

**THE BENEFICIATION OF CARBONATE RICH COAL SEAM
WATER THROUGH THE CULTIVATION OF *ARTHROSPIRA
MAXIMA (SPIRULINA)***

by

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Declaration

Student number: 42160944

I, Francois Michael Grove, declare that: **The beneficiation of carbonate rich coal seam water through the cultivation of *Arthrospira maxima (Spirulina)*** is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references.



SIGNATURE
(Mr) Francois M. Grove

2 June 2013
DATE

Abstract

Coal seams are commonly associated with poor quality water that requires treatment. Water treatment can be very expensive and can severely affect the profitability of mining projects. This study investigated the potential cultivation of *Arthrospira maxima* (*Spirulina*) in coal seam water to beneficiate coal seam water in order to effectively offset the water treatment cost. The study was conducted in Northern South Africa and formed part of a larger Coal Seam Water Beneficiation Project (CSWBP).

The study consisted of laboratory based Flask Studies and outdoor High Rate Algal Pond Studies. The Flask Studies that were carried out in the on-site field laboratory, found that the coal seam water could provide a suitable medium for *Spirulina* cultivation. In addition, it was found that the optimal pH for the selected strain ranged between 9 - 10.5 and that the addition of excess iron, up to 100 times the concentration found in defined growth media such as Schlösser's, to the culture media could enhance productivity.

The High Rate Algal Pond Studies (HRAP) were carried out over a period of 18 months. The studies showed that the coal seam water at the CSWBP is a valuable resource that can reduce media costs by 50% without affecting productivity. In a study encompassing 334 days it was shown that heating the culture through plate heat exchangers would result in a significant increase in productivity and a heated productivity of 19.86 g/m²/day was recorded. An unheated productivity of 14.11 g/m²/day was recorded.

Therefore, it was found that it would be economically feasible to beneficiate coal seam water found at the CSWBP through the cultivation of *Arthrospira maxima* (*Spirulina*).

Key Words: : *Arthrospira*, *Spirulina*, mass culture, beneficiation, coal seam water, HRAP, raceway ponds, algae, biotechnology, phycology, coal, mining

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CHAPTER 1: INTRODUCTION

1.1. Introduction

1.1.1. *Project Background*

Due to confidentiality considerations the specific project for which the research was conducted or any of the production methods used by the proponent will not be disclosed in this dissertation. The project will be referred to as the Coal Seam Water Beneficiation Project (CSWBP). This dissertation will describe a part of the algal study that formed part of the larger CSWBP. The dissertation focuses on the pilot level study on the use of *Spirulina* to beneficiate raw coal seam water.

1.1.2. *The Mine Water Problem*

Coal seams generally contain large volumes of associated water. As a result, large volumes of coal seam water must be pumped from the coal seam prior to production. This water ranges from fresh to highly alkaline in quality and can seriously influence the viability of mining operations (ALL Consulting, 2003). The water extracted at the CSWBP has an average total dissolved salts (TDS) of 5300 mg/l made up predominantly of sodium bicarbonate (NaHCO_3), and a pH ranging from 7.30 to 8.80. The water quality exceeds Class III of the SANS 241 drinking water standard and as such is not suited for domestic use or discharge (SANS, 2011). As a result, the water must be treated. This is accomplished through Reverse Osmosis (RO) at the CSWBP. RO produces two water streams 1) potable water 2) saline brine with an average TDS of ~ 15 000 mg/l and a pH ranging from 8.30 to 9.10 (Figure 1-1).

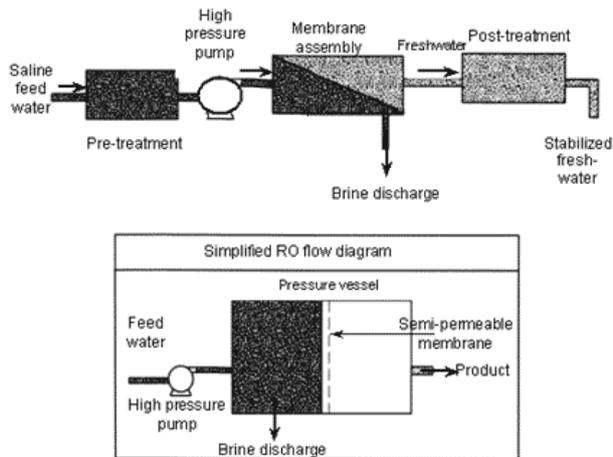


Figure 1-1: The Reverse Osmosis process (ALL Consulting, 2003)

The potable water stream is pumped to a shallow borrow pit where it serves as a fresh water source for the game and cattle in the area. The saline brine is stored in high density poly ethylene (HDPE) lined ponds. The water in the brine is allowed to evaporate leaving behind the salts that can then be safely removed to a hazardous waste facility.

RO is a very effective water treatment method but it becomes exorbitantly expensive for large volumes of water (ALL Consulting, 2003). As the CSWBP can produce as much as 28 000 m³ per day, RO alone is not an ideal solution. Because reverse osmosis is so expensive and the untreated water exceeds drinking water standards, the coal seam water poses a major environmental and financial risk. In order to mitigate the financial and environmental risk, a cost effective method was required to make beneficial use of the water.

1.1.3. Beneficial Water Use Options

The first step in identifying potential methods of treating or utilising the produced water at the CSWBP was to draw up a list of all common practises in the industry. Table 1-1 shows the most common options for dealing with produced coal seam water utilised in the USA.

Table 1-1: Most common options for dealing with produced coal seam water in the USA (ALL Consulting, 2005). (modified)

No.	Main Option	Sub-option No.	Sub-option
1	Treatment Technologies	a)	Freeze-Thaw/Evaporation
		b)	Reverse osmosis
		c)	Ultraviolet light
		d)	Chemical treatment (Chlorination, Iodine, Silver)
		e)	Ion Exchange
		f)	Capacitive Desalination (CD) or De-ionization
		g)	Electrodialysis Reversal (EDR)
		h)	Distillation
		i)	Artificial wetlands
2	Underground Injection	a)	Injection into a coal seam aquifer
		b)	Injection into a non-coal aquifer
3	Impoundments	a)	Wildlife and livestock watering impoundments
		b)	Fisheries (<i>aquaculture</i>)
		c)	Recharge ponds
		d)	Recreation
		e)	Evaporation ponds
		f)	Constructed wetlands
		g)	Secondary impoundment uses
4	Surface Discharge	a)	Direct discharge to surface water
		b)	Discharge to land surface with possible runoff
		c)	Discharge to surface impoundments with possible infiltration and subsurface discharge
		d)	Discharge to the salt pan in the area
5	Agricultural	a)	Stock watering
		b)	Irrigation
6	Industrial	a)	Coal mine use
		b)	Animal feeding operations
		c)	Cooling tower water
		d)	Field and car wash facilities
		e)	Enhanced oil recovery
		f)	Fisheries (<i>aquaculture</i>)
		g)	Fire protection
		h)	Other industrial uses
7	Domestic and Municipal	a)	Domestic use

No.	Main Option	Sub-option No.	Sub-option
	Use	b)	Municipal water use

Golder and Associates Environmental Consultants were contracted to evaluate the feasibility of these 32 options for the CSWBP in terms of constraints, practicality and economic factors. Only eight options were found to be viable. One of these was the cultivation of saline tolerant algae such as *Dunaliella salina* and *Spirulina spp.* in the CSW for fine chemical extraction i.e. abstraction of valuable cell components such as beta-carotene, or for aquaculture. Both organisms are commonly cultivated, harvested and retailed (Spolaore *et al.*, 2006).

1.1.4. Saline Tolerant Algae

The advantage of cultivating saline tolerant algae such as *Dunaliella salina* and *Spirulina spp.* is that they are able to withstand extreme conditions which most other organisms cannot. Such organisms are classified as extremophiles. The fact that they can tolerate extreme conditions allows them to be grown as a monoculture that is predominantly free of parasitic contamination in large outdoor ponds (Spolaore *et al.*, 2006). When compared to the large sterile bioreactors required to achieve a monoculture with other, less hardy micro algae, outdoor ponds are inexpensive and allow relatively inexpensive mass culture (Weldy & Huesemann, 2007). Mass culture in turn provides the economy of scale to make such operations economically viable (Weldy & Huesemann, 2007). The fact that parasitic contamination is minimized also increases productivity.

Both *Spirulina spp.* and *D. salina* are widely used as a human and animal food. They also contain large quantities of valuable compounds such as β -carotene, lipids and fatty acids (Spolaore *et al.*, 2006). Both these attributes make them economically attractive to cultivate, harvest, and sell.

1.1.4.1. *Dunaliella salina*

Dunaliella salina is a unicellular green alga that has become a model organism for the study of salt adaptation in algae (Oren, 2005). The species is commonly found in salt lakes and other hyper-saline sites and can withstand salt concentrations of up to 200 mg/l NaCl (Oren, 2005; Weldy & Huesemann, 2007). Due to this unique salt tolerance *Dunaliella spp.* are the dominant primary producers in hyper-saline environments (Oren, 2005).



Figure 1-2: *Dunaliella salina*.

D. salina is commonly cultivated for the β -carotene it produces; β -carotene concentration can reach up to 14% of dry weight (Brown *et al.*, 1997) It is therefore used as an ingredient in supplementary and functional foods (Spolaore *et al.*, 2006). Weldy and Hueseman (2007) found that under nitrate limiting conditions, *D. salina* produces a cellular content of between 16 and 44% lipids (wt), making it a viable source of bio-diesel.

1.1.4.2. *Arthrospira (Spirulina)*

Arthrospira spp. commonly known as *Spirulina spp.* are multicellular, filamentous blue-green algae (cyanobacteria) that are found in environments ranging from soils and marshes to thermal springs, the ocean and brackish water (Richmond, 1988; Gupta *et al.*, 2013). In general, *Spirulina spp.* occur in tropical and subtropical alkaline lakes with a high bicarbonate concentration and a pH of up to 11 (Durand-

Chastel, 1980; Vonshak *et al.*, 1981; Richmond, 1988; Tomaselli 1997; Borowitzka, 1999; Ravelonandro *et al.*, 2011). Here they form dense mats of nearly mono-algal growths that float on the surface (Durand-Chastel, 1980; Ciferri, 1983, Pandey & Tiwara, 2010; Gupta *et al.*, 2013).



Figure 1-3: *Spirulina* trichome (string of cells) (Photo taken by the author on 1 April 2007)

Spirulina biomass is a valuable commodity. Its economic potential stems from its remarkable composition, which consists of up to 65% proteins, ten vitamins, high levels (one of the highest known in nature) of β -carotene and fatty acids (Mühling *et al.*, 2005 a & b; Ravelonandro *et al.*, 2011). This makes it ideally suited for use as a human food and supplement, as well as being useful as animal feed (Li & Qi, 1997; Ravelonandro *et al.*, 2011; Teimouri *et al.*, 2013). It should be noted that several species of cyanobacteria are known to produce toxic substances known as cyanotoxins (Merel *et al.*, 2010; Vichi *et al.*, 2012; Pirez *et al.*, 2013). Cyanotoxins have increasingly become a risk to human and animal health (Vichi *et al.*, 2012; Pirez *et al.*, 2013). According to Vichi *et al.*, (2012) although *Spirulina* does not produce cyanotoxins, it is commonly associated in nature with cyanobacteria that do such as, *Microcystis*, *Anabaena*, *Nostoc* and *Planktothrix* species. However, in commercial scenarios where mono-algal conditions are maintained, contamination of *Spirulina* products with cyanotoxins are rare as demonstrated by the results of Vichi *et al.*, 2012 that found no levels of contamination in 16 commercially available products.

1.1.5. *Dunaliella* vs *Spirulina*

From the above sections, it is clear that both organisms are remarkable and have significant commercial value due to their extraordinary composition. Both organisms could thus be used as a means to beneficiate the coal seam water at the CSWBP for the following reasons:

- High quality *Spirulina* biomass may sell for 10-25 US\$ per kg (Rose *et al.*, 2006; Bulk Herb Store, 2013). *D. salina* biomass sells for a similar value. Certain extractables such as beta-carotene reach prices exceeding 1000 US\$ per kg (Rose *et al.*, 2006; Batista *et al.*, 2013). Therefore, both are valuable commodities.
- Both require an alkaline medium to grow (Richmond, 1988; Oren, 2005; Spolaore *et al.*, 2006). The cost of making this media can contribute up to 25% of production costs for *Spirulina* (Belay, 1997; Carlson *et al.*, 2007). However, the proposed project site has a pristine alkaline water resource. Using this water source as the growth medium could reduce the cost of media. The availability of this resource should effect major savings thereby decreasing algal production costs;
- Drying of biomass is one of the major costs involved in dry algal biomass production. This cost can be substantially reduced by using methane, produced on-site, as a combustion fuel;
- The proposed project site is located on the Tropic of Capricorn. Therefore, the environmental conditions in terms of solar irradiance and temperature are ideal for the cultivation of photosynthetic organisms. These environmental conditions should allow for higher than average algal productivity and solar evaporation.

However, the use of water produced at the CSWBP has major drawbacks with regard to the cultivation of *D salina*. Firstly, the coal seam water produced at the

CSWBP is deficient in the high concentration of NaCl which is required by *D. salina* for optimal growth. This would necessitate the addition of significant amounts of NaCl. Secondly, the optimal water temperature for *D. salina* cultivation is 28°C (Weldy & Huesemann, 2007) while the ambient temperature at the project site may reach as high as 44°C. This may result in water temperature exceeding the optimum growth level for the organism. Finally, in order to extract maximum value from *D. salina* it is necessary to extract the β -carotene, which is a costly process. That being said, *D. salina* does produce significantly more β -carotene than *Spirulina spp.* and as such, per kilogram, *D. salina* biomass is more valuable than that of *Spirulina* (Rose *et al.*, 2006).

In terms of pH, conductivity, ambient temperature and water temperature, *Spirulina* is better suited to be cultivated in the coal seam water produced at the CSWBP. However, the hyper-saline brine produced through reverse osmosis would be an ideal medium for *D. salina*.

Therefore, both organisms would add value to the CSWBP, but *Spirulina* is better suited to the environmental conditions and the untreated coal seam water quality. For this reason and practical considerations such as simplicity of production method and production cost, it was decided to initially experiment with the use of *Spirulina* on the beneficiation of raw coal seam water. This forms the thrust of the research project. At a later stage, it may become practical to experiment with the use of *D. salina* to beneficiate the hyper-saline brine.

1.2. Research Hypothesis and Questions

The hypothesis of the study was that *Spirulina* could be used to beneficiate the coal seam water produced at the CSWBP plant. In order to test the hypothesis the following research questions had to be answered:

- Can *Spirulina* survive in coal seam water produced at the CSWBP?
- Which strain of *Spirulina* is most suitable to be grown in coal seam water?
- What kind of medium will be most suitable for growth?

- Are the site-specific conditions suitable for *Spirulina* production?
- What steps can be taken to optimise *Spirulina* production?
- What production yields can be achieved at the proposed site?

Ultimately, the objective of the study would be to produce high quality, low cost *Spirulina* biomass in a sustainable manner as part of a holistic water management system. In order to reach this goal a pilot study was required to answer the research questions. Details of the bench-top (Flask Studies) and open-field trials (High Rate Algal Pond Studies) are discussed in Chapter 3.

1.3. Layout of Dissertation

The dissertation consists of five chapters. Chapter 1 serves as a general introduction.

Chapter 2 consist of the literature review conducted prior to the research. The literature review will summarise the history of the use of *Spirulina spp*, briefly describe their physiology and taxonomy, touch on the mechanics of photosynthesis and describe aspects of mass culture including methods and production considerations.

Chapter 3 details the research design and methodology used, both of the laboratory work and the pilot study. It will include a description of methods, equipment and experimental design.

Chapters 4 and 5 contain the results and discussion of the Flask and High Rate Algal Pond (HRAP) studies, respectively.

Chapter 6 presents the conclusions of the study.

CHAPTER 2: LITERATURE REVIEW

2.1. Introduction

This chapter contains information gathered from the available literature. Sources included peer-reviewed articles, textbooks, the internet and personal communications. The scope of the literature review includes all aspects of *Spirulina* mass culture.

Spirulina has achieved a great deal of fame or alternatively, infamy, as a human supplement in the nutraceutical market. It has been widely proclaimed as the new wonder weight loss drug treatment by some researchers, only to be comprehensively discredited by others who found that weight loss from *Spirulina* was insignificant and unsustainable (Anon, 2003). Unfortunately, this adverse publicity has served to detract from its very real potential.

Spirulina offers a rare opportunity to assimilate the often dissimilar goals of science and business. Scientifically its potential use as a simple model to elucidate the complex mechanisms of cyanobacterial metabolism, motility and genetics has gone widely unexplored. As cyanobacteria laid the basis for life on earth, an understanding of the principles governing these primitive organisms would add a huge amount to our knowledge concerning the very principles of life (Richmond, 2000; Gupta *et al.*, 2013). Because of the relative ease of obtaining a monoalgal culture of *Spirulina* its potential to be used in this manner far exceeds traditional model organisms such as *Nostock* and *Anabaena* (Vonshak, 1997a; Gupta *et al.*, 2013).

Spirulina's economic potential stems from its remarkable composition, which consists of up to 65% proteins, ten vitamins, and high levels (one of the highest

known in nature) of β -carotene as well as fatty acids such as γ -linoleic acid (Jimenez *et al.*, 2003; Mühling *et al.*, 2005a; Ravelonandro *et al.*, 2011; Gupta *et al.*, 2013). These compounds have medicinal qualities. Research has indicated that consumption of *Spirulina* biomass can be effective in treating high blood cholesterol, hyperlipidemia and some cancers (Deshnium *et al.*, 2000; Costa *et al.*, 2004; Gupta *et al.*, 2013). Kumar *et al.* (2009) found that *Spirulina* is effective in treating collagen-induced arthritis in rats. In addition, *Spirulina* extracts have had some success against the HIV-1 by inhibiting the replication of the virus in human cells through augmentation of the human immune system (Hirahashi *et al.*, 2002)

Economically, *Spirulina* has already proven to be a highly valuable microorganism. With an ever increasing world market approaching 4000 tons of dry weight per year it competes well with other microalgae such as *Dunaliella* and *Chlorella*. *Spirulina* sells for \$10-25 a kilogram, indicating a 100 million dollar industry, which is poised to grow. Market growth is due to various factors; firstly, it costs three times less than *Chlorella* to produce; secondly, β -carotene extraction from *Spirulina* is much cheaper than from *Dunaliella*; and thirdly, technology for mass production of *Spirulina* is well tested and understood (Li & Qi, 1997; Cheng, 2001; Grobbelaar, 2009). These factors should bring about marked reductions in production costs. With a drop in production costs, the possibility of tapping into new, previously unexplored markets becomes very real. These markets include animal feeds, aquaculture and high-end nutraceuticals such as β -carotene (Desmorieux and Decaen, 2005; Grobbelaar, 2009; Ravelonandro *et al.*, 2011; Gupta *et al.*, 2013; Teimouri *et al.*, 2013).

The following sections of this literature review will cover the morphology, taxonomy, history, physiology and production methods of *Spirulina*.

2.2. Morphology and Taxonomy

2.2.1. Morphology

Figure 2.1 below depicts *Spirulina* - a multicellular, filamentous blue-green alga (cyanobacterium) (Richmond, 1986).



Figure 2-1: The figure shows a *Spirulina* trichome (string of cells). Taken by the author on 1 April 2007.

The arrangement of the multicellular trichomes in an open left handed helix along the entire length makes this genus highly recognizable (Durant – Chastel, 1980; Richmond, 1986; Tomaselli, 1997; Desmorieux and Decaen, 2005). The helical shape is only maintained in liquid media, on solid media it reverts to a true spiral (Ciferri, 1983). The diameter of the helix varies between 20-100 μm (Desmorieux and Decaen, 2005) whereas the diameter of the central filament is 1-12 μm (Richmond, 1986). Filaments with seven complete spirals measure about 1000 μm and include 250 to 400 cells (Durand - Chastel, 1980). These vegetative cells undergo binary fission in a single plane and display easily visible cross walls under light microscopy (Tomaselli, 1997). Blue-green filaments are free-floating, non-heterocystous and display gliding motility along their axes (Richmond, 1986; Tomaselli, 1997; Vonshak & Tomaselli, 2000).

The helix pitch can be determined by a simple formula given by Tomaselli (1997). Tomaselli (1997) found that the pitch varies between species and strains but is usually between 12 -72 μm . Temperature and physical and chemical conditions may also affect helix geometry (Van Eykelenburg cited by Tomaselli, 1997). Many cases have been noted where, after some time, a specific strain in culture loses its helicity (Lewin, 1980; Richmond, 1986; Vonshak, 1986). According to Richmond (1986), once these straight variants have formed they have no tendency to revert back to the helical form. Jeeji Bai (1985) maintains that this is due to mutations affecting certain trichomes under specific environmental conditions.

2.2.2. Taxonomy

While algae are eukaryotic, cyanobacteria are prokaryotic; it is therefore rather strange that these organisms should be classified as algae. The reason given by Chapman and Gellenbeck (1989) is that, because of the ecological niche the cyanobacteria occupy, they are on a macroscopic level, virtually indistinguishable from other eukaryotic algae. In addition, according to Harlin and Darlin (1988), cyanobacteria contain a different photosynthetic pigment (chlorophyll a), while bacteria mostly contain bacteriochlorophyll or rhodopsin. Cyanobacteria use H_2O as an electron acceptor, produce oxygen and use photosystems I and II for photosynthesis. This same photosynthetic process is found in all contemporary photosynthetic eukaryotes (Harlin & Darlin, 1988).

The genus *Spirulina* falls under the order Oscillatoriaceae (Richmond, 1988; Tomaselli, 1997). Most members of the Oscillatoriaceae have unbranched, cylindrical filaments of indefinite length (Lewin, 1980). During the 1850s, Stizenberger and Gomont (both cited by Tomaselli, 1997) placed forms with regularly coiled filaments and visible septa within the genus *Arthrospira*, and those with invisible septa within the genus *Spirulina*. Bergey's Manual of

Systematic Bacteriology (Castenholtz, 1998) accepted this view of placing the two forms into different genera. “The separation of these two genera has been repeatedly affirmed, on the basis of many characteristics such as, helicity and trichome size (Desikachary, 1959; Hindak, 1985), cell wall structure and pore pattern (Guglielmi & Cohen-Bazire, 1982a, 1982b), gas vesicles, thylacoid pattern (Guglielmi *et al.*, 1993), trichome motility and fragmentation (Agnostidis & Komarek, 1988) and oligonucleotide catalogue of 16S rRNA (Guglielmi *et al.*, 1993)” (Tomaselli, 1997). According to Múhling *et al.* (2005a) the lack of γ -linoleic acid in all *Spirulina* strains and its presence in all *Arthrospira*, could be used as a simple method to distinguish between the genera.

Despite this, many, if not most researchers, continue to use the more traditional classification of Oscillatoria as proposed by Geitler (1925) in which he unified all members of the Oscillatoriaceae with helically coiled trichomes into the genus *Spirulina*. The genus *Arthrospira* is thus commonly designated as *Spirulina*. This classification will be used throughout the dissertation.

The importance of a clear taxonomic classification to species level becomes apparent when new species are found and considered for human consumption (e.g. *S. subsalsa*). This species has been shown to be toxic (Tomaselli, 1997). If this organism were to be lumped together with *Arthrospira* species, the health implications could be catastrophic (Tomaselli, 1997).

2.3. Ecology

Richmond (1988) describes *Spirulina* as a ‘ubiquitous’ organism that can be found in environments ranging from soils and marshes to thermal springs, the ocean and brackish water. In general, *Spirulina* occurs in tropical and subtropical alkaline lakes with high bicarbonate concentrations and pH of up to 11 (Durand-Chastel, 1980; Richmond 1988; Tomaselli, 1997; Borowitzka, 1999; Jiménez et

al., 2003; Morais *et al.*, 2009). Here, they form dense mats that float on the surface in nearly monoalgal growths (Durand-Chastel, 1980; Ciferri, 1983; Pandey & Tiwara, 2010).

2.4. Historical Overview of *Spirulina*

2.4.1. Origin

The cyanobacteria, also known as blue-green algae, were some of the first organisms to evolve. Since blue-green microfossil algae were found in the oldest known sedimentary rock samples dated at 3.5 billion years old (Durand-Chastel, 1980), the cyanobacteria are assumed to have evolved around 3 billion (Chapman and Gellenbeck 1989) to 3.5 billion (Durand – Chastel, 1980; Desmorieux and Decaen, 2004) years ago. On the other hand, some cyanobacteria facultatively use sulphide as an electron donor under anaerobic conditions (Padan, 1979). This suggests that the cyanobacteria evolved from organisms resembling modern non sulphur, purple, photosynthetic bacteria. This puts the time-frame for their evolution at 1.5 billion years ago (Harlin and Darlin, (1988).

“The release of oxygen and its subsequent accumulation in the atmosphere resulted in the most profound and enduring impact algae have ever exerted on life on this planet” (Margulis and Sagan, 1986). The release of oxygen, which was toxic to all living organisms except the oxygen evolving autotrophs, forced the adaptation of the anaerobic organisms. Two options were available to these organisms, either to find anaerobic environments (where we still find them today) or evolve a metabolism that could nullify the effects of the toxic oxygen. In this manner, aerobic metabolism, the Krebs cycle and electron transport, evolved. Harlin and Darlin (1988) give a comprehensive explanation of how the ever

increasing oxygen levels eventually created the ozone layer, shielding the earth from harmful solar radiation, and thereby, allowing the evolution of terrestrial life forms.

2.4.2. History of Human Usage

Humans have been using microalgae as a food source for more than 2000 years, starting with the use of *Nostoc* by the Chinese to stave off famine (Spolaore *et al.*, 2006)

Spirulina was first discovered by the West when Cortez reached Mexico early in the 16th century (Furst, 1978). According to the account by Cortez, the Aztecs ate a 'dried mud' in large quantities and traded it with any willing merchants. The tecuitlatl (*Spirulina*) was eaten over maize in a sauce called Chimolli, which was made by combining the tecuitlatl with tomatoes, chilli peppers and various spices (Durand-Chastel, 1980; Ciferri, 1980).

After this early account, which only became known in the mid-sixties, the identity and classification of the Aztecs tecuitlatl remained a mystery for more than four hundred years (Durand-Chastel, 1980). It was not until 1940 that this organism and its use as a human food was rediscovered. This rediscovery occurred during an expedition to Chad where it was discovered that the blue-green cake that the Kanenbou people, living around Lake Chad, ate on a daily basis was, in fact, *Spirulina* (Ciferri, 1980). The Kanenbou called it dihé and ate it much the same way as the Aztecs. Details of harvesting methods, cultural effects and even recipes of both the Kanenbou and Aztecs are described in Durand-Chastel (1980) and Ciferri (1983).

For various reasons, most notably the Second World War, dihé or tecuitlatl remained unknown to the Western world until the early 1950s when the ever-

growing human population sparked massive interest into novel sources of protein (Richmond, 1988). Large-scale algal cultures were identified as one of the most promising means of reducing the world's perceived protein deficit (Durand–Chastel, 1980). In 1964, dihé was again “discovered” by a member of the Belgian Trans–Saharan expedition, botanist Jean Léonard (Furst, 1978). Léonard's reports of a blue-green algal cake being eaten by the Kanenbou people of Central West Africa attracted the attention of the French Petroleum Institute (Richmond, 1986). Genevieve Clément (1967), a member of the French Petroleum Institute, was the first person to study these cakes under laboratory conditions (Durand – Chastel, 1980). Her results were only published in 1967 and outlined methods to produce *Spirulina* on a commercial scale (Soeder, 1980).

Around this time, a company Sosa Texcoco, requested the French Petroleum Company to study the appearance of a large algal bloom occurring in the evaporation ponds of their bicarbonate production facility (Vonshak, 1997a). This was found to be *Spirulina* and prompted the first in-depth study of the organism, by Zarouk (cited in Belay, 1997), and consequently the first production plant of this alga (Vonshak, 1997b).

2.5. *Spirulina* as a Source of Sustenance

The photosynthetic process is a very efficient means to utilise solar energy to produce useful organic compounds (Matsudo *et al.*, 2012). Therefore, microalgae present an efficient means to convert solar energy into protein and other useful organic compounds (Spolaore, 2006).

Because *Spirulina* is rich in high quality protein, vitamins, minerals and many biologically active substances it is ideally suited to be used as a human and animal food and supplement (Li *et al.*, 1997; Morais *et al.*, 2009; Kim *et al.*, 2013).

In addition, production methods of *Spirulina* are relatively cheap and simple when compared to other microalgae. As such, it is a perfect candidate for mass production.

2.5.1. *Spirulina* as a Source of Protein

Initial interest into the mass production of *Spirulina* was sparked by its exceptionally high protein content, which varies between 60-70% of dry mass in response to environmental factors (Jassby, 1988; Costa *et al.*, 2000; Pelizer *et al.*, 2003; Jiménez *et al.*, 2003; Desmorieux and Decaen, 2005, Morais *et al.*, 2009; Ravelonandro *et al.*, 2011; Teimouri *et al.*, 2013). Jassby (1988) states that *Spirulina* compares favourably with the best traditional protein sources when measured on a usable protein basis. As a protein source the cyanobacteria outperforms cellulose rich algae and plant sources, because of the high levels of polysaccharides in their cell walls, which make the proteins easily digestible (Li *et al.*, 1997; Kim, 2013; Teimouri *et al.*, 2013). Moreover, Richmond (1988), maintains that *Spirulina*'s already first class amino acid profile could be easily modified to increase the levels of sulphur containing amino acids that it lacks (Jassby, 1988; Gupta *et al.*, 2013).

An added advantage is that *Spirulina* has a low nucleic acid content of 4%, which is important, as an excess intake of nucleic acids has been known to cause gout and gallstones (Jassby, 1988).

2.5.2. *Spirulina* as a Source of Vitamins

Spirulina contains ten vitamins (Desmorieux and Decaen, 2005; Gupta *et al.*, 2013) with extraordinarily high levels of vitamin A (vit A) and B12 (Jassby, 1988;

Jiménez *et al.*, 2003) and E (Gupta *et al.*, 2013). Vitamin A deficiency (VAD) is a serious problem in 118 countries worldwide causing a variety of adverse effects, ranging from blindness to premature death. According to the World Health Organisation (WHO), VAD is the leading cause of preventable blindness in children (WHO, 2013). As such, the WHO had set goals to eradicate VAD by 2010 however, these goals were not reached and have been extended to 2015 (WHO, 2013).

According to Jassby (1988), fully formed vit A is not found in plants, but *Spirulina* contains high levels of provitamin A carotenoid pigments that are transformed into vit A in the gastrointestinal tract. These carotenoid pigments consist primarily of β -carotene, which makes up between 52% (Cysewski & Lorenz, 2004) and 79% (Palla & Busson, 1969) of total carotenoids. Seshadri *et al.* (1991), maintain that natural β -carotene has anti-carcinogenic qualities. Compared to other natural sources, *Spirulina* together with *Dunaliella* offers the simplest and most cost effective means to produce β -carotene that is ready for consumption (Romay *et al.*, 1998; Jiménez *et al.*, 2003).

2.5.3. *Spirulina* as a Source of Minerals and Valuable Chemicals

A large amount of iron is found in most *Spirulina* spp. It contains the highest concentration of any biological source; up to 1 g/kg (Jassby, 1988; Gupta *et al.*, 2013). The iron found in *Spirulina* is exceptionally digestible. Jassby (1988) speculates that this is caused by the presence of a linear tetrapyrrol phycocyanobilin. This, according to Hirata (2000), closely resembles bilirubin that is found in humans. The phycocyanobilin is thought to bind iron, thereby increasing its absorbability several-fold (Jassby, 1988). Although the exact process is speculative, studies conducted by Johnson and Schubert in Lewin (1986), indicate that iron from *Spirulina* is readily available for uptake by animals tested.

Phycocyanobilin forms part of the widely studied pigment phycocyanin, and is responsible for its deep blue colour (Hirata, 2000; Pandey & Tiwara, 2010). This colour in turn makes phycocyanin useful as a natural colorant (Kato, 1994; Mortensen, 2006; Batista *et al.*, 2013). This is only one of the pigment's useful properties. Romay (1998) describes its use as an antioxidant and anti-inflammatory agent. His work is supported by the findings of Hirata (2000), who states that: "phycocyanin acts as an effective antioxidant in the human body". Liu *et al.* (2000), has shown that phycocyanin has the ability to inhibit growth in K562 human leukaemia cells. Zhang *et al.* (2011) also showed the anti-cancer effects of phycocyanin in bench top experiments.

γ -linoleic acid, a polyunsaturated fatty acid, is known to reduce the level of serum triglycerides and low density lipoproteins and it stimulates the immune system (Leaf and Weber, 1988; Gupta *et al.*, 2013). According to Mühling *et al.* (2005a), γ -linoleic acid constitutes up to 29.4% of the total fatty acid content in *Spirulina* (*Arthrospira*), which is also affected by environmental factors, such as temperature (Mühling *et al.*, 2005a; Ravelonandro *et al.*, 2011).

2.6. Factors Affecting Productivity

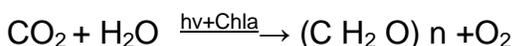
High production cost is the biggest hurdle to overcome if *Spirulina* is to achieve its potential for mass culture (Richmond, 1988; Vonshak, 1997b; Richmond, 2000; Spolaore, 2006; Pandey & Tiwara, 2010, Ravelonandro *et al.*, 2011). These high costs are caused mainly by the low productivity achievable in large scale operations (FAO, 2009; Grobbelaar, 2009).

