass yield of the Zoo Lake microalgae, it was necessary to establish

what relation between flow rate and CO_2 concentration would result in optimal growth. Five CO_2 gas concentrations, 5, 10, 25, 50 and 100%, were chosen for these experiments. The flow rates of the gases bubbled through was 20, 50 and 100ml/min.

I. Gas flow rate of 20ml/min

The flask that received a CO_2 gas concentration of 5% yielded the highest dry biomass of 1 055.51mg/l and a specific growth rate of 1.05 per day (Figures 3-18 and 3.21). The total dry biomass yield decreased as the concentration of CO_2 increased. The dry biomass obtained for the CO_2 gas concentrations of 10%, 25%, 5% and 100% in the other four flasks were found to be 809.69, 764.15, 705.83 and 366.21mg/l respectively on day 12 of culturing (Figure 3-18). While the specific growth rates were 1.42, 0.99, 0.89, and 0.70 per day (Figure 3-21).

Because the addition of CO_2 gas affected the pH of the culturing medium, it was checked throughout the experiment. The culturing flask exposed to a CO_2 gas concentration of 5% had an average pH of 6.41; while 10% CO_2 had a pH of 6.16; those with 25 and 50% an average pH of 5.81 and 5.30 respectively; and that with 100% an average pH of 5.01.

When atmospheric air was added to a microalgal culture at 20ml/min the growth rate was very low, at an average of 0.085 per day. A maximum biomass yield of about 68.00mg/l was obtained on day 12 of culturing. The average pH of the flasks bubbled with atmospheric air at 20ml/min was recorded at 6.85.

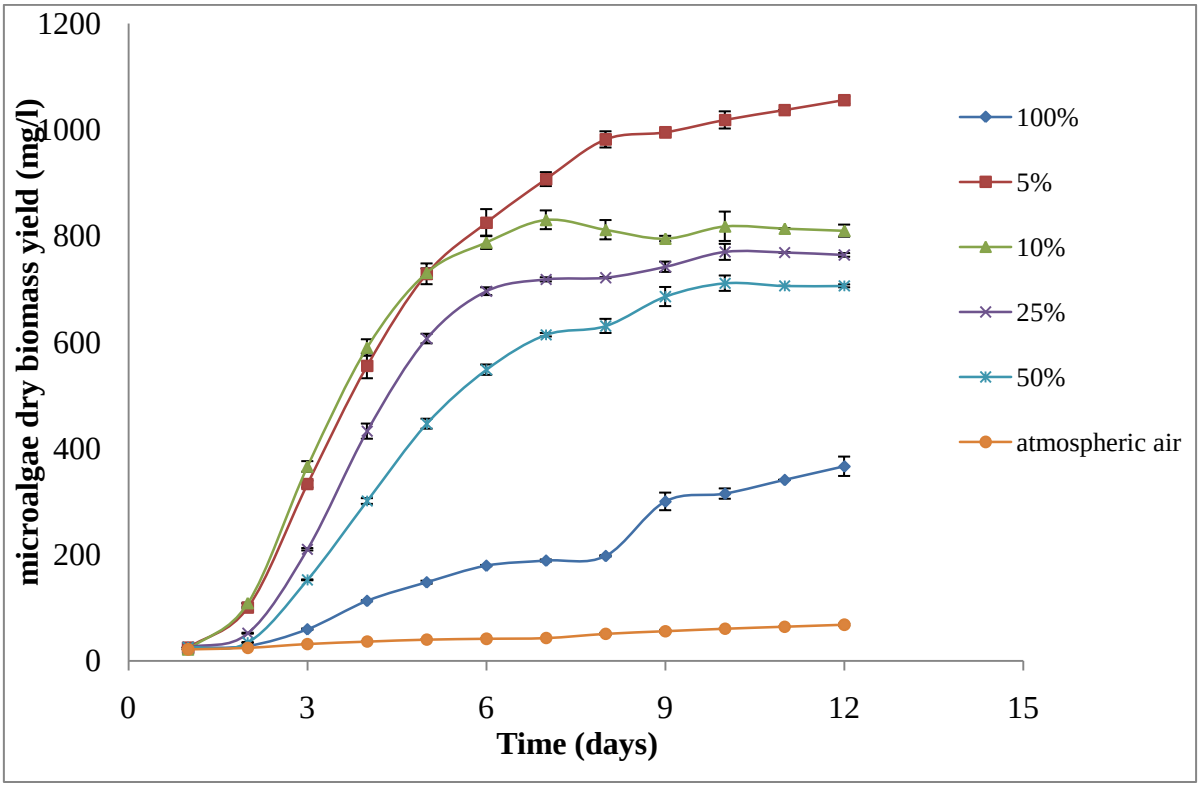

Figure 3-18: Effect of bubbling different CO_2 concentrations at 20ml/min. Microalgal cultures were bubbled with atmospheric air, 5, 10, 25, 50 and 100% of CO_2 at 20ml/min for 24 hours a day under a light intensity of 10 000lux for 24 hours a day in a nutrient medium containing 300mg/l nitrogen ions and 500mg/l of total phosphate ions. The highest dry biomass of 1 055.51mg/l was obtained using 5% CO_2 while the lowest was achieved using atmospheric air with a dry biomass of 68.00mg/l. 100% CO_2 recorded a dry biomass of only 366.21mg/l.

II. Gas flow rate of 50ml/min

When CO_2 was bubbled through the culturing flask at 50ml/min, the overall dry biomass and growth rate improved greatly. A maximum dry biomass of 1 200.63mg/l had been obtained with an average growth rate of 2.00 per day when a CO_2 gas concentration of 5% was used; but when the gas concentration was 10% the biomass yield was 1 080.31mg/l and the growth rate was 1.98 per day (Figures 3-19 and 3-21). Carbon dioxide gas concentrations of 25% and 50% yielded biomass of 846.13 and 794.38mg/l and specific growth rates of 1.89 and 1.61 per day respectively (Figures 3-19 and 3-21). Bubbling with pure CO_2 gas (100%) resulted in

a total biomass of 496.81mg/l and a low specific growth rate of about 1.26 per day. The average pH measured in cultures bubbled with CO_2 gas at 5, 10, 25, 50 and 100% was 6.36, 6.24, 5.86, 5.47, and 5.13 respectively. When atmospheric air was used at 50ml/min, the average growth rate was 0.106 per day, the overall biomass yield after 12 days of culturing was 86.67mg/l, and the average pH was 6.7.

III. Gas flow rate of 100ml/min

Bubbling pure CO_2 at 100ml/min resulted in a lag phase for the initial 3 days; thereafter microalgal growth was in the exponential phase. When CO_2 was bubbled at 100ml/min, the overall dry weight yield over the culturing period decreased slightly when compared with that of 50ml/min. Nevertheless both 5% and 10% concentrations resulted in a high dry biomass yield of 1 001.39 and 928.93mg/l, while their specific growth rate was 1.31 and 1.25 per day respectively (Figures 3-20 and 3-21). Total biomass yield declined with higher concentrations of CO_2 at 25 and 50% the resultant biomass was 883.22 and 773.35mg/l, with an average specific growth rate of 1.08 and 0.82 per day. The growth rate was greatly reduced to about 0.3 per day in 100% CO_2 gas, while biomass fell to as low as 279.46mg/l (Figure 20 and Figure 21).

