Identification and quantification of bacteria associated with cultivated Spirulina and impact of physiological factors

A thesis submitted for the degree of Master of Science in Engineering

By

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Plagiarism declaration

I know the meaning of plagiarism and declare that all the work in the document, save for that which is properly acknowledged, is my own.

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Abstract

Research into the use of 'algal' biomass for human consumption is receiving increased attention due to their favourable nutritional value, photosynthetic efficiency, and lower requirement of land and fresh water as compared to terrestrial crops. The Spirulina species, also known as *Arthrospira*, is of particular interest due to its high protein content and nutritional value. Open raceway pond systems are popularly used for commercial industrial scale cultivation of microalgae due to their economic feasibility. These open cultivation systems are, however, susceptible to contamination by other microorganisms. This raises concerns relating to suitability for human ingestion and the need to control bacterial growth to prevent contamination by pathogens and to minimise the overall bacterial load. Further, bacterial contamination in processed (harvested and dried) Spirulina biomass has been reported, suggesting that some of these contaminants may end up in the market ready product where appropriate processing approaches are not used.

This study sought to identify the microorganisms that typically contaminate Spirulina cultivation ponds, to understand their interaction with Spirulina biomass during cultivation and to evaluate the vulnerabilities of these contaminants, in order to generate strategies for controlling their populations during open pond cultivation.

The main objectives of this study were therefore:

- To quantify the bacterial load in processed Spirulina powder from a single pilot facility to ascertain the presence of the contaminant in the final product derived from the outdoor pond system used as a case study, and to quantify the bacterial load in the outdoor cultivation cultures.
- To identify and characterize the bacteria associated with these Spirulina cultures and processed powder from a pilot operation carried out in Franschhoek, South Africa, with a particular focus on evaluating the likelihood for pathogens.
- To establish the dynamics of the relationship between Spirulina and bacterial growth under different environmental conditions including pH, salinity and temperature.
- To develop practical methods to control and minimize contamination.

The bacterial contaminants were isolated from a sample taken from a commercial scale Spirulina cultivation pond during a pilot study, and identified by analysing their genetic finger prints by 16S rRNA sequencing. Various experiments were designed and carried out in the outdoor commercial scale open raceway ponds owned by BioDelta, laboratory scale open raceway ponds and closed airlift reactors. A modification of the cultivation medium recipe designed for commercial Spirulina production was used as a base case for all cultivation experiments. The medium was altered in various ways to assess the susceptibility of the contaminants to changing environmental conditions, thus, informing potential population control strategies. During the experimental campaigns, samples were analysed for Spirulina biomass concentration, bacterial load (counts) and medium composition in order to establish the growth characteristics and requirements of the bacterial contaminants during Spirulina growth cycle.

Bacterial load in Spirulina powder produced from the BioDelta raceway ponds was determined by the standard agar plating method. The results indicated bacterial load of up to 1.6×10^7 cfu.g^{-1} of dry powder of Spirulina using fresh (nutrient) agar medium. When the same batches of Spirulina powder were analysed simultaneously on salty (salt enriched) agar, the highest bacterial load obtained was 6.2×10^7 cfu.g^{-1} which was between 2 and 4 fold higher than on the fresh agar medium used in CeBER laboratory. The results obtained from the fresh agar plates were compared to those obtained by SwiftSilliker food laboratory which used a similar medium. SwiftSilliker food laboratory obtained 1.6×10^6 cfu.g^{-1} as their highest bacterial load. The salts added to make up the salty agar are those present in the BioDelta medium recipe. This suggested that, the BioDelta medium salt composition was potentially favourable to the growth of the bacterial contaminants i.e. that these are halophiles and not pathogens. The nutrient agar cultivation supported no general pathogen threat with only one instance of a single opportunistic pathogen being found.

The dominant bacterial contaminants were identified through clone libraries of the 16S rRNA gene fragments. The eleven dominant bacterial species included *Alkalimonas delamerensis, Cecembia lonaresis, Pseudomonas mendocina, Paenibacillus camelliae,*

members of the genuses *Halomonas, Mesorhizobium, Idiomarina* and *Micrococcus*, with three unidentified species. These contaminants were largely halophiles and not the typical pathogens likely to pose danger to consumers. Only one instance of an opportunistic pathogen was found; *Pseudomonas mendocina* has been found to be pathogenic in immune compromised patients. All bacterial contaminants were associated with natural high salt environments: the soil, lakes and salterns (i.e. high pH and salt concentration).

The commercial scale experiment to investigate the association between Spirulina and bacterial contaminants was carried out in a 50 000 L outdoor raceway pond over 67 days. Continuous harvesting of the Spirulina biomass started on day 17 after inoculation. During that initial 17 days of batch cultivation, the Spirulina biomass concentration increase from 0.08 $g.L^{-1}$ to 1.28 $g.L^{-1}$, while the bacterial load increased from 3.7×10^4 $cfu.mL^{-1}$ after inoculation to 1.26×10^6 $cfu.mL^{-1}$. Across the entire experiment, the growth of Spirulina correlated closely with growth in bacterial numbers. Owing to the oscillatory nature of the bacterial counts observed, repeat data was collected to investigate the quality of the results and their trends.

A similar experiment to the 50 000 L raceway pond experiment was carried out in 80 L pilot scale open raceway pond in a greenhouse over 71 days with periodic biomass harvesting. Spirulina grew from 0.06 $g.L^{-1}$ to 1.50 $g.L^{-1}$ over a period of 41 days. The bacterial load increased from 6.73×10^4 $cfu.mL^{-1}$ to 5.56×10^6 $cfu.mL^{-1}$. From the comparative analysis between the 50 000L and 80 L pond, it was found that harvesting kept the bacterial load relatively low. Fluctuations were observed as with the 50 000 L pond.

The effect of pH control agents (1M HCl and $NaHCO_3$) on the growth of Spirulina culture and associated bacterial load was investigated in airlift reactors in the CeBER laboratories. Spirulina grew relatively the same across all reactors. However, the bacterial load was higher in cultures adjusted with HCl than with $NaHCO_3$. The pH of cultures adjusted with $NaHCO_3$ was better controlled.

In order to minimize the bacterial load in open outdoor Spirulina cultivation systems, the effect of temperature, salinity and pH were investigated to provide potential strategies that

could manipulated. Experiments to address this objective were carried out in shake flasks using temperature controlled incubators where necessary. Of the temperatures investigated (25°C, 30°C and 35°C), it was found that 35°C had a negative effect on Spirulina biomass productivity. Spirulina grew best at 30°C. There were no significant effects of temperature on the bacterial load. Increase in salinity of the media by addition of salts did not increase the Spirulina growth or biomass concentration but increased the cost of the production. The bacterial load was lowest at low salinity and bacteria thrived at high salinity towards the end of the cultivation period. Cultivation at salinity of 7 ppt was best for minimising bacterial contamination and cost of Spirulina. The effect of the various pH (9, 10 and 11) levels tested did not show any significant effect on Spirulina growth. Bacterial load was to best controlled at pH 11. It was concluded that of the variables studied, pH was the best controller of contaminating bacteria in the Spirulina culture.

The effect of drying on bacterial load in Spirulina biomass was investigated. The experiment was carried out in an oven- and freeze-dryer. It was shown that a short drying cycle minimises contamination as does drying under conditions that do not favour bacterial growth. Bacterial numbers increased rapidly on ambient storage of biomass and in the early stages of low temperature drying. These results indicate that both cultivation conditions and post- cultivation processing are important in minimising bacterial load in Spirulina biomass. Through effective control, the bacterial load can be minimised and pathogens avoided.

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Abbreviations

ALR	Airlift reactor
C	Carbon
CeBER	Centre for Bioprocess Engineering Research
cfu	Colony forming units
CO_2	Carbon dioxide
COD	Chemical oxygen demand
dH_2O	Distilled water
DCW	Dry cell weight
EDTA	Ethylenediaminetetraacetic acid
HCl	Hydrochloric acid
$NaHCO_3$	Sodium bicarbonate
OD	Optical density
ppm	Parts per million
ppt	Parts per thousand ($g.L^{-1}$)
PBR	Photobioreactor

Nomenclature

X	Biomass concentration ($g.L^{-1}$)
μ	Specific growth rate (day^{-1})
Pmax	maximum productivity ($g.L^{-1}.d^{-1}$)

1 Introduction

1.1 Context and scope of the study

Algal biotechnology continues to be one of the fastest growing sectors of the bioeconomy, focusing on the generation of microalgal biomass from CO_2 and mineral salts for the production of nutraceuticals, animal feed, value added products and energy (Costa *et al.,* 2003; Chisti, 2006; Çelekli *et al.,* 2009; Thirumala, 2012). Traditionally, microalgal and other photosynthetic unicellular biomass were harvested from their natural environment and used as a natural sources of health food and animal feed (Chisti, 2006). One particular photosynthetic species is Spirulina*,* a free-floating, multicellular and photosynthetic filamentous cyanobacterium that grows in the alkaline saline environments (Vonshak, 1997a). Spirulina is made of compounds with high nutritional value, including proteins, vitamins, minerals, pigments, lipids and polysaccharides. It has various biotechnological applications including the production of biomass to be used as food for human consumption, animal feed, fertilizers, energy source and wastewater treatment or bioremediation (Costa *et al.,* 2003; Mata *et al.,* 2010; Chu, 2012).

Commercial cultivation of microalgae has been carried out in both open and closed systems. The open systems are usually shallow ponds in which cultures are naturally exposed to solar irradiation. A paddlewheel is used to keep the culture circulating and enhance nutrient transfer (Algae-Biomass-Organization, n.d.; Chaumont, 1993; Chu, 2012). Closed systems are typically enclosed illuminated vessels designed for controlled production of photoautotrophic cultures (Costa *et al.,* 2003; Griffiths, 2011). These are kept in circulation through water jets, airlifts or mechanical pump mixing (Chaumont, 1993). Commercial cultivation of microalgae for biomass production is primarily done in outdoor open raceway pond systems because of their enhanced, technical environmental and economic feasibility, despite low productivity (Richardson, 2011). Several studies have reported open systems as cost-effective and showing an acceptable net energy recovery (NER) for the production of microalgal biomass on an industrial scale (Chisti, 2007; Lam & Lee, 2012; Sudhakar & Premalatha, 2012).

Although outdoor open pond systems are relatively cheap to construct and operate, light, water evaporation and temperature cannot be controlled. This makes them susceptible to the influence of environmental conditions (Bhatnagar *et al.,* 2013). They cannot be operated in a sterile manner and their large production capacity makes them challenging to manage and maintain. Inherently, contamination by other microalgal species, predators, competing and non-competing bacteria is inevitable (Belay 1997; Vonshak & Richmond 1988; Mahadevaswamy & Venkataraman 1981).

The generation of biomass meant for consumable products or pharmaceuticals needs to be pathogen free and to comply with the microbiological standards, guidelines and specifications put in place for food. Due to the nature of open systems, it is challenging to meet these specifications and obtain such cultures, especially because microalgal growth naturally occurs together with growth of other microorganisms (Cole, 1982; Reynolds, 1994; Stenuite *et al.,* 2009); hence specific approaches are required to achieve this. Common strategies employed to maintain algal or bacterial monocultures include cultivation at extreme environments, for instance high alkalinity (particularly for *Spirulina*), salinity and defined nutrition (Lee, 1986). Even though these conditions do not necessarily eliminate bacteria and other biological contaminants, growth of the desired species is favoured more than contaminants (Lee, 2001). Control of bacterial contamination requires knowledge and understanding of bacterial communities. Fundamental understanding of the community structure and the various interactions of microorganisms within the microalgal culture can help to alleviate some of the risks and disadvantages of open systems (Lakaniemi *et al.,* 2012). Quality control tests, inspections and monitoring of process conditions, which often include cell counts, are a common way to ensure the quality of products. The success and profitability of products achieved from cultures depends entirely on their quality. Therefore, it is important to understand the relative growth kinetics of desired and undesired species that take place within these systems and the effect of process conditions and parameters on cultures.

1.2 Objectives of the dissertation

This project set out to study the process of contamination during outdoor pond cultivation of Spirulina by identifying the contaminants, investigating the nature and quantity of the

contaminants and seeking approaches to minimise contamination. Understanding the nature of the contaminating bacteria gave insights into their effect on the Spirulina culture quality as well as their vulnerabilities. This led to understanding of the conditions which may make them thrive or perish.

The study sought to both quantify the contamination problem and to recommend possible methods to control or reduce bacterial loading guided by the understanding of the effect of process conditions and environmental requirements of these bacteria. The study intended to evaluate the effectiveness of the various potential methods for bacterial load reduction.

1.3 Dissertation structure

This thesis is divided in several chapters. Chapter 1 is an introduction to the thesis which presents the context and rationale for the study. In Chapter 2 a review of literature related to the problem statement is presented to identify knowledge gaps and provide the bases for the hypotheses. Bacteria reported to be commonly found in Spirulina cultures are described with special attention given to the relationships and interactions within these systems. Chapter 3 outlines the materials used and the methodology followed to carry out the investigations. It also includes the experimental approach followed. Chapter 4 presents growth kinetics of Spirulina and associated bacteria cultivated in outdoor open raceway ponds. Findings relating to the identification of bacteria found in Spirulina cultures and commercially processed powder are included. Chapter 5 centres on the effect of process conditions on the relative growth of Spirulina and associated bacteria in airlifts and shake flasks which inform management of bacterial load in Spirulina cultures. Further, the prevalence of contaminating bacteria as a function of the drying conditions of Spirulina is also presented in Chapter 5. The conclusions and recommendations of the study are presented in Chapter 6.

2 Literature review

2.1 Introduction

The current study was concerned with contamination of the Spirulina product by other bacteria during the production process. The study sought to identify and characterize the contaminating bacterial communities with the aim of proposing control measures to minimize the contamination. In this chapter a summary of literature relevant for the fundamental understanding and motivation of the study is presented.