To increase productivities, it is essential to have an understanding of the factors affecting it. Principal among these is the effect of light.

2.6.1. *Photosynthesis and the Effect of Light*

In its simplest form, photosynthesis can be described as the use of light energy to split a water molecule with the concurrent fixation of CO₂ and the production of oxygen.

Photosynthesis occurs according to the following reaction.



The photosynthetic pigments of *Spirulina*, namely chlorophyll a, phycocyanobilins and carotenoids, capture light energy, photons, from the sun (Mohanty *et al.*, 1997; Matsudo *et al.*, 2012). The captured photons then excite an electron, which in turn reduces a series of electron acceptors, such as ferredoxin, all along an electron transport chain to the reaction centre, where it is converted into chemical energy. This energy is used to fix CO₂ (Grobbelaar, 2000; Matsudo *et al.*, 2012). In general, 12 photons are needed to fix one mole of CO₂ (Grobbelaar, 2000; Matsudo *et al.*, 2012).

In addition to Photosystem I and Photosystem II (the reaction centres of photosynthesis that contain specialised chlorophylls that can undergo charge separation), *Spirulina* and other cyanobacteria also have phycobilisome (PBsome) (Hirata, 2000). The PBsome is a pigment protein complex, consisting of phycocyanobilin, which can absorb light at wavelengths where chlorophyll a cannot (Mohanty *et al.*, 1997; Matsudo *et al.*, 2012).

Because solar radiation (light) is the energy source for photosynthesis, light intensity is one of, if not the, most important factors affecting photosynthetic

microorganisms (Vonshak, 1997; Bezerra *et al.*, 2011; Ravelonandro *et al.*, 2011).

According to Vonshak (1997a), the most common method used in the study of the effect of light on photoautotrophic metabolism is the Photosynthesis-irradiance (PI) curve (Figure 2-2). According to Kirk (1994), PI curves provide a method of measuring the rate of oxygen production or carbon dioxide fixation as determined at a given light intensity. Light intensity is measured as irradiance ex. W/m^2 or MJ/m^2 . Carbon fixation is normalised to biomass and in the case of algae, dry weight (Kirk, 1994). Kirk (1994) provides various methods of determining the rate of oxygen generation including chemical analysis, the use of an oxygen electrode and manometry. Therefore, rates of photosynthesis are expressed in rates per units biomass, typical units for the specific photosynthetic rate (P) are $\mu\text{moles CO}_2$ (or O_2) or $\text{mg C per mg chl a per hour}$ (Kirk, 1994).

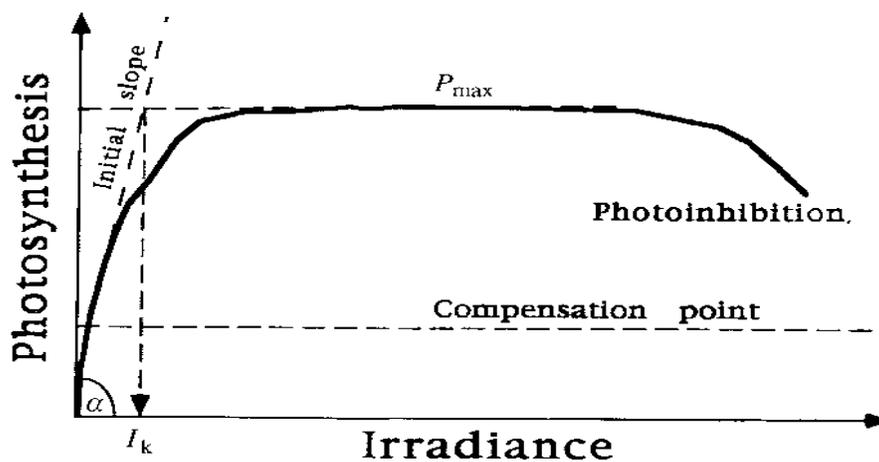


Figure 2-2: A typical photosynthesis versus irradiance (PI) curve (Vonshak, 1997a). P_{max} represents the maximal photosynthetic rate, I_k is the irradiance derived when extrapolating the initial slope of the IP curve to where it crosses P_{max} , i.e. the transition between light-dependant and light-saturated photosynthesis and α is the slope of the light limited phase of the PI curve and provides an indication of efficiency with which light is harvested, the compensation point represents the irradiance where respiration rate equals photosynthetic rate.

The PI curve represents a typical growth response to substrate availability where the substrate availability is represented by light intensity measured as irradiance (Richmond, 2000). According to Figure 2-2, cultures experience negative growth in the dark or at very low light intensities. Negative growth occurs because although some photosynthesis occurs, at low light intensities the rate of respiration i.e. oxygen consumption and carbon dioxide generation through the metabolism of biomass, exceeds the rate of photosynthesis (Kirk, 1994).

As irradiance increases, a point is reached where the photosynthetic rate is balanced by respiration rate. This is called the compensation point (Vonshak, 1997a). Thereafter, growth becomes linear in response to increasing substrate/light availability. Growth then follows an exponential curve which slowly tails off as the saturation process is initiated in which, “an increasingly higher light flux is needed to affect a given response” (Richmond, 2000).

According to Kirk (1994), at the saturation point the maximum potential photosynthetic rate P_{max} , is reached. At P_{max} , where the photosynthetic machinery of the cell is fully saturated, there is no further net response to increasing irradiance. The irradiance at which photosynthesis is saturated I_k can be estimated from the intersection of the initial slope with P_{max} , or more accurately as P_{max}/α . Alpha is the slope of the light limited section of the PI curve and is an indication of the efficiency of light harvesting. Kirk (1994) describes α as estimate of the amount of photosynthesis per incident photon.

If the photon flux continues to increase, the culture will start to experience negative growth, a phenomenon known as photoinhibition (Vonshak, 1997a; Grobbelaar, 2009). Photoinhibition is defined as “a loss of photosynthetic activity due to an increase of photon flux density (PFD) beyond the saturation point of photosynthesis” (Vonshak, 1997a). It is thought that this is caused by the accumulation of toxic H_2O_2 .

Vonshak *et al.* (1988) showed that photoinhibition, or the effect thereof, varies between strains and with differing growth conditions. If strains grown under diverse light intensities are compared, those strains grown under higher PFD show a higher resistance to light stress (Vonshak, 1997a). Cultures grown in low light exhibit a lower photosynthetic maximum, as well as a lower overall efficiency (amount of incident light used). This is due to the acclimation to different light intensities found in all algae (Vonshak, 1997a).

Grobbelaar and Kurano (2003) who corroborated and expanded on Vonshak's (1997a) findings, found that high light (HL) acclimated algae exhibit:

- Higher (than low light) photosynthetic rates
- Low contents of chlorophyll but higher levels of auxiliary pigments such as carotenoids
- Low maximum photosynthetic efficiencies i.e. lower α values
- High I_k values

The exact opposite of the above mentioned characteristics are found in low light adapted strains (Grobbelaar and Kurano, 2003).

It is important to know the state of a particular culture as this will influence its reaction to a change in light conditions. Vonshak and Lu (1999) showed that low light (LL) adapted cells are more prone to photo-damage if exposed to high solar irradiation, than are HL adapted cells.

In the PI curve, light intensity is given as the sole limiting factor in a light-limited system. According to Richmond (2004), this only holds true in optically thin cultures, where all other parameters are optimised. This situation does not occur in mass cultures as the maintenance of optically dense cultures is crucial if the high photon flux experienced outdoors is to be utilised (Richmond, 2004).

In mass culture, it is the amount of light available to each cell, or the cells light regime, that is of paramount importance (Richmond, 1988). This light regime is influenced by a number of factors. The cell density of a given culture is one such factor.

2.6.2. Cell Density

2.6.2.1. Self-Shading

In dense cultures, cells tend to shade each other, a phenomenon known as self-shading (Richmond *et al.*, 1980; Vonshak, 1997b; Matsudo *et al.*, 2012). According to Richmond (1988), at the average cell density occurring in mass culture (400-500 mg/l), self-shading can cause up to 80% of the culture to be in complete darkness, even while experiencing full solar irradiance. Under self-shading circumstances the culture is light-limited as well as being low light adapted. In fact, it has been shown that population density is inversely related to photosynthetic potential (Richmond, 1988; Matsudo *et al.*, 2012).

2.6.2.2. Optimal Cell Density

Richmond and Zou (1999) have demonstrated the existence of an optimal cell density (OCD), this is the density at which, for a given set of conditions and a given strain, the highest productivity is obtained. They found that any deviation from the OCD resulted in a loss in productivity. The cell density provides the only means to control the amount of light received by individual cells, and can be controlled with relative ease through harvesting (Richmond, 1988; Matsudo *et al.*, 2012). Cell density directly influences the light regime experienced by individual cells through its effect on the light dark cycle.

2.6.2.3. Light - Dark Cycles

Rather than enduring a constant PFD, cells experience a light dark (L-D) cycle which in dense cultures can vary from a fraction to a few seconds (Richmond, 2009; Ravelonandro *et al.*, 2011). This cycle is a function of the speed with which the cells move from the dark lower layers (dark volume) to the illuminated higher levels (photic volume). This in turn, is influenced by the light path, culture depth, turbulence and cell density (Richmond, 2009; Ravelonandro *et al.*, 2011).

Reducing the light path increases the rate of the L-D cycle, which corresponds to an increase in photosynthesis (Richmond, 2004; Bezerra *et al.*, 2011). This occurs because at high densities, cells are exposed to relatively short bursts of light and long periods of darkness. Increasing the frequency of the oscillation between the photic and the dark volumes would therefore result in a higher light utilization efficiency (Grobbelaar *et al.*, 1996; Bezerra *et al.*, 2011), i.e. the amount of incident light used by photosynthesis (Vonshak, 1997b; Bezerra *et al.*, 2011). In a bioreactor, the light path is directly proportional to the culture depth (Vonshak & Grobbelaar, 1986, Bezerra *et al.*, 2011).

One of the most common and effective means to increase the frequency of the light dark cycle is by increasing turbulence.

2.6.2.4. Turbulence

Turbulence has a three-fold role to play in maximizing productivity. Firstly, it moves the cells between the photic and dark volumes that occur in the pond, thereby increasing the rate of the L-D cycle and distributing the PFD more evenly

among the cells (Richmond & Vonshak, 1980; Richmond & Grobbelaar, 1986; Borowitzka & Borowitzka, 1989; Richmond, 2009; Ravelonandro *et al.*, 2011). Secondly, it breaks down the nutrient barrier that forms around individual cells, which can seriously affect cell growth (Richmond and Grobbelaar, 1986; Richmond, 2004; Carlson, 2009). Thirdly, it allows the degassing of inhibitory oxygen to take place (Vonshak, 1997b; Carlson, 2009).

Richmond and Vonshak (1980), found that for any given temperature and light range, an increase in turbulence resulted in an increase in output rate and OCD. During a similar study conducted in 1986, they found that increasing the rate of stirring facilitated the maintenance of a higher cell density at peak performance. The rate of mixing is limited by the resultant energy costs and fragmentation of trichomes (Belay, 1997; FAO, 2009).

2.6.2.5. Interdependence

The above-mentioned factors should not be viewed in isolation since they are interdependent. This makes solving the low productivity issue extremely complex.

Richmond (2004), who studied this interrelationship found that at low PFD, increasing mixing rate (turbulence) had no effect on either OCD or output rate, as light intensity was the limiting factor. Doubling the PFD at a constant mixing rate virtually doubled the OCD and tripled productivity. Keeping the PFD constant at the higher level and doubling the mixing rate resulted in a doubling in OCD and an increase in output rate.

This shows that when PFD is low, light is the limiting factor. With increased PFD light would become saturating but is prevented from doing so by an increase in optimal density. Self-shading now occurs and light would become limiting again, but for the turbulence, which shortens the L-D cycle and light path which in turn

increases both OCD and output rate. Thus, at ultra high cell density and high turbulence it becomes possible to efficiently utilise the high photon flux density (HPFD) available outdoors. All these factors are intricately linked to the next topic of discussion, temperature.

2.6.3. Temperature

Spirulina is a mesophilic organism with a temperature optimum of between 35 and 40 degrees Celsius (Richmond, 1988; Morais *et al.*, 2009; Markou & Georgakakis, 2011). However, there is a marked difference in temperature tolerance between strains (Vonshak, 1997a; Morais *et al.*, 2009). Finding the optimum temperature for the specific strain being used is therefore of the utmost importance.

Richmond (1988) found that the lower limit for growth is well defined at approximately 18°C, with culture deterioration occurring at 12°C. Jiménez *et al.* (2003) have refuted these findings. The study conducted in southern Spain achieved viable productivities at temperatures as low as 9°C. This confirms the finding by Vonshak and Tomaselli (2003) that various strains vary in their optimal temperature ranges and that the organism is highly adaptable to experienced conditions. They found that certain strains had an optimum temperature range between 24-28 °C and other between 40-42 °C.

At night, the organism can survive much lower temperatures even down to 0°C (Vonshak & Richmond, 1980; Jiménez *et al.*, 2003). This occurs because when temperature is low during the day it severely limits the photosynthetic rate, as certain enzymes cannot function, notably ATPase, and energy transfer is less effective. With lowered photosynthetic activity growth does not occur.

Low night temperatures are an advantage, as it curtails the so called 'dark losses' due to respiration (Vonshak, 1997b; Carlson *et al.*, 2007; FAO, 2009).

The Siam Algal Company, located in Thailand where temperatures remain high at night, have reported losses of up to 30% of biomass produced during the day, due to respiration at night (Vonshak, 1997b).

Richmond and Vonshak have repeatedly shown that temperature has an impact on the effect of light and visa versa. In winter, when temperatures are low, raising PFD has a very slight effect on output rate because temperature is limiting. Changing temperature while the PFD is low also has little effect on productivity, since light is the limiting factor. Consequently, the highest productivity is achieved when both parameters are optimised. Vonshak (1997b) showed that at low temperatures cultures experience more damage due to photoinhibition than cultures that are heated. This occurs since higher temperature increases metabolism and the damage done by photoinhibition can be repaired more quickly.

However, even at optimal temperature and light regimes, cultures are unable to survive without the correct nutrition.

2.6.4. Media and Nutrition

The first step in the successful culturing of *Spirulina* is making up media with nutrient concentrations that supports growth. This is a vital and expensive part of mass producing algae and can constitute up to 25% of production costs (Belay, 1997, Spolaore et al., 2006; Carlson et al., 2007; FAO, 2009; Markou & Georgakakis, 2011).

The most important aspect of the media is that it should be highly alkaline (Richmond, 1988; Markou & Georgakakis, 2011). This is essential as it provides the organism with the necessary high pH to achieve a monoalgal conditions in

outdoor cultures (Richmond, 1988; Belay, 1997; Borowitzka, 1999; Lee, 2001; Grobbelaar, 2009).

This alkalinity is provided by high concentrations of sodium bicarbonate; up to 0.2M (Richmond, 1988). The sodium bicarbonate serves a dual purpose in that it also buffers the media effectively. This is important since Richmond (1988) found that *Spirulina* cultures react adversely to sudden changes in pH. Ravelonandro *et al.* (2011) found that culture health is severely affected by rapid changes in pH.

Traditionally nitrates have been used as the main source of nitrogen (Jassby, 1988; Costa *et al.*, 2001; Matsudo *et al.*, 2009; Markou & Georgakakis, 2011). Using different sources of nitrogen can, however, be just as effective. Costa *et al.* (2001) showed that comparable growth is achievable using ammonium and urea. Matsudo *et al.* (2009) made a similar finding and also found that the use of urea can bring about a significant reduction in media costs.

As *Spirulina* is a photoautotroph it has long been thought that it could not grow heterotrophically in the dark (Richmond, 1988; Lee, 2001; Andrade & Costa, 2007). In a study conducted by Mühling *et al.* (2005a), it was found that out of a total of 35 strains, 34 could utilise glucose and 24 fructose heterotrophically in the dark. None could use polysaccharides. For that reason, some producers add small amounts of glucose to the medium to enhance growth; this could be risky as it may increase the bacterial load on the system to dangerous levels (Lee, 2001). Andrade and Costa (2007) found that molasses produced as a waste product from sugar cane could be used effectively to stimulate growth.

Spirulina is able to survive under relatively high sodium concentrations, of up to 18 g/l if the ratio of K^+ to Na^+ is greater than five. Both nutrients are indispensable to growth (Richmond, 1988, Costa *et al.*, 2003; Matsudo *et al.*, 2009).

Because *Spirulina* is often cultured at high temperatures (34-40°C), gas solubility in the medium is low (Gordillo *et al.*, 1999). Consequently, this leads to the culture becoming carbon limited (Borowitzka, 1999; Carlson *et al.*, 2007, Ravelonandro *et al.*, 2011). In these situations, CO₂ is added as an additional inorganic carbon source (Gordillo *et al.*, 1999; FAO, 2009; Ravelonandro *et al.*, 2011). The CO₂ also serves as an effective pH control (Hu *et al.*, 1996; Richmond, 2009). Gordillo *et al.* (1999) found that the addition of CO₂ above saturation levels did not cause any increase in the maximal growth rate but did decrease maximum biomass yield. Ravelonandro *et al.* (2011) made a similar finding showing that CO₂ addition up to 1% improved productivity by 60%. Thereafter, no measurable difference could be determined.

2.6.5. pH

Spirulina is an alkaliphile and as such thrives in a pH range between 9.5 and 12 (Richmond, 1988; Spolaore *et al.*, 2006; Ravelonandro *et al.*, 2011; Gupta *et al.*, 2013). According to Vonshak and Grobbelaar (1986) this is mainly due to two reasons: gas exchange and exclusivity of the culture.

Gas exchange between the culture and the atmosphere is dependant on the gradient of the partial pressures of the gasses. Increasing the pH increases the CO₂ gradient, thereby allowing the uptake of free carbon from the atmosphere (Vonshak and Grobbelaar, 1986; Belay, 1997; Ravelonandro *et al.*, 2011). Exclusivity is self-evident, and will be discussed in more detail under the maintenance of a monoalgal culture.

Under laboratory conditions, *Spirulina* is reported to survive in a large pH range (Richmond, 1988; Vonshak, 1997a; Belay, 1997). Defining an optimum is once

again complicated since it varies between strains, at different population densities and with environmental conditions.

Vonshak and Grobbelaar (1986) found that productivity was unaffected up to pH 10.5 after which a sharp decline could be seen. On the other hand, Richmond (1988), found no measurable decrease in growth up to pH 12, after which both teams of researchers observed the gradual death of the culture. However, Pandey and Tiwara (2010) recorded no growth at a pH of 12 for a strain of *S. maxima*. Ogbonda *et al.* (2007) found maximum productivity for a strain of *Spirulina* isolated from the Niger Delta at pH 9. Belay (1997) recommends growing the culture at no higher than pH 10.5, as higher levels result in the precipitation of calcium carbonate.

2.7. Production Technology

2.7.1. Methods Used for Mass Production

Two general culture systems are used in algal mass production, namely, open and closed reactors (Lee, 2001; Spolaore *et al.*, 2006; Carlson *et al.*, 2007; FAO, 2009; Morais *et al.*, 2009). Each has its own advantages and disadvantages. Various factors need to be considered when deciding upon a suitable system. These include biology of the alga; cost of land, labour, energy, water, nutrients; climate (for outdoor production) and the type of final product (Borowitzka, 1999; Carlson *et al.*, 2007; FAO, 2009). Finally, the correct choice for an economically viable outcome becomes a compromise between all these factors (Borowitzka, 1999; Carlson *et al.*, 2007; FAO, 2009).

2.7.2. Closed Reactors

During the last 50 years, two main types of enclosed photobioreactors have gained favour commercially. These are flat plate and tubular bioreactors (Carlson *et al.*, 2007; FAO 2009; Matsudo *et al.*, 2012). The latter consists of straight horizontal tubes, made from transparent material such as Plexiglas (Torzillo, 1997; Wang & Lan, 2012), which are connected in a myriad different ways as described by Lee (2001) and by Wang and Lan (2012).

Flat plate photobioreactors, as the name indicates, are constructed of a shallow (flat) tray which is then covered with a transparent material (Tredici and Zittelli, 1997; Spolaore *et al.*, 2006; Carlson *et al.*, 2007; Wang & Lan, 2012). These flat reactors are then placed at an angle with the horizontal in order to achieve maximal control over the light path (Lee, 2001; Wang and Lan, 2012). Flat plate reactors provide the advantage of a large illumination to volume ratio (Wang & Lan, 2012). Large-scale plate reactors of up to 1000 L have been used successfully in the production of *Spirulina* (Borowitzka, 1999).

Various authors favour closed systems for *Spirulina* production (Tredici and Zittelli, 1997; Torzillo, 1997; Borowitzka, 1999). The ability to produce “clean” algae with a high light utilisation efficiency, temperature control and the ability to be used outdoors, are but some of the apparent advantages of closed systems. Add to this the small spatial requirement, the ability to control culture conditions and the corresponding consistent and high quality product and the apparent advantages of closed bioreactors are clear (Borowitzka, 1999).

Lee (2001) notes that capital and energy costs in enclosed bioreactors are high, therefore in order to deliver a product that is priced competitively, the volumetric biomass production needs to be much higher than is currently achievable. Furthermore, it is difficult to rid closed reactors of bacterial infections; therefore, most products produced in these systems do not meet the Good Manufacturing

Practise for pharmaceuticals (Lee, 2001). Consequently, the only option available for commercially produced products of closed photobioreactors becomes food, feed and additives. All of which are low in price and production volume. Wang and Lan (2012) conducted a complete review of closed systems including flat plate, tubular, membrane and disposable plastic bag PBRs. They concluded that although promising, especially in the field of CO₂ capture, the technology is not yet feasible for large scale cultivation.

It is thus not surprising that open-air culture systems are by far the dominant system in the mass cultivation of *Spirulina* (Tredici & Zittelli, 1997; Spolaore et al., 2006; Carlson et al., 2007; FAO, 2009; Morais et al., 2009; Markou & Georgakakis, 2011; Zhu *et al.*, 2013). The reason is simple economics.

2.7.3. Open Air Systems

Four main open air systems predominate in the production of microalgae, namely, big shallow ponds, tanks, circular ponds and raceway ponds (Borowitzka, 1999; Carlson *et al.*, 2007; FAO, 2009; Zhu *et al.*, 2013). According to Borowitzka (1999) *Spirulina* culture requires good mixing conditions. This excludes shallow big ponds which are mainly used in the production of *Dunaliella*. Tanks and circular ponds are limiting with regards to the volume they can produce (Lee, 2001) and, in the case of the circular ponds shown in Figure 2-3, due to the energy costs associated with the rotating scraping arm needed in this system (Lee, 2001). As a result, the largest pond size recorded only has a 50 m radius.

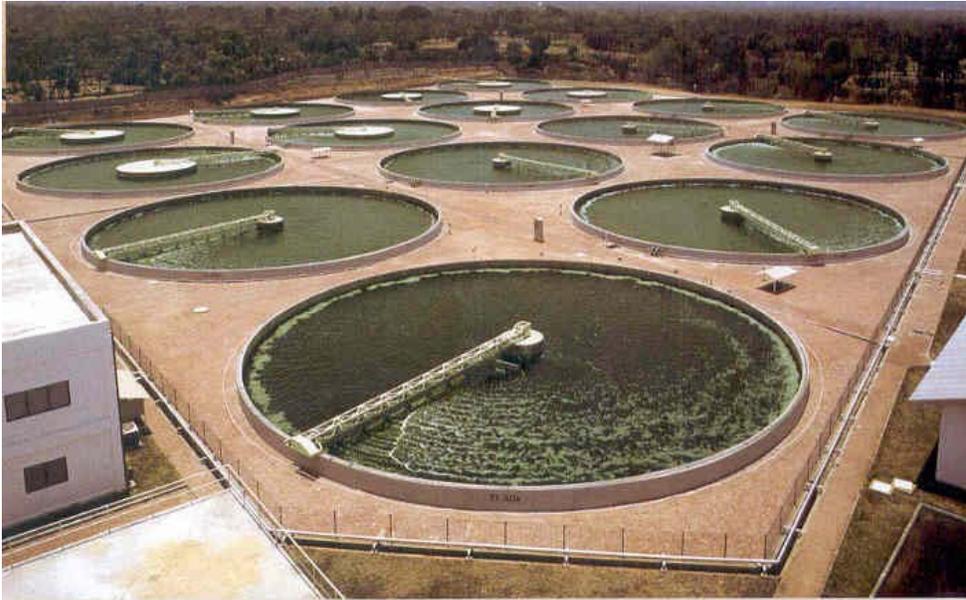


Figure 2-3: Circular ponds of Sun Chlorella Corporation, Shimogyo-ku, Kyoto, Japan (Rose *et al.*, 2006)

Tank systems are mainly employed for the in-house culture of microalgae in the aquaculture industry.

By far the most common and widely used system for mass production of *Spirulina* is the raceway pond (Richmond, 1988; Belay, 1997; Borowitzka, 1999; Cheng, 2001; Lee, 2001; Carlson *et al.*, 2007; FAO, 2009; Grobbelaar, 2009; Zhu *et al.*, 2013). This system will be discussed below in much greater detail.

Raceway ponds shown in Figure 2-4 and 2-5 are shallow constructions 15-30 centimetres deep (Vonshak, 1997b; Carlson *et al.*, 2007; FAO, 2009; Zhu *et al.*, 2013). Some ponds are constructed from concrete; the Siam Algal company of Taiwan use concrete lined ponds (Vonshak & Richmond, 1988; Vonshak, 1997b;) Most producers prefer using shallow earthen constructions lined with a cheap hardy material such as PVC (Vonshak & Richmond, 1988; Richmond, 1988; Belay, 1997; Carlson *et al.*, 2007; FAO, 2009; Zhu *et al.*, 2013). Earthen lined ponds achieve a significant reduction in construction costs of the ponds.



Figure 2-4: Earthrise farms California. The figure shows the raceway production ponds (Rose *et al.*, 2006).



Figure 2-5: Cyanotech: Hawaii. The figure shows the raceway production ponds (Rose *et al.*, 2006) .

Richmond (1988) suggests that raceway shaped ponds are stirred by a paddlewheel at speeds of 50-70 cm/s. Grobbelaar (2009) has utilised a linear flow rate of 23 cm/s with some success. Evidence exists that the paddlewheel is not an effective method for creating turbulence (Vonshak, 1997b; Richmond, 2004), but does in fact create laminar flow, which achieves little or no increase in

the light dark cycle of individual cells. This absence of turbulence could be remedied by the introduction of vortex creating baffles (Laws *et al.*, 1983 in Vonshak, 1997; Carlson *et al.*, 2007; FAO, 2009).

Pond sizes can vary from 0.1 to 0.5 hectares (Vonshak & Richmond, 1988; Zhu *et al.*, 2013). Size is determined by optimal pond depth, mixing velocity, energy requirements and construction material (Borowitzka & Borowitzka, 1989; FAO, 2009). Cell concentrations of 0.1-0.5 g/l can be maintained correlating to a productivity of 25 g/l/d (Richmond *et al.*, 1990, Borowitzka, 1999). These low productivities mean that production costs of \$8-\$15 per kilogram are high when compared to other excellent protein sources such as soy-meal at \$1 per kilogram (Lee, 2001). Production costs of this order are comparable to other culture systems, but the large volumes of product produced makes open raceway cultivation economically more attractive.

One of the major drawbacks of the open culture systems is the reliance on favourable environmental conditions (Borowitzka, 1999; Morais *et al.*, 2009). This means that there is little control over optimal conditions and the quality of the final product. Borowitzka (1999) explains that most outdoor cultures are light- and carbon dioxide limited. Morais *et al.* (2009) found that covering the ponds with a greenhouse structure effectively mitigated these drawbacks.

2.8. The Production Process

To appreciate the factors affecting mass culture it is imperative to have an understanding of the basic production process. Belay (1997) describes mass cultivation as having four distinct phases; culturing, harvesting, drying and packaging. Each phase has its own unique challenges which are discussed in detail by Vonshak (1997) and Belay (1997).

2.8.1. *Culturing / Culture Maintenance*

2.8.1.1. pH

As discussed before, maintaining a high and constant pH is integral in the production of *Spirulina*. In industrial-scale operations pH levels are maintained by supplying CO₂ to the medium (Belay, 1997; Spolaore et al., 2006; Carlson *et al.*, 2007; FAO, 2009; Ravelonandro *et al.*, 2011; Zhu *et al.*, 2013). The specific system employed is a compromise between efficiency of gas transfer, capital and production costs (Belay, 1997). Maximal efficiencies of 80% CO₂ transfer have been reported in laboratory studies (Eriksen *et al.*, 1998). These kinds of numbers are, however, unknown in mass production, where 60% is considered extraordinary (Vonshak and Richmond, 1988; Belay, 1997; Carlson *et al.*, 2007; FAO, 2009).

2.8.1.2. Nutrient Concentration

During culture growth, nutrient concentrations may vary significantly due to precipitation, leaching from the system and uptake by the organisms themselves. This in turn, is determined by output rate and culture density (Belay, 1997; Zhu *et al.*, 2013). Belay (1997) describes a loss of phosphorous and iron from the medium that cannot be accounted for by growth. It is, therefore, very important to routinely monitor the levels of all main nutrients i.e. nitrogen, phosphorus, potassium and magnesium.

2.8.1.3. Monoalgal Culture

Maintaining a pH higher than 9.5 is mandatory to sustaining a monoalgal culture (Richmond and Grobbelaar, 1986; Richmond, 1988; Vonshak and Richmond, 1988; Belay, 1997; Morais *et al.*, 2009). *Spirulina* cultures are most susceptible to contamination during inoculation when the culture is very dilute, weakening its allelopathic capabilities (Belay, 1997; Pelizer *et al.*, 2003).

In industrial applications where the medium is constantly recycled, the build-up of organic matter stimulates the growth of green algae (Richmond, 1988) resulting in a type of autoinhibition (Belay, 1997).

Chlorella is the main contaminant of *Spirulina* cultures (Vonshak and Richmond, 1988). Blooms of these algae can be controlled by the addition of 1mM ammonia (Richmond and Boussiba in Vonshak and Richmond, 1988). Higher concentrations of ammonia (2 mM) have been shown to be effective in controlling grazers such as ciliates, rotifers and cladocerans (Lincoln *et al.*, 1982; Vonshak and Richmond, 1988).

2.8.2. **Harvesting and Drying**

2.8.2.1. Harvesting

Because of the length of *Spirulina* trichomes and their tendency to bio-flocculate, harvesting the biomass is hugely simplified in comparison to other commercially produced microalgae (Richmond, 1988; Laliberté *et al.*, 1997; Morais *et al.*, 2009).

According to Vonshak (1997), inclined or vibrating screens are used to harvest the biomass. Inclined screens usually consist of a 380-500 µm mesh with a

surface area of 2-4 m². Efficiencies of up to 95% with a capability of 10-18 m³ of culture per hour can be obtained from such a setup (Vonshak and Richmond, 1988). Vibrating screens may also be used. They are usually placed in double or triple stacks and can achieve the same volumes and efficiencies as inclined screens while using only a third of the surface area (Vonshak, 1997).

Vonshak and Richmond (1988) note that in both harvesting systems mechanical shearing forces produced by pumping can cause fragmentation of trichomes. The resultant smaller trichomes are then able to move through the screen and enter the culture. Smaller microalgae can also pass through the harvesting screen. In this way, harvesting can lead to a culture that is dominated by short trichomes and contaminants. Any fragmentation gives a competitive advantage to both mixotrophic and heterotrophic organisms (Grobbelaar, 2009). Additionally, vibrating screens may allow narrow trichomes to pass through the harvest screen and contaminate the culture and are considered by Vonshak and Richmond (1988), not to be ideal for the harvesting of *Spirulina*. Morais *et al.* (2009) utilised the unused harvest liquid to feed fish, thereby, effectively recycling the liquid.

2.8.2.2. Drying

Drying poses a significant financial outlay, as it can constitute up to 30% of production costs (Richmond, 1988; Borowitzka and Borowitzka, 1989; FAO, 2009). With *Spirulina*, it is imperative to wash the slurry with acid water to remove absorbed carbonates, so that the ash content is not higher than 7% (Richmond, 1988; Vonshak, 1997; Belay, 1997).

Three methods of drying predominate.

- Spray drying, which is the most expensive and effective (Vonshak, 1997b),

- drum drying, which is effective but produces a flake that is not easily formed into a tablet (Richmond, 1988), and;
- sun drying, the least expensive but recommended only in the animal feed market because the resultant bacteriological load is not suitable for human consumption (Richmond, 1988).

2.9. Conclusion

Algal mass production is now, as it has been for the last 50 years, biotechnology with great potential. From the review of the literature, it is apparent that in mass culture the low productivity and resultant high production cost is a serious barrier to market. It was found that the maintenance of optimal pH, cell density, and nutrient concentration are crucial to achieve economically viable production. It is also evident that the specific strain utilised will affect all the aforementioned parameters and therefore strain selection is critical to any mass culture endeavour. Finally, the effect of environmental conditions, especially incident light and ambient temperature was found to be a key driver of productivity especially in open outdoor cultures.

CHAPTER 3: METHODOLOGY

3.1. Research Design

The investigation comprised of quantitative experimental research in a real world setting. Internal validity was assured by using a post-test control only experimental design. External validity was assured through the real world setting (Leedy and Ormrod, 2005). All laboratory experiments were hypothesis based.

In order to answer the research questions two experimental approaches were used. The two methodologies were Flask Studies and High Rate Algal Ponds (HRAP).

3.1.1. *Flask Studies*

The flask studies were bench top batch culture investigations that were carried out under laboratory conditions.

The flask studies were used for strain selection and to determine optimum levels for:

- nutrient supplementation,
- optimum pH for growth in Coal Seam Water Media, and the;
- effect of iron concentration on growth.