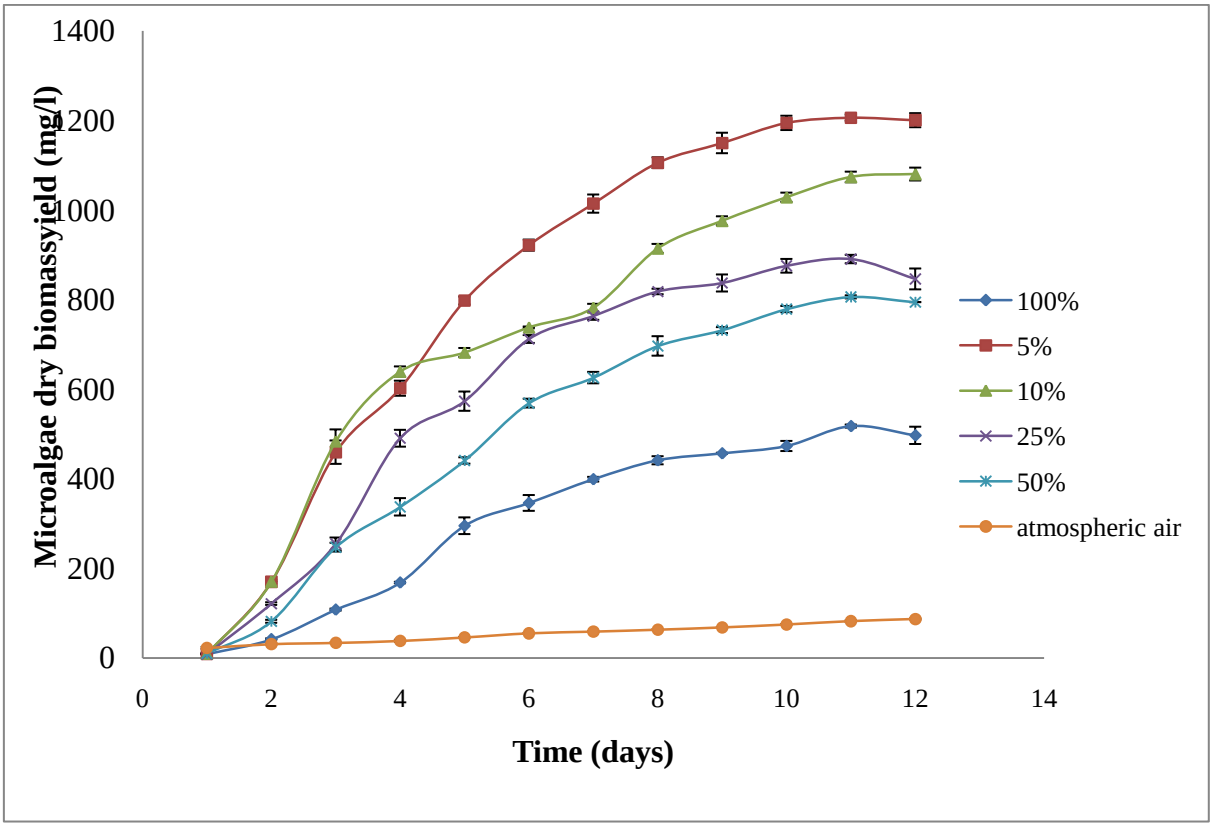

Figure 3-19: Effect of bubbling different CO_2 concentrations through the cultures at 50ml/min. Ambient air, 5, 10, 25, 50 and 100% of CO_2 were bubbled at 50ml/min for 24 hours a day under a light intensity of 10 000lux for 24 hours a day in a nutrient medium containing 300mg/l nitrogen ions and 500 mg/l of total phosphate ions. The culture bubbled with 5% CO_2 achieved the highest dry biomass of 1 200mg/l and 100% CO_2 produced a dry biomass of 496.81mg/l, while that bubbled with atmospheric air recorded the lowest dry biomass of 86.67mg/l.

Generally the pH decreased as the CO_2 concentration increased. An average pH of 6.14 was recorded using 5% CO_2 concentration, and 5.84 in the flask bubbled with 10% CO_2; 5.38 and 5.19 in the 25 and 50% CO_2 gas cultures; and the lowest pH of 5.09 in the 100% CO_2 gas concentration. When atmospheric air was bubbled at 100 ml/min the growth rate was 0.24, a maximum biomass yield of 382.10mg/l was recorded on day 12 of culturing, and the average pH was 6.6.

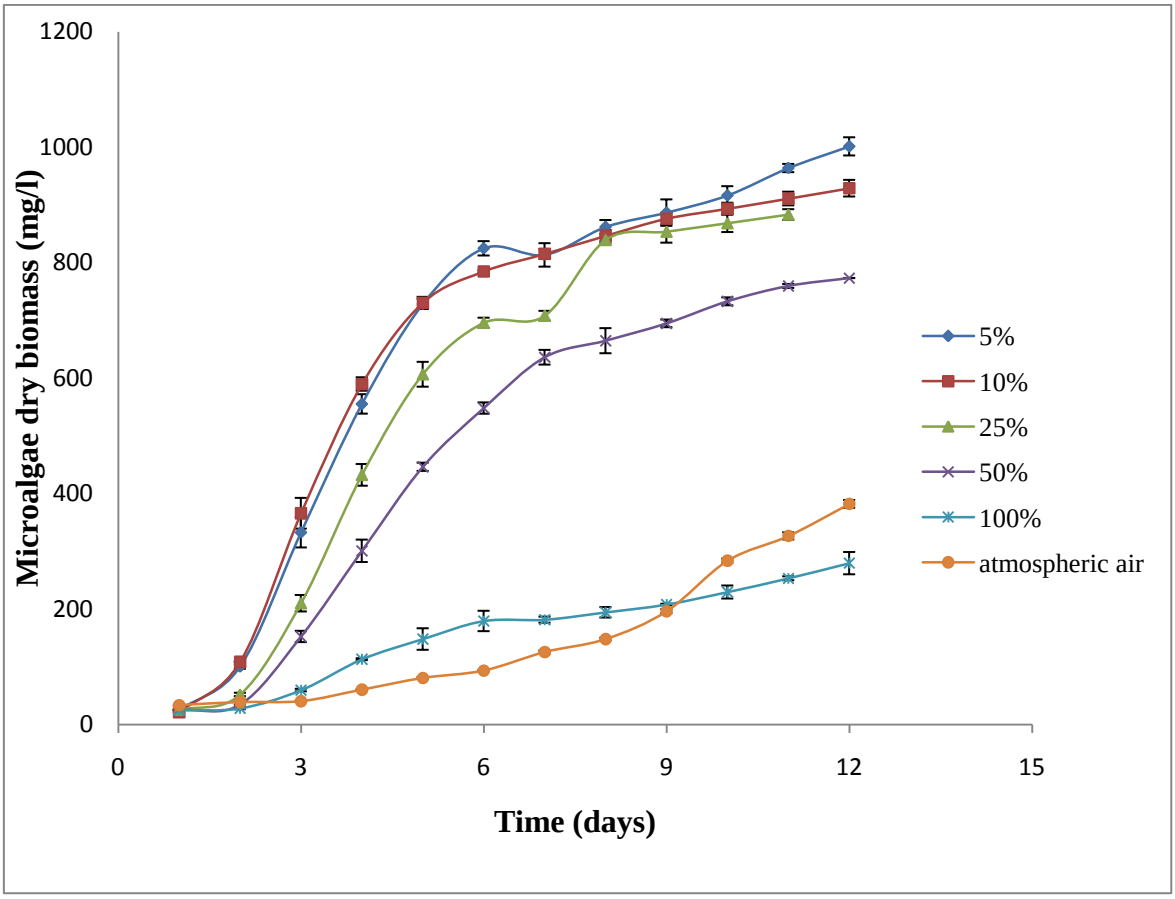

Figure 3-20: Effect of bubbling different CO_2 concentrations at 100ml/min. Microalgal cultures were bubbled with atmospheric air, 5, 10, 25, 50 and 100% of CO_2 at 100ml/min for 24 hours a day under light intensity of 10 000lux for 24 hours a day in a nutrient medium containing 300mg/l nitrogen ions and 500mg/l of total phosphate ions. The culture containing 5% CO_2 yielded the highest dry biomass of 1 001.31mg/l while 100% recorded a dry biomass of 279.46mg/l, which was lower than that obtained with atmospheric air at 382.00mg/l.