Section 2.2 gives a brief history of the commercial exploitation of microalgae and cyanobacteria, highlighting their benefits to mankind. An account of the history and taxonomy of Spirulina is also presented in the section. Section 2.3 focuses on the characteristics and commercial applications and benefits of Spirulina in particular. Section 2.4 discusses various factors affecting the growth of Spirulina and their implications to productivity at commercial scale. Section 2.5 focuses on the commercial production of Spirulina, describing the various steps involved and cultivation systems used. The discussion is presented partly with respect to the growth factors presented in Section 2.4, thereby highlighting some of the problem areas addressed by the current study. The section is concluded with a review of current challenges involved in commercial cultivation including the design of the reactor systems, inoculation, nutrient provision and contamination issues. Section 2.5.3 focuses on the product quality and safety issues concerning Spirulina. Section 2.5.4 describes bacteria previously found in Spirulina cultures and their interactions. A summary of literature relevant for the fundamental understanding and motivation of the study is presented in Section 2.6. Section 2.7 presents the importance and focus of the work done in this study. The objectives and aim of the project, as well as the hypothesis and key questions are also presented.

2.2 The industrial exploitation of microalgae and cyanobacteria

2.2.1 History and importance of microalgal biotechnology

Biotechnological exploitation of microalgae and cyanobacteria started between 1940 and 1952 and has grown rapidly with focus on benefit to mankind (Burlew, 1953; Gantar &

Svirčev, 2008) owing to their high content of valuable nutrients (Ciferri, 1983; Shimamatsu, 2004; Spolaore *et al.,* 2006; Mata *et al.,* 2010). Among other advantages over terrestrial plants, microalgae and cyanobacteria are more efficient photosynthetically, and require less land mass and fresh water per unit biomass (Chelf *et al.,* 1993; Packer, 2009; Griffiths, Dicks, *et al.,* 2011).

Production of biomass from these resources has led to new developments in many aspects relating to ecology, physiology and biochemistry (Apt & Behrens, 1999; Jimenez *et al.,* 2003). Literature estimates that 200 000 to 800 000 microalgal species may be found in nature (Norton *et al.,* 1996), but only about 50 000 have been described (Guiry, 2012). From these, 15 000 unique biological compounds have been defined (Cardozo *et al.,* 2007). Cyanobacterial species are estimated at 2000 to 8000 (Nabout *et al.,* 2013). In addition to the natural production, over 10 000 tons of microalgae (including cyanobacteria) biomass is said to be commercially cultivated globally per annum (Benemann 2009) and there is a desire to increase this output for a range of products. Despite this, microalgae are still not well studied and only 15 species have been used extensively in the biotechnology industry (Table 2.1) (Borowitzka, 1995; Mata *et al.,* 2010). Most of these species have been cultivated commercially due to their value in the food and pharmaceutical industry and production of energy (Borowitzka, 1999). Of these, Spirulina has emerged as being particularly interesting to its high content of protein and other nutrients (Chu, 2012; Costa *et al.,* 2003; Danesi *et al.,* 2002; Mata *et al.,* 2010).

Table 2.1: Species exploited in biotechnology and their typical cultivation systems (Borowitzka, 1999; Carvalho *et al.,* 2006; Chisti, 2007; Spolaore *et al.,* 2006; Tredici *et al.,* 2009; Priyadarshani & Rath, 2012; Pulz & Gross, 2004).

Species	Products	Application	Cultivation systems	Annual production (tons)*	Producers
Cyanobacteria					
Spirulina	Biomass, protein, phycocyanin	Nutraceuticals, cosmetics, animal feed, additives	OPs, Lakes	3000	USA, China, Japan, Myanmar, India
Aphanizomenon flos-aquae	Mycosporine-like amino acids (MAA), protein,	Nutraceuticals, cosmetics	Natural lakes, Closed PBRs	500	USA
Nostoc	Protein, pigments (echinenone, myxoxanthophyll)	Nutraceuticals	Closed PBRs	600	China, Japan, Asia
Microalgae					
Chlorella vulgaris	Biomass, protein, Ascorbic acid	Nutraceuticals, feed (aquaculture) and cosmetics	OPs, Closed PBRs	2000	Germany, Japan, Taiwan
Dunaliella salina	Carotenoids, B-carotene	Nutraceuticals, feed and cosmetics	OPs, lagoons	1200	Germany, Japan, Australia, China, Israel
Haematococcus pluvialis	Carotenoids, astaxanthin	Nutraceuticals, feed, additives	OPs, Closed PBRs	300	USA, Israel, India
Porphyridium cruentum	Polysaccharides, pigments	Pharmaceuticals, cosmetics	Closed PBRs	-	

Open ponds: OPs, Photobioreactors: PBRs, PUFA (polyunsaturated fatty acids) *: based on dry weight

2.2.2 History and taxonomy of Spirulina (*Arthrospira*)

The interest in Spirulina lies in the fact that it is rich in nutrients. It has gained popularity in the health food industry due to its high protein content and nutritional profile (Spolaore *et al.,* 2006), and has been used widely in the algal biotechnology space. Spirulina or *Arthrospira* is photosynthetic, multicellular and filamentous cyanobacterial species that grows naturally in brackish water, saline lakes with high pH (alkaline) and warm environments rich in bicarbonate or carbonate (Costa *et al.,* 2003; Vonshak, 1997a). The use of Spirulina biomass as food was first documented by a member of the Hernan Cortez's troops, Bernal Diaz de Castillo who took notice of Spirulina in 1521 at a then Tenochtitlan market, now Mexico City (Ciferri, 1983; Vonshak, 1997a). Due to the limited knowledge, he referred to what he saw as "dihe made from some sort of ooze which they get out of the great lake (Lake Texcoco), from which they make bread having a flavour something like cheese." Subsequent to this, Spirulina went unnoticed by the western cultures for over 400 years from the sixteenth century until 1940 (Durand-Chastel, 1980). Lake Chad which is situated 10 000 kilometres (km) away from Lake Texcoco represented another natural source of Spirulina. In 1940, Dangeard re-discovered that the Spirulina from Lake Chad was being consumed by the people of the Kanembu tribe as their main source of protein (Dangeard, 1940; Leonard, 1966; Ciferri, 1983). In 1967, Spirulina was named a "wonderful future food source" by the International Association of Applied Microbiology (Sasson, 1997). Following this, the first commercial production took place in 1970 (Ciferri, 1983).

The name Spirulina was mistakenly given to the organism due to misclassification of *Arthrospira* by Geitler in 1932 (Vonshak, 1997a). Nübel et al. (2000) later corrected this by classifying Spirulina as *Arthrospira* not only by its morphological appearance under microscope but also using sequencing analysis of the conserved 16S rRNA gene which is more reliable in identifying microorganisms. Although *Arthrospira* and *Spirulina* have been accepted as separate genuses in the Bergey´s Manual of Systematic Bacteriology, the product commonly referred to as Spirulina belongs to the genus *Arthrospira* and these terms are used interchangeably (Castenholz, 2001). Because of the long use and marketing of the products as Spirulina, the name Spirulina is commonly used for the two most important *Arthrospira, A. platensis* and *A. maxima*. These are used as dietary

supplements (nutraceuticals) and as food and animal feed (Belay, 2013). For uniformity, the name Spirulina is used throughout this study. Table 2.2 shows the classification of *A. platensis,* the species considered in the current study.

Table 2.2: Classification of *Arthrospira platensis,* commonly known as Spirulina.

Taxonomic classes	Common names
Domain	Bacteria
Kingdom	Eubacteria
Phylum	Cyanobacteria
Class	Cyanophyceae
Sub-class	Oscillatoriophycideae
Order	Oscillatoriales
Family	*Oscillatoriaceae*
Genus	*Arthrospira*
Species	*Platensis*

Spirulina is often grouped with microalgae due to its ability to photosynthesize and similar appearance by eye, despite being a bacterium (Cannell, 1993). Microalgae are microscopic, usually simple, aquatic plants. They are classified according to their pigments, cell wall and biochemical constituents (De Pauw & Persoone, 1988; Bhattacharya & Medlin, 1998; Sheehan *et al.,* 1998). Cyanobacteria (the phylum of which Spirulina is a member), also known as blue-green algae, are prokaryotic microorganisms with microalgal morphology and properties that are commonly associated with photosynthetic bacteria. The biochemistry and cell structure of cyanobacteria resemble that of gram-negative bacteria (Moore *et al.,* 1998), however, cyanobacteria contain the chlorophyll *a* pigment, while bacteria contain bacteriochlorophyll or rhodopsin (Bold & Wynne, 1985).

2.2.3 Commercial production of Spirulina

The first commercial production of Spirulina was done in Lake Texcoco in 1970 (Vonshak, 1997a; Belay, 2008). Since then, there have been efforts on developing and improving technologies used in algal biotechnology. The production and use of Spirulina has increased since the start of the production in Lake Texcoco (Belay, 1997). The

annual worldwide commercial production of Spirulina has increased from 3000 to 5500 tons between 2005 and 2013 (Belay, 2013).

The main commercial producers of Spirulina are located in the United States of America (USA) and Asia. Earthrise Nutritionals, located in USA, is the world largest Spirulina producing (450 tons) facility while China is estimated to produce (350 tons) 10% of the world production, at Hainan DIC Microalgae Co. Ltd, formerly known as Hainan Simai Enterprising (Rosa, 2013) (Table 2.3).

Table 2.3: Some of the commercial producers of Spirulina in the form of either food, feed or biological products (Belay, 1997; Vonshak, 1997a; Pelizer *et al.,* 2003).

Company	Country	Production (year: ton)
Spirulina Production Facility Project	SA (Cape Town)	2004
Earthrise Nutritionals	USA (California)	1995: 360; 1996: 400; 2002: 450
Hainan DIC Microalgae Co. Ltd.	China	2002: 350
Neotech Food Co.	Thailand	1995: 30; 1996: 40
Cyanotech corporation	USA (Hawaii)	1995: 250; 1996: 300
Genix	Cuba	2001: 100
Myanmar Microalgae Biotechnology Project	Myanmar	1995: 32; 1996: 300
Solarium Biotechnology	Chile	2000: 4.5; 2001: 28.6; 2002: 13
Nan Pao Resins Chemical Co.	Taiwan	1995: 70; 1996: 80; 2000: 150
Siam Algae Co.	Thailand	2002: 135
Sosa Texcoco	Mexico	130
Ballarpur Industries Ltd	India	1994-1995: 25; 1995-1996: 80
Blue Biotech	Germany	Not determined
Pannol	Australia	Not determined
Spirulina Mexicana	Mexico	2 tons (daily)
Blue Continent Co. Ltd.	Taiwan	100
Koor Foods Co.	Israel	10
Nippon Spirulina; DIC Lifetec	Japan	30
EID Parry	India	180

South Africa, along with Latin America, Northern Australia and South Asia are some of the countries with abundant sunlight and large areas that could be used for the construction of raceway ponds. Technologies enabling the production of Spirulina, such as open pond systems, have potential to grow the economy and mitigate poverty and malnutrition especially in Africa as whole. Getting into a space where these technologies are operated proficiently in industry will not only create jobs but also be able to augment the prominent players in the agricultural sector.

2.3 Characteristics and applications of Spirulina

2.3.1 Morphology

Spirulina is characterized by the loosely coiled cylindrical filaments or trichomes, with a width varying between 3 and 12 μm and a length range of 300-500 μm (Ciferri, 1983). The cells that make up the filaments contain gas vacuoles that give Spirulina its free floating property (Tomaselli, 1997; Belay, 2008). The structure of the filaments can be influenced by environmental conditions, mainly temperature, and because of this, different shapes of filament (loosened spiral and straight filaments) have been reported (Jeeji Bai & Seshadri, 1980; Vonshak, 1997b). The filaments are planktonic and display a gliding motility (Mishra *et al.,* 2013).

Spirulina reproduces through fragmentation and elongation where long mature trichomes self-destruct into fragments, then grow and increase in length by binary fission and concurrently take the helical shape (Figure 2.1) (Ciferri, 1983; Vonshak, 1997b). Details on growth conditions conducive for Spirulina growth are given in Section 2.4.

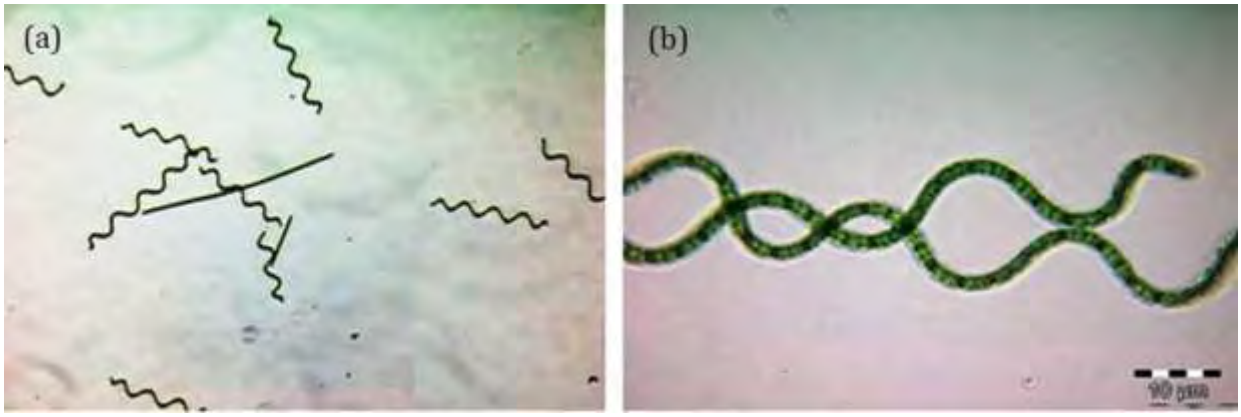


Figure 2.1: Microscopic pictures of Spirulina *(Arthrospira platensis)* under: (a) 10X and (b) 100X magnification.

2.3.2 Biochemical composition

Spirulina is a valuable functional food or dietary supplement because of its exceptional nutrient composition and digestibility (Costa *et al.,* 2003; Chisti, 2006; Çelekli et al, 2009; Thirumala, 2012). Spirulina contains a high protein content of 60-70%, 15-20% carbohydrates, 4-7% lipids, 7% minerals and vitamins (Ciferri, 1983; Shimamatsu, 2004; Spolaore *et al.,* 2006). It is commercially available in the market in the form of powder, granules or flakes and as tablets or capsules. Table 2.4 shows the chemical composition of Spirulina. The level of biochemical composition is influenced by environmental factors (Vonshak & Richmond 1988; Vonshak et al. 1983; Vonshak et al. 1996; Vonshak 1997; De Oliveira et al. 1999; Walach et al. 1987).