Each of these studies will be discussed in detail in subsequent sections.

Data collection and statistical methods are described under the analytical methods section (section 3.5.3 and 3.5.4) of this chapter.

3.1.2. *High Rate Algal Ponds (HRAP)*

Four small raceway ponds, each with a surface area of $\sim 20 \text{ m}^3$, were constructed at the project site. Using various operating protocols the research questions and hypotheses, were tested.

As the ponds were outdoors, it was not possible to control environmental conditions. However, all controllable parameters such as pH, conductivity, depth and turbulence were kept constant at levels prescribed by the ruling operating protocol.

The HRAP represent the pilot phase of the project. These studies consisted of the following.

- Batch Culture
- Fed Batch Culture
- Semi Continuous Culture
 - Nutrient Optimisation
 - Condition Optimisation (Pond Heating)

3.2. Research Methodology

Data was captured on a daily basis; this included qualitative and quantitative data.

3.2.1. *Quantitative Data*

3.2.1.1. Culture Parameters

The following culture parameters were recorded on a daily basis

- pH,
- Conductivity (mS/cm),
- Temperature (°C)
- Depth (mm)
- Salinity (NaCl g/l).

3.2.1.2. Growth Data

The following growth data were determined analytically:

- Culture density as chlorophyll a ($\mu\text{g/l}$) and;
- Productivity as Δ dry weight (g/l).

3.2.1.3. Environmental Conditions

All environmental conditions were monitored by an onsite Campbell Scientific (CR1000.Std.05) automated weather station that measured:

- Total solar irradiance (MJ/m²),
- Minimum and maximum temperature (°C),
- Wind speed and direction (m/s),
- Precipitation (mm), and;
- Relative humidity (%).

3.2.2. Qualitative Data

Qualitative data regarding physiology and the health of the culture was captured through microscopy. Parameters were evaluated subjectively these included:

- Colour (blue green),
- Helicity,
- Length,
- General condition, and;
- Motility.

3.3. Flask Studies

3.3.1. Strain Selection

A wide variety of *Spirulina* strains may be considered for cultivation, each with its own characteristics in terms of optimum growth conditions, productivity and chemical composition. Selecting the right strain for a specific application and site is thus of critical importance in the commercial success of a *Spirulina* biomass operation.

3.3.1.1. Materials and Methods

Each potential strain was grown under laboratory conditions and tested for robustness and photosynthetic activity.

The following strains were evaluated in this study:

- UTEX LB 2340 *Spirulina platensis*
- UTEX LB2721 *Arthrospira sp*
- CCALA Czech Republic: Trion. *Arthrospira maxima* Setchell & Gardner Lefebvre 1963/m-132-1
- Three South African environmental isolates of unknown origin Named SA1, SA2 and SA3.

Strains were grown as batch cultures in Schlösser's medium (Vonshak, 1997a). Cultures were sub-cultured into media on a 1:1 ratio of culture to fresh medium using aseptic methods and then placed in low light conditions to prevent photoinhibition. The initial volumes used were 3 ml media and 3 ml inoculum. Scale up commenced at a ratio of 1:5 for subsequent dilutions. Once all strains achieved 375 ml volume and a chlorophyll a concentration of ~ 6000 µg/l, triplicate Erlenmeyer flask were made up to a total volume of 250 ml consisting of Schlösser's media and a 10% inoculum. The remaining master cultures were amended to 1000 ml using fresh Schlösser's media. Subsequently the chosen strain was scaled up as described before to achieve a 1000 l volume in a photobioreactor (PBR).

Growth was measured using chlorophyll a determination as described in the analytical methods section of this dissertation. Cells were also evaluated subjectively through microscopy; parameters noted were colour, motility, helicity and trichome length.

3.3.2. Nutrient Supplementation Study

3.3.2.1. Introduction

Media development was central to the feasibility of the project. The composition of nutrient media can constitute up to 25% of production costs (Belay, 1997). Therefore, if the coal seam water could provide a suitable growth medium, and result in a significant nutrient cost reduction, the mass culture of *Spirulina* would be an attractive proposition. The potential use of CSW and the amendments required to optimise the water as a growth medium were investigated in both flask and HRAP studies. The following section describes the flask studies.

To create an optimal growth medium for *Spirulina*, based on the coal seam water found at the site, the following hypotheses were established.

Nutrient Supplementation Hypothesis 1

Coal seam water is a suitable medium for cultivating *Spirulina* without the intervention of addition of further chemicals, and there will be no meaningful differences in growth performances compared to that of *Spirulina* cultivated in an optimal growth medium such as Schlösser's medium.

Nutrient Supplementation Hypothesis 2

Spirulina grown in coal seam water amended to Schlösser's medium (ingredients added to well water so that final chemical composition is similar to Schlösser's medium), will have similar biomass yields or growth performances compared to that of *Spirulina* cultivated in only Schlösser's medium.

Nutrient Supplementation Hypothesis 3

Spirulina cultivated in amended coal seam water medium, but without the addition of micronutrients, will show no difference in growth performances

compared to that of *Spirulina* cultivated in CSW with the full suite of Schlosser's additives.

Nutrient Supplementation Hypothesis 4

Spirulina cultivated in coal seam water with the addition of potassium, nitrate and phosphate to a similar concentration to that of Schlösser's medium will show no differences in growth performances compared to that of *Spirulina* cultivated in only Schlösser's medium.

3.3.2.2. Materials and Methods

A range of concentrations of Schlösser's medium was made up using well water in-stead of distilled water (with the exception of the micronutrient mix which was made up in distilled water). The percentage Schlösser's medium after dilution was: 100% (Treatment 1); 75% (Treatment 2); 50% (Treatment 3); 25% (Treatment 4); 0% (Treatment 5). The final volumes were made up to 100 ml in 250 ml conical flasks. The control (Treatment 6) comprised Schlösser's medium made up in distilled water.

To investigate whether amended coal seam water would substitute for Schlösser's medium, a treatment containing 100 ml of coal seam water was amended with constituents of Schlösser's medium. This was made up so that the final concentration of salts in the treatment approached that of Schlösser's medium. This was called Treatment 7. A medium volume of 100 ml was dispensed into 250 ml conical flasks.

A similar treatment to Treatment 7 was set up with the exception that no micronutrients were added, this was called Treatment 8.

To investigate the possibility that coal seam water was only nutrient limiting, a final treatment, Treatment 9, consisting of 100 ml coal seam water was made up

in 250 ml conical flasks. The only salts added were potassium, nitrate and phosphate in amounts equivalent to that of Schlösser's medium. A complete description of the treatments is provided in Table 3-1.

Table 3-1: Treatment composition

Sample ID	Makeup
Treatment 1	100% Schlösser's media in CSW
Treatment 2	75% Schlösser's media in CSW
Treatment 3	50% Schlösser's media in CSW
Treatment 4	25% Schlösser's media in CSW
Treatment 5	0% Schlösser's media in CSW
Treatment 6	Control: Schlösser's media in distilled water
Treatment 7	CSW amended to Schlösser's media
Treatment 8	CSW amended to Schlösser's no micro nutrients.
Treatment 9	CSW with addition of NPK at Scholssers concentrations

Schlösser's media was made as described in Vonshak (1997a). Tables 3-2 and 3-3 provide a detailed description of the media recipe.

Table 3-2: Schlösser's medium macro nutrients solution

Constituent	Weight (g)
NaHCO ₃	13.61
Na ₂ CO ₃	4.03
K ₂ HPO ₄	0.50
NaNO ₃	2.50
K ₂ SO ₄	1.00
NaCl	1.00
MgSO ₄ ·7H ₂ O	0.20
CaCl ₂ ·2H ₂ O	0.04
FeSO ₄ ·7H ₂ O	0.01
Chelaton III	0.08

Table 3-3: Schlösser's medium micro nutrient solution recipe added to 981ml distilled water.

Constituent	Solution Strength	Quantity
ZnSO ₄ .7H ₂ O	0.1%	1.0 ml
MnSO ₄ .4H ₂ O	0.1 %	2 ml
H ₃ BO ₃	0.2 %	5 ml
CO(NO ₃) ₂ .6H ₂ O	0.02 %	5 ml
Na ₂ NoO ₄ .2H ₂ O	0.02 %	5 ml
CuSO ₄ .5H ₂ O	0.0005%	1 ml
FeSO ₄ .7H ₂ O		0.7 g
Chelaton III		0.8 g

All treatments were carried out in triplicate. A 10 ml inoculum of *Spirulina* from the outdoor Photo Bio-Reactor (PBR) was added to each flask to give a final volume of 110 ml. The culture flasks were placed on an orbital shaker exposed to natural light. The flasks were randomly rearranged on the shaker every day.

At T (hours) = 96, T = 192, T = 288 and T = 480, a sample was drawn from each of the flasks and the chlorophyll a concentration determined. Daily microscopic examination was carried out on all the treatments.

3.3.3. Effect of Iron Concentration on Growth

3.3.3.1. Introduction

Iron limitation of algal growth has been widely reported (Fogg, 1991) and sequestration of iron in pond systems can result in poor growth productivity performance (Vonshak 1997b). According to Vonshak (1997b) two possible scenarios appear in *Spirulina* mass culture: 1) low iron availability in the culture medium manifests as low growth rates and susceptibility to photoinhibition and 2) contaminating algae may acquire a competitive advantage over *Spirulina* in

sequestering scarce iron resources. Thus, in theory, iron limitation in *Spirulina* culture can be overcome by ensuring availability through extra enrichment of iron. However, it is also important to determine toxicity thresholds. To this end, an experimental protocol was established to determine the effect of iron concentration and to investigate the following hypotheses.

Iron Concentration Hypothesis 1

Iron in excess can be used to boost growth of *Spirulina*.

Iron Concentration Hypothesis 2

Spirulina can be successfully cultivated in concentrations of iron of up to 12 mg.L⁻¹ iron.

3.3.3.2. Materials and Methods

Schlösser's medium was made up in distilled water (including the addition of micronutrients) to pH 10.5, and 225 ml was added to each of 18 1L conical flasks. Each flask was inoculated with 25 ml growing *Spirulina* culture from the PBR. Six treatments were used, each with 3 replicates, consisting of a control (normal Schlösser's medium) and iron enrichment of 10 x, 20 x, 30 x, 50 x and 100 x, the iron concentration in Schlösser's medium. Treatments and their makeup are shown in Table 3-4 below.

Table 3-4: Effect of Iron on Growth Flask Study: Treatment description

Sample ID	Media	Iron Concentration (mg/l)
Treatment 1	Control: Schlösser's media	0.12
Treatment 2	Schlösser's media: [iron] X10	1.2
Treatment 3	Schlösser's media: [iron] X20	2.4
Treatment 4	Schlösser's media: [iron] X 30	3.6
Treatment 5	Schlösser's media: [iron] X 50	6
Treatment 6	Schlösser's media: [iron] X 70	8.4
Treatment 7	Schlösser's media: [iron] X 100	12

The appropriate amount of Na₂EDTA a chelating agent was added to ensure the solubility of iron in solution.

In addition to the normal micronutrient component of Schlösser's medium, 6 ml of the appropriate Fe-enriched solution was added to the appropriate treatment. To the control treatment, 6 ml distilled water was added in place of the iron-enriched solution.

Growth was recorded at T (hours) = 96, 168, 264 and 408 by measuring chlorophyll a concentration and dry weight. Aliquots of 2 ml were drawn for chlorophyll a measurements and 10 ml aliquots drawn for dry weight measurements. GF/A filters were used for dry weight measurements. pH was recorded at the start and at the end of the experiment. Dry weight and chlorophyll a determinations were conducted as described in the analytical methods section of this chapter.

3.3.4. Optimum pH for Growth in Coal Seam Water Media

3.3.4.1. Introduction

Spirulina is an alkaliphile and as such thrives in a pH range between 9.5 and 12 (Richmond, 1988; Jiménez *et al.*, 2003; Ravelonandro *et al.*, 2011; Gupta *et al.*, 2013). According to Vonshak and Grobbelaar (1986) this is mainly due to two reasons: gas exchange and exclusivity of the culture.

Gas exchange between the culture and the atmosphere is dependent on the gradient of the partial pressures of the gasses. Increasing the pH increases the CO₂ gradient, thereby allowing the uptake of free carbon from the atmosphere

(Vonshak and Grobbelaar, 1986; Belay, 1997; FAO, 2009). Exclusivity is achieved through the application of a high pH as this excludes potential competitors that are unable to withstand the physiological challenges presented by a high pH.

Under laboratory conditions, *Spirulina* is reported to grow in a large pH range (Richmond, 1988; Vonshak, 1997; Belay, 1997; Jiménez *et al.*, 2003; Morais *et al.*, 2009). Defining an optimum is complex since it varies between strains, at different population densities and with environmental conditions.

Vonshak and Grobbelaar (1986) found that productivity was unaffected up to pH 10.5 after which a sharp decline could be seen. On the other hand, Richmond (1988), found no measurable decrease in growth up to pH 12, after which both teams of researchers observed the gradual death of the culture. Belay (1997) recommends growing the culture at no higher than pH 10.5, as higher levels result in the precipitation of calcium carbonate.

Clearly, from the above, pH is a very important variable in mass algal culture and the pH range varies between various strains of *Spirulina*. For this reason, a pH study to determine the optimum pH level for the selected strain was conducted as described below.

The following hypothesis was made at the outset of the study.

Optimum pH Hypothesis 1

The pH optimum for the strain of *Spirulina* used is between 9.5 and 10.5.

3.3.4.2. Materials and Methods

Based on the research of Richmond (1988); Vonshak (1997) and Belay (1997), six pH values, ranging from 9.5 to 11.5, were investigated. Each treatment was carried out in triplicate and consisted of 225 ml Schlösser's medium made in CSW and pH-adjusted with a concentrated NaOH solution. The treatments are described in Table 3-5 below.

Table 3-5: Effect of pH on Growth Flask Study: Treatment description

Sample ID	Media	pH
Treatment 1	Control	9.5
Treatment 2	Schlösser's media	10
Treatment 3	Schlösser's media	10.4
Treatment 4	Schlösser's media	10.7
Treatment 5	Schlösser's media	11
Treatment 6	Schlösser's media	11.6

Each flask received 10% inoculum from the PBR to give a final volume of 250 ml. Treatment 1, was used as the control at a pH of 9.5. All cultures were placed randomly on an orbital shaker and randomly rotated daily. Flasks were sampled every 4 days at T (hours) = 96, 192 and 288 and growth was determined by chlorophyll a determination as described in the analytical methods section. The final pH was recorded on the final day of the experiment.

3.4. High Rate Algal Pond Studies

Four, high rate, raceway-type ponds were constructed, each with a surface area of 14 m² and a depth of 350 mm. Ponds were manufactured using a prefabricated galvanised metal base frame and lined with a durable PVC liner. Paddlewheels were constructed of stainless steel, and semi-circular inserts,

made of galvanised steel, were placed at each end of the pond to prevent settling of the biomass (Figure 3-1).



Figure 3-1: High Rate Algal Ponds and associated infrastructure.

Pumps, piping, electrical controls and valves were assembled and a field laboratory was constructed in a shipping container and then transported to site.

The *Spirulina* strain, *Arthrospira maxima* from the CCALA, was cultured at the Environmental Biotechnology Research Unit (EBRU), in a 1 m³ photobioreactor (PBR) using Schlösser's medium. After two weeks of growth in a controlled environmental room, the culture was harvested through a 50 µm nylon mesh and re-suspended in double strength Schlösser's medium. The harvested biomass was transported in cooler boxes from Grahamstown and inoculated into the PBR on site. Once the culture in the PBR had concentrated up, it was ready for inoculation into the raceway ponds (Figure 3-2).



Figure 3-2: High Rate Algal Pond first inoculation.

Harvesting commenced five months after initial inoculation producing the product shown below.



Figure 3-3: First harvested product.

The HRAP studies represented quantitative experimental research in a real world setting. Internal validity was assured by using a post-test control only experimental design. External validity was assured through the real world setting

(Leedy and Ormrod, 2005). In addition, the HRAP studies represented a pilot scale study which provided information on the potential up-scaling of the project.

3.4.1. *Batch Culture*

Initially the ponds were operated in a batch mode. In this instance, the batch mode was represented by the harvesting protocol. As such, nutrients and inoculums were added to the raceway ponds and harvested once a predefined cell density was achieved. After the harvest, the process would be repeated. This phase lasted for several months and consisted of three different operating protocols. The study evolved as understanding of the site-specific factors improved. In the first and second protocols, only one pond was utilised, and in the third protocol, all four ponds were utilised.

3.4.1.1. *Batch Culture Protocol 1 (Pond 1 First Inoculation): Materials and Methods*

Only Pond 1 was utilised during this experiment which represented a first pass at understanding the prevalent environmental conditions and their effect on algal growth.

The culture consisted of 4 m³ Schlösser's medium made up in RO water and 800 L culture inoculum (1002 µg/l Chl a) from the PBR. Initial pond parameters were based on Vonshak (1997b) and are shown in Table 3-6 below.

Table 3-6: Initial Operating Parameters of Batch Culture Protocol 1 (Pond 1 First Inoculation).

Parameter	Value
Temperature	29°C
pH	9.30

Parameter	Value
Cell Density	371 ug/l Chl a
Electrical Conductivity	23.1 mS/cm
Depth	300 mm

Because of the thin culture in the pond, 80% of the pond was covered with 80% shade cloth to prevent photoinhibition (Richmond & Zou, 1999). The shade cloth was removed in two stages. Firstly, 50% was removed after 5 days of incubation, and after 9 days of incubation, the entire shade cloth was removed.

Growth was estimated by chlorophyll a determination as described in the analytical methods section. Cell morphology was monitored on a daily basis using light microscopy. Conductivity, salinity, pH, depth and temperature were measured on a daily basis. Conductivity, salinity, pH, and depth were maintained at a constant level by adding RO water to replace the volume of water lost through evaporation.

3.4.1.2. Batch Culture Protocol 2 (Pond 2 First Inoculation): Materials and Methods

Pond 2 was initiated in a batch culture mode. The culture consisted of 4m³ Schlösser's medium made up in RO water and 800 L culture inoculum (6173 ug/l Chl a) from the PBR. Initial pond parameters are shown in Table 3-7.

Table 3-7: Initial Operating parameters of Batch Culture Protocol 2 (Pond 2 First Inoculation)

Parameter	Value
Temperature	27,6°C
pH	9.24
Cell Density	1458 ug/l Chl a
Electrical Conductivity	22.8 mS/cm
Depth	300 mm

Pond 2 was initiated in the same way as Pond 1 but with a higher initial cell density. The parameters used were based on Vonshak (1997) and were considered best practise. Initially the pond was covered by 80% shade cloth over 80% of the pond. The shade cloth was never removed.

Growth was estimated by chlorophyll a determination as described in the analytical methods section. Cell morphology was monitored on a daily basis using light microscopy. Conductivity, salinity, pH, depth and temperature were measured on a daily basis. Conductivity, salinity, pH, and depth were maintained at a constant level by adding RO water to replace the volume of water lost through evaporation.

3.4.1.3. Batch Culture Protocol 3 (All Ponds: Environmental Conditions: Materials and Methods)

All four ponds were used for this protocol. Inoculum was obtained by harvesting Pond 2 through a 45 µm mesh, which allowed all single celled contaminants to pass through, and supplemented with 800 L from the PBR. Each pond was made up of 4m³ of Schlösser's medium (made up in RO water) with the addition of the inoculum, so that the cell density in all cases approached 1000 ug/l chl a.

The experiment represented a group trial of the feasibility of growing algae at the experienced environmental conditions. The aim was to ensure that no specific conditions related to a single pond i.e. natural shading, proximity to potential pollution sources etc. would influence the results. Therefore, all growth parameters were kept at the published optimum across all four ponds.

Initial pond parameters are described in Table 3-8 below.

Table 3-8: Initial pond parameters Batch Culture Protocol 3

Parameter	Unit	Pond 1	Pond 2	Pond 3	Pond 4
Temperature	°C	26.6	26.2	25.8	25.8
pH	N/A	9.45	9.43	9.47	9.49
Cell Density	µg/l chl a	1046	898	1052	1274
Depth	mm	300	300	300	300

Growth was estimated by chlorophyll a determination as described in the analytical methods section. Cell morphology was monitored on a daily basis using light microscopy. Conductivity, salinity, pH, depth and temperature were measured on a daily basis. Conductivity, salinity, pH, and depth were maintained at a constant level by adding RO water to replace the volume of water lost through evaporation. In addition, sparging with CO₂ to control pH was initiated in Ponds 1 and 2 while Ponds 3 and 4 were used as the control.

3.4.2. Fed Batch Culture

During fed batch culture, the operating philosophy was to maintain nutrient concentrations at initial concentrations through the addition of nutrients but without any harvesting or removal of nutrients. In this way, no nutrient loss through metabolic uptake could occur which would result in the maintenance of optimal growth conditions. This phase of the pilot work consisted of two operating protocols. During the first protocol, only Pond 4 was utilised with the addition of Pond 2 during the second protocol. The fed batch culture represented the first attempt to pilot the growth of algae in the amended CSW.

3.4.2.1. Fed Batch Culture Protocol 1 (Pond 4 only): Materials and Methods

Only pond 4 was utilised during this protocol. Pond 4 was operated in the fed batch mode for 19 days. Based on the results of the Flask Studies, one cubic meter of coal seam water was enriched with constituents at 50% the concentration found in Schlösser's medium and the pH was adjusted to pH 10 with the use of 33-39% NaOH solution. In addition, based on results obtained during Batch Culture, and a further review of the literature, culture depth was reduced to 15.5 cm to allow a higher initial cell density of 2182 µg/l chl a. The completed medium was added to Pond 4 and inoculated with one cubic meter of culture from the PBR. Shade netting (80%) was placed over 70% of the pond to prevent photoinhibition. The shade netting was removed completely after day 7. Initial pond parameters are described in Table 3-9 below.

Table 3-9: Initial pond parameters fed batch culture protocol 2

Parameter	Value
Temperature	22,2°C
pH	9.94
Cell Density	2182 ug/l Chl a
Electrical Conductivity	23.4 mS/cm
Depth	155 mm

Growth was estimated by chlorophyll a determination as described in the analytical methods section. Cell morphology was monitored on a daily basis using light microscopy. Conductivity, salinity, pH, depth and temperature were measured on a daily basis. Conductivity, salinity, pH, and depth were maintained at a constant level by adding RO water to replace the volume of water lost through evaporation. No additional nutrients were added because the chemical analysis indicated that all nutrients except iron were present at optimal levels. Ten times the iron concentration found in Schlösser's media was added on day 16.

3.4.2.2. Fed Batch Culture Protocol 2 (High vs Moderate Conductivity): Materials and Methods

During this phase both Pond 2 and Pond 4 were utilised. It was hypothesised that maintaining highly saline conditions was integral to preventing contamination. Therefore, Pond 4 remained in operation but was altered to a high salinity pond with a conductivity of ~ 30 mS/cm through the addition of 1.15 kg K₂HPO₄; 5.75 kg NaNO₃; 2.3 kg K₂SO₄; 0.46 kg MgSO₄. Pond 2 consisted of coal seam water amended with the full concentration of Schlösser's salts with the exclusion of bicarbonate, carbonate and chloride. In addition, ten times the concentration of iron present in Schlösser's media was added to both ponds. Therefore, Pond 2 represented a moderate saline pond at a conductivity of ~20 mS/cm.

The duration of this protocol was 25 days. Additional chelated iron was added to both ponds on day 15. No additional nutrients were added as chemical analysis indicated that all nutrients with the exception of iron were present at the desired concentrations.

Initial pond parameters are described in Table 3-10 below.

Table 3-10: Initial pond parameters fed batch culture protocol 2

Parameter	Unit	Pond 2	Pond 4
Temperature	°C	23.2	23.3
pH	N/A	9.69	10.74
Cell Density	ug/l Chl a	1841	2768
Electrical Conductivity	mS/cm	21.8	31
Depth	mm	15.5	15.5

3.4.3. *Semi-Continuous Culture*

Semi Continuous Culture involved the daily harvesting of a predetermined volume from each pond and the subsequent replenishment of the harvested volume with fresh enriched coal seam water medium. The ponds were operated at 155 mm depth, and harvested at either 5 or 10% of the total volume depending on biomass density.

The ponds were harvested by gravitational flow to the sump and biomass recovered by filtering through a 30 μ m nylon mesh screen. The volume harvested varied for each pond and depended on steady state conditions established. All productivity figures reflect unwashed biomass.

Analysis of chlorophyll a, dry weight, harvest weight, cell counts, qualitative microscopy and general morphological examination were carried out daily. Daily productivity was calculated by determining the change in the daily biomass (dry weight).

The purpose of this phase of piloting was to determine whether semi continuous culture could be a feasible operating protocol. In addition, the experiment would be used to determine the optimal salinity i.e. nutrient concentration and to establish the upper cell density limits of irradiance/cell density interactions i.e. at which upper limit would self-shading prove detrimental to growth. To this end three operating protocols were utilised these will be described in the following sections.

3.4.3.1. Semi-Continuous Culture Protocol 1 (Biomass Concentration): Materials and Methods

Ponds 1 and 2 were commissioned on day 0 and operated for 63 days. Both ponds were made up in enriched coal seam water i.e. coal seam water with the addition of only NaNO_3 , K_2HPO_4 , K_2SO_4 and MgSO_4 to achieve Schlösser's medium concentration. Micro nutrients were added as in Schlösser's medium with the exception of that based on previous results; 20 times the iron concentration was added. Therefore, both ponds were identical with the exception of initial cell concentration. It was hypothesised that a higher initial biomass concentration would provide a competitive advantage and result in a higher growth rate and reduced contamination. To this end Pond 1 was established as the control with a normal average biomass concentration and Pond 2 was established as the high biomass experiment. Initial pond parameters are provided in Table 3-11 below.

Table 3-11: Initial pond parameters Semi Continuous Culture Protocol 1 (Biomass concentration)

Parameter	Unit	Pond 1	Pond 2
Temperature	°C	18.4	18.3
pH	N/A	10.12	10.13
Biomass Concentration	g/l (dry weight)	0.842	0.988
Electrical Conductivity	mS/cm	16.77	17.17
Depth	mm	15.5	15.5

Harvests were carried out on a daily basis. The volume of the harvest was determined by the biomass concentration. The target biomass concentration in Pond 1 was ~ 0.8 g/l dry weight. In Pond 2 the target cell density was ~ 1.4 g/l dry weight. Depending on growth rate between 0% to 10% of the pond volume was harvested to maintain the above target concentrations.

Evaporative loss was made up with RO water and harvest volume replenished with media.

Analysis of chlorophyll a, dry weight, harvest weight, cell counts, qualitative microscopy and general morphological examination were carried out daily. Daily productivity was calculated by determining the change in the daily biomass (dry weight) corrected for the loss to harvesting.

3.4.3.2. Semi-Continuous Culture Protocol 2 (Salinity): Materials and Methods

Protocol 1 continued throughout Protocol 2. As such, both Ponds 1 and 2 were operated as described in the above section. Ponds 3 and 4 were commissioned on day 38 of protocol 1 hereafter referred to as day 0. Pond 3 was commissioned as a low salinity pond at ~6% salinity. Low salinity was maintained by replenishing with the low salinity media (Table 3-12) after harvest and when necessary the addition of RO water. Pond 4 was commissioned as a high salinity pond at 18% salinity which was achieved with the addition of high salinity media (Table 3-12). Normal Schlösser's micronutrient concentrations and 20 times the iron concentration were added to all ponds. Table 3-12 below gives a detailed description of the medium makeup in all the ponds.

Table 3-12: Media makeup of all ponds during Semi-Continuous Culture: Protocols 1 and 2.

Pond(s)	1 & 2		Pond(s)	3		Pond(s)	4	
Multiplication factor	1		Multiplication factor	0.9		Multiplication factor	1.4	
Salts	kg/m³	kg	Salts	kg/m³	kg	Salts	kg/m³	kg
NaNO ₃	2.50	0.143	NaNO ₃	2.50	0.143	NaNO ₃	2.50	0.143
K ₂ HPO ₄	0.50	0.029	K ₂ HPO ₄	0.50	0.029	K ₂ HPO ₄	0.50	0.029
K ₂ SO ₄	1.00	0.057				K ₂ SO ₄	1.00	0.057
MgSO ₄	0.20	0.011				MgSO ₄	0.20	0.011
						NaHCO ₃	15.09	0.86
						Na ₂ CO ₃	5.64	0.322
						NaCl	0.59	0.034
pH control	kg/m³		pH control	kg/m³	kg	pH control	kg/m³	kg
NaOH	2.00	0.114	NaOH	0.67	0	NaOH	4.00	0.228
Micronutrients	l/m³		Micronutrients	l/m³	L	Micronutrients	L/m³	l
PIV	6	0.342	PIV	6	0	PIV	6	0.342
Chu	1	0.057	Chu	1	0	Chu	1	0.057
Vitamins	ml/m³		Vitamins	ml/m³	ml	Vitamins	ml/m³	ml
Vit B12	0.15	0.009	Vit B12	0.15	0	Vit B12	0.15	0.009
20 x iron	g/m³		20 x iron	g/m³	g	20 x iron	g/m³	g
FeCl ₃	11.64	0.663	FeCl ₃	11.64	0	FeCl ₃	11.64	0.663
Na ₂ EDTA	4.50	0.257	Na ₂ EDTA	4.50	0	Na ₂ EDTA	4.50	0.257

Both ponds 1 and 2 were maintained at a biomass concentration of ~ 0.8 g/l dry weight. Pond 1 functioned as the control at a salinity of ~9% however biomass concentration was maintained at the 0.65 g/l.

Harvests were carried out on a daily basis. The volume of the harvest was determined by the cell concentration. Depending on growth rate between 0%, to 10% of the pond volume was harvested to maintain the above target cell concentrations.

Evaporative loss was made up with RO water and harvest volume replenished with the appropriate media.

Analysis of chlorophyll a, dry weight, harvest weight, moisture content, cell counts, qualitative microscopy and general morphological examination were carried out daily. Daily productivity was calculated by determining the change in the daily biomass (dry weight). It also included the biomass harvested from the ponds.

Initial pond parameters are given in Table 3-13 below.

Table 3-13: Initial pond parameters Semi - Continuous Culture Protocol 2 (Salinity)

Parameter	Unit	Pond 1	Pond 3	Pond 4
Temperature	°C	10.7	10.2	10.3
pH	N/A	10.59	10.55	10.348
Cell Density	Ug/l chl a	4965	6987	6306
Depth	mm	155	155	155

3.4.3.3. Semi – Continuous Culture Protocol 3 (Nutrient Optimisation): Materials and Methods

Based on previously collected data it was hypothesised that a further reduction in nutrient concentration would not result in a loss of productivity. Further reduction of nutrient addition would realise a significant reduction in nutrient costs which contribute up to 25% of the production costs. To test the hypothesis, Pond 1 was commissioned with a half strength media i.e. coal seam water enriched with half the concentration of NPK present in Schlösser's media and then with a one third strength media. The media makeup is shown in Table 3-14 below

Table 3-14: Growth Medium Composition: Semi-Continuous Culture Protocol 3

Enriched coal seam water (EW) (Pond 4)		½ Enriched coal seam water (½ EW) (Pond 1)		⅓ Enriched coal seam water (⅓ EW) (Pond 1)	
Salts	kg/m³	Salts	kg/m³	Salts	kg/m³
NaNO ₃	2.50	NaNO ₃	1.25	NaNO ₃	0.83
K ₂ HPO ₄	0.50	K ₂ HPO ₄	0.25	K ₂ HPO ₄	0.17
K ₂ SO ₄	1.00	K ₂ SO ₄	0.50	K ₂ SO ₄	0.33
MgSO ₄	0.20	MgSO ₄	0.10	MgSO ₄	0.07
NaOH	2.00	NaOH	2.00	NaOH	0.67
Micronutrients	l/m³	Micronutrients	l/m³	Micronutrients	l/m³
PIV	6	PIV	6	PIV	6
Chu	1	Chu	1	Chu	1
Vitamins	ml/m³	Vitamins	ml/m³	Vitamins	ml/m³
Vit B12	0.15	Vit B12	0.15	Vit B12	0.15
20 x iron	g/m³	20 x iron	g/m³	20 x iron	g/m³
FeCl ₃	11.64	FeCl ₃	11.64	FeCl ₃	11.64
Na ₂ EDTA	4.50	Na ₂ EDTA	4.50	Na ₂ EDTA	4.50

Pond 4 was used as the control and contained full strength enriched coal seam water. All other parameters were kept constant and the semi-continuous culture operating protocol was followed. It should be noted that both ponds 1 and 4 were heated through heat exchange.

Both ponds 1 and 4 were maintained at a biomass concentration of ~ 0.70 g/l dry weight. Harvested biomass from Pond 1 was added back over the first 5 days to reach the required ~5000 ug/l chl a concentration.

Harvests were carried out on a daily basis. The volume of the harvest was determined by the cell concentration. Depending on growth rate between 0% and 10% of the pond volume was harvested to maintain target cell concentrations.

Evaporative loss was made up with RO water and harvest volume replenished with the appropriate media.

Analysis of chlorophyll a, dry weight, harvest weight, qualitative microscopy and general morphological examination were carried out daily. Daily productivity was calculated by determining the change in the daily biomass (dry weight). It also included the biomass harvested from the ponds.

Table 3-15 describes the initial culture parameters.