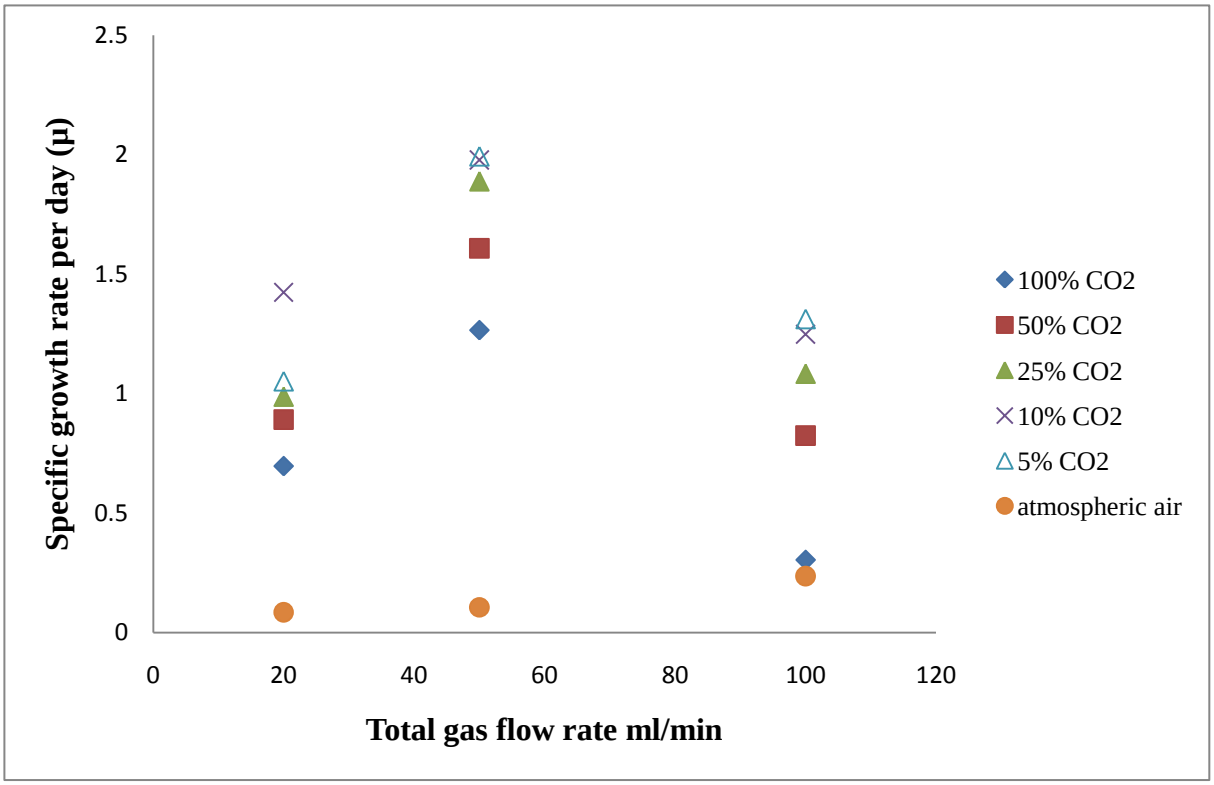

Figure 3-21: Effect of bubbling different CO_2 concentrations at different gas flow rates. Cultures containing 5, 10, 25, 50 and 100% of CO_2 were bubbled with different gas flow rates of 20, 50 and 100 ml/min under light intensity of 10 000lux for 24 hours a day in a nutrient medium containing 300mg/l nitrogen ions and 500 mg/l of total phosphate ions. The highest growth rate of 2.00 per day was recorded using 5 and 10% CO_2 at a flow rate of 50ml /min while the lowest growth rate of 0.3 per day was recorded at 100% CO_2 at 100ml/min.

3.4.5 Enhancing CO_2 tolerance and using higher flow rates

When pure CO_2 gas was bubbled at 250ml/min, almost no growth could be observed. Therefore an experiment was devised to investigate means of improving the growth of Zoo Lake microalgae at high CO_2 gas flow rates. When CO_2 was bubbled in the culture medium at 250ml/min for 0, 3, 6 and 9 hours a day there was a general increase in growth rates, which were 0.06, 0.85, 0.85 and 1.02 per day respectively, during the exponential growth phase. However at 12 and 15 hours a day, growth

rates of 0.68 and 0.75 per day were obtained. When pure CO_2 gas was bubbled continuously for 24 hours a day, the average growth rate reduced to 0.28 per day.

The highest total dry biomass of 350.40mg/l was obtained when pure CO_2 gas was bubbled for 9 hours a day. In contrast, the dry biomass yield was much lower at 70.01mg/l when CO_2 gas was bubbled continuously 24 hours a day. As in the previous experiments, one of the culturing flasks was not supplied with CO_2, but in this case it was simply exposed to the ambient air, that is, without any air being bubbled through it. This resulted in a very low biomass of 52.54mg/l. Exposing microalgae to higher flow rates of pure CO_2 gas for specific periods during the day raised the CO_2 tolerance: pure CO_2 gas bubbled for 3, 6, 12 and 15 hours a day yielded total dry biomass of 247.54, 296.78, 328.84 and 303.82 respectively. (Figure 3-22)

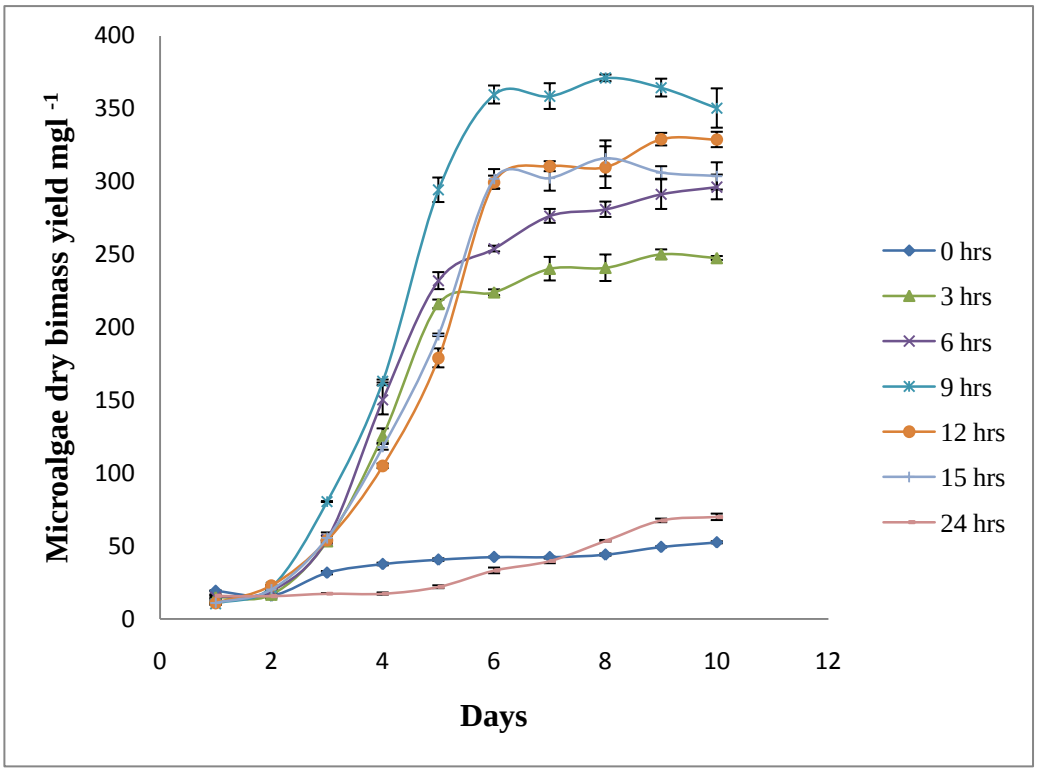

Figure 3.4.522: Effect of duration of pure CO_2 gas bubbling time under a light intensity of 10 000lux for 24 hours a day in a nutrient medium containing 150mg/l nitrogen ions and 1 000mg/l of total phosphate ions.

CHAPTER FOUR

4 Discussion

4.1 Establishing the effective culturing water

Johannesburg Zoo Lake is used for recreational purposes. It is supplied with water from Braamfonteinspruit River and other tributaries, which carry high levels of dirt, pollution and rich organic waste from the Johannesburg Zoo. It was expected that this water, supplemented with the nutrients in the Beijerinck medium, would support maximum microalgal growth better than distilled water supplemented with Beijerinck medium nutrients. However our results indicated that the Zoo Lake microalgae grew better in distilled water than in their native aquatic environment. As a result, we carried out all the subsequent experiments using Beijerinck medium supplemented with distilled water.