Spirulina comprises essential amino acids for cell regeneration and growth. In significant proportions of the total amino acids are leucine (10.9%), valine (7.5%) and isoleucine (6.8%) (Cohen, 1997). Cysteine, lysine and methionine are present in small amounts when compared to those in eggs and milk (Habib *et al.,* 2008). Carbohydrates are present as polysaccharides and lack cellulose, making them easy to digest and a quick source of energy (Becker, 2004). The lipids act as structural components for the cell membrane, as storage and a source of energy and also exist as metabolites.

Spirulina also contains a vast spectrum of vitamins essential for cell metabolism (Belay, 1997). It is a rich source of vitamin B12 which is important in treating anaemia (Becker, 1994a; Belay, 1997). It is also a good source of beta-carotene (β-carotene), being ten times more concentrated than carrots based on mass. β-carotene is known for its ability to stimulate the immune system, reduce harmful effects of radiation and chemotherapy and also improve eyesight (Seshadri, 1993).

Spirulina contains minerals such as potassium, sodium, calcium, manganese, iron, zinc, copper and chromium (Habib et al, 2008; Pandey et al, 2010). The minerals are easily absorbed by the body for growth and development. Spirulina is also rich in poly unsaturated fatty acids (PUFAs) and gamma-linoleic acid (GLA) which are medically beneficial (Hudson & Karis, 1974; Henrickson, 2006). GLA plays an important role in restoring and regulating body functions such as being a precursor in the synthesis of prostaglandins (Desai & Sivakami, 2004; Kovac *et al.,* 2013). Spirulina contains, in abundance, various pigments such as carotenoids, chlorophyll a and phycocyanin which

can serve as antioxidants. It also possesses a spectrum of valuable compounds with potential applications in many areas, some of which are discussed in Section 2.2.3.

Table 2.4: Biochemical composition of Spirulina based on dry biomass (Belay, 1997; Islam *et al.,* 2006; Koru, 2012a).

Components	**Percentage (%)**
Protein	55-70
Carbohydrates	15-25
Fatty acids	6 - 8
Minerals	7-13
Moisture	4 - 9

Vitamins (mg 100 g^{-1})	**Minerals (mg 100 g^{-1})**	**Fatty acids %**	**Pigments (mg 100 g^{-1})**
B-carotene 140	Potassium 1400	Palmitic acid 25.8-46.07	Phycocyanin 14000
Niacin (B_3) 14	Sodium 900	Palmitoleic acid 2.3-3.8	Chlorophyll a 1000
Riboflavin (B_2) 3.0-4.6	Calcium 700	Linolenic acid 11.1-12.0	Carotenoids 370
Thiamin (B_1) 3.5-5.0	Magnesium 400	γ-linoleic acid 17.1-40.1	
Vitamin (K) 2.2	Iron 100	Oleic acid 5.26	
Vitamin (B_6) 0.5-0.8	Manganese 5	Myristic acid 0.23	
Vitamin (B_{12})0.15-0.32	Zinc 3	Others 20.88	
Pantothenic acid 0.1	Copper 1.2		
	Chromium 0.28		
Biotin 0.01			
Folic acid 0.01			
Provitamin A 2.333.000 IU kg^{-1}			
Vitamin E 100 α-tocopherol eq.			

2.3.3 Applications of Spirulina

Applications of Spirulina are discussed in detail below, with focus on Spirulina in health and nutrition for both human food and animal feed, and in the food colorant industry (Ciferri, 1983; Khan *et al.,* 2005) (Figure 2.2).

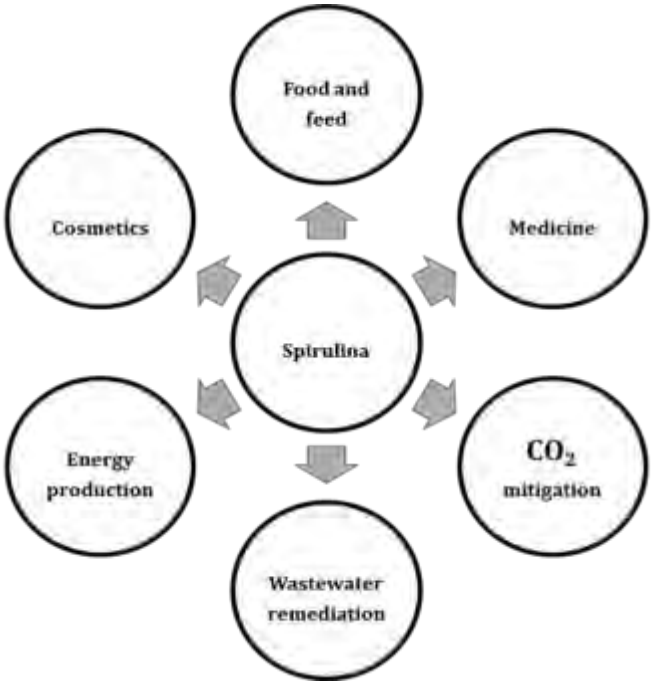


Figure 2.2: A schematic representation of various commercial application of Spirulina.

Spirulina as food and animal feed

Spirulina is commercially produced and sold worldwide as a food supplement with substantial amounts of protein than other plants and standard protein sources (Desai & Sivakami, 2004; Dejsungkranont *et al.,* 2012; Kovac *et al.,* 2013). Traditionally, it has been consumed as a food and dietary supplement due to its nutritional value. Currently, it is mostly incorporated into snack foods, pastas and beverages as well as nutraceutical powders and tablets or capsules (Liang et al. 2004; Yamaguchi 1997).

In addition to the benefits presented above, studies have shown improved growth rates, immune system, development and survival rates in animals on its inclusion in their feeds (Belay *et al.,* 1996; Li & Qi, 1997; Holman & Malau-Aduli, 2013). Further, most of the nutrients contained in the Spirulina are readily bio-available on ingestion due to easily digestible cell walls (Falquet, n.d.; Henrikson, 2010).

Source of phycocyanin

Spirulina contains C-phycocyanin, a phycobiliprotein for Spirulina which Spirulina is the main source (Bermejo *et al.,* 2002; Viskari & Colyer, 2003). This pigment has commercial value in the food industry where it can be used as a natural food colorant and additive (Del Campo *et al.,* 2000). It is also known for its health benefits which include acting as an anti-inflammatory and antioxidant. A study conducted by Source et

al. (2006) showed that mice with orally administered phycocyanin lived longer than those without phycocyanin. It is also used for treatment of Parkinson's and Alzheimer's diseases and has displayed abilities to decrease cholesterol levels by increasing high density lipoprotein (HDL) (Ruan et al. 1988; de Caire et al. 1995).

Other applications and benefits

Spirulina has also been used in the cosmetics industry as an anti-ageing and nourishing agent which promotes skin health (Spolaore *et al.,* 2006). The high levels of antioxidant in Spirulina scavenge free radicals or reactive oxygen species within the body and protect the skin from ultraviolet (UV) radiation (Poljšak & Dahmane, 2012; Mishra *et al.,* 2013). The phycocyanin in Spirulina has also been used in bioanalytical techniques (Borowitzka, 1988a) such as cytometry (characterization of cells) and immunoassay analysis because of its fluorescent properties (Spolaore *et al.,* 2006).

Subsequent to phycocyanin extraction from Spirulina cells, the remnants can potentially be used to produce energy as a secondary product or even as heavy metal remover in wastewater treatment. Spirulina also makes a good biofertilizer due to its biochemical composition, particularly the high nitrogen and phosphorus content. Further, its extracts are able to stimulate growth, development and resistance to pathogens in many plants (Song *et al.,* 2005).

Spirulina is able to fix carbon dioxide (CO_2) efficiently (10 to 50 times faster than terrestrial plants due to the fast growth rate) and converts it to biomass during photosynthesis (Usui & Ikenouchi, 1997; Skjånes *et al.,* 2007; Wang *et al.,* 2008). It can therefore contribute toward the mitigation of greenhouse gas emissions and their environmental effects by capturing CO_2 from industrial flue gases and converting it into chemical energy, which can be used to extract value added products and biofuels (Chisti, 2007; Wang *et al.,* 2008; Mata *et al.,* 2010). This can further reduce the impacts associated with the use of non-renewable fossil energy sources. In addition, despite the high volumes of water used in large-scale cultivation of Spirulina, especially in open pond systems, fresh water consumption remains lower than that required by terrestrial crops. This is due to the ability to recycle the water with supplementation by fresh or saline waters in either open and closed systems, whereas the cultivation of terrestrial crops loses water due to seepage into the soil. Both systems are susceptible to

evaporation. Further, Spirulina grows in salt water, thereby requiring less fresh water, while most terrestrial crops can only survive on fresh water. Therefore, the use of Spirulina can further alleviate the impact associated with supply of fresh water.

2.4 Factors affecting Spirulina growth

Large scale production of Spirulina biomass depends on numerous factors such the availability of nutrients and light, temperature, biology or strain of algae, and mixing and aeration of the culture (Borowitzka, 1999) with nutrients being the second major cost after labour (Vonshak, 2002).These factors, together with pH and pond depth, have been found to affect species dominance and influence the productivity and composition of the final product. Therefore, it is crucial to understand their interrelationships and influence on algal cultures. Understanding this may improve control over the composition of cultures (Mata *et al.,* 2010), and subsequently the quality of the final product.

2.4.1 Nutrients

Nutrients affect the metabolism and growth rate of microalgae and the composition of the final product. Carbon (C) is the principal element in the media composition for the cultivation of microalgae (Bumbak *et al.,* 2011), however, they also require inorganic compounds such as nitrates and micronutrients (Fe, Mg, Mn, etc). Nutrients need to be supplied in balance with what the specifications of the final products are. Nitrogen supplied in the form of nitrates (NO_3^-), nitrites (NO_2^-), ammonia (NH_4^+) or urea are important for the synthesis of amino acids for proteins and growth of microalgae (Borowitzka, 1988b; Zhila *et al.,* 2005; Choi *et al.,* 2011).

2.4.2 Light

Light serves as the main energy source for most photosynthetic microorganisms and plants (Vonshak, 1997a). Spirulina cells are able to capture light energy from the sun or artificial light and fix CO_2 while producing oxygen (O_2) (Grobbelaar, 2000; Matsudo *et al.,* 2009). Light limitation slows down the growth of microalgae. One challenge encountered within reactors during cultivation of microalgae is ensuring that individual cells receive maximum light. In photobioreactors (PBRs) with external lighting, cells closest to the surface of the reactor receive most of the light while the ones in the middle receive reduced light. As the cell density in the reactor increases light intensity

diminishes quickly with culture depth and growth becomes limited (Habib *et al.,* 2008; Langley *et al.,* 2012).

Light supply from the sun is subject to changes due to time of day, weather, season, and geography (Pulz & Scheibenbogen, 1998). Spirulina cells grown under natural sunlight experience three types of photoperiod: seasonal, diurnal and light and dark cycle (Fon Sing, 2010). All reactors using sunlight are subject to the absence of light during night-time. Light and dark cycles strongly influence the growth of algae. Biomass loss has been reported to occur at night due to dark respiration (Chisti 2007; Torzillo et al. 1991).

Conversely, when cells are exposed to too high a light intensity, they reach their saturation point where they are unable to utilize the excess light. Past this point, the cells are inhibited, a phenomenon commonly known as photoinhibition, which can be detrimental to cell growth (Vonshak *et al.,* 1988). According to Vonshak (1997), *A. platensis* and *A. maxima*, previously known as *Spirulina platensis and Spirulina maxima,* grow under high light intensities reaching saturation at a range of 150-200 μmol photons.m^{-2}.s^{-1} and 420-504 μmol photons.m^{-2}.s^{-1} respectively. This was further supported by findings reported by Kebede & Ahlgren (1996). The range is 5-10% of the 2000 μmol photons.m^{-2}.s^{-1} daylight usually measured on a sunny day. This corroborated with the data presented by Fraser (2011) showing that light intensities in *Scenedesmus* cultures attenuated down to 10% at 10 mm below the surface in a 3 g.L^{-1} culture. Further, the data demonstrated the effects of both culture depth and density where the depths for a 1.5 g.L^{-1} and 3.0 g.L^{-1} culture at 50% attenuation level were 5 mm and 3 mm respectively.

In open systems, photoinhibition commonly occurs in the early mornings when the incident light (light intensities) is high but the culture temperatures are still relatively low resulting in low metabolic activity. In such instances, it may be beneficial to heat up the cultures prior to sunrise (de la Noue & de Pauw, 1988; Vonshak *et al.,* 2001). Photoinhibition can also occur at the start of a culture when the culture is too dilute and not well adapted to the environment.

2.4.3 Temperature

Temperature plays an important role in the cultivation of Spirulina. Like any microorganism, growth rates of *A. platensis* and *A. maxima,* previously known as *Spirulina platensis* and *Spirulina maxima*, increase with increasing temperature (Vonshak & Tomaselli, 2000), to an optimum temperature of 30°C (De Oliveira *et al.,* 1999; Costa *et al.,* 2000). There are several studies supporting the optimum temperature as 30°C for Spirulina growth (Belay 1997; Jensen & Knutsen 1993; Danesi et al. 2002; Vonshak et al. 1996). Using three Spirulina isolates, Vonshak (1997) showed that the optimum temperature of Spirulina was species dependent. The minimum temperature that allows for growth of Spirulina is around 18°C and the culture is reported to deteriorate below 12°C (Vonshak *et al.,* 1982). While temperature is controllable at a laboratory scale or in closed systems, this is not economical in large-scale shallow outdoor open systems. Here, temperature depends on the environmental conditions which fluctuate according to the diurnal cycle and seasonal changes. However, cultivation in a greenhouse can reduce the influence of climatic conditions on the culture. Significant biomass losses occurring at night due to dark respiration have been reported (Torzillo *et al.,* 1991) and can be reduced by lowering the temperature of the culture at night (Vonshak & Richmond, 1988). High temperatures cause increased rates of evaporation (Vonshak & Richmond, 1988).