Table 3-15: Initial culture parameters: Semi Continuous Culture Protocol 3

Parameter	Unit	Pond 1	Pond 4
Temperature	°C	23.2	21.5
pH	N/A	10.024	10.104
Cell Density	ug/l Chl a	1333	4688
Electrical Conductivity	mS/cm	15.54	17.14
Depth	mm	155	155

3.4.3.4. Semi-Continuous Culture Protocol 4 (Temperature) Materials and Methods

Due to the change in seasons and in order to determine the effect of temperature on growth rate, gas fired heat exchangers were implemented in the HRAPs. All four ponds were modified by adding a plate heat exchanger to each pond. The heat exchangers were connected to a boiler heated with the methane produced on site.

It was hypothesised that increasing the temperature by 10 °C would result in a doubling of productivity.

Ponds 3 and 4 were utilised in the experiment which run concurrent with Protocol 3 but continued afterward. The Protocol lasted 365 days and represented a stable state production test. The growth medium in both ponds was enriched

coal seam water as described in Table 3-14. Pond 4 was heated and the temperature control set to a minimum of 30 °C and a maximum of 35 °C. Therefore, the gas heater would be on at any temperature below 30 °C and off above 35 °C. Pond 3 functioned as the control and was unheated.

All ponds were maintained at a cell concentration of ~ 5000 ug/l chl a.

Harvests were carried out on a daily basis. The volume of the harvest was determined by the cell concentration. Depending on growth rate between 0%, to 10% of the pond volume was harvested to maintain the above target cell concentrations.

Evaporative loss was made up with RO water and harvest volume replenished with the appropriate media.

Analysis of chlorophyll a, dry weight, harvest weight, qualitative microscopy and general morphological examination were carried out daily. Daily productivity was calculated by determining the change in the daily change in biomass (dry weight basis). It also included the biomass harvested from the ponds.

3.5. Analytical Methods

3.5.1. *Culture Parameters*

3.5.1.1. Conductivity, pH, Salinity and Temperature

Conductivity, pH, salinity and temperature were measured with a WTW multi 1970i multi meter as described in the operating manual provided with the device.

Calibration was carried out as needed or weekly as described in the operating manual using WTW standard pH solutions. Measurements in the ponds were taken as early as possible in the morning i.e. from 7-8 AM Both the parameter and the time was noted.

3.5.1.2. Depth

Pond depth was measured daily using a metal ruler. Fixed clamps were installed on all ponds. The ruler was then clamped into place, the paddle wheels switched off for 5 minutes and the depth read off the ruler taking the meniscus into account. The depth was used to calculate the evaporative loss. The calculated volume was then replaced with RO water.

3.5.1.3. Dissolved Oxygen

Dissolved oxygen was measured with a WTW oxi 1970i measuring device. Measurements were taken as early as possible in the mornings with a target time between 7 and 8 AM.

3.5.1.4. Chlorophyll a Determination

Chlorophyll a concentration was determined using the method described by Lichtenthaler (1987). The method is completed in four stages. First, 5 ml of sample was filtered through a GF/A glass fibre filter using a diaphragm vacuum pump (Vaccumbrand). Then the filter was crushed using a glass rod in a centrifuge tube with the addition of 5ml 100% acetone and stored in a deep fridge. After 24 hours, the acetone containing the filter paper was centrifuged at 5000 RPM for 20 minutes using a Universal 16 centrifuge. The supernatant was then decanted into a 5 cm cuvet and absorbance measured at 661.1 nm and

644.8 nm with a Helios ϵ Thermo Spectronic spectrophotometer. The absorbance values measured were used to calculate the chlorophyll concentration using the formula below.

$$[\text{Chl } a] \mu\text{g/l} = \frac{[(11.24 \times A_{661.6}) - (2.04 \times A_{644})] \times \text{acetone volume (ml)}}{\text{sample volume (ml)}} \times 1000$$

3.5.1.5. Dry Weight

Dry weight was determined by measuring the mass in grams of a 110 mm unused GF/A filter using a Metlor Toledo mass balance. Once the mass of the dry filter paper was determined, a 500 ml sample (10 ml for flask studies) was filtered through the filter paper using a Buchner funnel and diaphragm vacuum pump (Vacuum brand). The used filter paper was then placed in a petri dish and put in a Thermo laboratory oven at 60 °C for 24 hours until completely dry. The mass of the dry filter paper containing biomass was then determined using the mass balance. The dry weight was determined by subtracting the unused filter weight from the used filter weight and multiplying by the appropriate factor to provide a value in g/l. Therefore, for a 500 ml sample, the measured difference in dry filter paper weight was multiplied by two.

3.5.2. **Qualitative data**

Qualitative data was collected through microscopy. To this end a Carl Zeis microscope was used.

Microscopy was carried out daily by collecting a 500 ml sample from all active ponds. Three slides from each sample were made and the condition of the culture subjectively determined by evaluating colour, motility, trichome size and number. These parameters provided an estimation of general culture conditions

and served as an early warning of potential deficiencies in the culture before growth data became available.

3.5.3. *Data Analysis*

Various methods were used to analyse the data collected. This included statistical analysis and calculation of growth kinetics.

3.5.3.1. Growth Kinetics

The growth kinetic parameters determined were the maximum biomass concentration (X_{\max}), maximum specific growth rate (μ_{\max}) and doubling time (t_d). All parameters were calculated using the formulas described by Becker (1994) which are shown below.

Maximum biomass concentration (X_{\max}): Maximum concentration of biomass measured throughout the study period.

Doubling time (t_d): $g = 0.69t / (\ln M_f - \ln M_i)$ where:

g = doubling time

M_f = final biomass

M_i = initial biomass

Maximum specific growth rate (μ_{\max}): $\mu_{\max} = \frac{\ln 2}{g} = 0.69/g$

In order to use a proxy measure of growth such as chlorophyll a concentration to determine growth rates in batch culture it is essential that the culture is in balanced growth (Anderson, 2005). During balanced growth the average per cell concentration of major cell constituents remains stable and the rate of change of

these constituents is an indication of population growth. Stable growth in batch culture is only achieved during the exponential growth phase (Anderson, 2005). To ensure that the measured values used to determine the growth kinetic parameters are representative of the exponential growth phase the growth curves were plotted in semi log and only the linear portion of the curve used for calculation purposes (Anderson, 2005). Because no standard curve was developed and only chlorophyll a concentration was used as the estimation for growth during the majority of the flask studies, it is not possible to compare growth kinetic values amongst the various experiments or with other experiments in the literature. Far more rigour would have been required to make this possible. Anderson (2005) indicates that to ensure that the culture is in the exponential phase, several generations of algae must be grown in similar conditions, data should be collected regarding lag phase, doubling time etc. This information should then be used to design the sampling strategy as such that at least three data points in the exponential phase are available for use in growth calculations. It is unfortunate that the proper rigour was not followed during the design of the flask studies, this was due to the time constraints and the pilot level focus of the overall project. However, the growth kinetic data presented is valid as an indication of performance amongst treatments in the same experiment and is treated as such.

3.5.4. *Statistical Analysis*

For data with multiple nominal variables and a single measurement variable, statistical analysis was done using a two-way analysis of variance (ANOVA) with replication as described by Sokhal and Rohlf (1995). Excel was used to perform the calculations. Therefore, the two sided ANOVA with replication was used to analyse all algal growth data in experiments containing more than two treatments that were measured in triplicate over time. The ANOVA test provides three null

hypothesis one among both of the factors and one interaction term i.e. an indication of the significance of the interaction between the two factors. Any value of P below 0.05 indicates a significant relationship.

The student's t-test or a single ANOVA was used for data that contained single measurement and nominal variables such as the relationship between algal growth and solar irradiance and studies where only one measurement parameter, such as salinity, was altered between only two treatments. Excel was again used to perform the calculations. For the student's t-test the test statistic is t_s and any value below 0.05 indicates a significant relationship.

CHAPTER 4: FLASK STUDIES: RESULTS AND DISCUSSIONS

4.1. Introduction

This chapter presents results for studies carried out in batch flasks.

4.2. Strain Selection

A wide variety of *Spirulina* strains may be considered for cultivation, each with its own characteristics in terms of optimum growth conditions, productivity and chemical composition. Selecting the right strain for a specific application and site is thus of critical importance in the commercial success of a *Spirulina* biomass operation.

Growth results as determined from chlorophyll a concentration are provided in Figure 4-1.

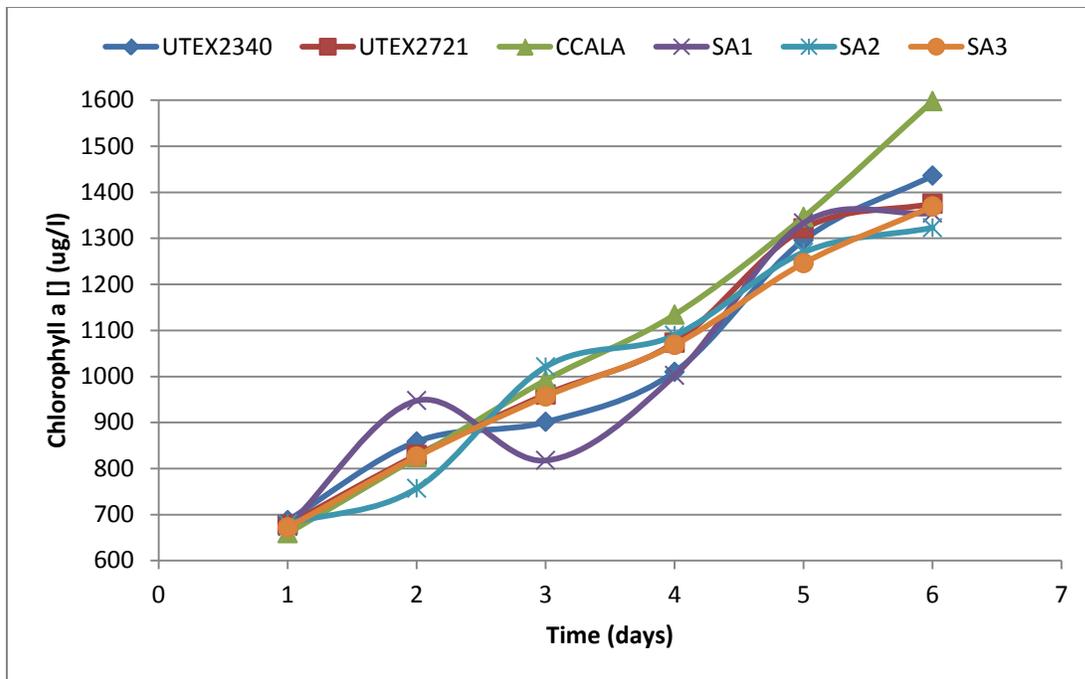


Figure 4-1: Chlorophyll a concentration of the different *Spirulina* strains recorded during the strain selection flask study. Each strain was grown in triplicate and consisted of 225ml Schlösser's media and a 10% inoculum with a concentration of ~ 6000 µg/l.

The above data indicated that growth occurred in all treatments. From the data it is apparent that the CCALA strain of *Arthrospira maxima* (Strain: Setchell & Gardner, 1963) sourced from CCALA in the Czech Republic achieved the highest final chlorophyll a concentration of 1598 µg/l. This is 11% better growth over the 6 day period than the second most prolific strain UTEX 2340 which achieved a final chlorophyll a concentration of 1436 µg/l. The third most prolific strain was UTEX 2721 which achieved a final chlorophyll a concentration of 1375 µg/l which is 16% less than the CCALA strain.

Although the results only represent a single experiment, all the strains were previously adapted to the culture conditions during the scale-up process from 6 ml to 375 ml cultures over a period of two weeks before use as inoculum in the current experiment. Therefore, the potential negative impact of adaptation to culture conditions may be discounted. As such and because the experiment was carried out in triplicate, the result should provide a fair representation of potential

growth. In addition, the subjective microscopy examinations indicated superior motility, helicity, colour (more blue green) and length in the CCALA culture. Based on these results and due to time constraints caused by the late arrival of strains from international culture collections and a due date imposed by the project sponsor, a judgement call was made to designate the CCALA strain as the strain of choice.

As such, this strain of *Arthrospira maxima* was cultured into a 1000 L photo-bioreactor before transport to the site for use in the pilot study. All other strains were cultured in a 1:1 glycerol solution and frozen for potential future use. As discussed in Chapter 2, traditional nomenclature will be utilised. Therefore, although the specific species used was identified as *Arthrospira maxima* by the culture collection it was sourced from, hereinafter, it will be referred to as *Spirulina* as is the common practise.

4.3. Nutrient Supplementation

The nutrient supplementation study aimed to determine whether CSW would provide a suitable medium for *Spirulina* cultivation and also whether using the CSW would allow a reduction in the nutrient addition requirements of optimised media.

The growth results as recorded in all treatments during the nutrient supplementation study are shown in Figures 4-2 and 4-4. Treatment 6 is the control in both graphs. Figure 4-2 contains the results for Treatments 1 – 6.

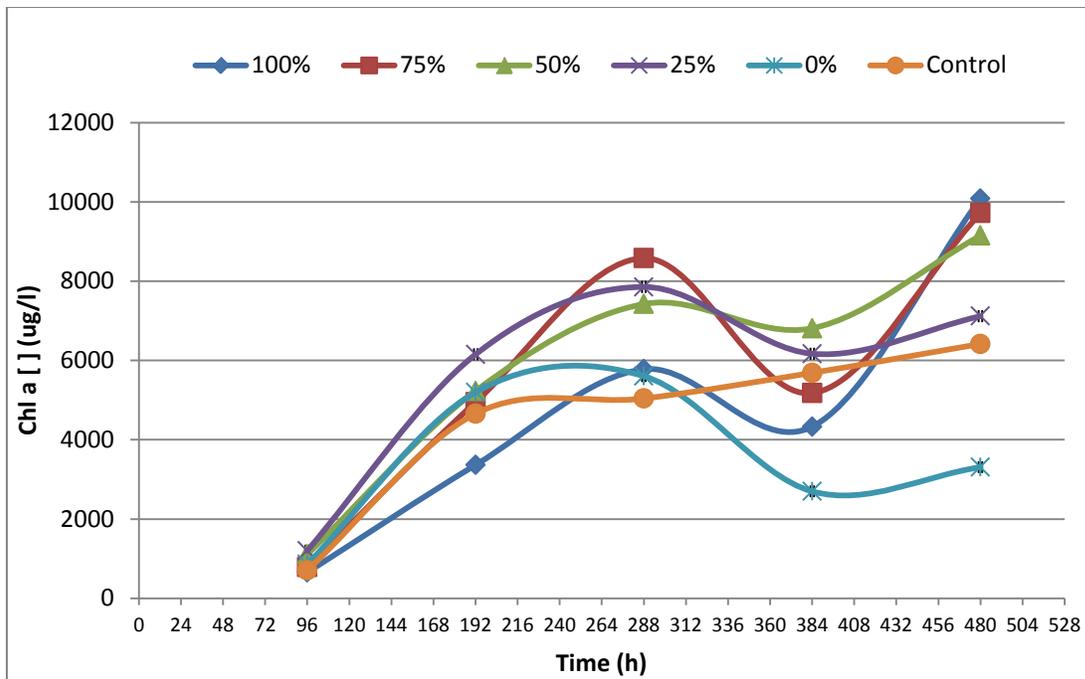


Figure 4-2: Chlorophyll a concentration recorded in Treatments 1-6 during the Nutrient Supplementation Flask Study (mean \pm standard deviation). All treatments were carried out in triplicate and consisted of CSW with the addition of Schlösser's media at varying concentrations. Treatment 1: 100% Schlösser's media; Treatment 2: 75% Schlösser's media; Treatment 3: 50% Schlösser's media; Treatment 4: 25% Schlösser's media; Treatment 5: 0% Schlösser's media and Treatment 6: Control, Schlösser's media made with distilled water.

Figure 4-2 indicates that after an initial lag phase from time 0 to 96 hours, logarithmic growth was achieved in all treatments between hours 96 and 192. This was as expected for growth in a non-limited system and conforms to results published by Chen (2011).

The results shown in Figure 4-2 indicate that although in most treatments the exponential growth phase ended at 192 hours, Treatment 2 exhibited exponential growth until 288 hours. Therefore, on average, the exponential phase duration was 96 hours from hour 96 to hour 192 but in Treatment 2 it was 192 hours. Costa *et al.* (2001) found an average exponential phase duration of 294 hours. However, in their control grown in defined media the exponential phase duration was 312 hours. Ravelonandro *et al.* (2011) also found an average exponential

phase duration of 96 hours which conforms to the average value determined during this experiment.

Between 288 and 384 hours, the biomass decreased markedly in all treatments except the control. This unexpected decrease could have resulted from poor solar irradiance and low temperatures as a result of cloudy conditions and precipitation recorded during this period shown in Figure 4-3 below.

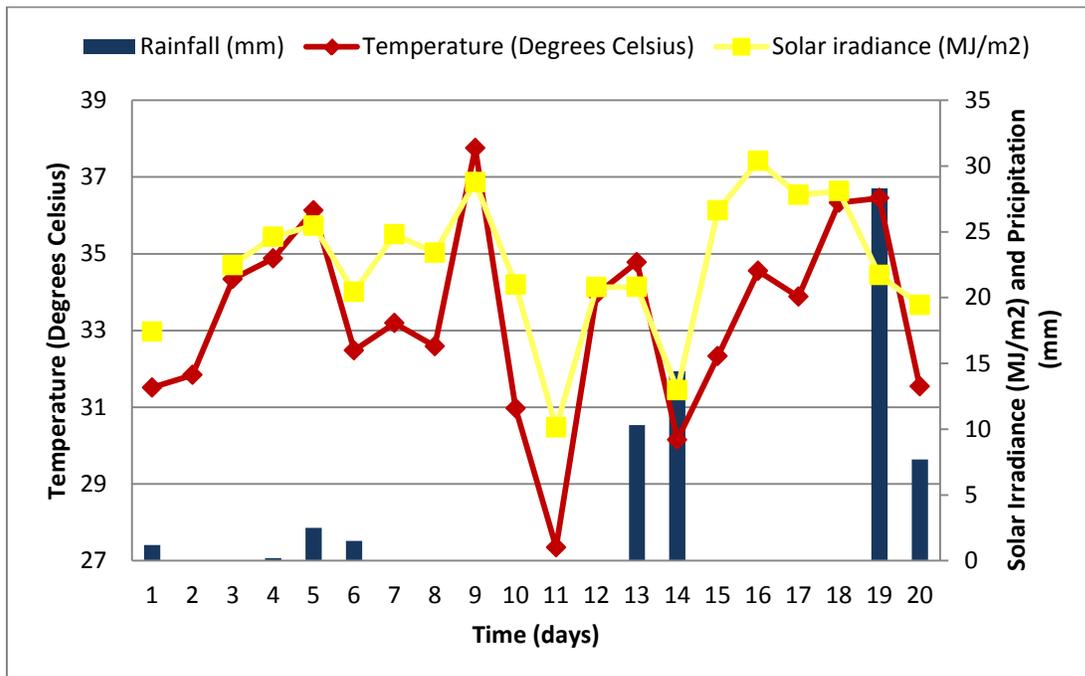


Figure 4-3: Ambient weather conditions as measured by the on site Campbell Scientific Automated Weather Station during the Nutrient Supplementation Flask Study.

The directly positive relationships between solar irradiance, temperature and algal growth are well established in the literature (Vonshak, 1997a).

As the experiment was carried out in the field laboratory, without the facility of a light and temperature controlled room, this impact was unavoidable. However, all treatments were exposed to similar conditions and positions randomised on a daily basis. The control did not exhibit a similar decrease. However, the control had the lowest biomass content at hour 288. Therefore, it is possible that the

effect of lower light conditions had a smaller effect on the control because the lower cell density resulted in lower self-shading.

With the return of favourable ambient weather conditions, growth in all treatments commenced again between hour 384 and 480. However, Treatment 5 showed a further decrease in chl a content. This could be expected as Treatment 5 contained only CSW and culture conditions could have become nutrient limited.

Table 4-1 indicates that in relation to Schlösser's, CSW is deficient in several critical growth nutrients. These include macro nutrients: nitrogen, potassium and sodium and micronutrients: magnesium, zinc, boron and molybdenum. However, CSW contains calcium, iron and manganese in excess of that found in Schlösser's and significant amounts of carbon in the carbonate and bicarbonate forms.

Table 4-1: Comparative chemical analysis of coal seam water and Schlösser's media.

Element	Schlösser's Medium	Coal Seam Water	Difference (CSW- Schlösser's)
	(mg/l)	(mg/l)	(mg/l)
Ca	14.45	23.64	9.19
K	673.27	11.70	-661.57
Mg	19.74	13.89	-5.85
Na	7692.29	2034.00	-5658.29
HCO ₃	12885.34	5057.38	-7827.96
CO ₃	2281.73	0.42	-2281.31
Cl	632.16	376.75	-255.41
NO ₃ (N)	1823.82	0.39	-1823.43
SO ₄	629.21	0.75	-628.46
PO ₄ (P)	272.63	2.47	-270.16
Fe ³⁺	0.12	1.99	1.87
Cu	0.00	0.00	0
Co	0.01	0.00	-0.01
Mn	0.07	0.27	0.2

Element	Schlösser's Medium	Coal Water	Seam	Difference (CSW- Schlösser's)
Zn	0.02	0.00		-0.02
Bo	0.11	0.00		-0.11
Mo	0.43	0.00		-0.43
TDS	26,925	7,524		-19401

Figure 4-4 shows the growth results obtained in Treatments 6 - 9.

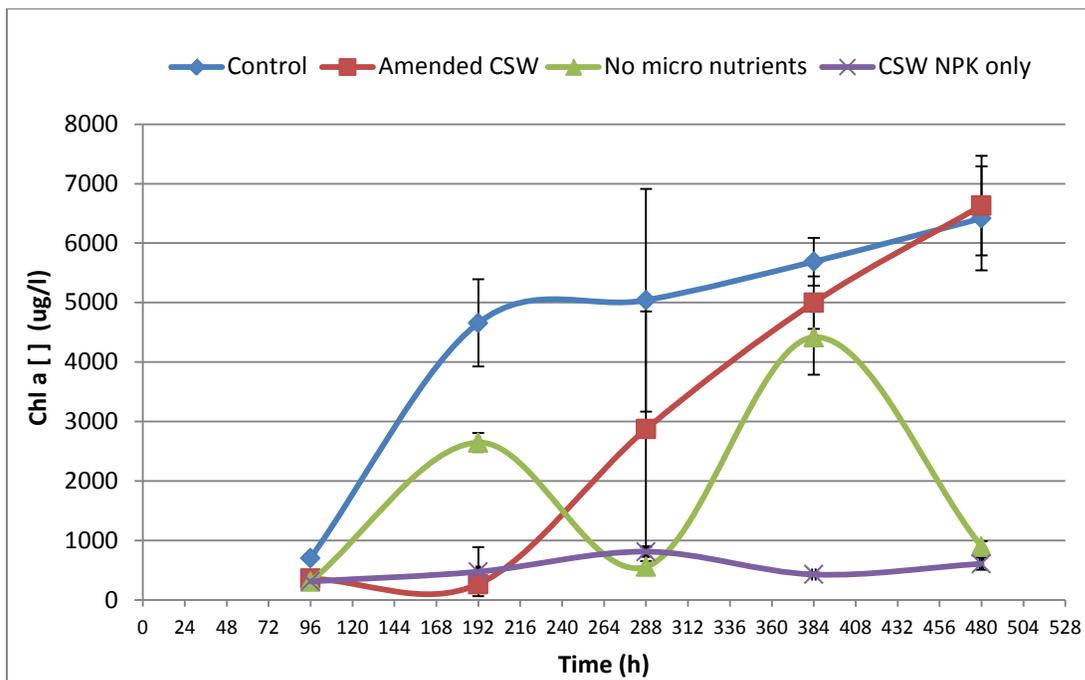


Figure 4-4: Chlorophyll a concentration recorded in Treatments 6-9 during the Nutrient Supplementation Flask Study (mean \pm standard deviation). All treatments were carried out in triplicate and consisted of CSW amended to Schlösser's media nutrient concentrations. Treatment 6 was the control; Treatment 7: CSW amended to Schlösser's nutrient concentration, Treatment 8: amended CSW without micro nutrients and Treatment 9: CSW with only NPK added to Schlösser's concentrations.

Figure 4-4 shows growth curves that do not conform to the findings obtained by Chen (2011) for growth in an optimised growth medium. This was as expected as all treatments except Treatment 7 were deficient in some essential nutrient. Treatment 7 showed an extended lag phase but thereafter growth continued

unabated. The effect of the poor ambient weather conditions was less pronounced in all these treatments. This reduction in affect could be as a result of the lower cell densities at hour 288 as explained previously.

Table 4-2 shows the results for maximum biomass concentration (X_{max}), maximum specific growth rate (μ_{max}) and doubling time (t_d). All parameters were calculated as described in Chapter 3. However, it must be noted that due to the lack of a standard curve as explained in the data analysis section of Chapter 3 the growth kinetic data cannot be compared to other studies. However, the data are relevant to the current study. In addition, as Treatment 9 never entered the exponential growth phase, no growth kinetic data are shown for this treatment.

Table 4-2: Growth kinetic parameters of the Nutrient Supplementation Flask Study: X_{max} : Maximum concentration of *Spirulina* (mean \pm standard deviation, g/l chl a); μ_{max} : Maximum specific growth rate (mean \pm standard deviation, g/h chl a); t_d : Doubling Time (hours); Δt (Length of exponential growth phase (hours))

Sample ID	Makeup	X_{max}	μ_{max}	t_d	Δt
Treatment 1	100% Schlösser's media in CSW	0.1001 \pm 0.00317	0.0170 \pm 0.00048	40.734	96
Treatment 2	75% Schlösser's media in CSW	0.0097 \pm 0.00071	0.0192 \pm 0.00122	36.18	96
Treatment 3	50% Schlösser's media in CSW	0.0099 \pm 0.00064	0.0160 \pm 0.00336	45.07	96
Treatment 4	25% Schlösser's media in CSW	0.0079 \pm 0.00101	0.0171 \pm 0.00141	40.81	96
Treatment 5	0% Schlösser's media in CSW	0.0056 \pm 0.00065	0.0183 \pm 0.00308	38.42	96
Treatment 6	Control: Schlösser's media in distilled water	0.0064 \pm 0.00088	0.0196 \pm 0.00193	35.60	96
Treatment 7	CSW amended to Schlösser's media	0.0066 \pm 0.00084	0.0222 \pm 0.01301	45.80	96

Sample ID	Makeup	X_{\max}	μ_{\max}	t_d	Δt
Treatment 8	CSW amended to Schlösser's no micro nutrients.	0.0044 ± 0.00063	0.2148 ± 0.00032	32.09	96
Treatment 9	CSW with addition of NPK at Schlösser's concentrations	N/A	N/A	N/A	N/A

Figure 4-5 provides a visual representation of the maximum specific growth rates (μ_{\max}) for all treatments.

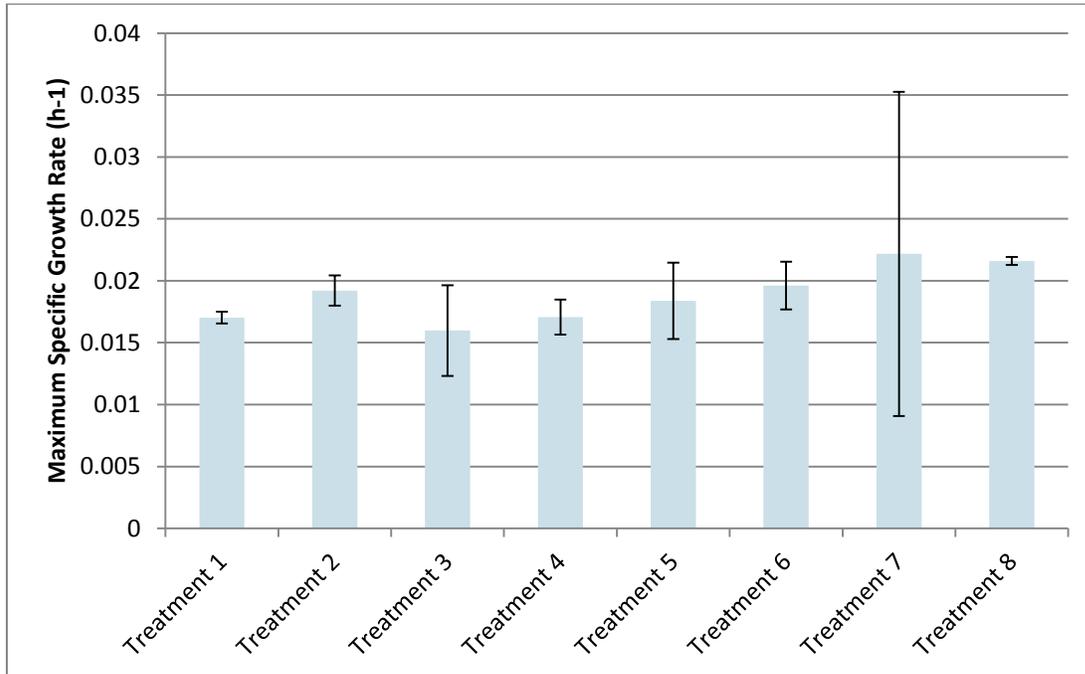


Figure 4-5: Maximum specific growth rate results for the Nutrient Supplementation Flask Study (mean \pm standard deviation)

From Figure 4-5 and Table 4-2 it is apparent that Treatment 7 achieved the highest specific growth rate. However, the large standard deviation may indicate that there was a measurement error. Interestingly, Treatment 5, which contained only CSW achieved a specific growth rate similar to the control. However, a

much smaller X_{\max} was attained; this could be explained by the nutrient limited conditions that were expected to exist in the media.

The results of the two-way ANOVA (with replication) statistical analysis are provided in Table 4-3 below.

Table 4-3: Nutrient Supplementation Flask Study Two Way ANOVA with replication statistical analysis of all treatments.

Source of Variation	SS	df	MS	F	P-value	F crit
Treatments	4.39E+08	8	54820301	64.01847	7.7E-34	2.042986
Time	4.36E+08	4	1.09E+08	127.3222	3.48E-36	2.472927
Interaction	2.86E+08	32	8952115	10.45417	7.91E-19	1.572028
Within	77068802	90	856320			
Total	1.24E+09	134				

SS= sum of squares; df = degrees of freedom; MS: Mean Squares; F: test statistic; P-value: Probability (if P greater than 0.05 null hypothesis rejected); F crit: Critical test statistic value (if F larger than F crit then null hypothesis rejected)

Table 4-3 shows a significant interaction term ($F_{1.57} = F 10.45$ and $P = 7.91 \times 10^{-19}$). This is a measure of the interaction/interrelationship between time and the amendments made to the treatments. Therefore, it can be stated that time does have an impact on growth. This statement is supported by the effect over time shown in Table 4-3 by the, $F_{2.47} = 127.32$ and $P = 3.48 \times 10^{-36}$, values derived from the analysis. The effect between treatments ($F_{2.04} = 64.02$ and $P = 7.7 \times 10^{-34}$) was significant. Therefore, the amendments made to the treatments did have a significant effect on growth. However, the above ANOVA analyses the entire dataset. In order to test the hypothesis established before the outset of the experiment, ANOVA analysis was conducted to compare each treatment to the Control.

Nutrient Supplementation Hypothesis 1

Coal seam water is a suitable medium for cultivating *Spirulina* without the intervention of further chemical addition, and there will be no meaningful differences in growth performances compared to that of *Spirulina* cultivated in an optimal growth medium such as Schlösser's medium.

Table 4-4 provides the results of the statistical analysis of Treatment 5 and the Control.

Table 4-4: Nutrient Supplementation Flask Study Two Way ANOVA with replication statistical analysis of Treatment 5 and the Control (Treatment 6).

Source of Variation	SS	df	MS	F	P-value	F crit
Treatment	7034729	1	7034729	8.26014	0.009382	4.351243
Time	82469644	4	20617411	24.20885	2.01E-07	2.866081
Interaction	21839994	4	5459999	6.411101	0.001727	2.866081
Within	17032952	20	851647.6			
Total	1.28E+08	29				

SS= sum of squares; df = degrees of freedom; MS: Mean Squares; F: test statistic; P-value: Probability (if P greater than 0.05 null hypothesis rejected); F crit: Critical test statistic value (if F larger than F crit then null hypothesis rejected)

With a $F_{4,35}$ of 8.2 and a P value of 0.0093 the result is significant. Therefore, the null hypothesis may be rejected and it may be stated that CSW requires nutrient amendment to achieve similar growth to that achievable in an optimal growth medium.

Nutrient Supplementation Hypothesis 2

Spirulina grown in coal seam water amended to Schlösser's medium (ingredients added to CSW so that final chemical composition is similar to Schlösser's medium), will have similar biomass yields or growth performances to that of *Spirulina* cultivated in only Schlösser's medium.

Table 4-5 provides the results of the statistical analysis for Treatment 7 and the Control.

Table 4-5: Nutrient Supplementation Flask Study Two Way ANOVA with replication statistical analysis of Treatment 7 and the Control (Treatment 6).

Source of Variation	SS	df	MS	F	P-value	F crit
Treatments	16341446	1	16341446	16.66573	0.00058	4.351243
Time	1.34E+08	4	33437667	34.10122	1.14E-08	2.866081
Interaction	20597763	4	5149441	5.251629	0.004664	2.866081
Within	19610833	20	980541.6			
Total	1.9E+08	29				

With a $F_{4,35}$ of 16.66 and a P value of 0.00058 the result is significant. Therefore, the null hypothesis may be rejected and it can be stated that CSW amended to Schlösser's medium nutrient concentration will not have a similar biomass yield to that grown in only Schlosser's. However, it should be noted that Treatment 7 achieved a higher μ_{max} and X_{max} than the control. Therefore, amended CSW does provide a suitable growth medium for the strain selected.