Although water analysis of Zoo Lake water was not done, the findings above suggest that probably the water contains some toxic substances that inhibit the growth of Zoo Lake microalgae. Because Zoo Lake is a recreational centre, the City of Johannesburg usually controls phytoplankton growth. However, when the samples were taken, the pH of the Zoo Lake water at the time was 9.2, and this could have a negative impact on growth. The species investigated later proved to grow well at a pH of 5–7, so very alkaline conditions in their indigenous environment might also have limited their growth. In addition, it is known that alkaline conditions mimic availability of CO_2 for the microalgae to feed on.

4.2 Establishing a culture with high CO_2 toleration

A well-known characteristic of microalgae is that they grow in highly selective environments (Borowozka, 1998). Microscopic observations indicated that a consortium of microalgae species was growing in the culture when ambient air was bubbled in the culture. However CO_2 was bubbled into the microalgae culture, a single species appeared to be dominant. The introduction of CO_2 has a tendency to reduce the pH, and this could have resulted in the growth of species that tolerate a slightly alkali environment.

4.3 Identification of the Zoo Lake microalgae

Desmodesmus sp is one of the most abundant coccoid green algal genera to be found in fresh to brackish water. These algae occur mainly as two-celled coenobia, but also as multiple and single cells of less than 10µm in length (Vanormelingen 2007). Species belonging to the *Scenedesmus/Desmodesmus* complex have been well documented as possessing a high degree of polymorphic variability, which makes taxonomic identification difficult (Lurling 2003). Species delimitation in the genus is also problematic because the structure of coenobia and the occurrence of spines, dents, ridges, and other such variations provide a very varied morphological character.

Observation of the species was done using a microscope in an attempt to identify the species of the Zoo Lake microalgae, and inconclusive results were obtained. Basing on the features observed it was suggested that the microalgae could be one of the *Chlorella sp* families. There were doubts on the accuracy of this general assumption,

because *Desmodemus sp* has similar phenotypic plasticity characteristics that vary environmental conditions and the need for a defense mechanism against predators (Lurling 2003). The molecular phylogenies of the genus, based on the 18S rDNA, focused on distinguishing between *Scenedesmus* and *Desmodemus*, while the ITS2 region proved to be a suitable marker of the delimitation of different species (Van Hannen *et al.*, 2000). In this investigation the ITS2 region was used for the DNA sequencing and identification, which is a technique that several authors have used to obtain conclusive results. The identification provided by the molecular analysis was confirmed by visual inspection using a microscope: the unknown species taken from Zoo Lake identified as *Desmodemus sp.*

4.4 Light intensity and photoperiod

Experimental findings established the optimal conditions for growing the green microalgae *Desmodesmus sp* from Zoo Lake. The most important environmental factor for the growth of photosynthetic unicellular microalgae is light, as it provides energy for the photoautotrophic process. However, the quality and duration of the light to which the algae are exposed in the natural environment is frequently at an insufficient level. The number of hours of daylight to which the microalgae are exposed influences the circadian rhythm of photosynthesis and their respiration, cellular division and growth rate (Hobison *et al.*, 1979; Redalje and Laws, 1983). Moreover, light also affects their enzymatic activities and macromolecule synthesis (Hobison *et al*., 1979; Foy and Smith, 1980). The growth of phytoplankton, which is

also photosynthetic, is controlled by both the spectral quality and quantity of the light source and the length of daylight (Striebel *et al.,* 2009).

A typical light intensity growth curve has three distinct phases: i) the light-dependent portion during which the growth rate increases in parallel with the light intensity; ii) the light-independent phase or plateau; and iii) the light-dependent period when the growth rate declines with a rise in light intensity (Sorokin and Krauss, 1958). These findings indicated a linear increase in both dry weight yield and growth rate as the light intensity increased in light limiting region. However at very high light intensities the maximum dry weight yield and growth rate almost reached a plateau (Figure 3-7). A further rise in light intensity to above 8 000lux did not make much difference to either the growth rate or the dry weight of the microalgae, suggesting that a light saturation point had been reached. Under prolonged irradiation at a supra-optimal level, photosynthetic rates usually decline from their light-saturated values, a phenomenon called photo-inhibition (Masojideck 2003).

The effect of day length and light intensity has a distinct influence on individual species. The growth rate in some may be reduced or cease altogether with changes in the duration of light, while others may flourish under continuous illumination (Brand and Guillard 1981). The particular species studied showed a proportional rise in both growth rate and dry weight yield at 6 000lux with an increase in day length. However, the growth rate at 20 and 24 hours was relatively similar. This suggests that the species requires a total of at least 120 000lux a day, but that any further increase in total light intensity has no meaningful effect on growth. Other researchers have reported that blue-green algae exhibit growth that is proportional to the duration of

the effective light period; that intermittent illumination of algae does not give as good a yield as continuous illumination; that they do not require diurnal alternation of light and dark; and that there is no evidence of circadian rhythms in algae (Lorenzen and Hesse, 1974). Bouterfas (2006) showed that *Selenastrum minutum, Coelastrum microporum f. astroidea* and *Cosmarium subprotumidum* had a light saturation point, even though the growth rate of these species of microalgae was higher than the obtained result. The differences could be due to the differences in species being cultured and culturing conditions. Weng *et al.* (2008) reported that the growth rates of *Cryptomonas sp.* were generally lower than 0.50 per day under optimal light intensity and iron concentrations.

This result implies that *Desmodesmus sp* from Zoo Lake do not require a dark period for maximal growth. It can also be concluded that for *Dsesmodesmus sp* to grow, it needs maximal exposure to light per day at an intensity of 6 000lux.

The results we obtained provided information that is vital to the design of efficient microalgal culturing systems. The natural sunlight on a sunny day provides about 8 000lux or more, and it is suggested that the microalgae species investigated can be grown using natural sunlight as a source of energy. This would mean culturing microalgae in an open pond system, which would combine cost effectiveness and a sustainable green house gas emission strategy.

4.5 Nutrients for microalgal growth

4.5.1 Nitrogen ions

Nitrogen ions are important nutrients for microalgae; they support both growth and biomass synthesis. It is widely accepted that nitrogen may be the limiting factor in phytoplankton productivity. Most of the microalgae species are able to utilize a wide range of both organic and inorganic nitrogen sources. Although it was done within limits, it was found out that increasing the concentrations of the nitrogen ions influenced the microalgal growth rate and dry weight yield positively during the culturing period. When no nitrogen ions were added to the culturing medium, very little growth was observed. This could have been caused by the nitrogen ions in the inoculums as well as organic nitrogen released by the dead cells of other microalgae. When the microalgae were grown at concentrations of 18.75 and 37mg/l nitrogen ions, there was an initial rapid growth phase in response to the presence of the nitrogen. However, on day four the growth slowed down because of the depletion of nitrogen ions in the medium.

A maximum growth rate and dry weight yield was achieved at a nitrogen ion concentration of 300mg/l. Nitrogen ion concentrations higher than 300mg/l resulted in a reduced growth rate, and it was also noted that at higher nitrogen concentrations the growth pattern did not stabilize, like the other growth curves, on day 12 of culturing. The slightly reduced growth rate is probably attributable to nitrate regulation, when there is an increase in the amount of nitrogen ions in the medium nitrate reductase activity is increase, which in turn leads to NH_4^+ production and accumulation. The nitrate regulation process induces inhibition of nitrate assimilation,

which probably slows down growth. In addition, higher nitrate reductase activity can lead to an accumulation of nitrite inside the cells that is toxic, and causes reduced nitrate uptake and inhibition of growth (Jeanfils, 1993). Other scientists have pointed out that when nitrogen ions are in excess, the nitrate uptake is slow (Shi *et al.,* 2007). Certain researchers have reported that nitrate concentrations in the range of 0.5–10mM had little effect on the growth rate of *D. vindis*, while higher concentrations of NH_4^+ > 2.5mM were lethal to *Dunaliella sp*. Some species are able to adapt to higher nitrogen ion concentrations regardless of whether it is in the form of NH_4^+ or NO_3^-, (Giordano and Beardall, 2008) although more than 45mg/l of nitrogen could inhibit the growth of most species of algae. A concentration of 2.0mM total ammonia could inhibit photosynthetic O_2 evolution by about 50% in most algal species, while higher concentrations of ammonia could lead to death of the algae.