Temperatures above 38°C can harm the cells (Hongsthong *et al.,* 2009). Hence maintenance and monitoring of temperature is very critical. It affects the structure of cell components (lipids and proteins) and metabolism of Spirulina cells (Cassidy 2011; Ogbonda et al. 2007; De Oliveira et al. 1999; Tomaselli et al. 1988). Some filaments can change their morphology from spiral to a straight shape as they adapt to high temperature (Jeeji *et al.,* 1980; Vonshak, 1997b). The benefit of the morphological adaptation to the cells has not been rigorously studied.

2.4.4 pH

Spirulina is an obligate alkaliphile (Grant *et al.,* 1990). The pH of the growth medium has a profound effect on the nutrient availability, physiological properties, production and quality of microalgal biomass (Chen & Durbin, 1994). Spirulina cells are sensitive to pH changes and require monitoring and control for optimal growth. Spirulina thrives at an optimum pH range between 9-10.5 (Belkin & Boussiba 1971).

At pH higher than the optimum range, microalgal growth can be inhibited (Richmond, 2000; Pandey *et al.,* 2010). At pH above 9, ions such as Mg^{2+} and Ca^{2+} may undergo precipitation following auto-flocculation (Vandamme *et al.,* 2015). Spirulina growth can be affected by limited availability of these nutrients. The amount of CO_2 available in the solution at any given time is dependent on the pH of the medium (see Figure 2.3). Temperature and nutrient concentrations also contribute to the accessibility of the carbon in the various forms indicated by Becker (1994a). Higher pH favours the forward reaction on Equation 2.1. At pH between 6.33 and 10.3, bicarbonate (HCO_3^-) exists as the dominant carbon species while (CO_3^{2-}) dominates at pH above 10.3 (Steeman, 1976; Weiner, 2010).

The pH of the medium increases during Spirulina growth. Sodium bicarbonate in the medium dissociates into Na^+ and bicarbonate (HCO_3^-). Some of the sodium ions (Na^+) are taken up and used as a micronutrient by Spirulina while HCO_3^- is converted to CO_2 and hydroxyl ions (OH^-) with the help of carbonic anhydrase (CA) (Richmond & Grobbelaar, 1986). The OH^- accumulated in the medium causes an increase in pH (Richmond & Grobbelaar, 1986; Grobbelaar, 2004). In poorly buffered systems, CO_2 gets taken up in exchange for OH^- ions that are secreted by the cells and released into the medium (Becker, 1994a). The excess OH^- causes a shift on the equilibrium, loss of CO_2 and ultimately a pH increase. According to Belay & Gershwin (2008), high pH and alkalinity of the culture medium inhibits the growth of most potential contaminating organisms, with the inhibitory conditions depending on the contaminant. Understanding the effect of pH on both the growth of Spirulina and its contaminants was, therefore, important for the purpose of the current study.

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-}$$ **Equation 2.1**

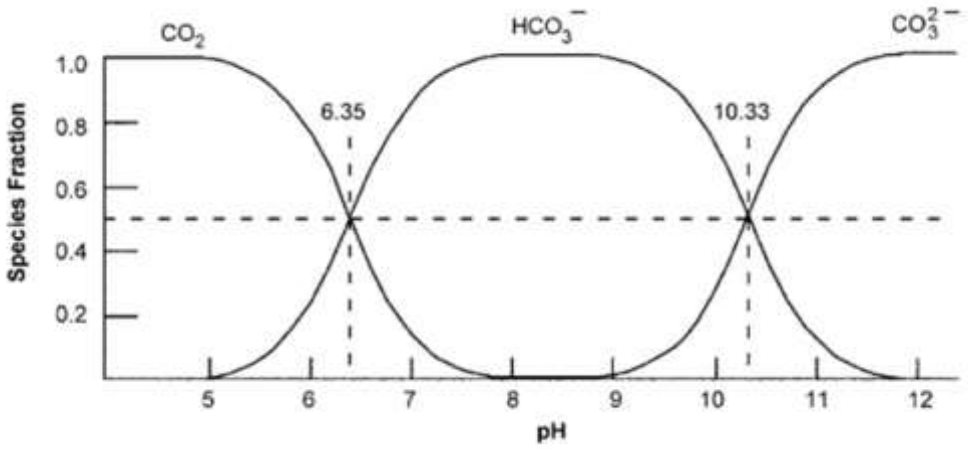


Figure 2.3: pH dependency of the carbonate species inn water (diagram reproduced from Weiner 2010).

2.4.5 Salinity

Salinity refers to the concentration of all dissolved salts in the algal media (Gordon *et al.,* 2004). Spirulina is tolerant of different levels of salinities and high pH environments making its environment unfavourable to bacteria (Sathe, 2010). However, some bacteria such as haloalkaliphiles are able to thrive in such conditions (Zhao *et al.,* 2014; El Hidri *et al.,* 2013; Sorokin *et al.,* 2014).

Salinity affects the growth, productivity, composition of Spirulina, metabolic activity and pathways enhancing or inducing the production biological compounds (Shalaby *et al.,* 2010; Sreevani *et al.,* 2011; Mutawie, 2015). Changes in morphology of cells and change in pigmentation are common under different salinity levels (Stam & Holleman, 1975; Vonshak *et al.,* 1996). Salt imbalance causes osmotic stress on the cells. However, Spirulina is able to tolerate varying levels of salinities through the accumulation of carbohydrates (compatible solutes) such as glucosylglycerol (Mackay *et al.,* 1984; Warr *et al.,* 1985). It is thus important to keep salinity at optimal levels for Spirulina growth (Chisti, 2007), while considering the alteration of salinity to inhibit the growth of the bacterial contaminant. Evaporation of water is not preventable in outdoor open ponds and increases the salinity of ponds. Water lost to evaporation must be replaced by adding freshwater (Boussiba *et al.,* 1987; Becker, 2004; Chisti, 2007) or using a purge suitable for the level of salt present in the water, otherwise salinity increases.

2.5 The Spirulina production process and product quality requirements

2.5.1 Spirulina production process

Mass production of Spirulina involves several steps, harvesting and processing (Soeder, 1980; Becker & Venkataraman, 1982). Figure 2.4 shows a schematic diagram of the production system. Generally, the process starts in the laboratory with inoculum cultivated in Schott bottles in a strictly controlled environment. When the inoculum is dense enough, it is transferred to medium scale reactors. The medium scale cultures in airlifts are then used as inoculum such that the initial OD in the raceways is 0.1 which is equivalent to 0.1 $g.L^{-1}$ dry weight (Figure 2.4). A series of inoculum steps of increasing scale is used for inoculation of large cultivation systems. An effective cultivation of Spirulina at large scale involves the provision of nutrients (including carbon source) and light at the appropriate temperature and pH. In open systems, light and heat are naturally supplied by the sun and climatic factors. Control of culture depth and cell density are ways of regulating light availability. Mixing of the culture is essential to keep nutrients evenly distributed to expose all the cells to light (Sathe, 2010). The filamentous biomass is harvested from raceways, usually by filtration, and washed subsequently to remove excess salts. Unlike many microalgal species, the size of Spirulina filaments is long enough to allow easy harvest by filtration, resulting in lower production costs. Microalgal cells that are too small (less than 20 μm) require an intense harvesting technique that is costly in terms of both money and energy (Becker & Venkataraman, 1982). Spirulina cells are usually filtered through a fine cloth on sloping or shaking screens making them easy to harvest (Sivakumar & Rajendran, 2013).

Processing of Spirulina biomass for human or animal consumption comprises drying, milling (if drying does not yield a powder) and packaging. The drying of the recovered biomass is done to remove moisture, preserve structure of cells and nutrients and provide a product with extended shelf life. Depending on the drying conditions, the drying process may increase bacterial load but it prevents further bacterial growth during storage to prolong the shelf life of product. The drying process is carried out until moisture level of 7% is reached. Various drying techniques have been reported (e.g. sun, oven, spray, vacuum-shelf and freeze drying (Becker, 1994a). The choice of the drying method depends on the species and use of the final product. Following the drying process, the dried biomass may require milling. This is then packaged as powder,

capsules or tablets. More information on drying is given by Dunford (2015). Finally, the product quality depends on the precautionary measures taken at each stage. The handling of the product at any stage may introduce contamination; as such, care must be taken accordingly. Further details on cultivation considerations are presented in Section 2.5.2.

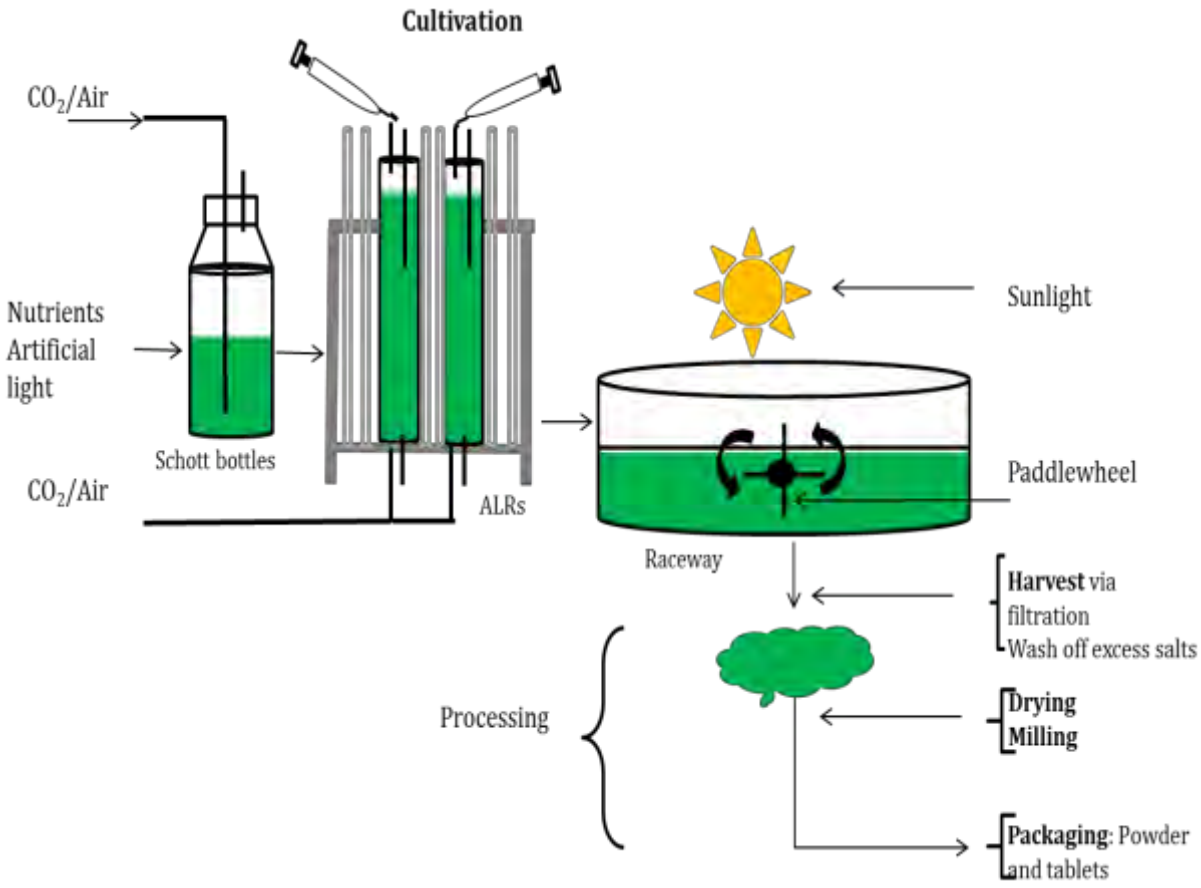


Figure 2.4: Schematic summary of the stages involved in the production of Spirulina.

2.5.2 Cultivation systems of Spirulina

Commercial cultivation of Spirulina can be carried out in either open or closed systems depending on the purpose of the biomass (Borowitzka, 1999). Open systems are usually shallow ponds in which cultures are naturally exposed to solar irradiation, with a paddlewheel to keep the culture circulating and enhance nutrient transfer (Allaboutalgae.com 2015; Chaumont 1993; Chu 2012). Closed systems are enclosed illuminated vessels designed for controlled biomass production of phototrophic liquid cultures. They come in the form of tubes, columns and plates able to transmit light through their outer surface (Costa et al. 2003; Griffiths 2011) and keep cultures in circulation through water jets, airlifts or mechanical pump mixing (Chaumont, 1993).

Large-scale cultivation of microalgae for biomass production is predominantly done in outdoor open raceway ponds because of they are economical, easier to construct and easy to scale up (Borowitzka, 1999).

Recent research efforts continue to be devoted towards outdoor open systems, for practical and economic reasons. Closed systems are being investigated for cultivation and production of high value products in the pharmaceutical industry. They are currently not economic where the sole objective is to produce large quantities of biomass for commodity products, mainly because of the high maintenance expenses associated with them and that they are not easily scalable (Costa *et al.,* 2003). Worth empathizing are the uncontrollable climatic conditions and the potential for contamination encountered in open systems, making them more effective for species that grow in selective environments and countries with hot climates and little rain (Borowitzka, 1999). Closed systems offer a more controllable environment with less evaporation and contamination (Grima *et al.,* 2000). Contamination of algal biomass targeted for food and feed by bacteria poses potential danger to cultures and consumers, depending on the contaminants, and must be carefully and monitored. Bacterial contaminants are not of major concern in energy production.

Reactor design and mixing

The design of reactors in which microalgae are cultivated depends mainly on the desired product and its use in terms of safety (Muñoz & Guieysse, 2006), as well as the physicochemical culture conditions. Material selected to construct reactors needs to allow for light penetration, not be toxic (leach chemicals toxic to culture) and provide a high surface to volume ratio to improve the photosynthetic efficiency (Carvalho *et al.,* 2006).

It is important to keep the specifications at hand and always refer back to them as a guide when a reactor is being designed. These include low system maintenance, capital and production cost, effective mixing and gas-liquid transfer (Chisti, 2007; Griffiths, 2013). Closed reactors generally have a gas exchange unit for the provision of CO_2 (Chisti, 2007) and provide active control of temperature, nutrients, mixing and pH . Commercial production of microalgal products using enclosed tubular and flat plate photobioreactors may be limited to those products of high value that are consumed as

pharmaceuticals, food, feed and additives as the high price associated with these products offsets the increased reactor costs (Lee, 2001).