Nutrient Supplementation Hypothesis 3

Spirulina cultivated in amended coal seam water medium, but without the addition of micronutrients (PIV, Chu, Vit B12), will show no difference in growth performances compared to that of *Spirulina* cultivated in CSW with the full suite of Schlosser's additives.

In order to test this hypothesis the growth results of Treatment 7 and 8 were statistically analysed. The results of this analysis are shown in Table 4-6.

Table 4-6: Nutrient Supplementation Flask Study Two Way ANOVA with replication statistical analysis of Treatment 7 and Treatment 8.

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Treatments	11908714	1	11908714	22.5843	0.000122	4.351243
Time	76955150	4	19238787	36.48542	6.33E-09	2.866081
Interaction	54428347	4	13607087	25.80518	1.19E-07	2.866081
Within	10546013	20	527300.6			
Total	1.54E+08	29				

With a $F_{4,35}$ of 22.58 and a P value of 0.000122 the result is significant. Therefore, the null hypothesis may be rejected and it may be stated that the addition of micro-nutrients to amended CSW is an absolute requirement.

Nutrient Supplementation Hypothesis 4

Spirulina cultivated in coal seam water with the addition of potassium, nitrate and phosphate to a similar concentration to that of Schlösser's medium will show no differences in growth performances compared to that of *Spirulina* cultivated in only Schlösser's medium.

In order to evaluate this hypothesis, the growth data for Treatment 9 and the Control must be analysed. This analysis data is shown in Table 4-7.

Table 4-7: Nutrient Supplementation Flask Study Two Way ANOVA with replication statistical analysis of Treatment 9 and the Control (Treatment 6).

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Treatments	1.19E+08	1	1.19E+08	229.3506	2.02E-12	4.351243
Time	32946523	4	8236631	15.94021	5.19E-06	2.866081
Interaction	26917274	4	6729318	13.02314	2.22E-05	2.866081
Within	10334404	20	516720.2			
Total	1.89E+08	29				

With a $F_{4,35}$ of 229.35 and a P value of 2.02^{-12} the result is highly significant. Therefore, the null hypothesis may be rejected and it may be stated that similar growth cannot be achieved in CSW with the addition of NPK as in optimised media. However, the large significance is most probably an over statement due to an unexplained lack of growth in Treatment 9.

Although no hypothesis was made prior to the experiment, the similarity in growth achieved in Treatments 1 - 4 and the Control may indicate an interesting relationship. The statistical analysis of this data is shown in Table 4-8.

Table 4-8: Nutrient Supplementation Flask Study Two Way ANOVA with replication statistical analysis of Treatment 9 and the Control (Treatment 1-4 and Treatment 6).

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Treatments	39463822	4	9865955	1.693055	0.166332	2.557179
Time	55468073	4	13867018	2.379661	0.064082	2.557179
Interaction	28514863	16	1782179	0.305832	0.994051	1.850315
Within	2.91E+08	50	5827309			
Total	4.15E+08	74				

With a $F_{2,56}$ of 1.69 and a P value of 0.17 the results are not significant and the null hypothesis, that no significant change in growth will occur due to the addition of varying concentration of Schlösser's media to CSW, is correct. This result may indicate that even at levels of 25% Schlösser's to CSW, nutrients are still available in excess and as such further reductions in nutrient concentrations could be made.

The above results indicate that alterations to nutrient concentrations do have a significant impact on growth. However, the results also show that amended CSW could serve as an optimal growth medium and that the addition of micro-nutrients are essential to growth.

4.4. Effect of Iron Concentration on Growth

Iron limitation of algal growth has been widely reported (Fogg, 1991) and sequestration of iron in pond systems can result in poor growth productivity performance (Vonshak 1997b). According to Vonshak (1997b) two possible scenarios appear in *Spirulina* mass culture: 1) low iron availability in the culture medium manifests as low growth rates and susceptibility to photoinhibition, and 2) contaminating algae may acquire a competitive advantage over *Spirulina* in sequestering scarce iron resources. Thus, in theory, iron limitation in *Spirulina* culture can be overcome by ensuring availability through extra enrichment of iron.

The growth results recorded during the Effect of Iron Concentration on Growth Flask Study as represented by increase in biomass determined on a dry weight basis are provided in Figure 4-6.

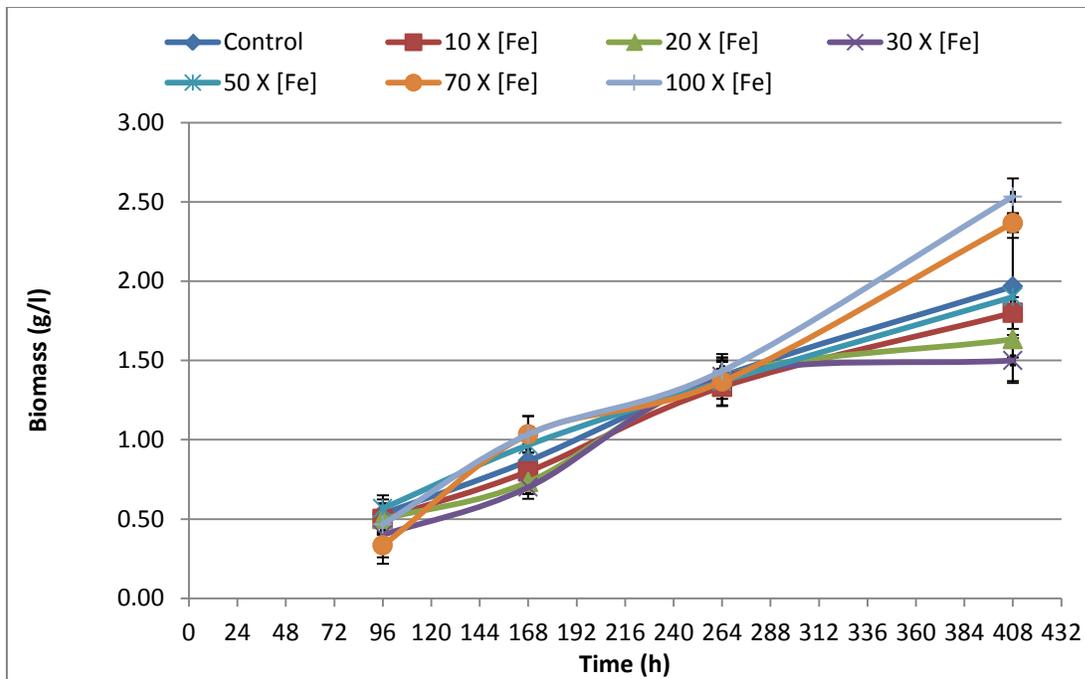


Figure 4-6: Growth as determined on a dry weight basis during the Effect of Iron Concentration on Growth Flask Study (mean). All treatments were carried out in triplicate in Schlösser's media made in distilled water with concentrations of iron varying from 10 to 100 times that found in Schlösser's media. Treatment 1, the control, consisted of normal Schlösser's media made in distilled water while Treatments 2-7 each had increasing iron concentrations as shown in the legend of the above graph Treatment 2: 10X; Treatment 3: 20X; Treatment 4: 30X; Treatment 5: 50X; Treatment 6: 70X; Treatment 7: 100X.

Figure 4-6 indicates that the control achieved a higher final biomass concentration than all treatments except Treatments Six and Seven which both outperformed the control.

To determine whether a significant relationship is indicated the results of the two-way ANOVA (with replication) statistical analysis are provided in Table 4-9 below.

Table 4-9: Effect of Iron Concentration on Growth Flask Study, Two Way ANOVA with replication, statistical analysis of all treatments.

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Treatments	0.00012	6	2.01E-05	8.108974	2.53E-06	2.265567
Time	0.002607	3	0.000869	350.8894	2.92E-36	2.769431
Interaction	0.000184	18	1.02E-05	4.123932	2.3E-05	1.791158
Within	0.000139	56	2.48E-06			
Total	0.00305	83				

Table 4-9 indicates a significant interaction term ($F_{1.79} = 4.12$ and $P = 2.3 \times 10^{-5}$) between time and growth (represented by the interaction row in the table). Therefore, time does have an impact on growth. This is supported by the effect over time shown in Table 4-9 by the, $F_{2.77} = 350.89$ and $P = 2.92 \times 10^{-36}$, values derived from the analysis. The effect between treatments ($F_{2.27} = 8$ and $P = 2.53 \times 10^{-06}$) was significant. Therefore, the iron amendments made to the treatments did have a significant effect on growth. As such, the statistical null hypothesis, that no meaningful difference would be detected, can be discounted. When the same analysis is made between the control and Treatments 2 – 5 the results as shown in Table 4-10 were obtained.

Table 4-10: Effect of Iron Concentration on Growth Flask Study, Two Way ANOVA with replication, statistical analysis of Treatments 1 to 5.

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Treatments	1.52E-05	3	5.06E-06	1.419103	0.255272	2.90112
Time	0.00119	3	0.000397	111.345	5.15E-17	2.90112
Interaction	1.45E-05	9	1.61E-06	0.452242	0.895247	2.188766
Within	0.000114	32	3.56E-06			
Total	0.001334	47				

Table 4-10 shows an $F_{2.90} = 1.42$ and a P value = 0.25 between treatments and as such no significant effect exists between Treatments 1 to 5. Therefore, Treatments 6 and 7 caused the significant effect as shown in Table 4-10. Thus, it may be surmised that the addition of less than 50 times the concentration of

iron found in Schlösser's media, has no significant impact on growth. However, the addition of 70 times and greater the concentration of iron found in Schlösser's does have a significant impact on growth. It is unclear why the impact on growth only manifests at above 70 times the iron level. The literature indicates similar results in terms of a limit under which no effect is measurable; Melnic *et al.* (2011) found this limit to be at 5 mg/l and Raksajit *et al.* at above 20mg/l. Some variation can be expected due to the use of different experimental designs and strains of *Spirulina*. Therefore, the effect is described but not explained in the literature and as such should be the target of further study.

The following hypotheses were made prior to the study.

Iron Concentration Hypothesis 1

Iron in excess can be used to boost growth.

From the results in Table 4-10 and 4-11, Hypothesis 1 was not disproven, with the caveat that the effect only manifests above the 50X iron concentration limit. The current experiment only indicates that growth is stimulated in the range between 50 and 100 times the level found in Schlösser's, that is between 3.6 and 12.0 mg/l iron. Further work will be required to determine the exact concentration that has a significant impact on growth.

Hypothesis 2: that Spirulina can be successfully cultivated in concentrations of iron of up to 12 mg.L⁻¹ Fe (i.e. 100x) was not disproven.

4.5. Optimum pH for Growth in Coal Seam Water Media

Spirulina is an alkaliphile and as such thrives in a pH range between 9.5 and 12 (Richmond, 1988). Çelekli *et al.* (2009) recorded optimal growth at a pH of 10. According to Vonshak and Grobbelaar (1986) the high pH requirements is mainly due to two reasons: gas exchange and exclusivity of the culture.

Gas exchange between the culture and the atmosphere is dependent on the gradient of the partial pressures of the gasses. Increasing the pH increases the CO₂ gradient, thereby allowing the uptake of free carbon from the atmosphere (Vonshak and Grobbelaar, 1986; Belay, 1997). Exclusivity is achieved through the application of a high pH as this excludes potential competitors that are unable to withstand the physiological challenges presented by a high pH.

Six pH values, ranging from 9.5 to 11.5, were investigated.

The growth results, as represented by chlorophyll a concentration, are shown in Figure 4-7.

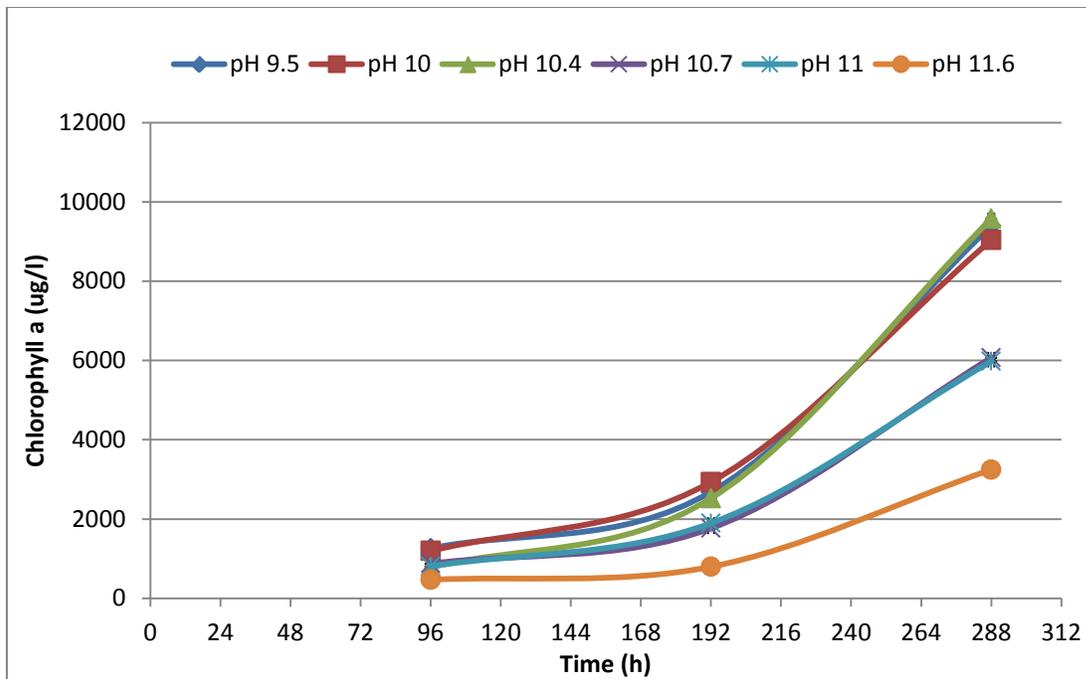


Figure 4-7: Chlorophyll a concentration as recorded during the Effect of pH on Growth in Coal Seam Water Media Flask Study (mean). All treatments were carried out in triplicate and consisted of Schlösser's media made in CSW and pH adjusted with a concentrated NaOH solution. Treatment 1: Control: pH 9.5; Treatment 2: pH 10; Treatment 3: pH 10.4; Treatment 4: pH 10.7; Treatment 5: pH 11; Treatment 6: pH 11.6.

Figure 4-7 indicates that Treatments 2 and 3 recorded similar growth results, both in terms of growth rate and maximum biomass to that in the control. Treatments 4 and 5 achieved similar growth rates and final biomass concentrations too each other, but both at a lower rate than the control. In addition, both Treatments 4 and 5 achieved a lower final biomass concentration than the control. At the upper end of the evaluated pH range (pH 11.6), growth was severely negatively affected in terms of growth rate and final biomass concentration as compared to the control.

Figure 4-7 indicates that growth was not affected in the range of pH between pH 9.5 to 10.4. In addition, Figure 4-7 indicates that growth is affected at a pH above 10.4. This could indicate that the pH optimum is between pH 9.5 and 10.4. To determine whether a significant relationship is indicated the results of

the two-way ANOVA (with replication) statistical analysis are provided in Table 4-11.

Table 4-11: Optimum pH for Growth in Coal Seam Water Flask Study, Two Way ANOVA with replication, statistical analysis of all Treatments.

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Treatment	55827347	5	11165469	21.45093	6.75E-10	2.477169
Time	3.99E+08	2	1.99E+08	383.0787	5.45E-25	3.259446
Interaction	48160616	10	4816062	9.252546	2.41E-07	2.106054
Within	18738434	36	520512.1			
Total	5.22E+08	53				

With a $F_{2,477} = 21.45$ and $P = 6.75 \times 10^{-10}$ between treatments the results were significant. As such, the statistical null hypothesis, that no meaningful difference would occur, can be discounted. Therefore, the pH amendment made to the treatments did have a significant effect on growth and it may be stated that pH does affect growth. However, to determine a pH optimum more focussed analysis is required. This is shown hereafter.

The analysis of the growth data of the control and Treatments 2 and 3 gave the following results.

Table 4-12: Optimum pH for Growth in Coal Seam Water Flask Study, Two Way ANOVA with replication, statistical analysis of control and Treatments 2 and 3.

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Treatments	110076.1	2	55038.07	0.069771	0.932858	3.554557
Time	3.44E+08	2	1.72E+08	218.1432	2.41E-13	3.554557
Interaction	1007603	4	251900.7	0.31933	0.861308	2.927744
Within	14199122	18	788840.1			
Total	3.59E+08	26				

Table 4-12 shows a $F_{3,55} = 0.69$ and a P-value = 0.93 between treatments which is not significant. As such, the null hypothesis, that no effect would occur, is upheld and it can be stated that altering the pH between 9.5 and 10.4 has no effect on growth. Therefore, pH in the range between 9.5 and 10.4 does not have a significant effect on growth and is suitable for *Spirulina* cultivation in CSW. This conforms well to the findings of Richmond (1988); Vonshak (1997a); Belay (1997) and Çelekli *et al.* (2009).

The analysis of the growth data of the Control and Treatments 4-6 gave the following results.

Table 4-13: Effect of pH on Growth Flask Study, Two Way ANOVA with replication, statistical - analysis of control and Treatments 4-6.

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	33893220	3	11297740	45.73993	4.43E-10	3.008787
Columns	1.88E+08	2	94015329	380.6296	6.64E-19	3.402826
Interaction	27005651	6	4500942	18.22247	7.32E-08	2.508189
Within	5927988	24	246999.5			
Total	2.55E+08	35				

Table 4-13 shows a P-value of 4.43×10^{-10} between treatments which indicates a highly significant relationship. As such, the null hypothesis, that no effect would occur, is rejected and it may be stated that a pH of higher than 10.4 does affect growth. This conforms well to the findings of Pandey and Tiwara (2010) who recorded a 47% reduction in dry weight concentration between *S.maxima* cultivated at a pH of 11 when compared to that grown at a pH of 10. In addition they found that, no growth occurred at a pH of 12.

The following hypothesis was made at the outset of the experiment.

Optimum pH Hypothesis 1

The pH optimum for the strain of *Spirulina* used is between 9.5 and 10.4.

The data did not disprove the hypothesis and it can be stated that the optimal pH range for growth of the selected strain of *Spirulina* was found to be between 9.5 and 10.4. However, based on the literature which indicates a pH optimum between 9 and 10.5 the probability that this may be extended to pH 10.5 is very high (Richmond, 1988; Spolaore *et al.*, 2006; Ravelonandro *et al.*, 2011; Gupta *et al.*, 2013).

CHAPTER 5: HIGH RATE ALGAL POND STUDIES: **RESULTS AND DISCUSSIONS**

5.1. Introduction

In this chapter, the results of the High Rate Algal Pond (HRAP) Studies are presented and discussed.

The High Rate Algal Pond Studies were carried out in the field. The first phase was operated in batch mode. Thereafter, the study progressed into a fed batch and then semi continuous culture operating protocols.

5.2. Batch Culture

5.2.1. *Batch Culture Protocol 1 (Pond 1 First Inoculation)*

Only Pond 1 was utilised during this experiment which represented a first pass at understanding the prevalent environmental conditions and their effect on algal growth.

The growth results, as determined from chlorophyll a concentration, are provided in Figure 5-1.

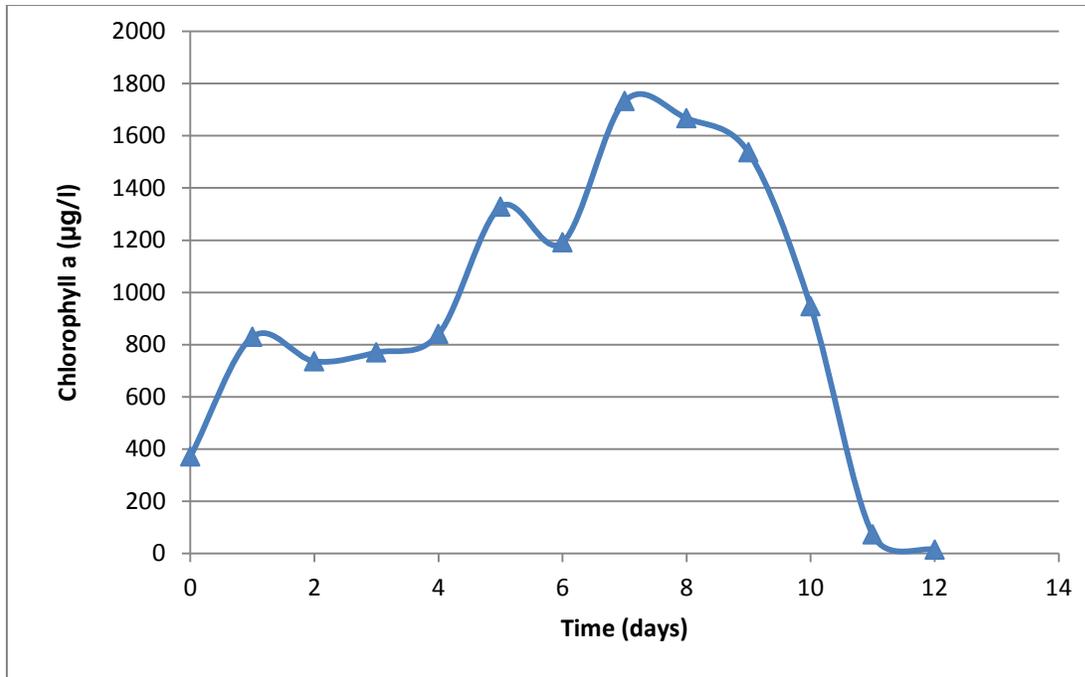


Figure 5-1: Growth results as determined from chlorophyll a concentration for Pond 1 during the HRAP: Batch Culture Protocol 1 (Pond 1 first inoculation).

Due to a lack of filter papers at the field laboratory and delivery problems over the festive period, chlorophyll a (chl a) determinations were not carried out in triplicate. Only single measurements were possible.

Growth in Pond 1 continued as expected until day 7 reaching a maximum chl a concentration of 1783 µg/l. After day 9, the culture entered a rapid death phase, reaching a minimum chl a concentration of 15 µg/l on day 12.

Microscopy examinations showed a culture in poor health from day eight until the end of the experiment. This poor health was characterised by a severe decline in colour quality from a bright blue green to brown by day 9. In addition, lyses of trichomes commenced on day 9 and continued unabated until complete culture death on day 12.

Weather conditions over the time under investigation are provided in Figure 5-2 below.

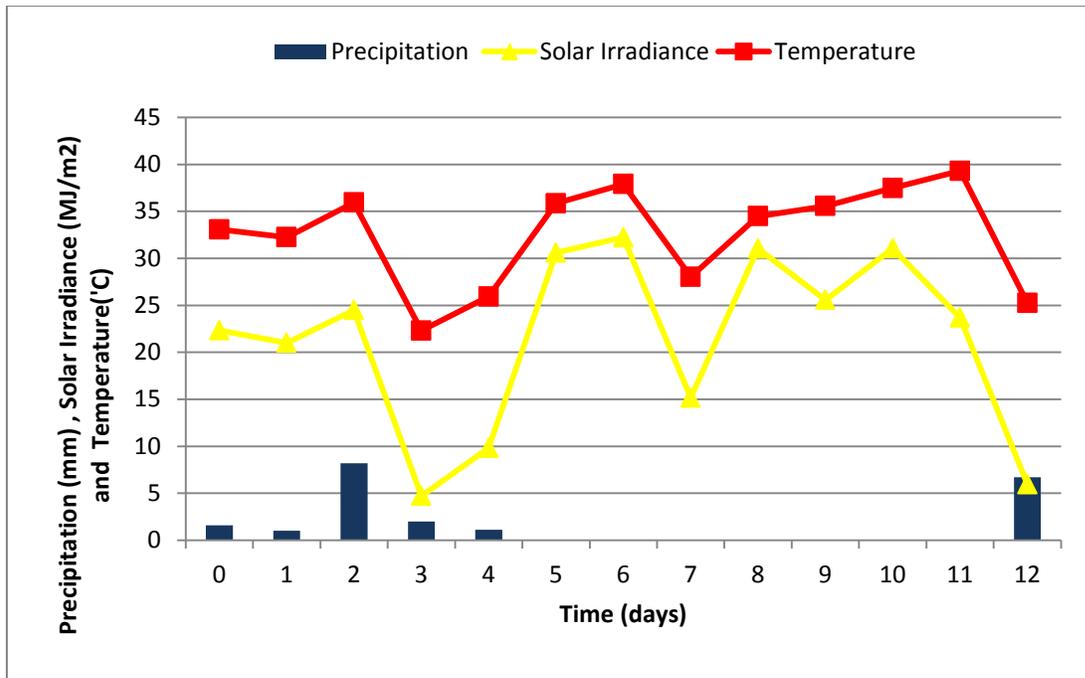


Figure 5-2: Ambient weather conditions as measured by the on site Campbell Scientific Automated Weather Station during the Batch Culture Protocol 1(Pond 1: First Inoculation Study).

The weather data indicates precipitation and cloudy conditions on day one until day four. Thereafter, solar irradiance and temperature increased on day five reaching of 32.22 MJ/m² on day six - the maximum value of solar irradiance measured for the study period. Cloudy conditions are indicated by the drop in solar irradiance on day seven. A sequence of very hot days with high solar irradiance starting on day seven and continuing until day 11 followed. Maximum temperature reached 39.30°C on day 11 and solar irradiance exceeded 31 MJ/m² on days eight and ten.

There are a number of possible causes for the growth pattern shown in Figure 5-1:

- The low solar irradiance values measured from day zero to day four and then again on day five should have resulted in a culture that was low light adapted.

- The combination of low light adapted culture, the removal of the shade netting on day nine and the bright conditions from day seven to day eleven, could potentially have caused photoinhibition resulting in photo-oxidative death as described by Vonshak (1997a).
- Nutrient limitation could also result in the crash. However, the Flask Studies indicated that nutrient limitation does not, on average, set in until between day 16 and 20. In addition, during an investigation into alternative nitrogen sources Matsudo *et al.* (2009) utilised a fed batch culture operating protocol with nutrient additions only after 13 days with no impact on growth. Similarly, Rodrigues *et al.* (2011) also used a 13 day feeding cycle for a fed batch system. The difference between the period before nutrient limitation sets in reported in the literature and that found in the flask experiments is related to different experimental setups and variations in strains used. However, both the literature and the flask experiments indicate that nutrient limitation after only 8 days is highly unlikely.
- An increase in salinity as a result of evaporative loss of water can also result in severe culture decline. Ravelonandro *et al.*, 2011 showed that an increase in salinity past 35 g/l NaCl can cause inhibition of growth. However, as evaporative loss was replenished through the addition of RO water on a daily basis this was not likely.

Therefore, photoinhibition was the most likely cause of the culture crash.

5.2.2. Batch Culture Protocol 2 (Pond 2 First Inoculation)

The growth results for the Batch Protocol 2 are shown in Figure 5-3.

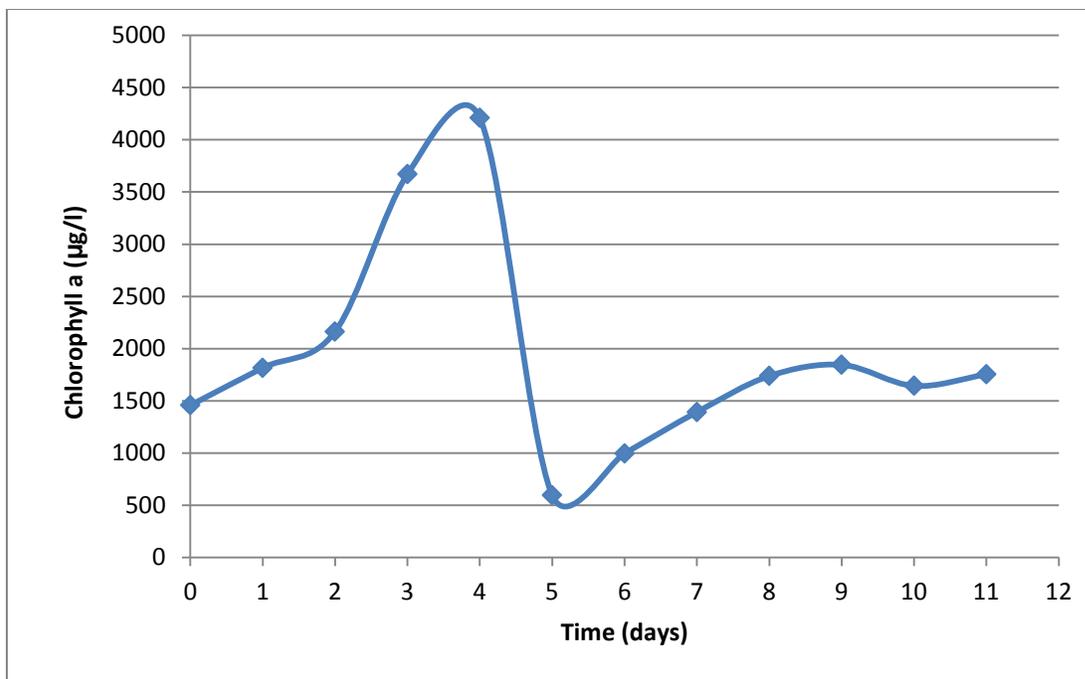


Figure 5-3: Growth as determined by chl a concentration for Pond 2 during the Batch Culture Protocol 2 (Pond 2 First Inoculation) (mean).

Figure 5-3 indicates a growing culture until day four. From day four to five a significant decline in chl a concentration was recorded. From day five to nine growth continued at a diminished rate.

Ambient weather data collected during this study are provided in Figure 5-4 below.

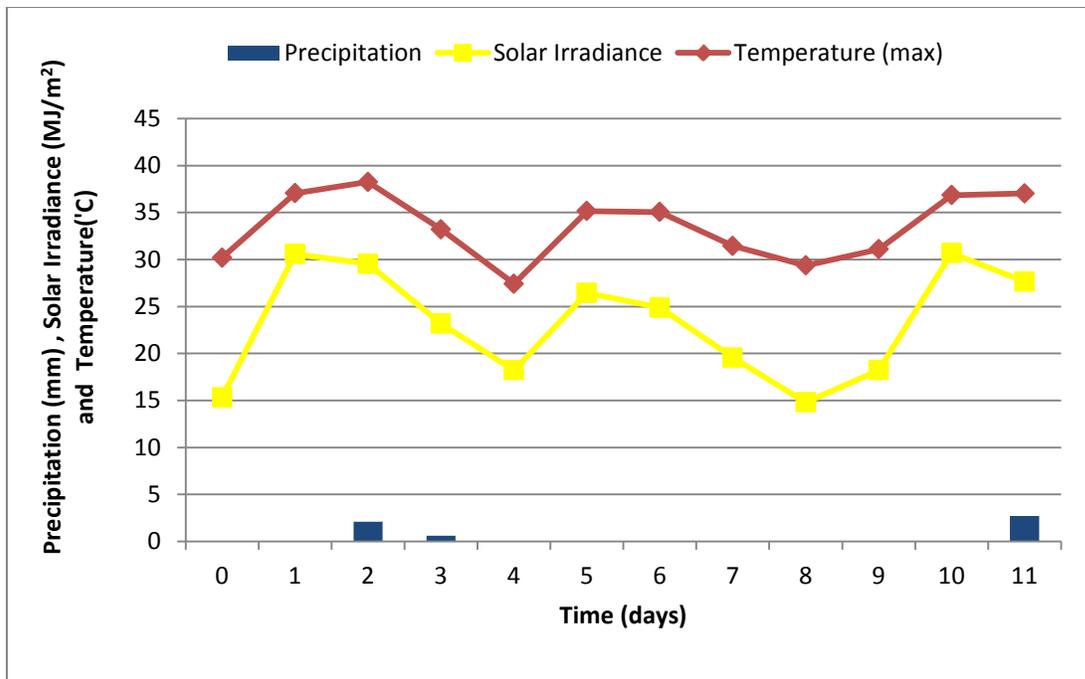


Figure 5-4: Ambient weather conditions as measured by the on site Campbell Scientific Automated Weather Station during the Batch Culture Protocol 2 (Pond 2 First Inoculation).

Figure 5-4 shows a significant drop in both solar irradiance and maximum temperature on day four which correlates well with the drop in growth shown in Figure 5-3.

Microscopy over the period indicated significant contamination by a helical single-celled alga from day five onwards (Figure 5-5).

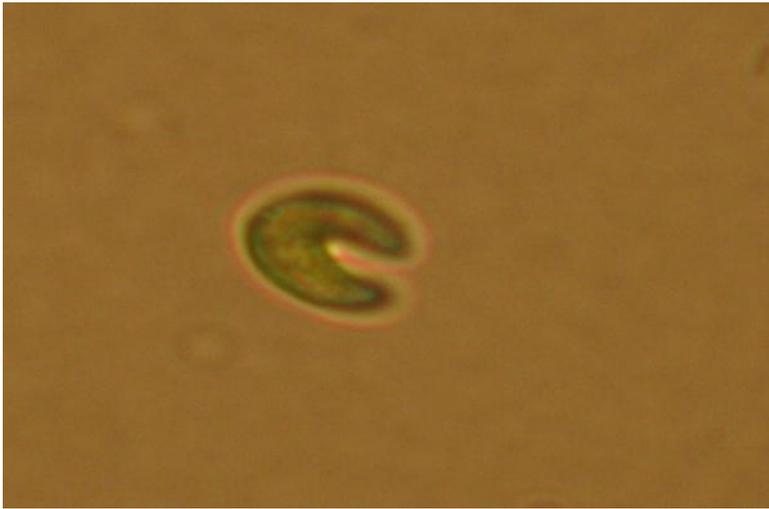


Figure 5-5: A single cell green alga that was found to contaminate ponds operated as batch cultures, provisionally identified as a *Kirchneriella*-type strain (400 X magnification) (Taken by the author).