Microalgae are known to remove nitrates and ammonium from wastewater efficiently (Mallick and Raj 1994). *T. uarea* has been found to be able to absorb between 60% and 80% of the ammonium ions in wastewater. However, in the experiments carried out by Mallick and Raj, the assimilation of nitrates and nitrites was at most 40% at the end of the culturing period of 40 days. In that study the general assimilation of nitrogen ions was estimated as below 80%, and the efficiency of uptake decreased as the concentration of nitrates in the medium increased. Other researchers have noted a 35% removal of ammoniacal nitrogen within 10 days (Singh and Dhar, 2007). Based on these results it was therefore recommended that microalgae can provide an effective secondary bioremediation strategy for wastewater treatment plants.

The main sources of the nitrogen used in experimental research are ammonium and nitrate salts and Syrette (1962) suggested that microalgae show a preference for

both types of salt, but reported that his laboratory used ammonium ions first. However, some species exhibit no preferential utilization of inorganic nitrogen ions, (Cain 1965) as was the case in the experiments we conducted. Although three types of nitrogen sources were tested, the microalgae under cultivation did not show any specificity or preferential utilization of the three nitrogen ion species used. Microalgae assimilate inorganic forms of nitrogen such as nitrate, ammonia and some organic forms like urea. The findings of Fidalgo *et al.* (1998) suggest that most algae at low concentrations of NH_4Cl, $NaNO_3$ and urea showed similar growth rates and dry biomass yields. Although the differences in the growth rates obtained in this study were insignificant, ammonium nitrate resulted in slightly higher growth rates than ammonium chloride and potassium nitrate. This can be explained by the fact that uptake of NH_4^+ in the medium slightly reduced the pH, which would make dissolving the CO_2 easier and result in higher yields. Given the lack of preference of most microalgae species for a particular source of nitrogen ions, it can be suggested that it would be reasonable to design a system that uses an organic source of nitrogen rather than an inorganic one, because the latter are carbon- intensive.

The sudden growth observed when nitrogen ions were added on day 12 to the flask that contained 18.75 and 37.5mg/l of nitrates confirmed that nitrogen is the main limiting factor for microalgae, and is essential for their growth. Addition of other nutrients on day 12 to the flask with no nitrates resulted in no growth and this shows that nitrogen ions are necessary for microalgae growth.

The microalgae investigated grow very well in high nitrate concentrations. Bucas (2008) claimed that natural uncontaminated waters anywhere in the world generally contain about 0.02 mg/l of NH_4^+ and about 0.01mg/l of NO_3^-. Contamination of natural

waters with anthropogenic nitrogen ions causes the concentration to reach levels as high as 10mg/l. The concentrations used in the experiments described in this dissertation are very high compared with those of natural levels. This proved that microalgae can be used both for nitrate remediation in wastewater and for absorbing CO_2.

4.5.2 Phosphate ions

The phosphate requirement for optimal growth differs considerably from species to species in microalgae. Phosphate ions are required by microalgae for most of their cellular activities, especially those involved in generating and transforming metabolic energy. Polyphosphate, which participates in metabolism, is also stored in the biomass and can be used when the external source of phosphates is insufficient. Fried *et al.,* (2003) found that a concentration of 7.47 X 10^{-6}M resulted in high microalgal growth levels, but they did not investigate the effect of very high concentrations of phosphate ions. Grobbelaar (2004) suggested that the approximate molecular formula for microalgae is $CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$ and that consequently phosphate ions are required in minute quantities to make biomass. At all the concentrations investigated, the growth rate appeared to increase continually and the biomass yield did not level off. This indicated that the cultures had not reached limiting conditions, and that *Desmodesmus sp* from Zoo Lake require low phosphate ion concentrations for growth.

The results obtained in this study showed that the microalgae under investigation required a concentration of 500mg/l of total phosphate ions. However a substantial concentration of phosphates is required in the medium because most of them

become complex and thus are not usable by the microalgae. Phosphate uptake depends on pH, redox conditions, and the presence of chelators, dilution and variable utilization of specific phosphorus compounds (Bostron *et al*., 1988).

Phosphate concentrations in natural water bodies are about 0.01mg/l, but when freshwater bodies are highly contaminated, they contain concentrations of up to 10mg/l (Bucas, 2008). Municipal wastewater usually has phosphate ion concentrations of 5–20mg/l. Microalgae therefore present a viable means of absorbing phosphate ions in wastewater treatment plants while at the same time sequestrating CO_2.

4.6 Effects of CO_2 of on microalgal growth

4.6.1 Effects of pH

One of the abiotic factors that affect the growth and abundance of microalgae is pH. A fluctuation in pH of 1–2 units means 10–100 fold changes in hydrogen ion activity. The concentration of hydrogen ions is known to affect numerous transport processes across the cellular membranes, and metabolic functions in the cytoplasm and cellular organelles. Further, pH influences the solubility, bioavailability and toxicity of ammonium/ ammonia, iron and heavy metals (Weisse and Stadler 2006). Changes in pH can affect algal growth in various ways. These include distribution of carbon dioxide species and carbon availability in water; alter availability of trace metals and essential nutrients (Chen and Durbin 1994). The findings from this study showed that the optimal pH for the microalgae was cultured at 6.5. Both a pH of 7 and a pH of less than 6.5 caused a reduction in growth. The reduced growth at the higher pH level may be attributable to carbon limitation and probably lack or reduced activity of

CCM carbon anhydrase in particular (Chinnasamy *et al.,* 2009). At a pH of 7 the dominant Ci species is HCO_3^-, and this form of carbon may not be readily usable by the microalgae under study, that is if the carbonic anhydrase has no activity or has reduced activity due to high levels of CO_2 in the medium (Papazi *et al.,* 2008; Chinnasamy 2009). On the other hand, an extremely low pH is known to cause physiological changes to the microalgae cells, and it is possible that the microalgae species under study does not easily adapt to the low pH, resulting in lower growth at pH 5.5. However, findings published by by Negoro *et al.,* (1991) point out that some microalgae are able to grow in low pH without the need for any pH adjustments.

4.6.2 Addition of pure CO_2

Yang and Gao (2003) suggest that rising CO_2 levels could increase primary productivity by ocean phytoplankton, and enhance the photosynthesis and growth of some microalgae. Photosynthesis by phytoplankton can be severely limited by the insufficient supply of inorganic carbon in lakes with algal bloom, where, because of the relatively low carbon dioxide content of atmospheric air, it is likely that the growth of phytoplankton cells will be limited and slow. The biomass of microalgae is known to contain at least 50% carbon (Globbelaar, 2004 and Powell, 2008). These findings showed that CO_2 is essential to microalgal growth. When pure CO_2 was added at 10ml/ min the growth rate increased almost five fold compared with that obtained with atmospheric air. These findings suggest that CO_2 is limiting at ambient air concentrations. *Demodesmus sp* investigated had very reduced growth when atmospheric air was bubbled at 50ml/min, this could be due to the fact that the flow rate was too low to supply carbon dioxide required for algal growth. Microalgae

understudy could have failed to make use of the CCMs since it has been reported that microalgae that tolerate high CO_2 concentrations might have lost their CCM capacity or have reduced carbonic anhydrase activity. Species with high activity of carbonic anhydrase flourishes in high pH or alkaline conditions. It is very possible that the *Desmodesmus sp* investigated could have lost its CCM capacity. Zoo Lake water pH was at an average of 9.2 and it is possible that this very high pH could make the CO_2 inaccessible to the microalgae under study and to support this there were no microalgae blooms at the lake.

4.6.3 Bubbling CO_2 at different concentrations and flow rates

Although many scientists have speculated that microalgae are susceptible to high CO_2 concentrations and cannot flourish under such conditions, some species are reported to grow rapidly in cultures with very high CO_2 content. An extreme CO_2 concentration that is about 1 000 times higher than in the ambient levels can easily be fixed to biomass by unicellular microalgae. Bubbling a high concentration of CO_2 through the cultures proved more effective in improving growth rates than doing the same with ambient air. However, the growth rate fell at very high concentrations of CO_2 and higher flow rates. Our findings suggest that the microalgae under study are able to tolerate high CO_2 levels.