In open raceway ponds, mixing is carried out using a paddlewheel. The culture depth varies from 10-50 cm (Dodd, 1986) and the paddlewheel is operated at a 50-75% depth (Pulz, 2001). Culture velocity is normally between 25 and 30 $cm.s^{-1}$ (Oswald, 1988). Mixing plays an important role in mass cultivation of Spirulina, ensuring that there is equal distribution of temperature, nutrients and light for all cells in culture (Terry & Raymond, 1985). Mixing also increases heat and mass transfer, growth rates and productivity of cultures. It is because of mixing that cells remain in suspension and do not settle down to form anaerobic zones that may later cause spoilage of cultures (Muñoz & Guieysse, 2006; Pandey & Tiwari, 2010). Insufficient and excessive mixing has adverse effects on the growth of cultures. The former often leads to self-shading, whereas the latter causes shear stress on the cultures (Eriksen, 2008) due to the long filamentous shape and lack of cellulosic cell wall (making the cells more susceptible to rigorous mixing). Paddlewheels in open ponds are the most common for mixing in large-scale cultivation. For stock culture maintenance, work done in the laboratory and in closed photobioreactors, mechanically mixing by shaking, stirring or rocking or pneumatic mixing by sparging with air or CO_2-enriched air e.g. airlifts (Venkataraman & Mahadevaswamy, 1992; Barbosa *et al.,* 2004).

Inoculum concentration

A healthy and rapidly growing inoculum should be used to minimise the lag period and the period of non-ideal conditions when starting cultures. Sufficient concentrations of inoculum are vital to ensure a short lag period and the survival of culture as too little may lead to loss due to photoinhibition.

Contamination of cultures

Although good control of the physicochemical factors contributes to the success of Spirulina cultivation, biological problems may arise, especially in open systems where cultures are susceptible to contamination by bacteria, predators and other algal species (Richmond 1986; Becker & Venkataraman 1982). Mechanical screening can be done to remove insects and other foreign particles from the final product. Highly selective growth conditions are necessary to promote the success of the species of interest

(Richmond *et al.,* 1982; Carvalho *et al.,* 2006), while minimising growth of contaminating species. Contamination by bacteria (especially pathogens) or other algae, is undesirable in cultures targeted for consumption and may result in product not being suitable for target market while contamination by predators or viruses can cause partial or complete loss of the final products or premature collapse of the culture.

Generally, bacterial contamination is avoided by maintaining sterile conditions. This is not feasible in large scale algal systems, whether open or closed, due to their size and the cost of associated sterilization and maintenance of aseptic conditions. Physicochemical treatments have been reported to be effective in reducing bacterial contamination (Richmond & Becker, 1986). Among them is the chemical treatment of the water to be used before cultivation of microalgae starts, but this will not prevent subsequent contamination in the open system. Cyanobacteria and bacteria have similar characteristics in that they are photosynthetic prokaryotes (for photosynthetic autotrophic bacteria) (Cole, 1982) or simply prokaryotes (for heterotrophic bacteria living on organic compounds released into the suspending media) so treatment of bacteria alone is almost impossible without harming the desired culture. A study by Choi et al. (2008) was able to eliminate contaminating bacteria through the use of specific antibiotics (neomycin, imipenem and cycloheximide) which target undesirable species based on their characteristics while no significant damage was done to Spirulina cells. Another study by Sena et al. (2011) was also able to obtain bacterial-free Spirulina using a mixture of antibiotics (ampicillin, penicillin, cefoxitin and meropenem).

Spirulina cultures can also be contaminated by heavy metals through the use of low quality nutrients or mineral salts and atmospheric fallout (Norsker *et al.,* 2010). Heavy metals such as mercury (Hg), lead (Pd), cadmium (Cd) and arsenic (As) have been reported as contaminants in Spirulina products (Belay, 1997). The accumulation of heavy metals in Spirulina cultures and products poses a health concern. Heavy metal contamination may be avoided by using high grade nutrients despite their high price. Routine monitoring of heavy metals in Spirulina cultures and products is critical for food grade Spirulina products (Belay, 1997).

2.5.3 Product safety requirements

The quality of the Spirulina product is the determinant factor for commercial success. The production process plays a critical role in terms of the nutritional quality and the public health (Norsker *et al.,* 2010). Inconsistency of the product can be minimized by employing good manufacturing practices (GMPs) together with routine analysis (i.e. microbiological standard tests, tests for biochemical composition and heavy metals) during the production period of the Spirulina (Antenna-Technologies, 2001; Belay, 2013). These guidelines are put in place to ensure the quality of the product and for consumer protection.

Table 2.5: Microbiological standards and quality requirements for some commercial producers of Spirulina (standard and quality requirements obtained from companies' websites and Dr Griffiths[1]).

Parameter	Companies			
	BioDelta	Cyanotech Corp.	Parry Nutraceutical	Hydrolina Biotech
Coliforms or Enterobacteria ($cfu.gL^{-1}$)	-	<10	0	<100
Salmonella ($cfu.gL^{-1}$)	0	0	0	0
Staphylococcus ($cfu.gL^{-1}$)	0	0	0	0
E. coli ($cfu.gL^{-1}$)	0	0	0	0
Mould and Yeast ($cfu.gL^{-1}$)	<100	-	<100	<100
Standard plate count ($cfu.gL^{-1}$)	<1000 000	<105	<50 000	<100 000
Moisture (%)	<7	<7	2.5-4.5	03-06
Heavy metals (ppm)				
Arsenic	<1.00	<0.50	<0.50	<1.00
Cadmium	<0.20	<0.20	<0.20	<0.05
Lead	<1.00	<0.20	<0.20	<1.0
Mercury	<1.00	<0.02	<0.05	<0.05

ppm: parts per million

[1] BioDelta: (Dr Melinda Grifths, personal communication)
www.cyanotech.com
www.parrynutraceuticals.com
www.hydrolinabiotech.com

As already mentioned in Section 1.1, production of Spirulina is carried out in outdoor open systems. This exposes the cultures to contamination by bacteria and heavy metals (Venkataraman & Becker, 1885; Richmond, 1986; Norsker *et al.,* 2010). Heavy metal accumulation is believed to occur when low grade nutrients have been used. Another concern is the potential production of β-methylamino-L-alanine (BMAA) and mycrocystis neurotoxins by most cyanobacteria which are known risk factors for neuro-degenerative diseases (Gilroy *et al.,* 2000; Gantar & Svirčev, 2008). Fortunately, BMAA and mycrocystis are not produced by Spirulina but contamination by other toxin producing cyanobacteria is possible (Shimamatsu, 2004).

2.5.4 Bacteria contaminants in cyanobacteria and microalgae cultures: interactions and behaviour

Interactions of bacterial contaminants with microalgal and cyanobacterial cultures

Growth of microalgae and cyanobacteria occurs naturally with growth of other microorganisms (Subashchandrabose *et al.,* 2011), especially heterotrophic bacteria (Cole, 1982; Reynolds, 1994; Stenuite *et al.,* 2009). During the period of living together, bacteria may foster either positive or negative relations with microalgae and cyanobacteria. Some of these interactions have been shown to be beneficial to algal cultivation and aquaculture (Fukami et al. 1997 and Goecke 2013). Microalgae and cyanobacteria provide bacteria with organic matter, as both metabolite-based extracellular organic matter (EOM) and extracellular polymeric substance (EPS), and O_2 in return for CO_2 generated by heterotrophic bacteria (Muñoz & Guieysse, 2006; Abed, 2010) (see Figure 2.5). Qu et al. (2014) showed that the growth of *Chlorella* can be enhanced when co-cultured with bacteria in low amounts, as opposed to the axenic culture, without bacteria. The interactions between heterotrophic bacteria and autotrophic microalgae or cyanobacteria are not only limited to the CO_2-O_2 exchange. This was supported by the findings of Sharp (1977), who observed the correlation between the organic concentration and bacterial numbers, where accumulation of microalgal organic compounds increased bacterial population size suggesting that bacteria are responsible for the removal of organic matter (e.g. EPS) which may affect the health of Spirulina cells if present in high concentration. Some bacteria are capable of reducing nitrates to nitrites, and producing siderophores which are molecules that facilitate

iron uptake and help promote growth of Spirulina (Vraspir & Butler, 2009; Vraspir, Holt & Butler, 2011; Baggesen, 2014).

The population size of the heterotrophic bacteria, which can be toxic to cyanobacteria and microalgae, can be regulated by the depletion of the organic compounds. This mutualistic relationship is most likely to be predominant in Spirulina cultures as it ensures the sustainability of both the bacterial and microalgal/cyanobacterial communities.

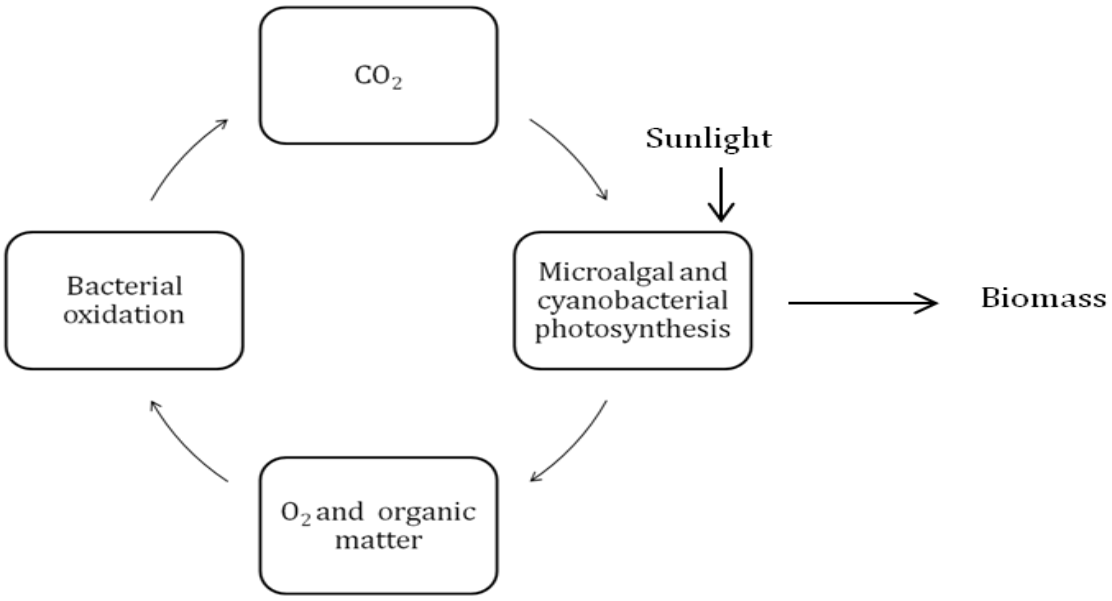


Figure 2.5: Microalgal-bacterial interactions adapted from Muñoz & Guieysse (2006).

A negative interaction may occur during growth of microalgae when an increase in parameters such as pH, temperature and dissolved oxygen concentration (DOC) inhibits growth of bacteria (Wolfaardt *et al.,* 1994; Oswald, 2003; Schumacher *et al.,* 2003). Bacteria can secrete algicidal extracellular metabolites harmful to microalgae (Fukami *et al.,* 1997) where the opposite is also true (Dor, 1980). Furthermore, bacterial-algal interactions may lead to flocculation of algal biomass for some species and its removal from suspension (Dicks 2012). Predator-prey interactions may occur where bacteria or other biological organisms feed off of microalgae or compete for nutrients. All these factors are important in shaping the bacterial community (Palmer, 1999; Collier, 2008; Schmidt *et al.,* 2009). Microalgae and bacteria form highly dynamic and complex ecosystems (Holmström *et al.,* 2002). The interactions taking place within these areas

have not been extensively studied, mainly due to methodological reasons (Largo *et al.,* 1997). The recent advancements in molecular tools have made it possible for researchers and biologists to study and gain more insights into these areas.

Bacterial contaminants in Spirulina

Some cyanobacteria are known to produce toxins but Spirulina has been consumed by humans for many years and has been demonstrated to be toxin free (Habib et al. 2008; Koru 2006). A problem arises when Spirulina cultures become contaminated by toxin-producing cyanobacterial species, or with pathogenic bacteria. Mass production of Spirulina is generally carried out in outdoor systems, where they are easily contaminated by bacteria, microalgae and heavy metals. Bacterial contamination cannot be omitted from open mass cultures because of the nature of outdoor open systems. However, contamination by pathogens such as *Salmonella, Shigella* and *Clostridium* is undesirable and needs to be prevented through good pond management. The pathogens *Salmonella*, *Shigella* and *Clostridium* are common in 50% of food-borne illness cases (Becker, 1994b). Microbiological analysis using the standard plate count (SPC) is important and commonly used for monitoring conditions within cultures. Unusually high cell numbers on the SPC or detection of the presence of coliforms and pathogens can be used as indicators of an unhealthy state of cultures and poor hygiene or pathogens (Payment *et al.,* 2003). Microbiological analysis done on samples of commercially produced Spirulina has shown that the presence of coliforms was uncommon in Spirulina cultures (Jassby, 1988). Another study by Sultan et al. (2014) reported that no pathogens and coliforms were found in dried Spirulina products investigated during analysis. Routine analysis of Spirulina cultures at Earthrise Spirulina Farms (1986) has demonstrated an absence of pathogens: *Shigella, Salmonella* and *Staphylococcus;* these are pathogens that raise most concern.

Staphylococcus aureus does not survive under Spirulina culture conditions. Therefore its presence in Spirulina is uncommon and occurs when cultures come into contact with the human skin during processing (Becker, 1994b). It is thought that the high alkalinity of the medium in which Spirulina is grown serves as a barrier against yeast and bacterial contamination (Vermorel *et al.,* 1975). A study by Hoekstra et al. (2011) reported the presence of *Clostridium* species in Spirulina products during their investigation. Five species of *Clostridium* were isolated which were the source of the

endospores detected. A recent study by Vardaka et al. (2016) isolated bacteria affiliated to the genera *Pseudomonas, Flavobacterium, Vibrio, Aeromonas, Clostridium, Bacillus, Fusobacterium, Enterococcus* from commercially processed Spirulina products. Findings such as these are indicative of the fact that bacteria and some pathogens are able to survive within the cultures and processing of the biomass. However, growth parameters can be manipulated to favour growth or be selective towards the species of interest and also improve the quality of the products (Kim et al. 2013; Vonshak & Richmond 1988; Vonshak 1997).