By day 6, *Kirchneriella*, was the dominant organism in the culture and growth shown is attributed to it.

It would appear that once *Spirulina* growth was affected by lower than optimal ambient weather conditions, its competitors were able to outcompete it for resources.

5.2.3. Batch Culture Protocol 3 (All Ponds: Environmental Conditions)

Growth results obtained during the study are shown in Figure 5-6.

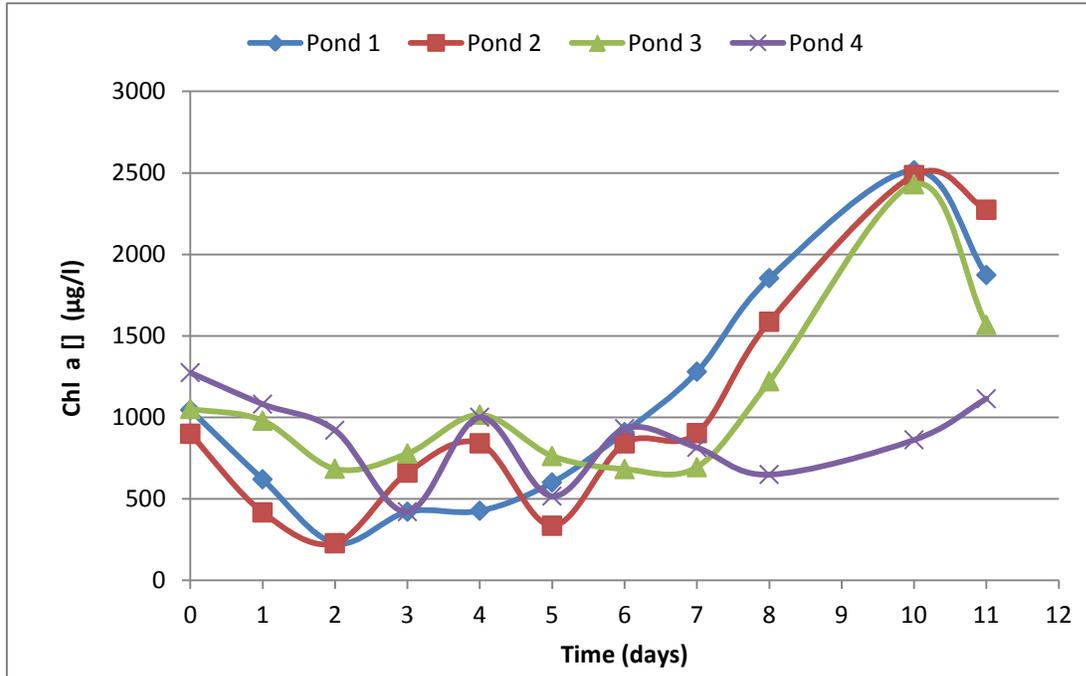


Figure 5-6: Growth results as determined by chl a concentration for all ponds during the Batch Culture Protocol 3 (All ponds: environmental conditions) (mean).

Figure 5-6 indicates very lacklustre growth over the first six days of the study. Thereafter, growth improved reaching maximum chl a concentrations in all ponds except Pond 4 by day ten.

The ambient weather data are provided in Figure 5-7.

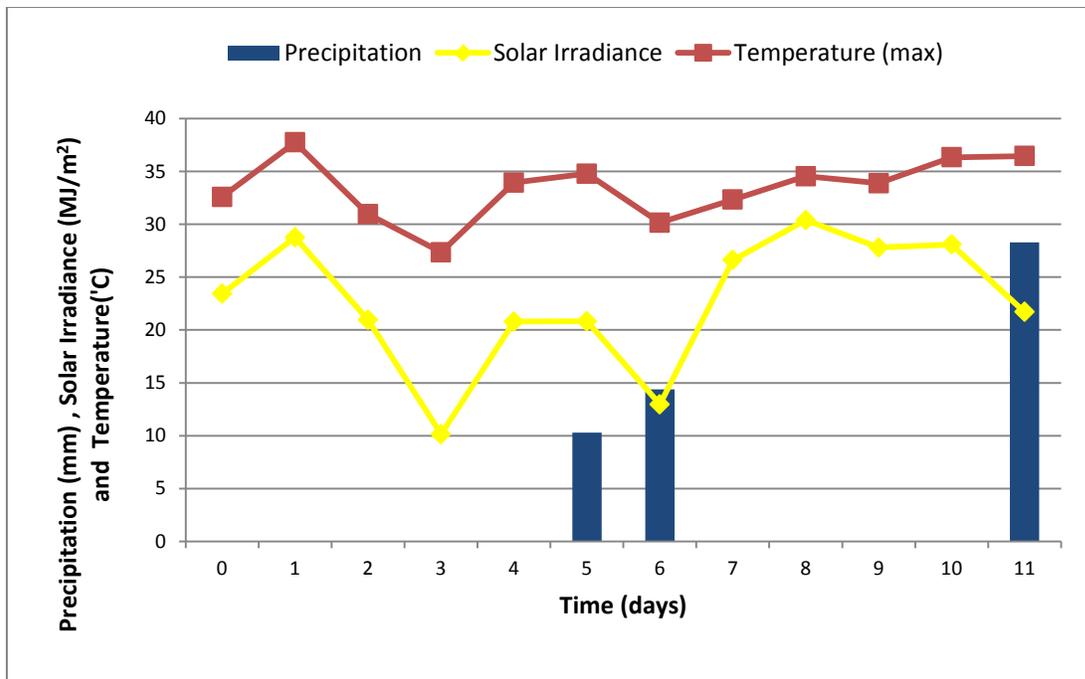


Figure 5-7: Ambient weather conditions as measured by the on site Campbell Scientific Automated Weather Station during the Batch Protocol 3 (All ponds: environmental conditions) study.

The weather data indicates that poor conditions for photosynthetic growth occurred from day one to day six. This was manifested as low solar irradiance as a result of cloudy conditions. Two precipitation events occurred on day five and six and then again on day 11.

Microscopy investigations indicated severe contamination from day five onwards in all ponds. Further increases in chlorophyll a concentrations are attributed to contaminants. The diluting effect of the precipitation altered the media to a point where it was no longer optimised for *Spirulina*. Salinity reduced to below 1.9 NaCl g/l which is within the optimum range for *Kirchneriella* (Feroni *et al.*, 2007). Therefore, the dilution effect of the rain may have provided a competitive advantage to this naturally occurring algal species. In addition, due to a lack of biomass, the inoculum concentration utilised was well below the optimum for *Spirulina* at 1 g/l biomass as described by Morais *et al.* (2009). Average inoculation concentration was 0.001 g/l chl a. This inoculum size was

necessitated by the low availability of biomass and may have impacted on the ability of the culture to maintain mono-algal conditions.

5.3. Fed Batch Culture

5.3.1. *Fed Batch Culture: Protocol 1 (Pond 4 Only)*

Growth results for the above protocol are provided in Figure 5-8.

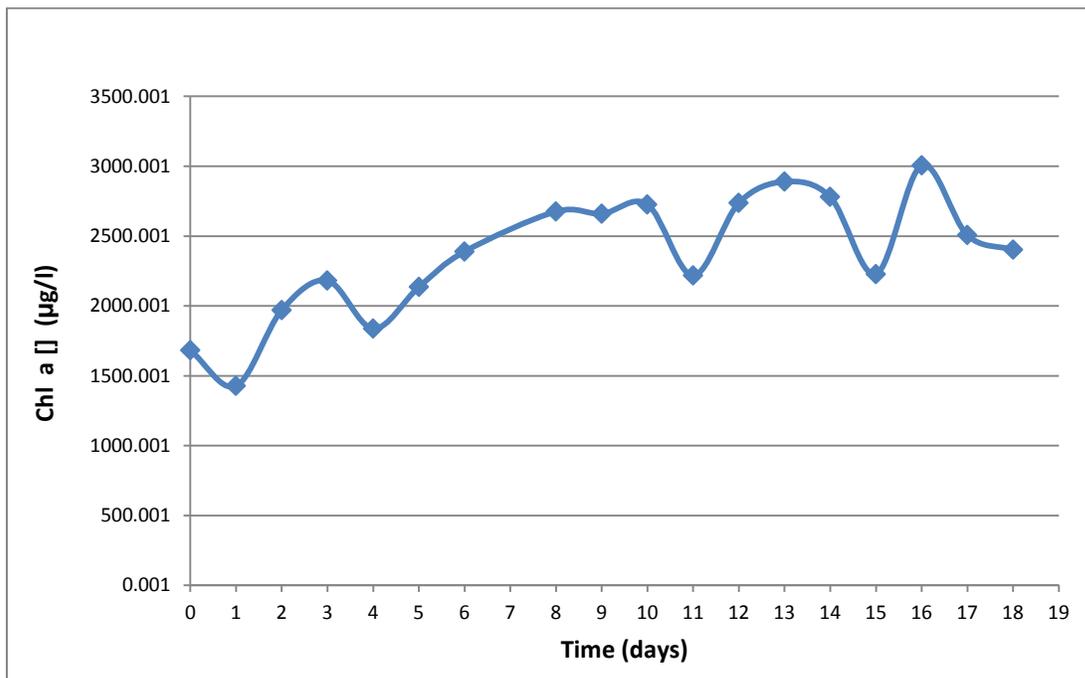


Figure 5-8: Growth results as determined by chl a concentration during the Fed Batch Culture Protocol 1 (Pond 4 Only) (mean).

No samples were taken on day seven due to it being a public holiday.

The growth curve shows growth over the full 18 days of the experiment. However, ambient weather conditions, shown in Figure 5-9, specifically

precipitation events and commensurate decreases in solar irradiance, appeared to depress growth.

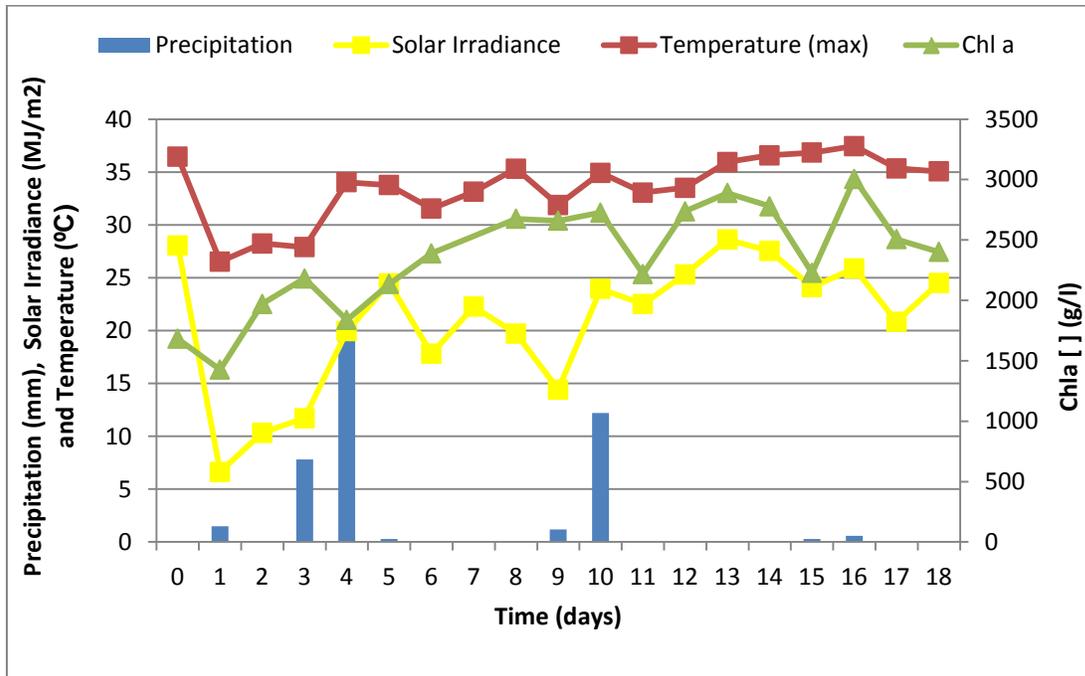


Figure 5-9: Growth (chl a concentration) plotted together with solar irradiance, temperature and precipitation during the Fed Batch Culture Protocol 1 (Pond 4 only).

The graph (Figure 5-9) indicates a directly proportional linear relationship between and growth and solar irradiance with a Pearson product moment correlation coefficient, $r = 0.50$. Statistical analysis of the significance of the relationship between solar irradiance and growth calculated a student t-test P-value of 8.34×10^{-17} . As such, the relationship is significant. The P-value calculated when comparing maximum ambient temperature and growth is 7.35×10^{-23} , and $r = 0.54$. As such growth exhibited a highly significant directly proportional linear relationship with both temperature and solar irradiance. Therefore, any change in solar irradiance or ambient temperature would result in a similar change i.e. increase or decrease in growth rate.

The culture did not fail after ten days as had all previous cultures done in the HRAP experiments. Interventions to preclude such a crash were:

- Decreased depth of the culture from 300 mm to 155 mm (Belay, 1997),
- Addition of 10X the iron concentration found in Schlösser's (based on results of the Flask Studies),
- The addition of nutrients on day 15 (Matsudo *et al.*, 2009),
- Starting pH of above 10 (Richmond, 1988; Panday & Tiwara, 2010).

These interventions were aimed either at providing a competitive advantage or to improve the light exposure of the culture. As minimal contamination was noted through microscopy and better growth achieved than any previous pond experiment these interventions were seen as successful. The study indicated that it would be possible to grow the chosen *Spirulina* strain in amended CSW at on-site environmental conditions.

5.3.2. Fed Batch Culture: Protocol 2 (High vs Moderate Conductivity)

The growth results recorded during the study are provided in Figure 5-10.

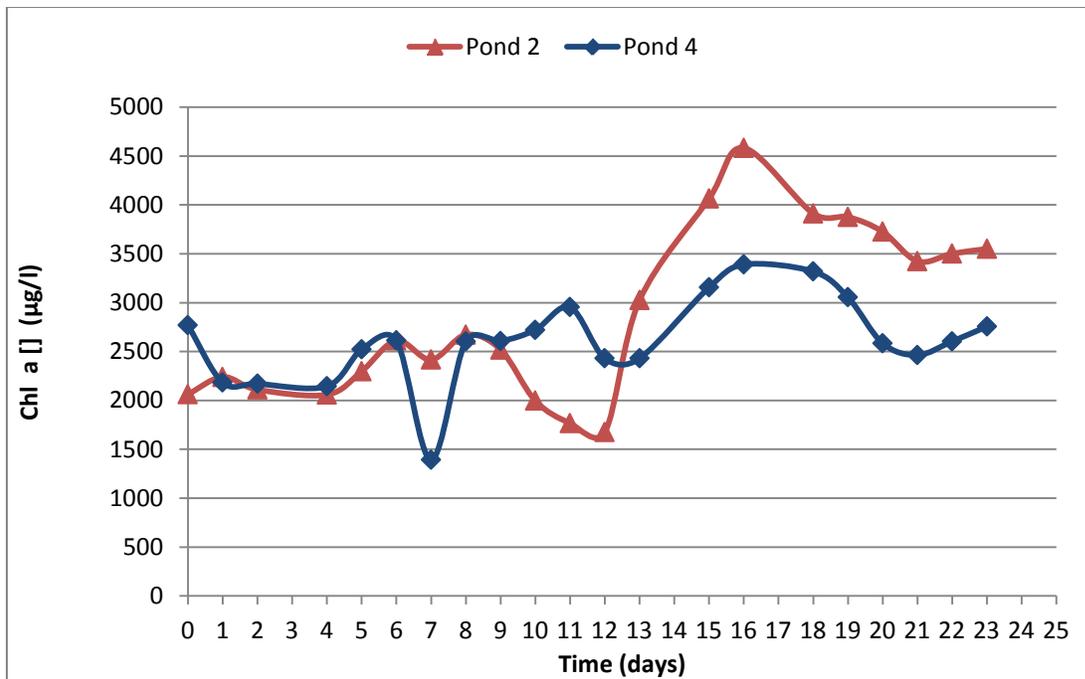


Figure 5-10: Growth results as determined by chl a concentration in Ponds 2 and 4 during the Fed Batch Culture Protocol 2 (High vs Low Conductivity) (mean). Pond 2: moderate conductivity pond (~ 20 mS/cm) no bicarbonate, carbonate or chloride added. Pond 4: high conductivity (~30 mS/cm) pond.

Figure 5-10 indicates that no samples were taken on days three, 14 and 17 due to unforeseen circumstances.

From Figure 5-10, it can be seen that growth was suppressed from day zero to 12. After day 12 growth occurred until day 16 more rapidly in Pond 2 than in Pond 4. After day 16 both cultures declined until day 21 and then exhibited growth at a low rate until day 23.

Figure 5-11 shows a total of 156 mm of precipitation fell from day zero to day seven; a total of 172 mm fell during the study period.

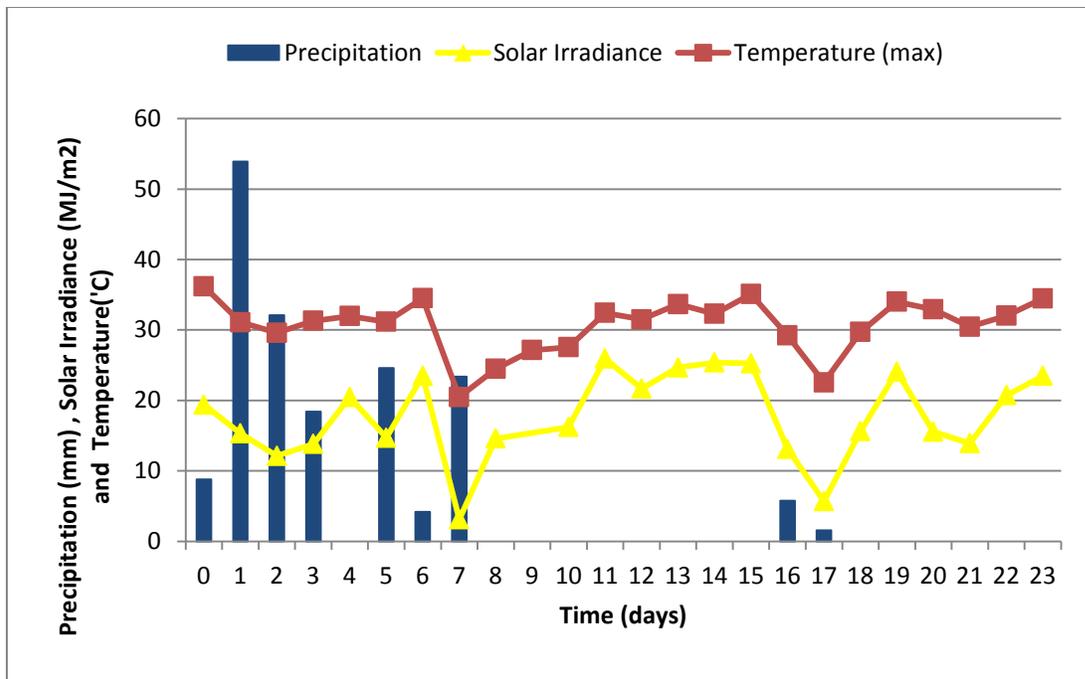


Figure 5-11: Ambient weather conditions as measured by the onsite Campbell Scientific Automated Weather Station during the Fed Batch Protocol 2 (High vs Low Conductivity) study.

Solar irradiance was suppressed until day eight and then increased from day eight until day 12 thereafter, irradiance remained relatively stable at the 25 MJ/m² level for three days until day 16. From day 16, solar irradiance decreased reaching a low of 5.69 MJ/m² on day 18. After day 18, solar irradiance increased reaching 24.47 MJ/m² on day 23.

Maximum ambient temperature averaged 30.66 °C over the study period.

As a result of the cloudy conditions and the accompanying precipitation recorded during the study period, solar irradiance was suppressed and conditions were poor for algal growth until day 12 and then again on days 16, 17 and 18.

The heavy rainfall also had an effect on culture conditions as illustrated in Figures 5-12 and 5-13.

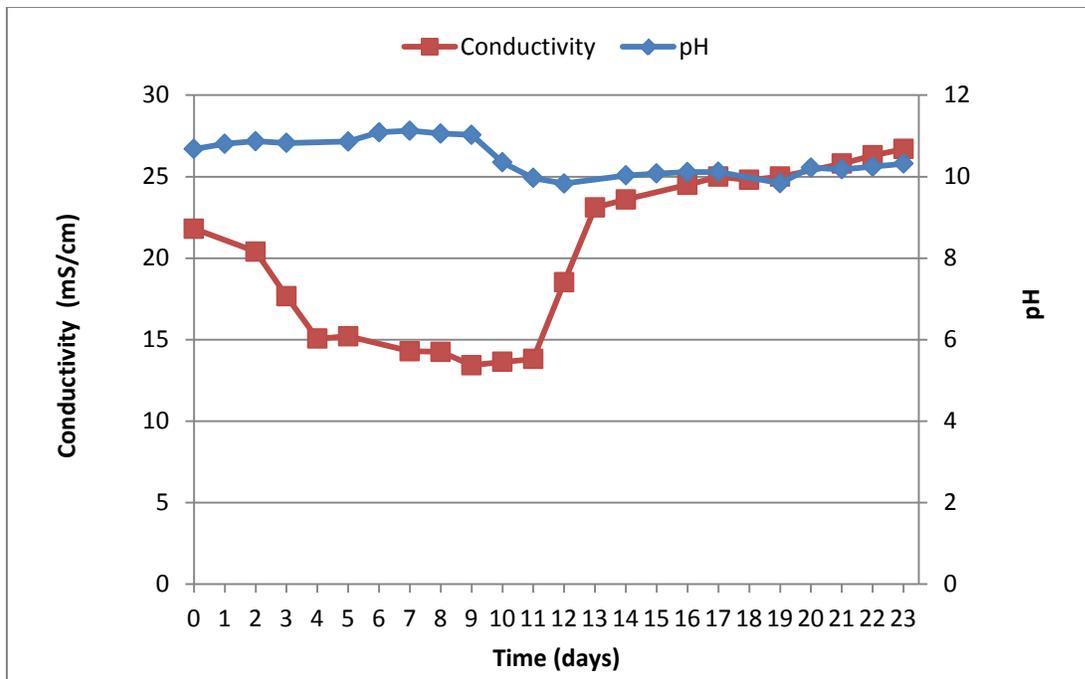


Figure 5-12: pH and conductivity measured in Pond 2 during the Fed Batch Culture Protocol 2 (High vs Low Conductivity).

The target conductivity in Pond 2 was ~20 mS/cm; this was achieved as average conductivity during the study period was 20.43 mS/cm. However, from day three to day 12 average conductivity was 15.09 mS/cm. Conductivity was suppressed from day zero and only started to increase after day 12. The initial increase was caused by the addition of salts on day 13 and again on day 14, thereafter, the increase was a result of evaporation. Morning addition of RO water was suspended due to increased culture depth as a result of precipitation.

The large concentration of sodium bicarbonate ensured that the culture medium was very well buffered. Therefore, pH remained relatively stable throughout the study. The decrease in pH from day nine to 11 was a result of CO₂ sparging carried out in Pond 2. pH was decreased from 11.27 (day 9) to 9.83 (day 12) through sparging and then increased as a result of photosynthesis to 10.11 on day 17. pH was effected by the precipitation on days 16 and 17 resulting in a decrease in pH to 9.83 on day 19. From day 19 to 23 pH again increase as a result of photosynthesis.

In Pond 4 a similar trend was seen as shown in Figure 5-13.

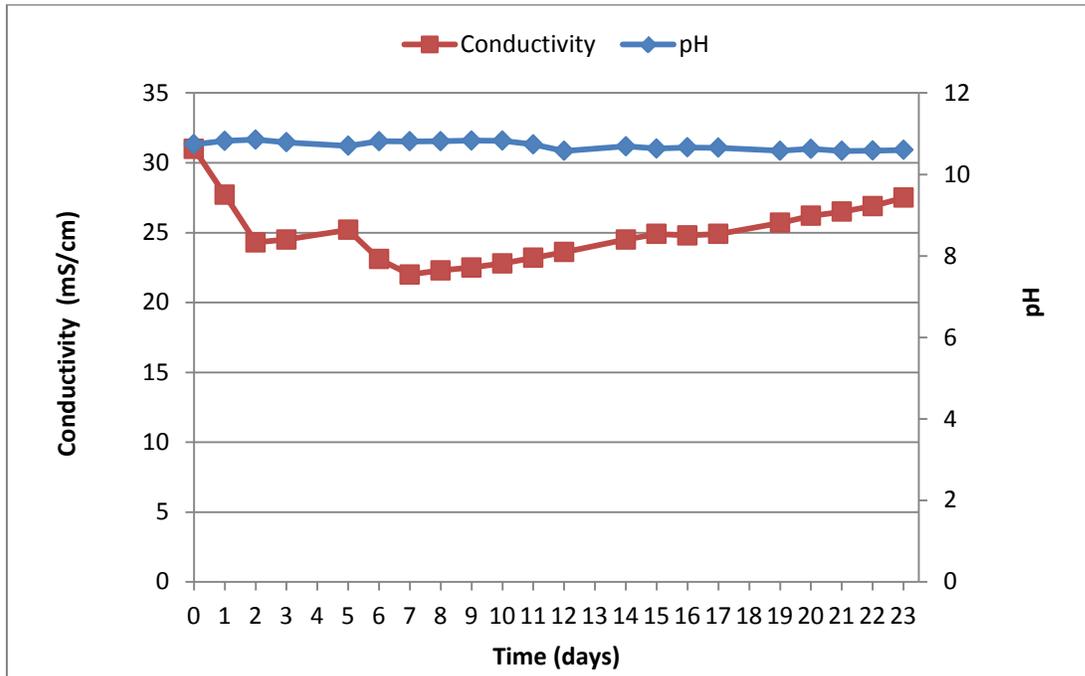


Figure 5-13: pH and conductivity measured in Pond 4 during the Fed Batch Culture Protocol 2 (High vs Low Conductivity).

Figure 5-13 indicates that conductivity started at 31 mS/cm and decreased to 22 mS/cm on day seven. This is a direct result of the diluting effect of the precipitation from day zero to day seven. Conductivity then steadily increased reaching 27.5 mS/cm on day 23 as a result of evaporation.

However, it was intended that Pond 4 would be a high salinity pond in order to test whether that would affect contamination. Unfortunately maintaining a high conductivity in Pond 4 was not possible. The final conductivity of 22 mS/cm was closer to the original target conductivity in Pond 2, the moderate salinity pond. As such, it was not possible to test the hypothesis that a high conductivity would control contamination.

High levels of contamination were observed from day 12 onwards in both ponds as shown in Figures 5-14 and 5-15.

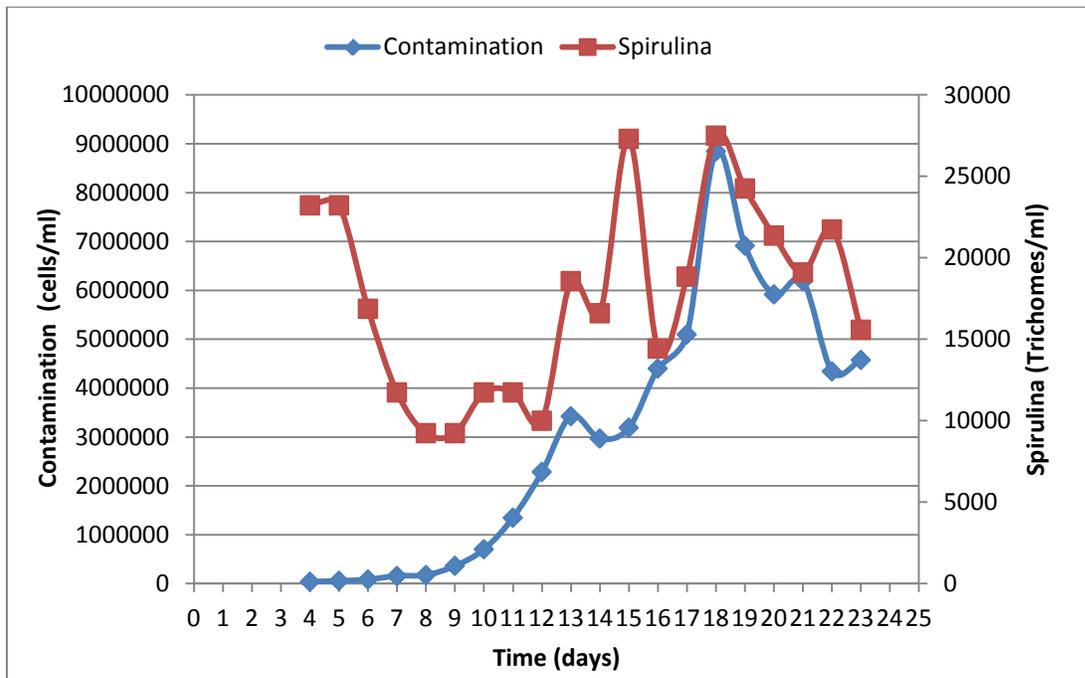


Figure 5-14: Results of cell counts in Pond 2 during the Fed Batch Culture Operating Protocol 2 (High vs Low Conductivity).

Figure 5-14 indicates that no cell counts were carried out in Pond 2 until day four. This was because microscopy indicated no contamination until day three. Contamination remained at low values until day nine. After day nine, contamination increased rapidly until day 13. A slight decrease in contaminant cell numbers was recorded on days 14 and 15 after which numbers again increased reaching maximal levels on day 18 of 8 848 000 cells per ml.

The figure also indicates that *Spirulina* trichome numbers decrease markedly from day four until day nine. For the next four days trichome numbers remained relatively stable before showing a rapid increase from day 12 to day 15. The concentration of *Spirulina* trichomes then decreased in a similar manner as that of the contaminants. This decrease in both contaminant cell number and trichome numbers was most probably related to the precipitation and low solar irradiance recorded on day 16 and 17.

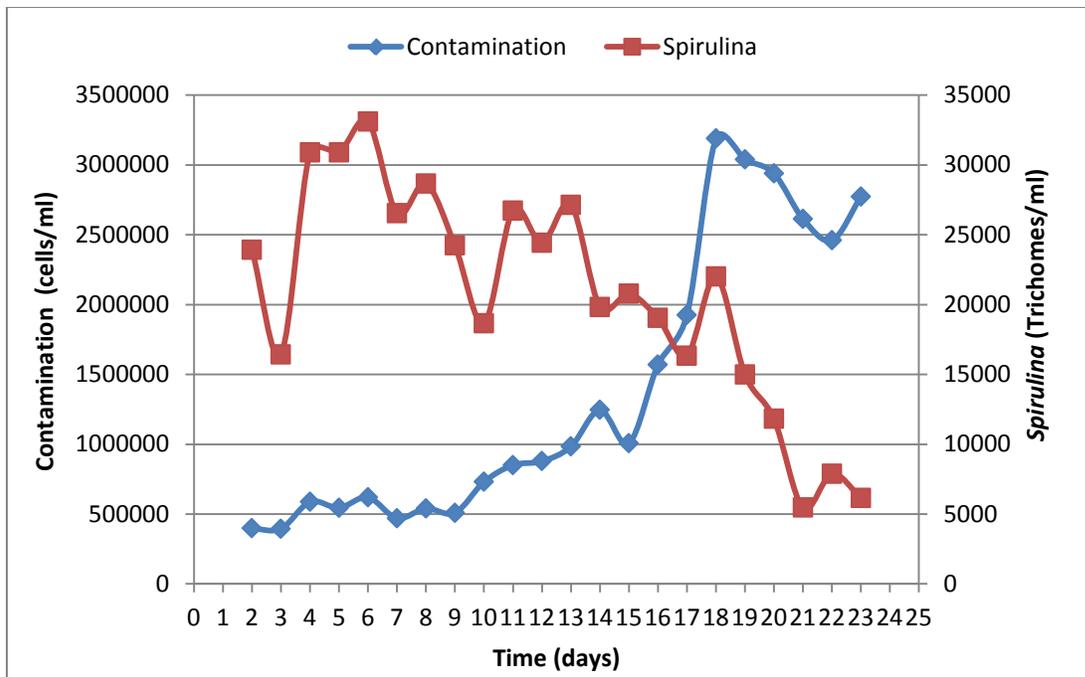


Figure 5-15: Results of cell counts in Pond 4 during the Fed Batch Culture Operating Protocol 2 (High vs Low Conductivity).

Figure 5-15 indicates that contaminant concentration reached countable levels on day two. From day two to nine contaminant concentration remained relatively stable around the 500 000 cells/ml level. After day nine contaminant cell concentration increased steadily reaching a maximum of 3 189 000 cells/ml on day 18. After day 18 contaminant cell concentration decreased until day 22. Trichome numbers reached a maximum of 33 117 trichomes/ml on day 6, thereafter a decreasing trend was observed until day 23.

From the above data it can be seen that it was not possible to maintain the culture conditions at the optimum for *Spirulina* because of the excessive precipitation. The cloudy conditions also resulted in depressed solar irradiance for much of the study period. Because conditions were not optimised for *Spirulina*, it was possible for contaminants to proliferate and, in the case of Pond 4, out compete *Spirulina*.

Interestingly in both ponds, contaminant concentrations increased significantly between day 12 and 14 which was also when both pH and conductivity were at the lowest levels in both ponds.

The severe weather condition experienced during this experimental protocol made testing the hypothesis, that a higher conductivity would prevent contamination, impossible because it was not possible to maintain a high conductivity.