Nevertheless, a reduced but not complete inhibition was noted when pure CO_2 was used at low flow rates. An extreme flow elevated CO_2 concentration can increase microalgal carboxylating and repress the oxygenating Rubisco activity, resulting in increased photosynthesis. Furthermore, CO_2 enrichment might improve photosynthetic electron transport between PSII and PSI, in this way raising the

photon yield and generating energy for CO_2 fixation (Dubnsky *et al.,* 1986). Papazi *et al.,* 2008 suggested that high levels of carbon dioxide cause reorganization of the photosynthetic apparatus resulting in enhanced photosynthesis. They reported that high levels of CO_2 affects polyamines and specifically increased the thylakoid bound putrescine level, that results in high number of reaction center density together with decrease in light harvesting oligomers/ light harvesting momomers.

Scientific research into the effect of an elevated CO_2 concentration on phytoplankton in freshwater has shown that it inhibits the rate of cell division. Nielson (1955) reported the decrease in photosynthetic rate of *Chlorella pyrenodosa*, and offered the hypothesis that CO_2 in high concentrations could act as a toxin in saturating light levels. Using the stepwise adaptation technique, Kodama *et al*. (1993) showed that *Chlorococcum littorale* could grow under 60% CO_2 conditions. Another species highly tolerant of CO_2 is *Euglena gracilis.* Its growth was improved by adding concentrations of CO_2 between 5–45%, with the best growth rate observed with a 5% CO_2 concentration. However, the species did not grow when the CO_2 concentration was above 45%. Hirata *et al.* (1996a 1996b) reported that *Chlorella sp.* UK001 could be cultured successfully under 10% CO_2 conditions, and Hanagata *et al,*. (1992) claimed that the same species could be cultivated in 40% CO_2. Furthermore, Maeda *et al.* (1995) found a strain of *Chlorella* sp. T-1 that could grow under 100% CO_2, although the maximum growth rate occurred under a 10% concentration. Research by Hanagata *et al,.* (1992) established that *Scenedesmus sp.* could grow under 80% CO_2 conditions, although the maximum cell mass was observed in 10–20% CO_2 concentrations. Seckbach *et al.* (1971) and Graham and Wilcox (2000) also reported

that *Cyanidium caldarium* and some other species of *Cyanidium* can grow in pure CO_2.

An increase in concentration of the CO_2 regardless of the flow rate resulted in a decrease in pH. When dissolving in water, CO_2 equilibrates into into $CO_{2\,(aq)}$, $HCO_3^-{}_{(aq)}$,and $CO^{3-}{}_{(aq).}$This lowers the pH, whereas at a pH of 6 and lower, $CO_{2\,(aq)}$ is dominant. At a pH of 6–9, $HCO_3^-{}_{(aq)}$ becomes more pronounced, and at a pH of 9 and above, CO_3^{2-} becomes predominant. (Ota *et al.,* 2009) It is possible that the low pH observed when pure CO_2 was used could have been the reason for the reduced growth rates. Contrary to our findings are those of Negoro *et al.* (1991), who examined the tolerance of several strains of microalgae to 5–15% CO_2, and concluded that some strains flourish with pH adjustments. Negoro *et al,.* (1991) also showed that *Scendemus* and *Chlorella* had a long lag phase in very high concentrations of CO_2. They further suggested that the growth rate was not affected by variation in the flow rates of air containing elevated CO_2. This was the result of their use of sea water, which has a strong buffering capacity. In contrast, findings in this study showed that flow rate has a great influence on both growth and dry biomass yield because fresh water has a weak buffering capacity, with the result that the pH drastically decreases when CO_2 gas is added.

These results imply that microalgae can tolerate high CO_2 at low flow rates. Therefore, when designing CO_2 sequestration systems for microalgae, engineers should ensure the flow rate is maintained at low levels, to allow maximum CO_2 mass transfer into microalgal biomass. The fact that most of the flue gases produced by

most industries contain 5–15% CO_2 concentration gives microalgae the advantage of being the best candidate for creating a sustainable carbon sink.

4.6.4 Enhanced growth at very high CO_2 flow rate

Extreme high flow rates of pure CO_2 have effectively reduced the growth of *Desmodesmus sp* under study. This could be probably due to toxicity of carbon dioxide to all phototrophic organisms when present in very high concentrations. The growth rate increased with increased number of hours of bubbling CO_2 at 250ml/min from 0 hours to 9 hours day and starts to decrease. Very low pH of 4.5 was recorded during the time pure CO_2 was bubbled and the pH would rise during the time when there was no bubbling of CO_2. The pH in the flask that were bubbled for one hour a day rose the highest of around 8.0 during the time when no bubbling was done. The deep fluctuations in pH due to bubbling pure CO_2 intermittently could have caused the low growth.

Bubbling for nine hours a day resulted in improved growth rate s per day as pH did not greatly fluctuate. Those that were bubbled 24 hours a day has very little growth because the pH was very low at an average of 4.5 and the investigated microalgae could not withstand these acidic conditions.

CHAPTER FIVE

5.1 Overall Conclusions

The investigations carried out have established the fundamental aspects that need to be taken into account when a microalgal CO_2 sequestration system is being designed.

As far as the culturing medium is concerned, *Desmodesmus sp.* was found to grow best in supplemented distilled water. It can therefore be cultured in any water that does not contain any growth inhibitors.

The experiments provided detailed information about the light requirements of *Desmodesmus sp*. It was fund out that, by using plant gro lamps at light intensity of at least 6 000lux per day is required. The results of the light duration tests led to the conclusion that although the growth of *Desmodesmus sp.* is not limited by the photoperiod, maximal biomass production occurs with a photoperiod of 24 hours a day.

Nutrient required to cultivate microalgae were investigated. It was found out that nitrogen ions are vital for microalgal growth and that nitrogen requirements best suited to the growth of *Desmodesmus sp*. was at a concentration of 300mg/l of nitrogen ions. It was discovered that very high (above 300mg/l) concentrations of nitrogen ions reduce microalgal growth but does not inhibit growth. The experiments also proved that the addition of nitrogen ions to a nitrogen-depleted or limited medium causes a resumption of growth. Another critical aspect of our findings was that *Desmodesmus sp.* does not have any preference for any type of nitrogen ion source.

Desmodesmus sp. requires a total phosphorus concentration of 500mg/l for optimal growth. It was also found out that phosphorus does limit microalgae growth, and that they phosphates are converted into microalgae biomass inefficiently.

A number of experiments were carried out to test the viability of using microalgae to absorb CO_2 into biomass. *Desmodesmus sp.* demonstrated that it tolerates high CO_2 concentrations. However the interaction between CO_2 concentration, CO_2 flow rate and pH balance has to be seriously considered when designing a CO_2 algal absorption system. It was found out that *Desmodesmus sp.* prefers higher concentrations of CO_2 than atmospheric air, and that a flow rate of 50 ml/min and a CO_2 concentration of 5–10% were the most effective combination for supporting maximal microalgal growth. *Desmodemus sp.* demonstrated that it could grow at higher flow rates of 100 ml/min with pure CO_2, but the growth was greatly reduced at 250ml/min. Attempts to improve growth at the higher flow rate of 500ml /min were made and bubbling pure CO_2 for 9 hours a day proved to support maximal microalgae growth.

5.2 Recommendations

The ultimate goal of this research project was to outline the best conditions for microalgal cultivation. Based on these results it was suggested that the microalgae should be grown in open ponds situated near small power plants or sources of CO_2 emission, and where their source of light would be natural sunlight. This would not only be a very good source of energy for microalgae but it would reduce operational costs. An artificial lighting system could be used at night only when the need arises.

Our findings on the nitrogen requirements of *Desmodemus sp*. lead us to recommend that an engineer designing a microalgal culturing system takes into account that a constant supply of 300mg/l of nitrogen ions should be maintained in the medium for maximal growth. A gradual and continuous addition of nitrogen ensures it is converted into biomass efficiently. Also, because this species does not discriminate between nitrogen sources, this species could be supplied with organic sources of nitrogen such as sewage rather than carbon-intensive nitrates.