Molecular methods for analysis of bacteria

Microorganisms are abundant in nature; this includes everything on earth, the soil, water and air. However only a small fraction (<1%) have been characterised (Torsvik *et al.,* 1990). This is attributed to their inability to grow in laboratory spaces. The recent advancements in molecular tools allow for new discoveries without relying on culture-dependent techniques. These techniques have given microbiologists insights into bacterial diversity and community structures. Microbes, previously hidden, are now being revealed (Marchesi, 2011).

Like any living thing, microorganisms have preferences toward environments that are abundant in nutrients, are beneficial to them and do not pose any harm. The ecology of an inhabitant is influenced by chemical and physical parameters. They affect the ability of microorganisms to withstand or tolerate changes in their surrounding which later impact their distribution (Death & Winterbourn, 1995).

2.6 Summary of the literature review

Biotechnological exploitation of microalgae and cyanobacteria for human consumption presents a number of advantages over terrestrial plants relating to nutritional value. Among others are ecological and environmental benefits with regards to the utilization of fresh water and land mass, higher photosynthetic efficiency than terrestrial crops and potential alleviation of impacts associated with use of fossil-based energy sources. Spirulina in particular has received much attention as a source of nutrients for humans owing to its high protein content. Further, unlike other unicellular microalgae, Spirulina is multicellular and easy to harvest. Sterile cultivation of Spirulina (and other microalgae) in closed systems is currently technically feasible but not yet economically

viable. At present, large scale production of microalgae (and Spirulina) has only been demonstrated commercially in open pond raceway systems for commodity products with the use of closed bioreactors for commercial products largely constrained to high value products. However, the open pond cultivation systems are prone to bacterial contamination, which may make the product unfit for the purpose of human consumption. The fitness for purpose depends in the identity of the contaminating bacteria (some bacteria are not harmful to human health) and the bacterial load according to the product safety requirements (Section 2.5.3). In response to these issues, the current study sought to investigate potential strategies of minimizing the bacterial load in open outdoor Spirulina cultivation systems. This was done with the ultimate aim of making the Spirulina product derived from these systems safe for human consumption. To achieve this, it was important to identify the contaminating bacteria and characterize their behaviour during cultivation to inform their load and community composition and to investigate their vulnerabilities to establish key parameters that can be manipulated to reduce contamination load while maintaining satisfactory productivity of the main product, Spirulina.

2.7 The importance and focus of the project

This study focuses on the identification and control of bacteria associated with Spirulina cultures cultivated in outdoor open ponds, using a local case study. The objective of this project is to develop strategies that minimize the bacterial load in Spirulina cultures during cultivation. In order to minimise the bacterial load in the cultures, these bacteria need to be identified and their behaviour within the cultures and under associated culture conditions characterized. The kinetic relationship between bacteria and Spirulina and their response to physicochemical factors give insights into their vulnerabilities.

2.7.1 Project objectives

The objectives of the current study are therefore:

- To quantify the bacterial load in processed Spirulina powder from a single pilot facility to ascertain the presence of the contaminant in the final product derived from the outdoor pond system used as a case study, and to quantify the bacterial load in the outdoor cultivation cultures.

- To identify and characterize the bacteria associated with these Spirulina cultures and processed powder from a pilot operation carried out in Franschhoek, South Africa, with a particular focus on evaluating the likelihood for pathogens.
- To establish the dynamics of the relationship between Spirulina and bacterial growth under different environmental conditions including pH, salinity and temperature.
- To develop practical methods to control and minimize contamination.

2.7.2 Key questions

Bacterial characterisation

- What are the types of bacteria found and in what quantity are they present in both the Spirulina culture in the production facility and the dried Spirulina samples?
- Are these bacteria pathogenic?

Relative bacterial response to physicochemical factors

- What physicochemical growth parameters can be used to minimise the growth of bacteria associated with Spirulina cultures relative to Spirulina itself?
- How do Spirulina and associated bacteria respond to varying these environmental factors?
- Which unit operations are the biggest sources of contamination in the process (growth pond, nutrient sources, harvesting and drying)?
- Which factors in the production process are the biggest sources of contamination in production of Spirulina biomass (wind, physicochemical conditions, culture health, Spirulina excreting organic material)?
- How does the above information help to control the bacterial population?

3 Materials and Methods

3.1 Introduction

This chapter presents details of the materials and methods used to carry out experiments aiming to investigate the project key questions outlined in Chapter 2. Materials used during this study are presented in Section 3.2, while analytical methodologies are detailed in Sections 3.3 and 3.4. Section 3.5 outlines the specific experimental approaches followed to achieve the aims of this study.

3.2 Materials

3.2.1 Chemicals and preparation of media and solutions

All chemicals and media components used during this study were purchased from Sigma-Aldrich, Merck, Biolab or Saarchem. Solutions and growth media for cultures were prepared using deionised water and, where appropriate, sterilised by autoclaving at 121°C for 20 min.

3.2.2 *Spirulina* and its culture

A cyanobacterial species belonging to the Spirulina, also referred to as *Arthrospira*, genus was used in this study. It was isolated from an abandoned wastewater treatment pond at Western Tanning Company outside Wellington, South Africa by Dr R P van Hille. DNA sequencing of the 16S rRNA gene of this Spirulina species was performed by Mr N van Wyk. This sequence was used to construct a 16S rRNA phylogenetic tree showing the evolutionary relatedness of the 16S rRNA gene sequence of this species to those from previously sequenced Spirulina species. Sequences included in phylogenetic analyses were chosen based on lowest E-values obtained after a nucleic acid blast (BLASTn) of the 16S rRNA gene sequence from the Spirulina isolate. The 16S rRNA sequence from a bacterial iron oxidising species, *Leptospirrilum ferriphilum,* was used as outlier. A multiple sequence alignment was performed using ClustalW (Thompson *et al.,* 1994). The evolutionary history was inferred using the Neighbour-Joining method (Saitou & Nei, 1987).

A stock of this cyanobacterium is maintained at the Centre for Bioprocess Engineering Research (CeBER) in the Department of Chemical Engineering, University of Cape Town. Stock cultures were cultivated in 200 mL bottles containing 150 mL culture in Zarrouk's

medium (Zarrouk, 1966). Cultures were aerated with humidified air supplied after filtration through a 0.22 μm syringe filter. Cultures were illuminated by fluorescent lamps (approx. 120 μmol photons.$m^{-2}.s^{-1}$). The composition of Zarrouk's medium used was: 18 g.L^{-1} $NaHCO_3$, 0.5 g.L^{-1} K_2HPO_4, 0.75 g.L^{-1} $NaNO_3$, 1 g.L^{-1} K_2SO_4, 0.04 g.L^{-1} $CaCl_2.2H_2O$, 0.2 g.L^{-1} $MgSO_4.7H_2O$, 1 g.L^{-1} NaCl, 0.08 g.L^{-1} Na_2EDTA, 0.01 g.L^{-1} $FeSO_4.7H_2O$ and 1 mL.L^{-1} each of micronutrient and trace element solution. The micronutrient solution consisted of 2.86 g.L^{-1} H_3BO_3, 1.81 g.L^{-1} $MnCl_2.4H_2O$, 0.22 g.L^{-1} ZnSO4.$7H_2O$, 0.08 g.L^{-1} $CuSO_4.5H_2O$ and 0.014 g.L^{-1} $Na_2MoO_4.2H_2O$, while the trace element solution was comprised of 0.0466 g.L^{-1} K_2CrO_7, 0.0478 g.L^{-1} $NiSO_4.7H_2O$ and 0.042 g.L^{-1} $CoSO_4.7H_2O$. Stock cultures were refreshed every two weeks by transferring approx. 10% (v/v) of the culture to a clean, autoclaved bottle and adding Zarrouk's medium to make it up to a total volume of 150 mL. These stocks were used as inocula for larger (2 L) cultures, cultivated under the conditions described above. These cultures were used as inocula for all experiments detailed in Section 3.5.

All *Spirulina* cultivation experiments were performed in BioDelta medium, used for the large scale commercial production of Spirulina at the BioDelta farm, Simondium, Franschhoek, South Africa. BioDelta medium consists of 4 g.L^{-1} $NaHCO_3$, 2 g.L^{-1} $NaNO_3$, 0.1 g.L^{-1} K_2HPO_4, 1.5 g.L^{-1} K_2SO_4, 0.2 g.L^{-1} $MgSO_4.7H_2O$, 6 g.L^{-1} NaCl, 0.025 g.L^{-1} EDTA, 0.04 g.L^{-1} $CaCL_2.2H_2O$, 0.01 g.L^{-1} $FeSO_4.7H_2O$ and 1 mL.L^{-1} of micronutrient solution stock (2.86 g.L^{-1} H_3BO_3, 1.81 g.L^{-1} $MnCl_2.4H_2O$, 0.22 g.L^{-1} ZnSO4.$7H_2O$, 0.08 g.L^{-1} $CuSO_4.5H_2O$ and 0.014 g.L^{-1} $Na_2MoO_4.2H_2O$).

3.2.3 Media formulation for the study of bacterial contaminants

Media used for isolation and cultivation of bacteria from Spirulina cultures are given in Table 3.1. The generally used lysogeny broth (LB) has also been referred to as Luria-Bertani medium, after the author of the original paper citing the use of this medium (Bertani, 1951). For the preparation of liquid medium, the agar was omitted from the solution.

Table 3.1: Recipes for media used in this study. Numbers indicate g.L^{-1} of each component required.

Components	Plate count agar (PCA) (Lab, 2013)	Salty medium (Griffiths, 2013)*	Lysogeny broth (LB) or agar (Bertani, 1951)
$NaHCO_3$	_	4	_
$NaNO_3$	_	2	_
NaCl	_	6	5
MAP or $NH_4H_2PO_4$	_	0.1	_
K_2SO_4	_	1.5	_
$MgSO_4.7H_2O$	_	0.2	_
Enzymatic or pancreatic digest	5	5	_
Yeast extract powder	2.5	2.5	5
Glucose	1	1	
Bacteriological agar	15	15	15
Tryptone	_	_	10

*: Personal communication

3.2.4 Algal reactor types

Algal growth reactors of varied scale including outdoor open pond raceways (70 L, 50 000 L and 500 000 L working volumes), indoor raceways (70 L), airlift photobioreactors (ALRs; 3.2 L) and shake flasks (200 mL) were used to conduct experiments. These are specified below.

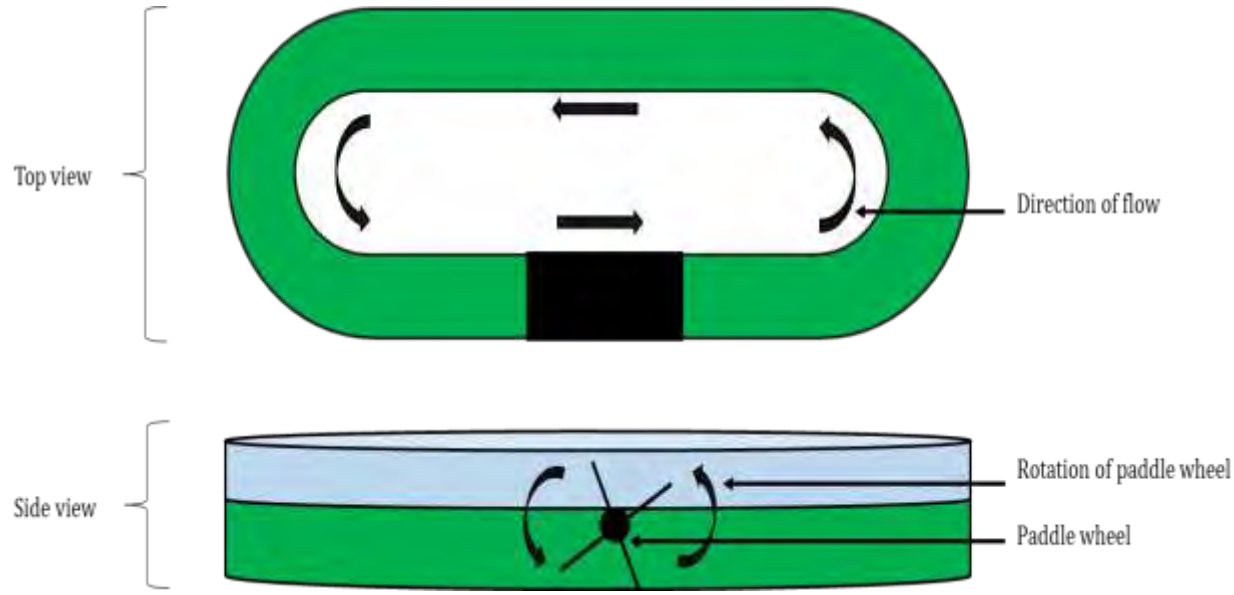
3.2.4.1 Raceway ponds

Two 80 L perspex raceway ponds, with a 70 L working volume, were used to cultivate *Spirulina* in the laboratory (Figure 3.1, Table 3.2). Two 36 W fluorescent bulbs attached to each side provided continuous light of 250 µmol m^{-1} s^{-1} photosynthetically active radiation (PAR) at the perspex surface. Circulation of the Spirulina culture was achieved by a four bladed paddle wheel at 13.8 ± 2°C. A similar raceway pond, situated in a greenhouse on Upper Campus, University of Cape Town (UCT), was used for comparative outdoor experiments.