The study showed that operating the ponds in a fed batch mode would be inappropriate because the operating methodology provided insufficient flexibility to deal with especially precipitation resulting in poor control of the critical culture parameters. Ravelonandro *et al.* (2011) reported similar problems with fed batch culture. In addition Bezerra *et al.*, (2011) found several advantages of semi continuous operations including enhanced control of culture conditions and maintenance of non-limiting nutrient and inhibitor levels. For this reason a semi continuous culture protocol was established.

5.4. Semi-Continuous Culture

5.4.1. *Semi-Continuous Culture Protocol 1 (Biomass Concentration)*

Biomass concentrations as determined by dry weight measurements are shown in Figure 5-16. All further experimental growth data will be presented as productivity. This is required because the semi continuous culture protocol targets maintaining a consistent biomass concentration. As such, the biomass concentration does not provide any information with regard to growth.

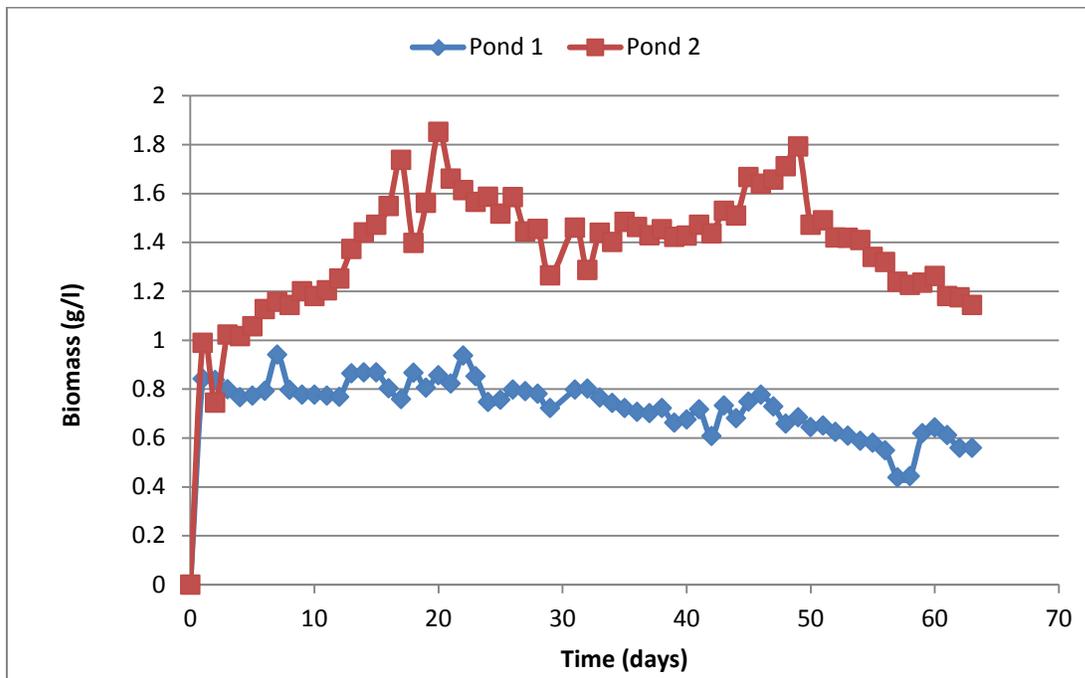


Figure 5-16: Biomass concentration (dry weight) during the Semi-Continuous Culture Protocol 1 (Biomass concentration) study (mean). Pond 1 was established as the low density pond at a target biomass concentration of ~0.8 g/l. Pond 2 was established as the high density pond and maintained at ~ 1.4 g/l.

Figure 5-16 indicates that biomass concentration in Pond 2 was maintained at a higher level than that in Pond 1 for most of the study period.

Growth results determined through dry weight determination are shown in Figure 5-17 below.

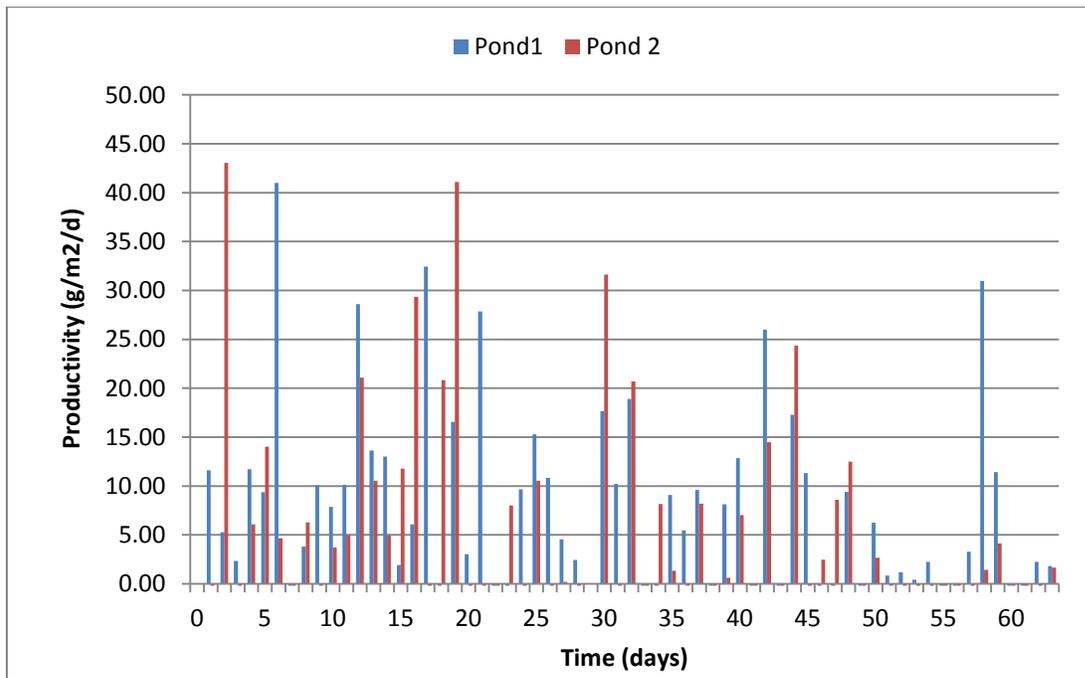


Figure 5-17: Productivity recorded during the Semi-Continuous Culture Protocol 1 (Biomass Concentration) (mean). Pond 1: low biomass concentration and Pond 2: high biomass concentration.

Figure 5-17 indicates that positive productivity was achieved over most of the study period. However, lower productivities were achieved during the latter part of the study compared to the beginning (Table 5-1).

Table 5-1: Average productivity recorded in Pond 1 and Pond 2 during the Semi-Continuous Culture Protocol 1 (Biomass Concentration) study.

Period	Pond 1: Average productivity (g/m ² /day)	Pond 2: Average Productivity (g/m ² /day)
Entire Study	8.32	6.32
First 30 days	10.92	9.4
Last 33 days	6.03	3.6

Table 5-1 indicates that average productivity over the entire study period was 8.32 g/m²/day in Pond 1 and 6.32 g/m²/day in Pond 2. In addition, average productivity in Pond 1 was 44% greater over the first 30 days compared to the

last 33 days. In Pond 2, average productivity was 61% greater over the same two periods.

The ambient weather data collected during the study period is provided in Figure 5-18.

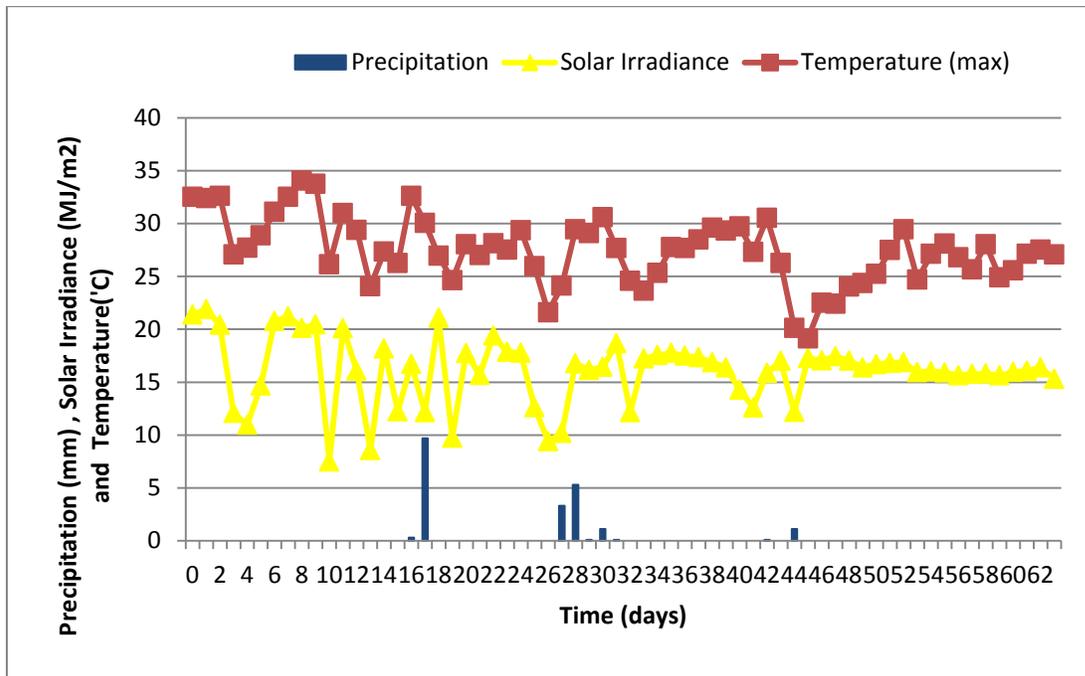


Figure 5-18: Ambient weather conditions as measured by the onsite Campbell Scientific Automated Weather Station during the Semi Continuous Culture Protocol 1 (Biomass Concentration).

Figure 5-18 indicates that average solar irradiance remained relatively stable throughout the study period and averaged around 16 MJ/m^2 per day throughout. However, from day 30 onwards, solar irradiance never exceeded 17 MJ/m^2 and recorded a very flat profile. As such incident light decreased throughout the study period. Maximum temperatures showed a decreasing trend throughout as expected as the study was carried out from April to June and as such reflects the change from autumn to winter. Total precipitation of 21.1 mm was recorded.

The effect of ambient weather conditions on morning culture temperatures are shown in Figures 5-19 and 5-20.

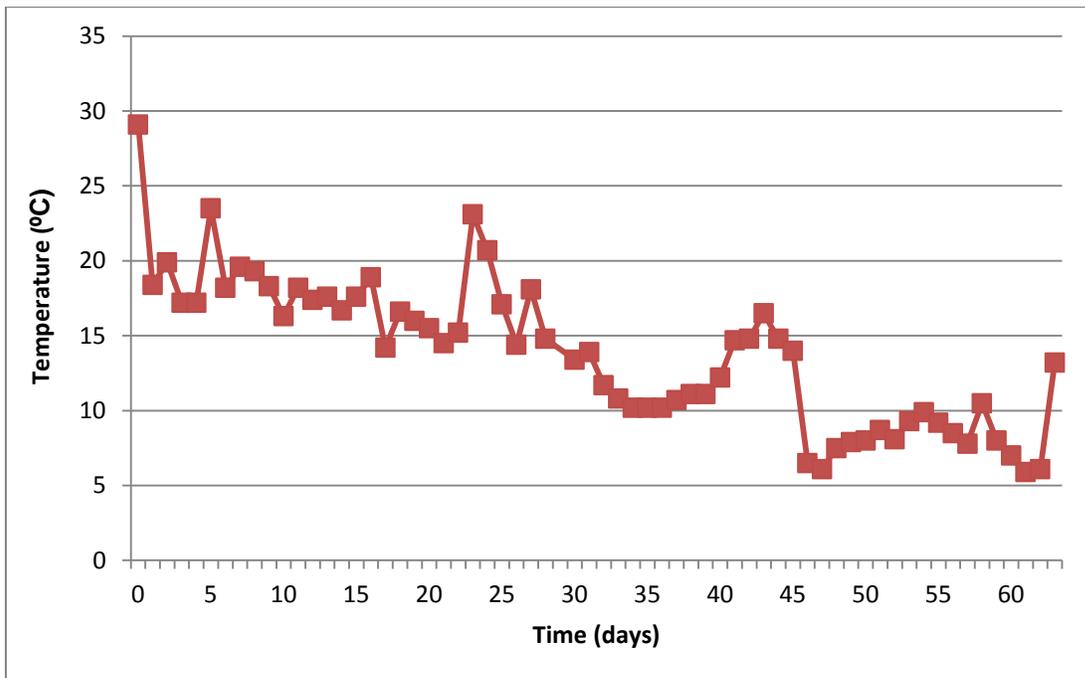


Figure 5-19: Morning culture temperature recorded in Pond 1 during the Semi-Continuous Culture: Protocol 1 (Biomass Concentration).

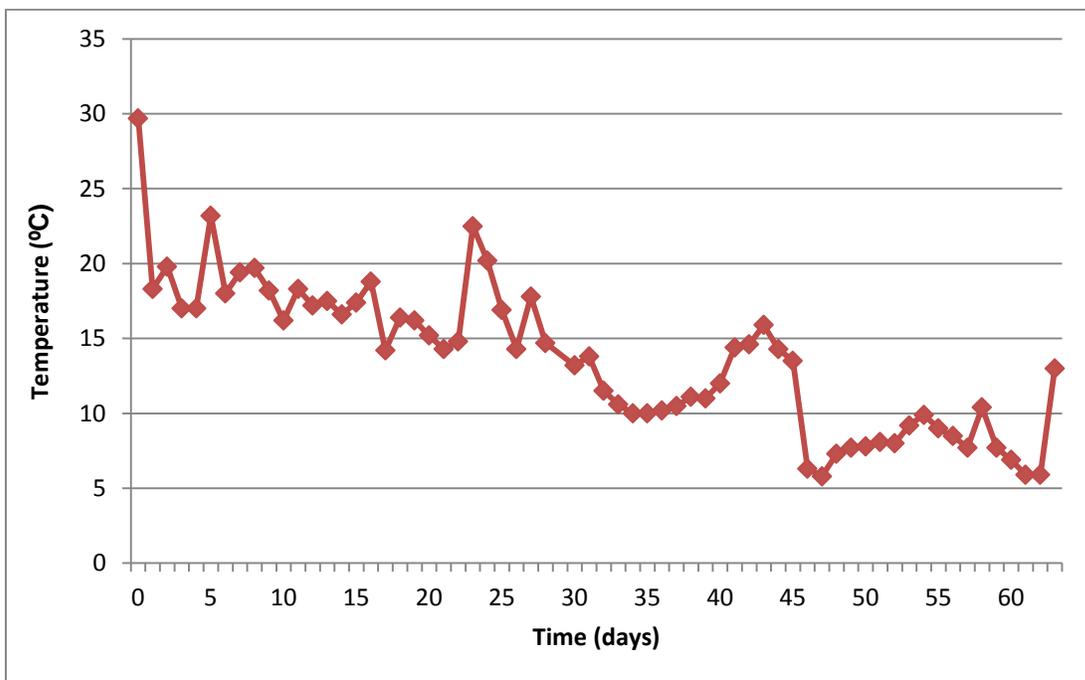


Figure 5-20: Morning culture temperature recorded in Pond 2 during the Semi-Continuous Culture: Protocol 1 (Biomass Concentration).

Figures 5-19 and 5-20 show that culture temperature followed an almost identical trend in both Pond 1 and Pond 2. In addition, morning culture temperature decreased throughout the study period.

The data collected indicates that Pond 1 (low biomass concentration) outperformed Pond 2 (high biomass concentration) in terms of productivity. To determine whether this difference in productivity was significant a one way ANOVA statistical analysis was carried out (Table 5-2).

Table 5-2: Semi-Continuous Culture Protocol 1 (Biomass Concentration), One Way ANOVA, statistical -analysis of productivity between Pond 1 and Pond 2.

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1374.49	1	1374.49	6.501181	0.012019	3.918816
Within Groups	25793.44	122	211.4216			
Total	27167.93	123				

SS = sum of squares; df = degrees of freedom; MS: Mean Squares; F: test statistic; P-value: Probability (if P greater than 0.05 null hypothesis rejected); F crit: Critical test statistic value (if F larger than F crit then null hypothesis rejected)....

With an $F_{3,06} = 6.5$ and a $P\text{-value} = 0.012$ the null hypothesis that biomass concentration would have no effect on growth can be discounted. As such, it may be stated that biomass concentration did have a significant impact on productivity.

The average biomass concentration in Pond 1 was maintained at ~0.8 g/l and in Pond 2 at 1.4 g/l. According to Travieso *et al.* (2001) the optimal biomass concentration for *Spirulina* cultivation ranges between 0.5 and 0.7 g/l. Morais *et al.* (2009) operated similar ponds as used in this study in a range between 0.5 to 1.24 g/l with the higher end of the range used during the summer months. According to Chojnacka and Noworyta (2004) biomass concentrations above 1.0 g/l may result in excessive shelf-shading resulting in photolimitation and reduced productivity. Therefore, at an average biomass concentration of 1.4 g/l self-

shading and photolimitation is the likely cause of the reduced productivity recorded in Pond 2. The decrease in productivity was exacerbated by the reduction in temperature and solar irradiance during the latter end of the study as indicated by the disparity in average productivity shown in Table 5-1.

Microscopy indicated that contamination remained below countable levels through-out the study period. The low contamination is thought to be a result of the high pH and constant cell density that it was possible to maintain due to low rainfall and additional control provided by the semi-continuous culture protocol. No difference in contamination between Pond 1 and 2 could be seen.

The study indicated that the semi-continuous operating methodology did provide a sustainable method of *Spirulina* cultivation. In addition, it showed that the correct biomass concentration is integral to achieving optimal productivity and that optimal biomass concentration varies seasonally as a result of varying solar irradiance.

5.4.2. Semi-Continuous Culture Protocol 2 (Salinity)

Productivities achieved and daily measured salinities are shown in Figures 5-21 and 5-22.

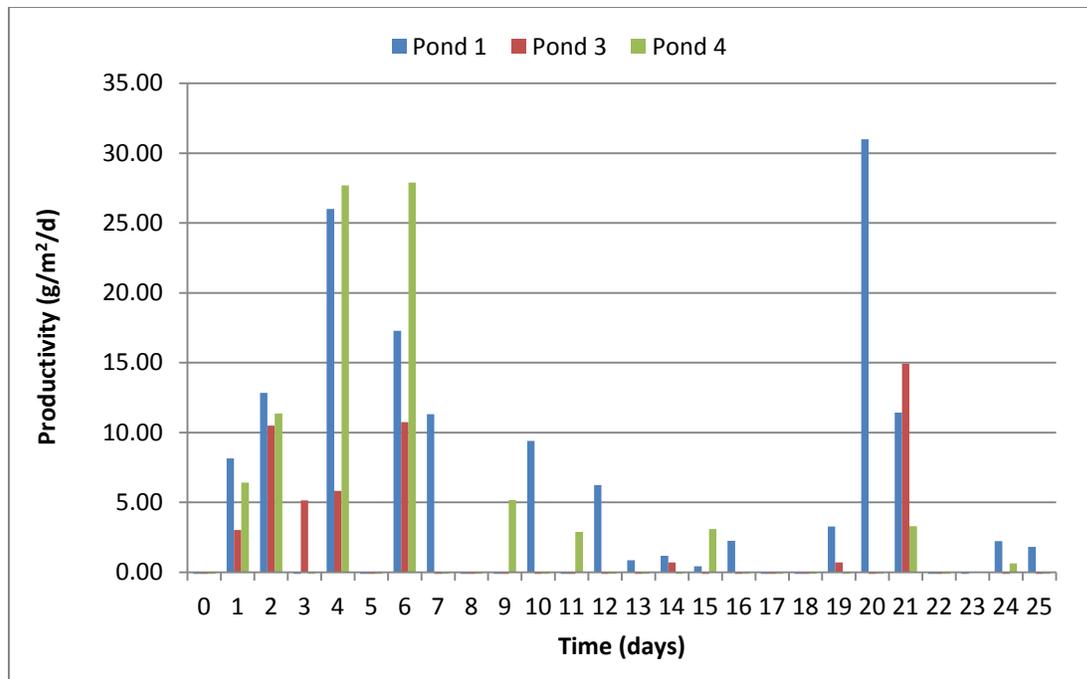


Figure 5-21: Productivity achieved during the Semi-Continuous Culture Protocol 2 (Salinity). Pond 1: Control 9% salinity, Pond 3: Low salinity 6%, Pond 4: High salinity 18%.

Figure 5-21 indicates that productivity was highly volatile throughout the study. Over a large part of the study zero productivity was recorded.

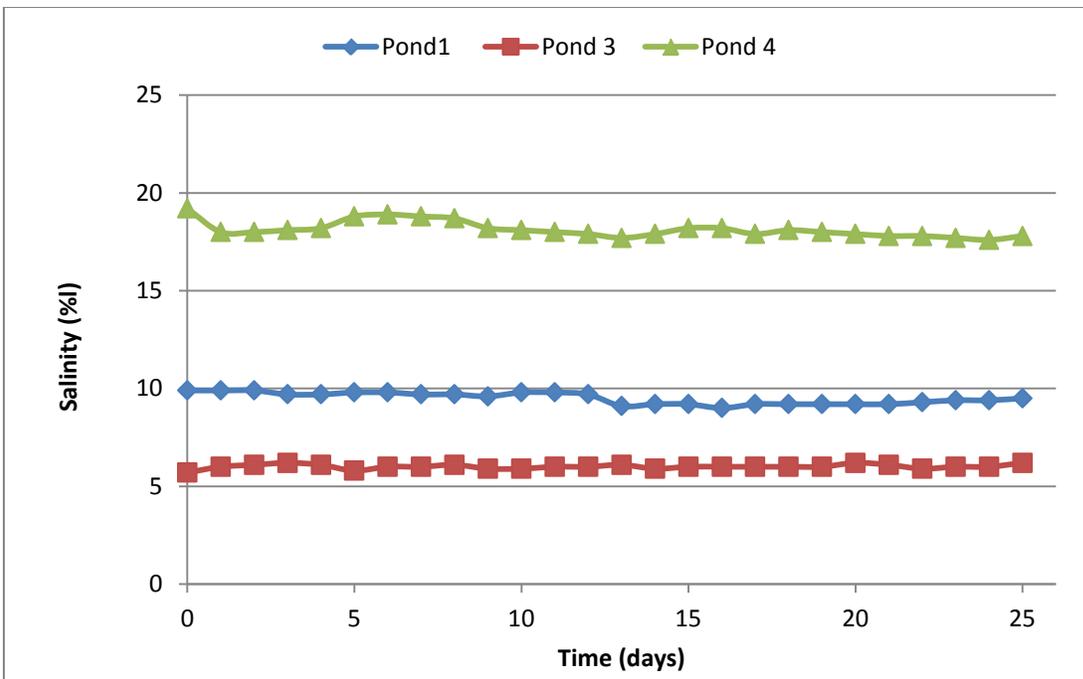


Figure 5-22: Daily measured salinities during the Semi-Continuous Culture: Protocol 2 (Salinity). Pond 1: control salinity ~9%, Pond 3: salinity 6% and Pond 4 salinity 18%.

Figure 5-22 indicates that the target salinities were mostly maintained without change throughout the study. Average salinity in Pond 1 was 9.5%, Pond 3 was 6% and Pond 4 was 18%.

Biomass concentration is shown in Figure 5-23.

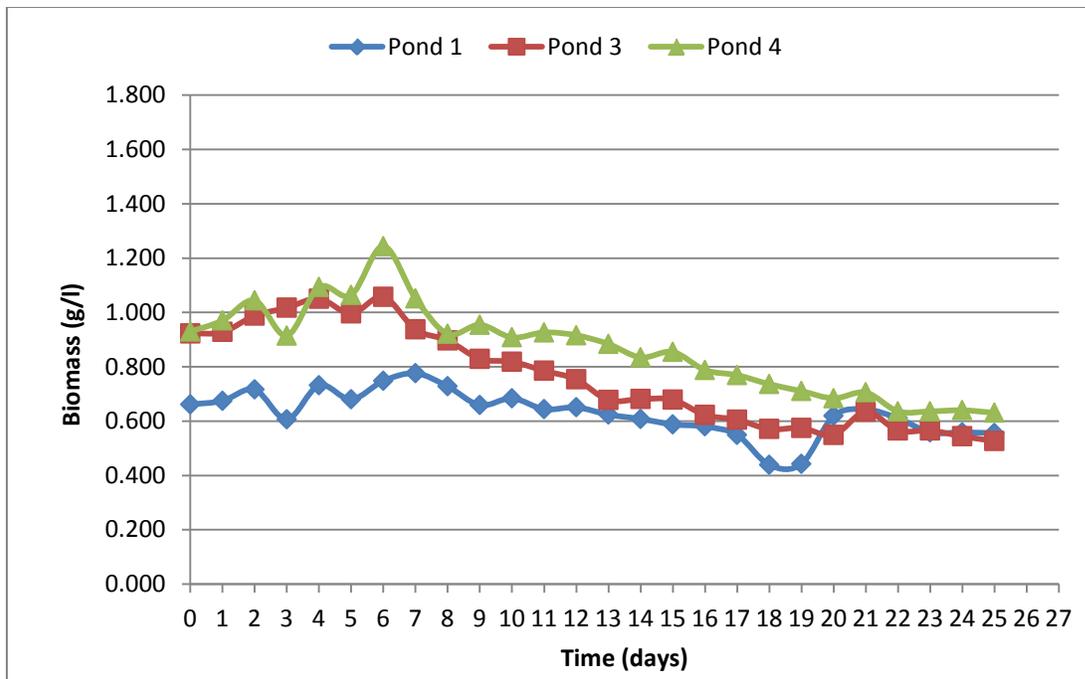


Figure 5-23: Biomass concentration recorded during the Semi-continuous Culture Protocol 2 (Salinity) study.

The target biomass concentration in Ponds 3 and 4 was ~ 0.8 g/l. Figure 5-23 indicates that the target was not achieved over the entire period. However, mean biomass concentrations were 0.761 g/l in Pond 3 and 0.863 g/l in Pond 4. Therefore, both Pond 3 and 4 were well within the target biomass concentration range at 4% and 7% variance from the target respectively. Pond 1 fulfilled a dual function as control in the current protocol and also as the low density pond in the Semi-continuous Culture Protocol 1. As such, biomass concentration was not maintained at 0.800 g/l level.

Ambient weather conditions are shown in Figure 5-24.

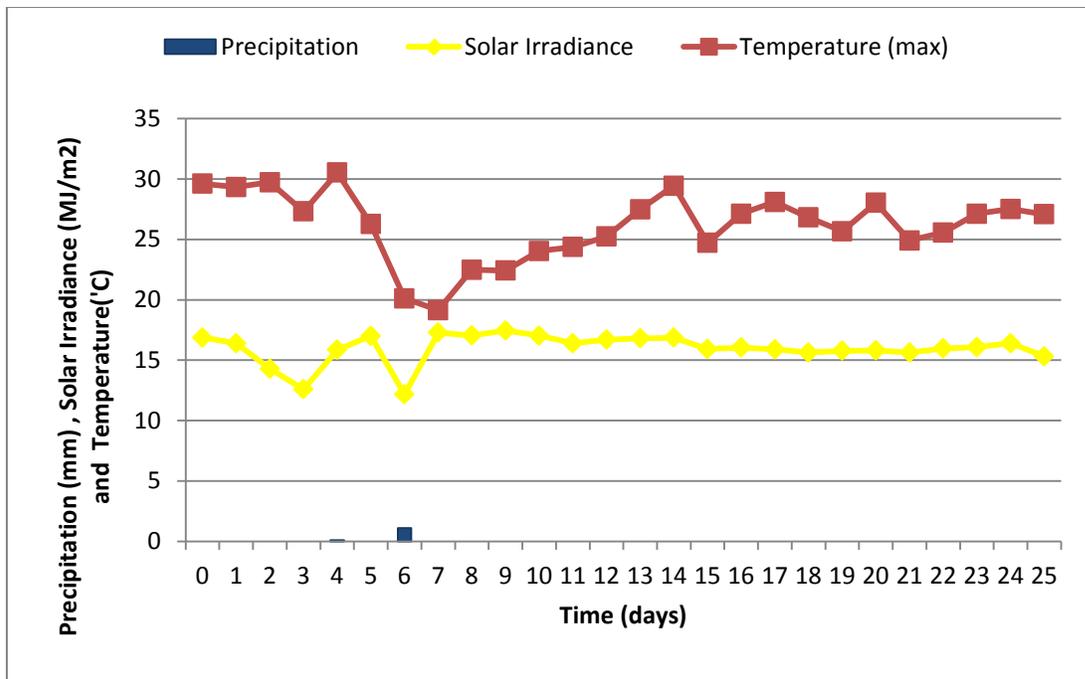


Figure 5-24: Ambient weather conditions as measured by the onsite Campbell Scientific Automated Weather Station during the Semi Continuous Culture Protocol 2 (Salinity).

Figure 5-24 indicates that relatively low maximum average temperature and solar irradiance predominated over the last part of the study. Solar irradiance remained very stable and is indicative of winter conditions. This was as expected as the study was carried out during May and June.

Table 5-3 below provides a summary of average productivities recorded and average biomass concentration.

Table 5-3: Summary of mean productivity during the Semi-continuous Culture: Protocol 2 (Salinity).

	Pond 1	Pond 3	Pond 4
Average productivity (g/m ² /d)	5.60	1.98	3.4
Average biomass concentration as dry weight (g/l)	0.628	0.761	0.863

Table 5-3 indicates that the control (Pond 1) outperformed both Pond 3 and Pond 4 in terms of productivity. Although some variation in cell densities was shown,

the magnitude of the variation should not adversely affect the growth data. This statement is supported by the fact that Pond 4 outperformed Pond 3. When taken in conjunction with the results from the Semi-Continuous Culture Protocol 1, if the effect of the density variation were significant, the lower density pond should have outperformed the highest density pond. However, Pond 4 outperformed Pond 3 with a lower biomass density.

The statistical analysis used to assess the significance of the result is provided in Table 5-4 below.

Table 5-4: Semi-Continuous Culture Protocol 2 (Salinity), Two Way ANOVA without replication, statistical -analysis of productivity between Pond 1, Pond 3 and Pond 4.

Source of Variation	SS	df	MS	F	P-value	F crit
Rows	3841.843	3	1280.614	16.66253	2.16E-08	2.726589
Columns	3826.251	25	153.05	1.991388	0.011905	1.653206
Error	5764.196	75	76.85594			
Total	13432.29	103				

With an $F_{2.72} = 2.73$ and a P-value = 2.16×10^{-8} the results are significant and the null hypothesis can be discounted. As such, it may be stated that salinity does affect productivity. However, based on the average productivities, the Control achieved the greatest productivity. Therefore, the optimal salinity for production of the specific *Spirulina* strain at the experienced environmental conditions is between 9 and 18%. However, considering the published optimum of 9% (Vonshak, 1997b) and cost considerations, the salinity will be maintained at 9% during all subsequent investigations.

5.4.3. Semi-continuous Culture Protocol 3 (Nutrient Optimisation)

Productivities recorded are shown in Figure 5-26 below.

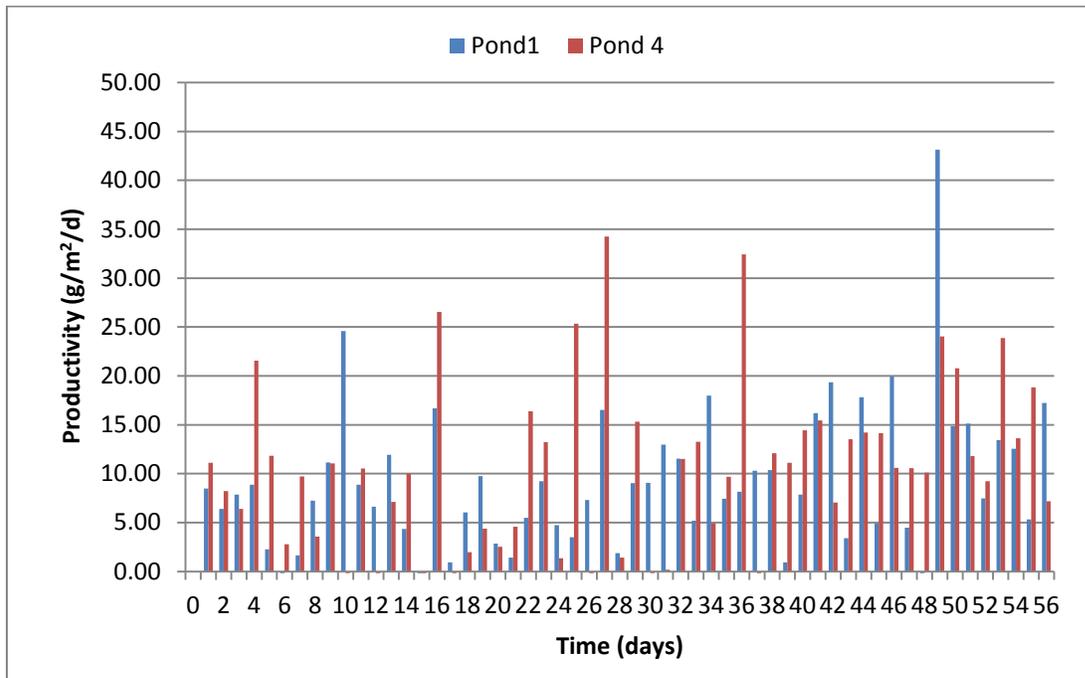


Figure 5-25: Productivity achieved during the Semi-Continuous Culture Protocol 3 (Nutrient Optimisation). Pond 1: half enriched CSW and Pond 4: Control = normal enriched CSW.