The results of this investigation shows that microalgae cultured is not efficient in using phosphate hence it is recommended that very dilute concentrations of phosphate ions should be used. *Desmodesmus sp* flourish very well when cultured from waste/effluent water as a secondary or tertiary waste water treatment

These findings confirm that *Desmodesmus sp* is a viable and sustainable means of absorbing CO_2 that would greatly help developing countries like South Africa to offset their carbon emissions.

5.3 Future work

This study has established the basic parameters required when designing a biological CO_2 absorption system. The next step would be to test the effect of a mixture of gases with gas composition equal to the industrial flue gas emission. Further experiments using actual flue gas from a local power plant on *Desmodesmus sp.* to see whether it can in practice are used to absorb flue gas emissions.

The experiments carried out were done in batches on a continuous basis. It was necessary to carry them out as part of a cumulative process that would allow us to

determine the nutrients required, their amount and the best way of adding them to the culturing medium that would result in optimal biomass production.

The data presented in this dissertation provide a preliminary basis only. In future they could be used to make calculations of the amount of CO_2 that can be absorbed by a microalgal species in a given time, and the amount of open pond space necessary to cultivate sufficient algae to absorb the flue gas emissions from a particular power plant.

Very little information has been published on the uses of *Desmodemus sp.* biomass. It is recommend that a careful analysis of the constituents of its biomass be carried out to establish the potential uses of this species, with a special focus on its application in bioremediation of waste from biodiesel and heavy metals.

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APPENDECES

Appendix A 1 Standard Beijerinck medium composition

Stock solution 1

NH_4NO_3	1.5g/l
K_2HPO_4	0.2g/l
$MgSO_4.7H_2O$	0.2g/l
$CaCl_2.2H_2O$	0.1g/l

Stock solution 2

KH_2PO_4	9.07g/l

Stock solution 3

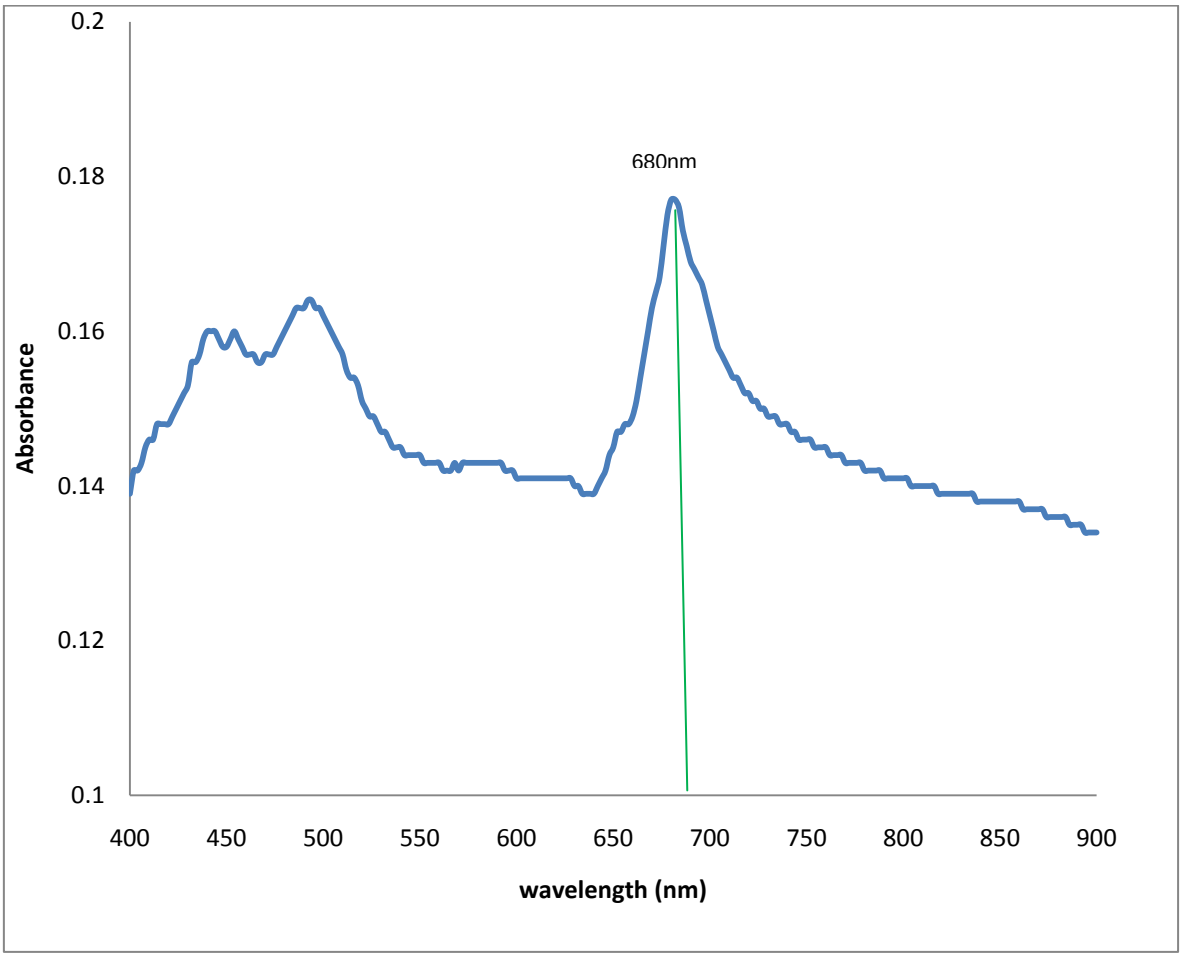
K_2HPO_4	11.61g/l

Micronutrients

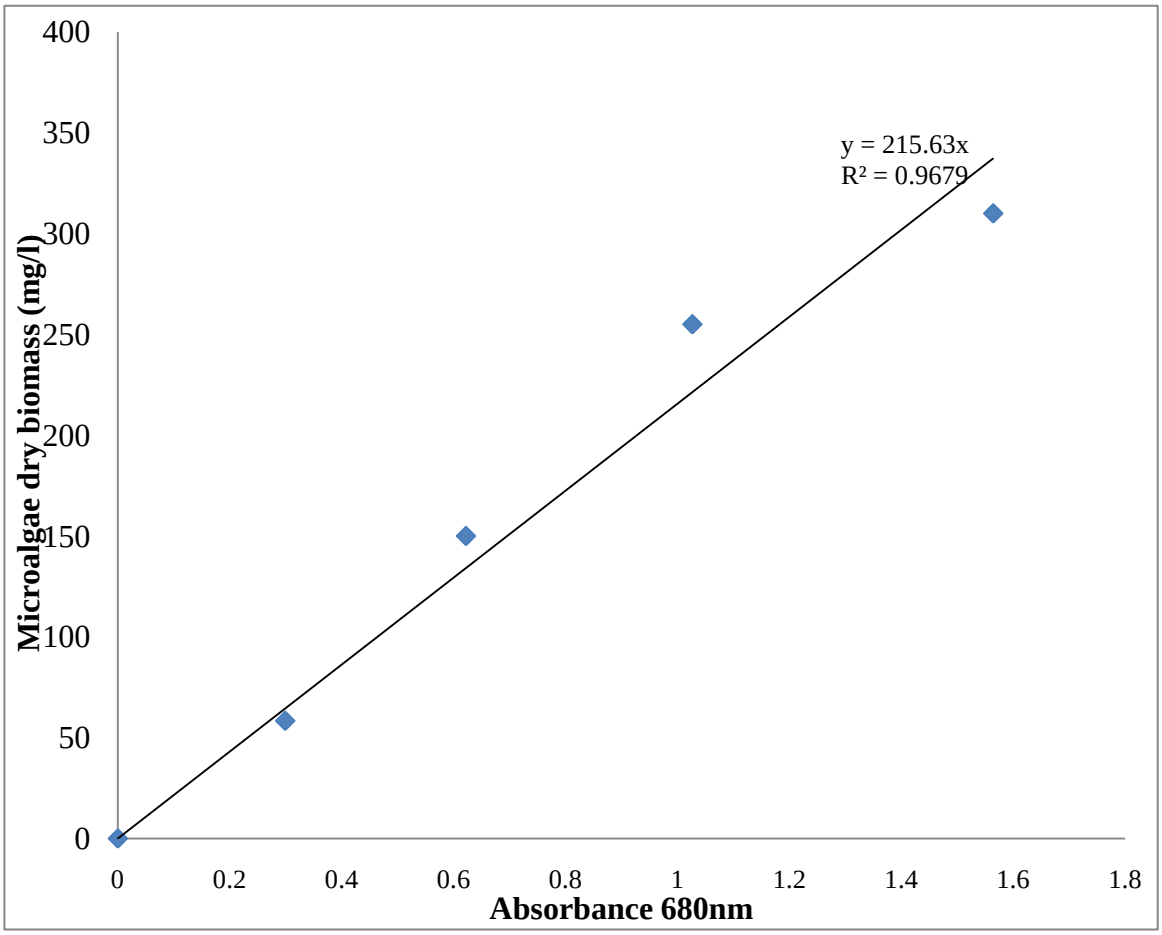
H_3BO_3	1.0 g/100ml
$CuSO_4.5H_2O$	15 g/100ml
EDTA	5.0 g/100ml
$ZnSO_4.7H_2O$	2.2 g/ 100ml
$MnCl_2.4H_2O$	0.5 g/100ml
$FeSO_4 .7H_2O$	0.5 g/100ml
$CoCl_2.6H_2O$	0.15 g/100ml
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.10 g/100ml