For larger scale outdoor raceway pond experiments, raceway ponds of similar structure, with either 50 000 L or 500 000 L working volumes, situated at BioDelta farm (GPS coordinates: -33.8378365 18.956717) were used. These are shown in Figure 3.1b, with dimensions given in Table 3.2. These larger raceways were constructed from cement and lined with black high density polyethylene (HDPE) plastic. Each pond was covered by a greenhouse structure. The 50 000 L pond had the following dimensions: width 11.29 m; working depth 0.2 m; length 32 m. The 500 000 L pond had dimensions of width 22 m, depth 0.2 m and length 124 m. The paddle wheel provided a mixing velocity of 25 $cm.s^{-1}$ in the 50 000 L pond. The mixing times were 960 ± 240 s and 6000 ± 1200 s for the 50 000 L and 500 000 L ponds respectively (MJ Griffiths, Personal communication, 20 September 2015). Outdoor cultivated *Spirulina* cultures were dependent on sunlight to drive photosynthesis and growth.

Table 3.2: Physical characteristics of raceway ponds used in the study (MJ Griffiths & Matthew Burke, Personal communication, 20 September 2015).

Dimensions	**70 L**	**50 000L**	**500 000L**
Length (m)	1.75	32	124
Width (m)	0.515	11.29	22
Depth (m)	0.2	0.2	0.2
Working volume (L)	70	50 000	500 000
Width of central island (m)	0.2	0.38	0.38
Surface area of pond(m^2)	0.590	334	2624
Paddlewheel speed (rpm)	13.8± 2		
Mixing velocity of contents ($cm.s^{-1}$)	19± 2	25	22
Mixing time (s)	181	960 ± 240	6000 ± 1200
Circulation time (s)	20	240	1080



(a)

(b)

Figure 3.1: (a) A schematic diagram of a raceway pond (not drawn to scale) (b) 50 000 L raceway pond at BioDelta farm (Simondium, Cape Town).

3.2.4.2 Airlift photobioreactors

Airlift photobioreactors (ALRs) used in this study had a total height of 600 mm and a working volume of 3.2 L. These reactors were constructed from glass cylinders. The outer cylinder was 100 mm wide with a height of 600 mm, while the inside cylinder (draught tube) was 50 mm wide with a height of 400 mm (Langley, 2010). The outer

cylinder was held in place by two steel plates, located at the bottom and top of the reactor. The top plate contained a sampling port (with a 3.18 mm diameter) and a gas outlet. The bottom plate contained a drainage port (with a diameter of 6.35 mm) and a gas inlet port (see Figure 3.2).

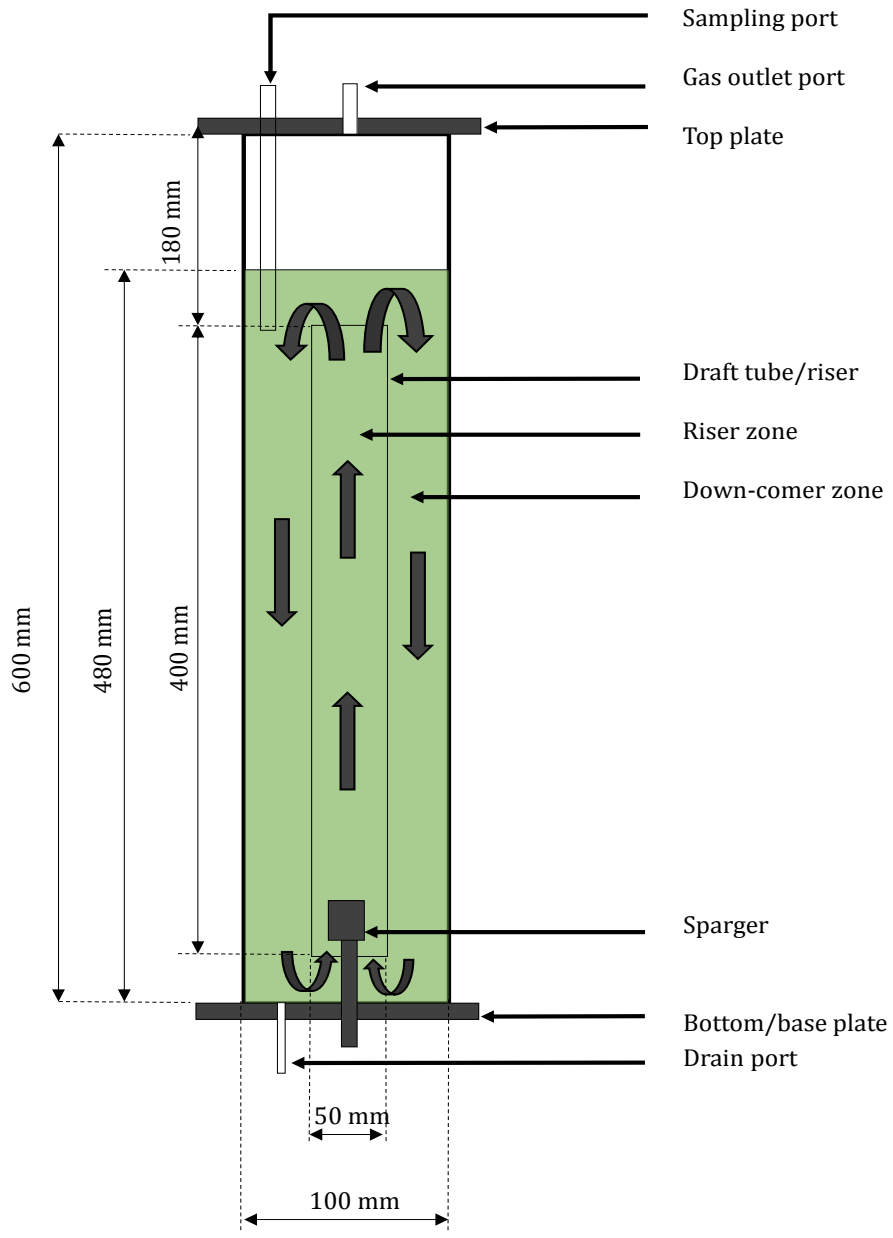


Figure 3.2: Diagram of airlift photobioreactor, adapted from Langley (2010).

Spirulina was cultured in these reactors without the addition of dispersion spargers to the gas inlet of the ALR. This prevented the formation of small air bubbles capable of lysing the fragile cyanobacterial cells. ALRs, containing the selected media, were sterilised by autoclaving at 121°C for 20 minutes prior to use. Reactors were placed vertically, close to each other, but separated by aluminium foil to help reflect more light.

Illumination was supplied by three 18 W white fluorescent bulbs providing ± 250 μmol photons.m^{-2}.s^{-1} on one side of the reactor.

Spirulina was cultured at room temperature (24 ± 2°C) with filtered air (0.45 μm) supplied continuously at a flow rate of 2 L.min^{-1}. The total circulation time in the reactor was approximately 7 ± 0.5 s, with a riser time of 1 ± 0.5 s and a down-comer time of 6 ± 0.5 s (Langley, 2010). Sterilized deionised water was added daily to correct for evaporation.

3.2.4.3 Shake flasks

Spirulina was cultivated in Erlenmeyer flasks on shaking platforms (Thermo Scientific) when multiple environmental parameters (temperature, salinity and pH) were investigated. The effect of pH and salinity on growth of Spirulina and bacteria were investigated at room temperature (24 ± 2°C), on an orbital shaker agitated at a speed of 145 rpm. Light was provided from above by eight cool white fluorescent bulbs emitting 88±2 μmol photons.m^{-2}.s^{-1} measured at the surface of the Spirulina culture (Figure 3.3).

Figure 3.3: Spirulina cultivation in glass Erlenmeyer flasks agitated at 145 rpm and room temperature under fluorescent lights.

Shake flasks were sterilised prior to use and closed with cotton wool to minimize evaporation and contamination of the cultures with airborne microorganisms. While facilitating gas-liquid mass transfer experiments investigating the effect of temperature

on Spirulina and bacterial growth were carried out in a temperature controlled shaking incubator (Labcon), fitted with fluorescent bulbs to provide illumination. Light intensity and agitation were similar to that for the salinity and pH experiments.

3.3 Analytical methods used for process monitoring

3.3.1 Dry cell weight (DCW) and biomass of *Spirulina* cultures

The growth of Spirulina cultures was measured by optical density (OD) at a wavelength of 750 nm (Helios α spectrophotometer, Thermo Scientific). At this wavelength the interference from chlorophyll is at its lowest and therefore the measurement would be indicative of the turbidity of the sample rather than the amount of pigment present (Griffiths *et al.,* 2011). OD measurements were performed using 3 mL samples, in triplicate. Where OD readings exceeded 1.0, samples were diluted with sterile medium to ensure OD measurements were within the linear range of the spectrophotometer. The average of the triplicate readings was taken as the OD of the reactor. The percentage error between the triplicate OD readings was 4%. OD measurements were converted to dry cell weight (DCW) by constructing a standard curve. Samples were taken at the end of the growth cycle, serially diluted, and the OD at 750 nm measured spectrophotometrically to determine the OD of each dilution. From each dilution, 20 mL was filtered onto pre-weighed cellulose nitrate membrane filters (0.45 µm, Sartorius) and dried overnight at 80°C, in triplicate. Filters were cooled to room temperature in a desiccator and weighed to determine the DCW. Both OD and DCW measurements were used to generate a standard curve allowing the conversion of OD values to biomass concentration. Biomass concentration and OD were related according to Equation 3.1:

$$y = 0.8472x \qquad \textbf{Equation 3.1}$$

where y is the optical density at 750 nm and x is biomass concentration expressed as g DCW.L^{-1}.

3.3.2 *Spirulina* cell morphology

Microscopic examination (Olympus BX40) was done using a 100x objective to confirm the identity and cell structure of the stock cultures before the initiation of experiments. The cell morphology was also examined regularly during the algal growth cycles.

3.3.3 pH measurement

pH measurements on Spirulina liquid cultures were performed daily with a pH meter (Cyber scan 2500, Wirsam Cape Town Scientific). The pH probe was stored in 3M ($mol.L^{-1}$) KCl buffer solution at room temperature and was calibrated against two standard buffer solutions of pH 4.0 and 7.0 before use.

3.3.4 Temperature control and measurement

During experiments in the 70 L outdoor raceway pond, the temperature of the culture was maintained at 29°C using a 300W aquarium heater (EHEIM JAGER, Germany). The temperature was monitored daily with an ethanol thermometer. The daily temperature fluctuations in the outdoor 50 000 L raceway were measured using a Seneye Reef aquarium monitor (Seneye Ltd, Norwich, England). The temperature experiments were carried out in a temperature controlled shaking incubator (Labcon),

3.3.5 Total alkalinity test

Alkalinity was measured according to the method described by Snoeyink & Jenkins (1980). Sample volumes of 20 mL were used and titrations were performed using 0.05 M (0.1 N) sulphuric acid (H_2SO_4). The initial pH of the sample and the volume of acid in a burette were recorded. A temperature probe connected to the pH meter used for titrations was used to allow for fluctuations in the temperature during the addition of acid to the sample. Samples were continuously mixed on a magnetic stirrer plate and the change in pH monitored during the addition of acid. The titration was done in two phases where the first phase comprised of addition of acid to the sample until the pH was between 4.3 and 4.6. This was followed by the slow addition of acid until a pH of 3.6 was reached. The second phase consisted of careful drop wise addition of acid and the recording of pH. Approximately five pH values between pH 3.5 and 3.0 were recorded, along with the volume of acid used. The values obtained from the five points recorded during titration were plotted to provide a graph which gave a linear equation that was used to calculate the volume of titrant at equivalence point (A in mL). The procedure is given in detail in Section 8.1.2. These results were used to calculate the total alkalinity expressed as the bicarbonate equivalent in $mg.L^{-1}$ ($Eq.L^{-1}$) using Equation 3.2.

$$Total\ alkalinity\ (\frac{Eq}{L}) = \frac{A \times N}{V} \qquad \textbf{Equation 3.2}$$

where A is the volume of standard acid (H_2SO_4) or titrant at equivalence point (mL); N is the normality of the standard used and V is the volume of the sample (mL).

3.3.6 Chemical oxygen demand and total organic carbon analyses

The chemical oxygen demand (COD) of the algal filtrate was determined to measure the amount of organic compounds contained in the liquid samples. These tests were conducted using COD reagents obtained from Merck chemicals. COD ($mg.L^{-1}$) measurements were conducted following the method from Black (2009) using the high range reagents (1500-10 000 $mg.L^{-1}$ COD). Briefly, 2.2 mL of solution A and 1.8 mL solution B were added to a test-tube respectively, followed by 1 mL of sample. Tubes were incubated at 150°C for 2 hours in a COD digest block. Once the samples had cooled down to room temperature, the OD of the samples was measured at a wavelength of 610 nm. To generate a standard curve, standard solutions of potassium hydrogen phthalate were used. A 10 $g.L^{-1}$ COD solution was prepared using 8.5 g L^{-1} potassium hydrogen phthalate. This solution was diluted to 1.0, 2.0, 4.0, 6.0 and 8.0 $g.L^{-1}$ COD mL to generate a standard curve. The spectrophotometer was blanked (zeroed) with a sample prepared using de-ionized water (0 $g.L^{-1}$ COD) treated the same way as the samples. Assuming that the reagents were able to oxidize all organic matter within the samples, the dichromate consumed by the samples, measured spectrophotometrically as a colour change from orange to blue-green, was equivalent to the amount of O_2 required to oxidize the organic matter.

The total organic carbon (TOC) in the Spirulina culture supernatant was analysed by A.L Abbott & Associates (PTY) Ltd (Woodstock, Cape Town). Samples were filtered to remove cells and solid matter, and the supernatants stored in 50 mL sterile tubes (Falcon 50mL conical centrifuge tube, Fisher Scientific) at -20°C until analysis.

3.4 Microbiological methods

3.4.1 Bacterial loads in *Spirulina* cultures

The bacterial load present within Spirulina cultures was determined by plating the culture supernatant onto salty medium (Table 3.1) agar plates prepared by the addition of 1.5% (w/v) bacteriological agar to the medium preceding autoclaving. Spirulina cultures were filtered using 50 µm soft nylon plankton net to remove the majority of algal biomass and the resulting filtrate serially diluted with sterile 0.1 M phosphate

buffer, pH 7. The dilutions chosen for plating depended on the expected bacterial counts based on the growth phase of the bacteria and the experimental treatment. To achieve countable colony forming units (CFUs) on agar plates, 0.1 mL of the diluted samples was spread-plated onto salty medium agar plates. Inoculated plates were incubated at 37±2°C for 72 hours. CFUs were determined by counting all the colonies observed on plates post incubation. Results were reported as $cfu.mL^{-1}$ for liquid culture and $cfu.g^{-1}$ for powdered Spirulina product while taking the relevant dilutions into account. Only plates with 30-300 colonies were counted and regarded as acceptable.