Figure 5-25 indicates that good productivities were achieved in both ponds throughout the study period. This is as expected as the study was carried out throughout spring (August, September and October). In addition, both ponds were heated; therefore, temperature was maintained at optimum growth levels (Figure 5-26).

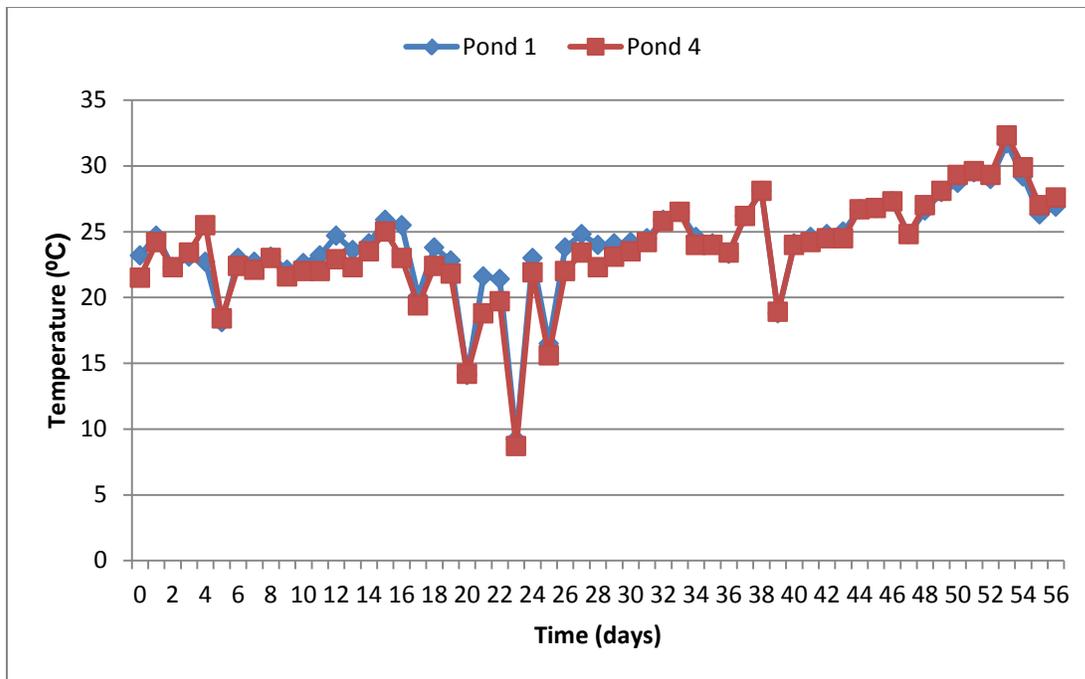


Figure 5-26: Culture temperatures as recorded in the mornings during the Semi-continuous Culture Protocol 3 (Nutrient Optimisation)half enriched CSW.

Figure 5-26 shows morning pond temperatures recorded throughout the study were very similar in both ponds. Although ponds were heated with the heat exchangers, some variation in culture temperature did occur as a result of ambient temperature variation. The drop in morning culture temperature shown on day 20 and 24 was a result of a failure of the heat exchangers. Morning culture temperatures showed an increasing trend related to an increase in ambient temperature shown in Figure 5-27.

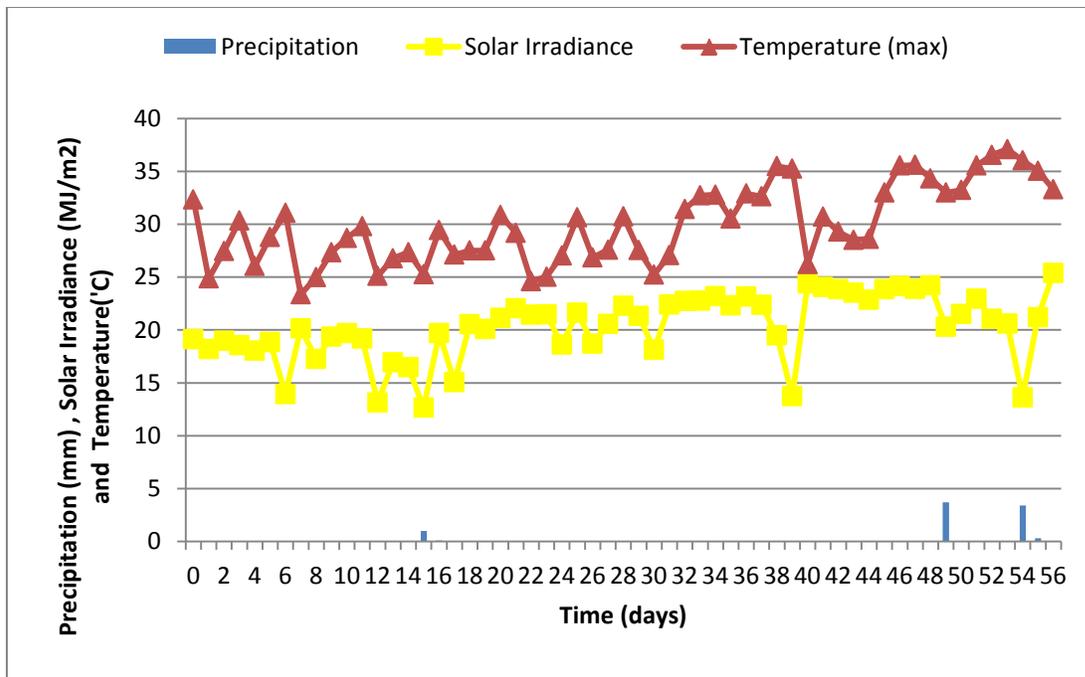


Figure 5-27: Ambient weather conditions as measured by the onsite Campbell Scientific Automated Weather Station during the Semi Continuous Culture Protocol 3 (Nutrient Optimisation): half enriched CSW.

Figure 5-27 indicates progressively increasing ambient maximum temperature and solar irradiance; this was as expected as the study progresses through August into September and October.

Previous studies indicated that biomass concentration can have a significant effect on productivity. Therefore the biomass concentrations recorded during the protocol are shown in Figure 5-28.

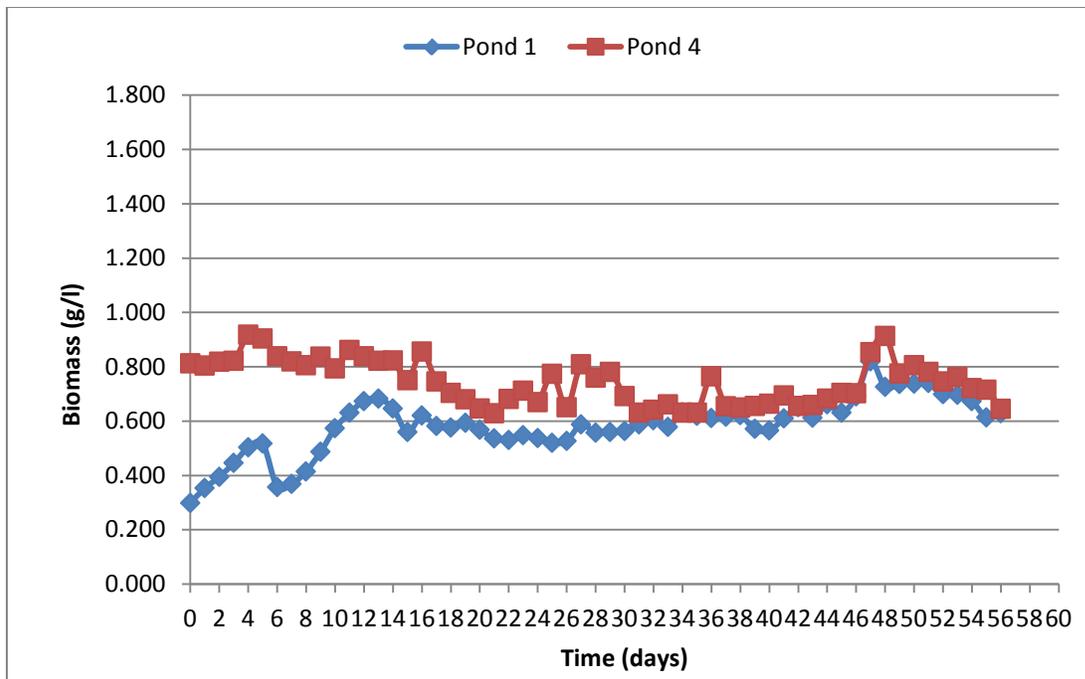


Figure 5-28: Biomass concentration recorded during the Semi-continuous Culture Protocol 3 (Nutrient Optimisation) study $\frac{1}{2}$ enriched CSW.

Figure 5-28 indicates that initially harvested biomass was added back into Pond 1 to achieve the target biomass concentration of ~ 0.7 g/l. However, for most of the study period biomass concentration in both ponds were maintained at similar levels.

Pond 1 achieved an average productivity of 9.34 g/m²/day. Pond 4 achieved a productivity of 10.64 g/m²/day. In order to investigate whether a significant difference in productivity exists between the two ponds, a one way ANOVA statistical analysis was carried out.

Table 5-5: Semi-Continuous Culture Protocol 3 (Nutrient Concentration): ½ nutrient concentration, One Way Anova, statistical analysis of productivity between Pond 1 and Pond 4.

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	54.14222	1	54.14222	0.638724	0.425895	3.927393
Within Groups	9324.277	110	84.76616			
Total	9378.419	111				

With a $F_{3.92} = 0.63$ and a P-value = 0.426 the null hypothesis can be accepted. Therefore, it may be stated that, enriching CSW with NPK at half the concentration available in Schlösser's media does not have a significant effect on productivity. Therefore, it may be possible to achieve a low nutrient/cost *Spirulina* growth media using CSW as a base.

Upon completion of the above study, Pond 3 was altered to a third nutrient concentration. The productivities achieved during this run are shown in Figure 5-29.

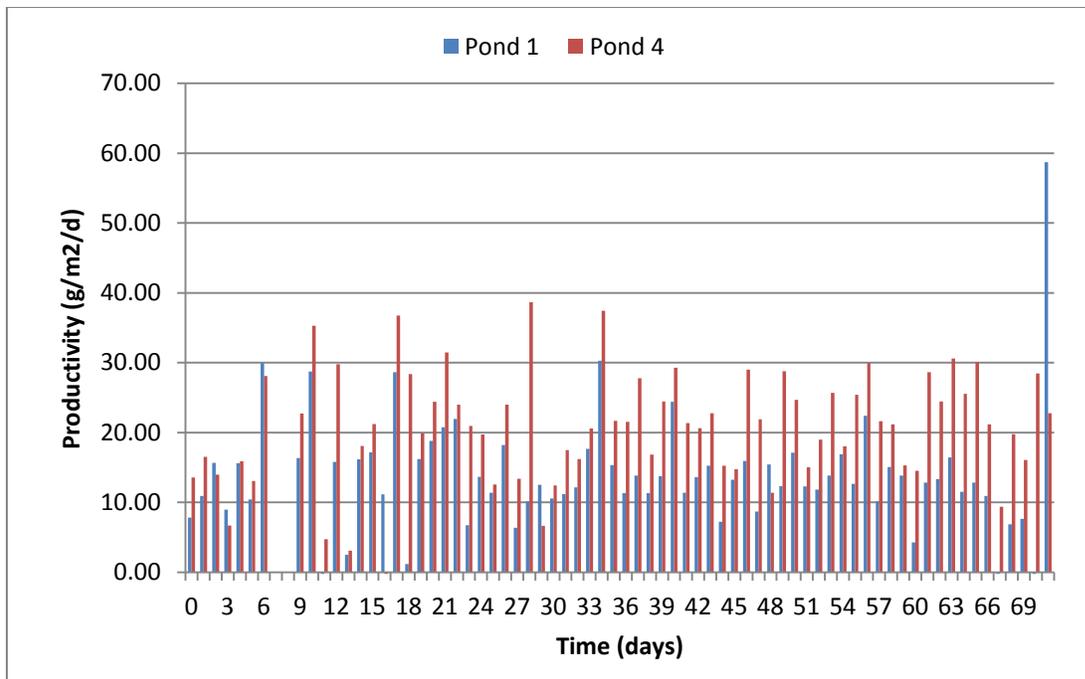


Figure 5-29: Productivity achieved during the Semi-Continuous Culture Protocol 3 (Nutrient Optimisation): Pond 1: 1/3 nutrient concentration enriched CSW, Pond 4: Control normal enriched CSW.

Figure 5-29 indicates that both ponds were productive throughout the study period. This is as expected as the study was carried out in the summer months October, November and December. Therefore, environmental conditions were optimal as illustrated in Figure 5-30. In addition, both ponds were heated and as such culture temperatures were optimised.

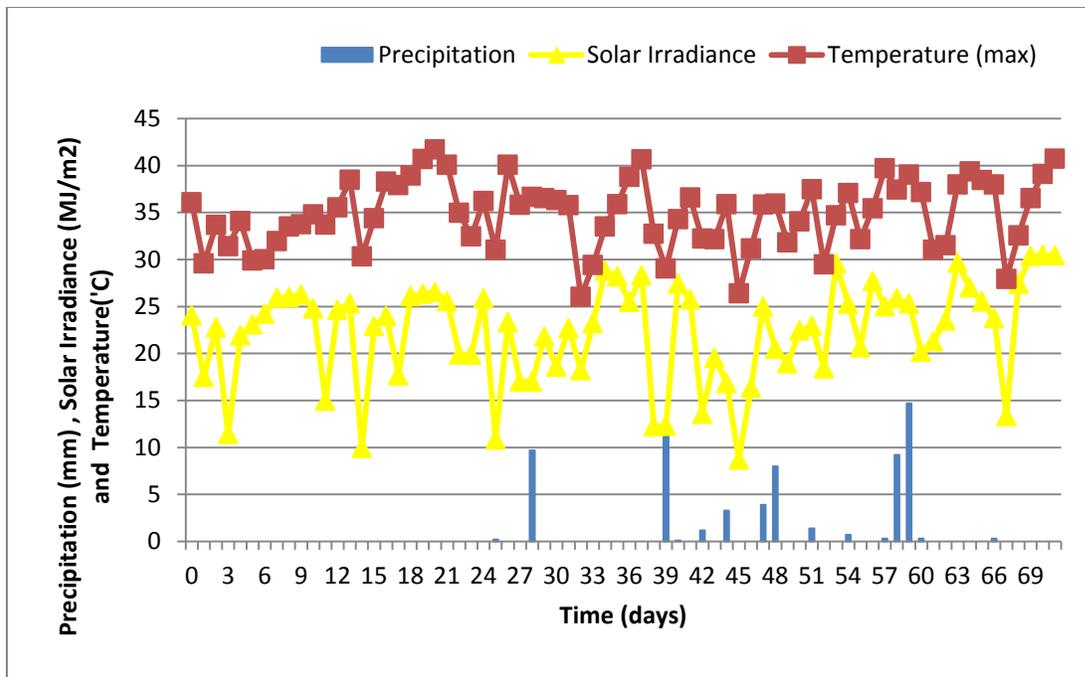


Figure 5-30: Ambient weather conditions as measured by the onsite Campbell Scientific Automated Weather Station during the Semi Continuous Culture Protocol 3: 1/3 nutrient concentration.

Figure 5-30 indicates that both solar irradiance and maximum ambient temperature increased throughout the study period as expected from the time of year. The figure also shows that a total of 66 mm of precipitation was recorded and both maximum temperature and solar irradiance were affected by these events.

Pond 4 achieved an average productivity of 20.87 g/m²/day and Pond 1 achieved an average productivity of 13.97 g/m²/day. To determine whether productivities were significantly different in the two ponds, a One Way ANOVA statistical analysis was carried out on the data set. This data are provided in the Table 5-6 below.

Table 5-6: Semi-Continuous Culture Protocol 3: 1/3 nutrient concentration, One Way ANOVA, statistical analysis of productivity between Pond 1 and Pond 4.

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1673.668	1	1673.668	24.62716	2.02E-06	3.909729
Within Groups	9378.514	138	67.96025			
Total	11052.18	139				

With a $F_{3,90} = 24.62$ and a $P\text{-value} = 2.02 \times 10^{-6}$, the null hypothesis can be rejected. As such it may be stated that reducing the nutrient concentration to 1/3 does have a significant impact on productivity.

The study indicated that although it is possible to reduce the nutrient addition to CSW by half of that which would be required in potable water, reducing the nutrient concentration by two thirds affects growth negatively. Therefore, using CSW as the basis for the growth media used for *Spirulina* cultivation can reduce the amount of nutritive chemicals required to make the growth media by half. Therefore, CSW will achieve a significant cost reduction when compared to pure water.

5.4.4. Semi-Continuous Culture Protocol 4 (Temperature)

The productivities achieved during the study on the effect of temperature are shown in Figure 5-31.

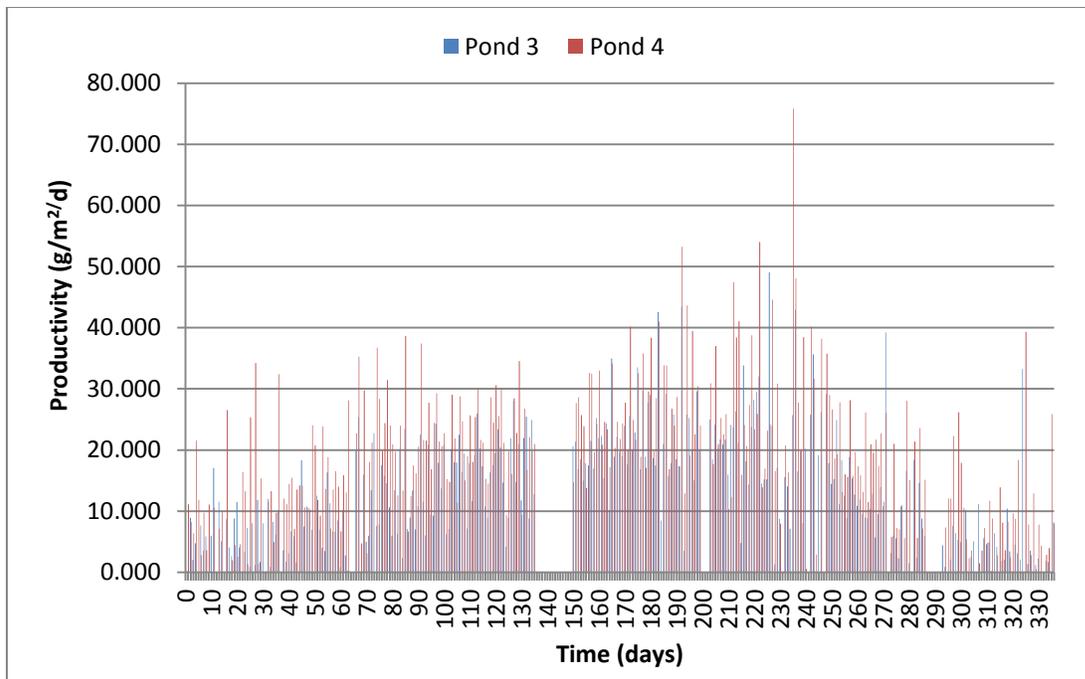


Figure 5-31: Productivity achieved during the Semi-Continuous Culture Protocol 4 (Temperature): Pond 3: unheated, Pond 4: heated.

Figure 5-31 contains productivity data for 336 days. The protocol was started in winter (August) and ended in winter (July). Missing data between days 136 and 149 represent the festive season. As such no measurements were taken over that period as the project team was not on site. From Figure 5-31 it is apparent that Pond 4 outperformed Pond 3. Pond 4 achieved an average productivity of $19.68 \text{ g/m}^2/\text{day}$ over the entire study period while Pond 3 achieved $14.11 \text{ g/m}^2/\text{day}$. This was as expected because Pond 4 was heated throughout this period while Pond 3 was not. The daily measured culture temperature is provided in Figure 5-32 below.

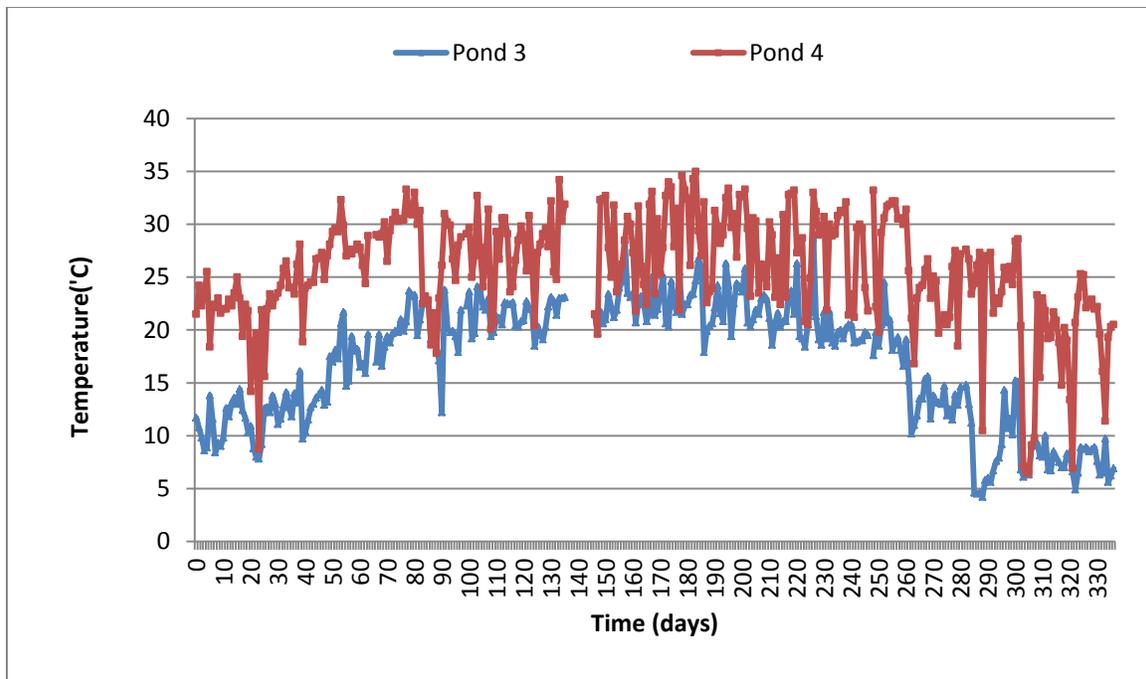


Figure 5-32: Daily measured morning culture temperature recorded during the Semi-continuous Culture Protocol 4 (Temperature) (measurements taken at ~ 7:00AM).

Figure 5-32 indicates that temperatures in Pond 4 were maintained at a higher level than those in Pond 3. The effect was more pronounced between day zero and 70 and then again between days 260 and 330 as this represents the winter months i.e. lower ambient temperatures. Figure 5-33 shows the ambient weather conditions recorded during the study period.

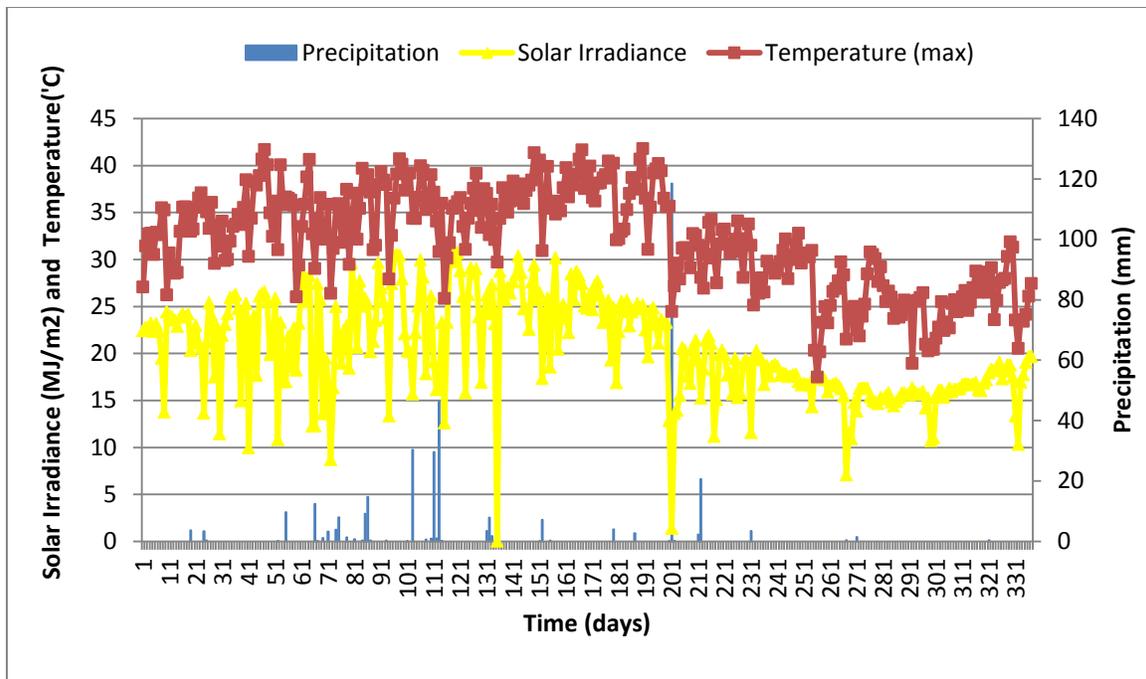


Figure 5-33: Ambient weather conditions as measured by the onsite Campbell Scientific Automated Weather Station during the Semi Continuous Culture Protocol 4 (Temperature).

Heavy rainfall of 118 mm was recorded on day 200. This affected productivity as shown in Figure 5-33. However, using the semi-continuous harvest protocol it was possible to manage culture parameter i.e. depth, conductivity etc. effectively and return all to target levels within a few days. Positive productivity was recorded by day 203.

In order to determine whether the heating of Pond 4 had a significant impact on productivity, a One Way ANOVA statistical analysis was carried out. These results are shown in Table 5-7 below.

Table 5-7: Semi-Continuous Culture Protocol 4 (Temperature): One Way ANOVA, statistical analysis of productivity between Pond 3 and Pond 4.

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	4378.093	1	4378.093	28.21488	1.52E-07	3.856574
Within Groups	95739.67	617	155.1696			
Total	100117.8	618				

Table 5-7 indicates a $F_{3,86} = 28.21$ and a P-value of 1.52×10^{-7} , therefore, the null hypothesis may be rejected. Therefore, heating of Pond 4 did have a significant impact on productivity.

However, the hypothesis made at the onset of the study was to determine whether a 10°C change in temperature would result in a doubling of productivity. In order to see whether this did occur, the average temperature and productivities were calculated from day 1 to day 32 (Table 5-7). These days were used as they represented winter conditions during which time it was possible to raise the pond temperature by 10°C in Pond 4 as compared to Pond 3.

Table 5-8: Average morning culture temperatures and productivities of Ponds 3 and 4, from day one to day 32 of the Semi-Continuous Culture Protocol 4 (Temperature).

	Pond 3	Pond 4
Average Temperature ($^{\circ}\text{C}$)	11.17	21.56
Average Productivity ($\text{g}/\text{m}^2/\text{day}$)	4.98	8.43

The above data indicates that the hypothesis holds true. This is also in accordance with the Arrhenius principle which states that for chemical reactions at room temperature, the reaction rate doubles for every 10 degree Celsius increase in temperature (Laidler, 1997).

From the growth data obtained over the 336 day study an average unheated productivity of $14.11 \text{ g}/\text{m}^2/\text{day}$ was determined. Over the summer months

productivity averaged at $\sim 21 \text{ g/m}^2/\text{day}$. This summer productivity rate compares well with the $19.78 \text{ g/m}^2/\text{day}$ reported by Grobbelaar (2009) for similar sized open race way ponds during summer in Musina. The yearly average productivity of $14.11 \text{ g/m}^2/\text{day}$ is much lower than the $21.59 \text{ g/m}^2/\text{day}$ found by Morais *et al.* (2009) in southern Brazil. However, Morais *et al.* (2009) covered all ponds with a greenhouse structure, as such, the $21.59 \text{ g/m}^2/\text{day}$ reported by them has more bearing on the heated pond productivity of $19.86 \text{ g/m}^2/\text{day}$, which compares well. Jiminez *et al.* (2002) reported a productivity $8.2 \text{ g/m}^2/\text{day}$ for *Spirulina* cultivation in southern Spain. Therefore, productivities achieved during the current study that equate to a yield of 51.50 t/Ha/y were at the top end of the range of productivities reported in the literature. As such, production of *Spirulina* in amended CSW is viable and potentially economically lucrative.

CHAPTER 6: CONCLUSION

The coal seam at the CSWBP required dewatering prior to production. The water associated with the coal seam does not meet discharge water qualities and as such requires treatment. The proposed treatment option by the project proponent was Reverse Osmosis. However, due to the expense associated with this water treatment option, methods of offsetting the cost i.e. beneficiating the CSW, were required. A desktop study indicated that due to the environmental conditions at the project site that is situated on the Tropic of Capricorn and the composition of the coal seam water (TDS of ~ 5000 mg/l predominantly from high carbonate and bicarbonate concentrations) saline tolerant algae cultivation could provide a feasible means of offsetting the cost of water treatment. The hypothesis of the study was that *Spirulina* could be used to beneficiate the coal seam water produced at the CSWBP.

Two potential saline tolerant algae were identified *Spirulina spp.* and *Dunaliella salina*. Both species are extremophiles and are able to grow in highly saline water. This ability made both species highly desirable, as the extreme culturing conditions would allow outdoor mass cultivation. In addition, both species are commonly cultivated and produce a saleable biomass. However, further investigation indicated that the lack of NaCl in the CSW and the prevailing environmental conditions at the site made the cultivation of *Dunaliella salina* less desirable than *Spirulina*. Therefore, as the CSW and environmental conditions were ideally suited to *Spirulina*, a pilot study was conducted consisting of laboratory based Flask experiments and outdoor High Rate Algal Pond experiments. This was the focus of the research/dissertation.

Flask studies indicated that the *Spirulina* strain *Arthrospira maxima* obtained from the CCALA would be suitable for up-scaling and cultivation at the CSWBP. Coal

seam water amended with salts found in Schlösser's media and appropriate micro-nutrients was found to be a suitable growth medium for *Spirulina* cultivation. It was also found that the addition of more than 50 times the concentration of iron found in Schlösser's media effectively boosted growth rate and maximum biomass concentration. In addition, the optimal pH for the *Arthrospira maxima* strain selected was found to be between 9.5-10.5. A pH of 10.5 was used as the target for outdoor cultivation.

The HRAP Batch Culture Studies indicated that the outdoor culture was susceptible to contamination by various naturally occurring algae species if culture conditions i.e. pH, conductivity, temperature, biomass concentration etc. were below the optimal. In addition, batch culturing did not provide the required flexibility to deal with precipitation that resulted in dilution of the culture and resultant contamination.

The Fed-batch Culture HRAP studies again showed that environmental conditions were crucial to algal growth and that precipitation and the resultant dilution and decrease in both temperature and solar irradiance were key constraints to algal productivity. It was found that decreasing culture depth to 150 mm, adding 10 times the iron concentration found in Schlösser's media, and maintaining a pH above 10 were critical to maintaining mono-algal growth. However, the fed batch culture methodology also proved ineffective in maintaining homeostatic culture conditions.

Semi-continuous culturing provided a sustainable method of maintaining homeostatic culture conditions. This in turn achieved productive growth and managed contamination to acceptable levels. It was shown that biomass concentration is a key driver of productivity and that high biomass concentrations i.e. 1.4 g/l had a significant negative impact on productivity. The optimal biomass concentration was found to vary as a function of incident light and that optimal biomass concentrations were in the range between 0.6 - 0.9 g/l.

The Semi-continuous Culture HRAP Study showed that productivity was unaffected in CSW amended with half the concentration of nutrient salts found in Schlösser's media. However, reducing nutrient concentrations to one third of that found in Schlösser's media did have a significant negative impact on productivity. As such, the CSW found at the project site will allow at least a 50% reduction in the cost of nutrient additions required for *Spirulina* cultivation.

Heating of the culture proved to be a very effective means of stimulating productivity. In addition, it was shown that the Arrhenius principle that predicts a doubling in productivity for every 10°C increase in temperature held true. Productivities achieved compared well with published productivities and indicated that the economic cultivation of *Spirulina* at the project site would be possible.

However, additional work will be required to establish the economics of potential mass cultivation. This work should include a development of a cost benefit model that analysis the Net Present Value and Internal Rate of Return of such an enterprise. In order to achieve this, a large scale, up to two hectare production facility including harvesting and drying infrastructure, will be required.

Additional research opportunities include optimisation of the production technique, potential genetic modification of *S. maxima* to improve photosynthetic efficiency, elucidation of the potential cultivation of *D.salina* and potential uses of waste algal biomass including:

- anaerobic digestion to produce methane that can be utilised to generate electricity and flu gas as a carbon source for algal growth,
- aquaculture including fish farming, brine shrimps and hydroponics.

The research completed has applicability in various other geographic areas. Immediately apparent is the Waterberg Coal Field. This coalfield is located in the north of South Africa and as such experiences ideal environmental conditions for

algal cultivation. In addition, the water associated with this coalfield is known to be alkaline as opposed to the acidic as in for instance the Witbank Coal Field. The cultivation of algae can also provide potential post-closure business cases for mines and in that manner alleviate the serious negative social impacts of closing mines by providing local communities with profitable enterprises.

In conclusion, it was found that it is possible to cultivate *Spirulina* in amended CSW at potentially economically viable productivities. In addition, the environmental site conditions and the composition of the CSW will allow substantial production cost reductions. As such, cultivation of *Spirulina* is an appropriate method of beneficiating the carbonate rich coal seam water found at the CSWBP and the original research hypothesis was substantiated.

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