100ml of stock solution, 40ml of stock solution, 60ml of stock solution 3 and 1ml of micronutrients were mixed with 749ml of distilled water in an Erlenmeyer flask.

Appendix A 2: **Zoo lake microalgae absorption spectrum**



Appendix A 3 Zoo lake Microalgae dry biomass calibration curve



Appendix A 4 Calculation of the growth rate

Calculation from a 50 % CO_2 bubbled at 20ml/min

Absorbance recorded

days	absorbance at 680 nm		
	1	2	3
1	0.119	0.119	0.114
2	0.163	0.161	0.156
3	0.703	0.707	0.711
4	1.395	1.37	1.42
5	2.02	2.09	2.1
6	2.5	2.59	2.535
7	2.83	2.86	2.85
8	2.975	2.855	2.94
9	3.165	3.105	3.27
10	3.22	3.345	3.325
11	3.273	3.273	3.273
12	3.285	3.26	3.275

Converted biomass from the calibration curve in Appendix A 3 using the formula

Recorded absorbance X the constant 215.63

days	biomass mg/l		
	1	2	3
1	25.65997	25.65997	24.58182
2	35.14769	34.71643	33.63828
3	151.5879	152.4504	153.3129
4	300.8039	295.4131	306.1946
5	435.5726	450.6667	452.823
6	539.075	558.4817	546.6221
7	610.2329	616.7018	614.5455
8	641.4993	615.6237	633.9522
9	682.469	669.5312	705.1101
10	694.3286	721.2824	716.9698
11	705.757	705.757	705.757
12	708.3446	702.9538	706.1883

Calculating the natural logarithm from the dry biomass obtained

days	natural logarithms of the dry biomass		
	1	2	3
1	3.244932	3.244932	3.202007
2	3.559559	3.547213	3.515665
3	5.021166	5.026839	5.032481
4	5.706458	5.688375	5.724221
5	6.076661	6.110728	6.115501
6	6.289855	6.325222	6.303758
7	6.413841	6.424386	6.420883
8	6.463808	6.422636	6.451974
9	6.525717	6.506578	6.558354
10	6.542945	6.581031	6.575034
11	6.559271	6.559271	6.559271
12	6.562931	6.555291	6.559882

Finding the average and standard deviations

days	average biomass	standard deviation
1	25.30059	0.62247
2	34.5008	0.777465
3	152.4504	0.86252
4	300.8039	5.39075
5	446.3541	9.399094
6	548.0596	9.782887
7	613.8267	3.293803
8	630.3584	13.30689
9	685.7034	18.00866
10	710.8602	14.47829
11	705.757	0
12	705.8289	2.713285

Using the formula (lnX2 – ln X1)/ (t2 – t1) at the exponential phase of phase

The exponential phase was from day 2 to day 5 of culturing period, so using average logarithms on day 2 and day 5

(6.1001- 3.541)3 = 0.856 there the growth rate was recorded as 0.86

Appendix A 5 DNA Extraction Procedure

CTAB-extraction (based on Saghai Maroof *et al.*, 1984):

1. Centrifuge 4ml of suspension at 16100g (Eppendorf 5415D, *Merck*) for 10 minutes in two 2ml Eppendorf Safelock (*Merck*) tubes.
2. Pool the pellets into a single Safelock tube.
3. Add 500*µ*l of Buffer PL1 (CTAB-based; NucleoSpin Plant II Kit, *Separations*) with 5*µ*l of proteinase K (10mg/ml).
4. Mix by vortexing with Vortex Genie 2 (*Lasec*).
5. Incubate in a waterbath at 60°C overnight.
6. Add an equal volume of chloroform:isoamylalcohol (24:1) to the sample.
7. Shake or mix by inversion for 5 minutes.
8. Centrifuge at maximum speed at 4°C (Eppendorf 58 10R).
9. Transfer supernatant (±400*µ*l) to a new 1.5ml tube.
10. Add an equal volume of chloroform:isoamylalcohol (24:1) to the sample.
11. Shake or mix by inversion for 5 minutes.
12. Centrifuge at maximum speed at 4°C (Eppendorf 5 810R).
13. Transfer supernatant (±300*µ*l) to a new 1.5ml tube.
14. Add 2_3.volume of ice-cold isopropanol to the supernatant.
15. Mix well by inversion.
16. Incubate overnight at -20°C
17. Centrifuge at maximum (Eppendorf 5415D) for 20 minutes.
18. Remove supernatant carefully.
19. Add 200*µ*l of 70% ethanol to wash pellet.
20. Mix by inverting until pellet comes loose.
21. Centrifuge at maximum (Eppendorf 5415D) for 10 minutes.
22. Remove supernatant carefully.
23. Remove excess ethanol by inverting tube and patting it on tissue paper.
24. Dry pellet at 55°C for 15 minutes.
25. Resuspend pellet in 50*µ*l ddH_2O.

Appendix A 6: DNA Sequence of the Zoo Lake Microalgae *(Desmodemus sp)*

Sequence (ITS800)

ATTGCAGATTCCGTGACCATCGAATCTTTGAACGCATATTGCGCTCGACCCCTCGGGGAAGAGCA
TGTCTGCCTCAGCGTCGGTTTACACCCTCACCCCACTTCCCTCACAGGAAGCGCTTGCTGCGTC
GTTTGACCAGCAACTGGGATGGATCTGGCCCTCCCAATCGAAGCAATTCGATTGGGTTGGCTGA
AGCACAGAGGCTTAAACTGGGACCCGTACCGGGCTCAACTGGATAGGTAGCAACACCCTCGGGT
GCCTACACGAAGTTGTGTCTGAGGACCTGGTTAGGAGCCAAGCAGGAAACGTGGAAACACGTAC
TCTGTATTCGACCTGAGCTCAGGCAAGGCTACCCGCTGAACTTAAGCATATCACTAAGCGGAGGA
AAAGAAACTAACAAGGATGCGCTTAGTAACGGCGAGCGAACCGCGCAAAGCCCAACTTGAAAAT
CTCCTTCGGAGAATTGTAGTCTATAGAAGTACCCTCTGCAACGGGCTGAGCTAAAGTCTCCTGGA
AAGGGGCGTCAGAGAGGGTGAGAACCCCGTAAGCTCACGCTTAGTTGCCTCACGAGGTGCTTTC
GAAGAGTCGGGTTGCTTGGGAATGCAGCCCAAATTTGGTGGTAAATCCCATCTAAGGCTAATACT
GGCGAGAGACCGATAGCGAACAAGTA