3.4.2 Preparation of competent *E.*coli cells for cloning of 16S rRNA gene fragments

E. coli cells used for transformation were prepared using a $CaCl_2$ method modified from Dagert & Ehrlich (1979). *E. coli* DH5α cells were plated from glycerol stocks (maintained in 15% (v/v) glycerol at -60°C) onto LB agar. Plates were incubated at 37°C, for approx. 16 hours. A single colony was selected and used to inoculate 10 mL LB liquid medium and incubated on a shaking platform (160 rpm) at 37°C for approx. 16 hours. This culture was used to inoculate 100 mL LB medium and cultures were incubated at 37°C with shaking (160 rpm) until an OD of approx. 0.4, measured at 600 nm, was reached. The culture was cooled on ice, transferred to pre-chilled centrifuge tubes and centrifuged at 3000 g for 5 min at 4°C. The pellet was suspended in 100 mL of pre-chilled 0.1 M $MgCl_2$, incubated on ice for 1 min and centrifuged as before. The cell pellet was re-suspended in 50 mL pre-chilled 0.5 M $CaCl_2$ by gently swirling, and incubated on ice for 1 hour. Following the incubation, cells were centrifuged as described before. The resulting cell pellet was re-suspended in 10 mL 0.5 M $CaCl_2$ supplemented with 15% glycerol. The cells were aliquoted (100 µl) into sterile Eppendorf tubes and stored at -60°C until use.

3.5 Experimental Methods

3.5.1 Isolation of bacteria from Spirulina biomass

The following experiments were conducted to assess the bacterial loads present within Spirulina cultures during cultivation in outdoor open ponds, and those present within commercially processed Spirulina powder. Spirulina processed powder from different batches was obtained from a pilot study of Spirulina cultivation in a 500 000 L outdoor

raceway pond carried out at the BioDelta Spirulina farm (Simondium, Cape Town). To assess the bacterial load present in the pond, liquid samples were collected and transported to the laboratory for analysis. The sample filtrate was diluted and bacterial load determined according to Section 3.4.1. Following the end of the cultivation season, the Spirulina biomass was harvested and dried at 45 to 50°C for 16-36 hours, using industrial scale equipment.

To assess the bacterial load present in the dried powder following these trial drying regimes, 0.1 g of factory processed powder was suspended in 10 mL of sterile 0.1 M phosphate buffer. Samples were mixed for 10 min (Vortex Genie 2 mixer, Scientific Industries), serially diluted and the bacterial load determined by plate counts as described in Section 3.4.1. Plates were also inspected to identify distinct colony morphologies, suggesting community diversity. Six morphologically different isolates were identified from plates of processed powder samples, and five from liquid pond samples. These bacterial isolates were cultured on salty agar plates to obtain pure isolates.

Concurrently, samples were sent to SwiftSilliker (Pty) Ltd food laboratory (Claremont, Cape Town) for analysis of total bacterial counts, total coliforms, yeast and mould and the presence of specific food pathogens such as *Salmonella* sp., *Bacillus cereus*, *Clostridium perfringes, Staphylococcus aureus,* and *Pseudomonas)*. The SwiftSilliker lab performed analyses using method number SWJM 35 based on APHA and ISO 4833 guidelines. For a comparative analysis of total bacterial counts between CeBER and SwiftSilliker food laboratory, the salty medium outlined in Section 3.2.3 was used as growth medium.

3.5.2 Identification of bacterial isolates associated with Spirulina cultures and processed powder

The 16S rRNA gene fragments from isolates were amplified to determine their identity based on highest similarity of the 16S rRNA gene sequences to those of known organisms. Genomic DNA (gDNA) was extracted from the 11 bacterial strains isolated from Spirulina pond cultures and processed powder using a method adapted from Krohn-Molt et al. (2013), described in detail in Appendix 8.1.3. The re-suspended DNA was stored at -20°C until further analysis.

Amplification of the 16S rRNA gene fragments were performed using the universal bacterial primers 27F (forward primer) and 1492R (reverse primer). The nucleotide sequences were: 5' GAG AGT TTG ATC TGG CTC AG 3' and 5' GTA CGC TAC CTT AGC ACT T 3' for 27F and 1492R for primers respectively (Lane, 1991). PCR products were analysed by agarose gel electrophoresis as detailed in Appendix 8.1.3. PCR products were excised from the agarose gel using a clean scalpel blade and the DNA extracted from the agarose using a Nucleospin® PCR and gel extraction kit (Whitehead Scientific, Cape Town). Extracted DNA gene fragments were cloned into the PJET1.2/blunt cloning vector (Promega) and transformed into freshly prepared $CaCl_2$ competent cells. The transformed cells were plated onto LB medium agar plates supplemented with ampicillin (100mg.mL^{-1}) and incubated at 37°C overnight. The resulting colonies were screened for the presence of plasmid DNA with 16S rRNA inserts by performing colony PCR's. Five colonies were chosen from each plate and sub-cultured on to fresh agar plates. The gDNA of the resulting pure cultures was extracted and included in PCR. Colonies containing plasmids and 16S rRNA gene inserts were picked and used to inoculate 10 mL LB medium supplemented with 100 mg.mL^{-1} ampicillin. Cultures were incubated at 37°C overnight on a shaker (150 rpm). Overnight cultures, 10 mL, were centrifuged to obtain cell pellets and the plasmid DNA extracted using the GenElute™ Plasmid Miniprep Kit (Sigma Aldrich) as per manufacturer's instructions. To quantify the concentration of plasmid DNA extracted, a Nanodrop® 2000 (Thermo Fischer Scientific, USA) was used. DNA sequencing was performed by Inqaba Biotechnical Industries (Pty) Ltd.

The sequence results of isolates were edited and analysed using Chromas version 2.01 (Technelysium, Helensvale Queensland, Australia) and DNAMAN version 4.13 (LynnonBioSoft). Sequences were compared and aligned with the GenBank reference sequences for known 16S rRNA sequences using the Basic Local Alignment Search Tool (BLAST) to identify sequences with the closest similarity to the isolated organisms. Alignments of the 16S rRNA fragments and several reference sequences were performed using the ClustalW (Thompson *et al.,* 1994) version 2.1 multiple sequence alignment program. To determine the accuracy of the phylogenetic tree (Figure 4.9), the bootstrapping method (1000 replicates) was used (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et*

al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 63 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1288 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.,* 2013).

3.5.3 Bacterial load determination of Spirulina cultivated in various algal growth reactors

The bacterial load associated with a commercial Spirulina culture, cultivated in a 50 000 L outdoor raceway pond at BioDelta farm between late January and early April 2014 was determined. Samples were collected twice or thrice weekly in sterile 50 mL Falcon tubes and transferred to the laboratory at UCT for bacterial analysis. Bacterial loads from these samples were determined as outlined in Section 3.4.1. The same methodologies employed on the 50 000L pond samples were used to determine bacterial loads from samples collected from ALRs, shake flasks and 70 L indoor and outdoor raceway ponds. Samples were collected in smaller volumes and dilutions were prepared accordingly. No new morphologically distinct bacterial colonies were observed on the bacterial count plates from the 50 000 L pond or other reactor samples.

3.5.4 The effect of time to analysis and travelling conditions on bacterial loads in Spirulina samples

Bacterial analysis of Spirulina samples from the BioDelta farm based in Franschhoek required transportation to the CeBER laboratories at UCT. The effect of time and travelling conditions on the bacterial load in Spirulina samples was investigated. Samples were collected from the 50 000 L raceway pond and stored in various ways prior to analysis, including incubating samples at either warm or chilled temperature: "S1" represented samples collected upon arrival and plated on-site, immediately before harvesting of biomass. This was used as a control as this would be indicative of the bacterial load in the pond at that time; "S2 no Inc" were samples collected after harvesting and plated on the farm; "S1 3 hr Inc-farm" represented samples incubated in the sun for 3 hours, then plated at the farm, while "S1 3 hr Inc-lab" represented the same sample (i.e. "S1 3 hr Inc-farm") plated at CeBER laboratories after 1 hour transportation from BioDelta; "S2 Room temp" and "S2 cooler" were samples left standing at room temperature and in a cooler environment respectively, while in transit to CeBER laboratories for about 1 hour for bacterial analysis (see Table 3.3).

Samples were collected in 50 mL Falcon tubes and divided into 3 samples for each treatment. Samples were serially diluted and analysed in triplicate.

Table 3.3: Treatment and handling of samples obtained from 50 000 L pond located at BioDelta farm.

Sample Code:	Description		
	Storage condition	**Storage duration**	**Plated at:**
S1 no Inc	-	0 hours	BioDelta
S1 3 h Inc-farm	Exposed to the sun	3 hours	BioDelta
S1 3 h Inc-lab	Exposed to the sun	3 hours in the sun, 1 hour in transit to CeBER	CeBER
S2 no Inc	-	0 hours	BioDelta
S2 room	Stored at room temperature	1 hour in transit to CeBER at room temperature	CeBER
S2 cooler	Stored in a cooler box	1 hour in transit to CeBER in a cooler box	CeBER

3.5.5 The control of pH in airlift reactors

Spirulina was inoculated into BioDelta medium and grown in ALRs (Section 3.2.3) to investigate relative growth of Spirulina and bacteria. The pH of cultures was adjusted by the addition of either 1M HCl or $NaHCO_3$. The effect of pH by these reagents on bacterial load in Spirulina cultures was investigated. Experiments were carried out in duplicate. Samples were taken daily for growth measurements and further analysis. All experiments were initiated from the same inoculum and left to run until day 5 where the pH was adjusted accordingly until the end of the experiment.

3.5.6 Parameters affecting bacterial load associated with Spirulina cultures

Further experiments were performed to determine the effect of salinity, pH and temperature on the bacterial load associated with Spirulina cultures. The process conditions used are given in Table 3.4. Cultures were grown in shake flasks in the standard BioDelta medium and agitated at 145 rpm (Figure 3.3). Temperature was controlled in a shaking incubator. The growth parameters were varied as shown in Table 3.4. Salinity was varied by halving or doubling all the components of the standard BioDelta medium (salinity of 14 parts per thousand (ppt)). For the pH experiments, cultures were allowed to increase in pH (Spirulina growth leads to pH increase due to uptake of CO_2 (see Section 2.4.4) until they reached the pH of interest, except for pH 9 which was reached by sparging of CO_2 into the medium. The pH in all flasks was maintained by the addition of 2% CO_2 once daily. Both salinity and pH studies were carried out at room temperature. Cultures were sampled (6-10 mL) daily over 14 days to determine algal and bacterial growth and pH. DNA extraction for bacterial speciation was done.

Table 3.4: Process parameters and how they were varied.

Variables	Treatment
Salinity	7, 14 and 28 parts per thousand(ppt)
pH	9, 10 and 11
Temperature	25, 30 and 35°C

Ppt: parts per thousand i.e. gL^{-1}

3.5.7 The effect of drying on bacterial load in processed Spirulina powder

The effect of drying conditions on bacterial load in Spirulina was investigated. Twenty five gram samples of wet Spirulina biomass were stored at either room temperature or 30°C for either 3 or 6 hours and then freeze dried overnight. Another set of wet biomass samples were oven dried at 45, 55 and 65°C without any pre-drying storage. Drying was done until the samples reached a constant mass. Tests were all done in triplicate. The resulting dried biomass from freeze- and oven-drying was ground to a powder before the bacterial analysis was carried out.

3.6 Research Approach

Spirulina naturally grows as a symbiont with a variety of bacterial species. This study focused on identifying the bacteria associated with Spirulina cultures, understanding the kinetics taking place during cultivation of Spirulina and associated bacteria in outdoor open raceway ponds and how these cultures are affected by process conditions such as temperature, pH and salinity. Growth of Spirulina and associated bacteria were monitored over time in a Spirulina cultivation pond, and the bacterial count in commercially processed Spirulina powder was also monitored. The method developed for analysis of bacterial count and diversity was by plating onto agar media. Formulation of medium able to capture most bacteria was paramount. Therefore, through the use and modification of APHA and ISO 4833 plate count agar method, various media compositions were tested and compared, together with the results that were obtained by an external laboratory, the SwiftSilliker food laboratory.

A phylogenetic analysis of the Spirulina isolate used in the project was carried out by a 16S rRNA gene study and is presented in Chapter 0. This is followed by quantifying the bacterial loads present within commercially processed Spirulina powder in the CeBER laboratories. A comparative analysis was done by sending a subset of samples of commercially processed Spirulina powder to the SwiftSilliker food laboratory, which uses the standard plating agar with no additional salts. In the CeBER laboratories, plate count analysis involved using salts from the BioDelta medium to supplement standard agar plates to assess the bacterial load in commercially processed Spirulina powder from a 500 000 L pond. Single colonies of bacteria with distinct morphologies were isolated from the agar plates used during the study of the powdered Spirulina and the 50 000 L pond operation between late January and early April 2014. The isolates were sub-cultured onto fresh agar plates to obtain pure bacterial isolates. Subsequently, growth kinetics and bacterial loads associated with commercially cultivated Spirulina were investigated. Growth measurements were taken daily using OD at 750 nm. These readings were converted to dry mass measurements using a standard curve constructed during similar study. To address sensitivity of methods to environmental conditions, samples from the 50 000 L ponds were exposed to different environmental conditions during transportation to CeBER laboratories for growth measurements and bacterial analysis. The results generated were verified by exposing Spirulina samples to different

environmental conditions: high temperature (in the sun) and in a cooler box with ice-blocks (Table 3.3). Growth measurements of Spirulin*a* were taken daily OD at 750nm. Bacterial loads associated with Spirulina cultures were determined by plating onto agar plates. These investigations were carried out in 50 000 and 80 L raceway ponds located outdoors and also in 3.2 L airlift reactors in the CeBER laboratories. The bacteria isolated from the 50 000 and 500 000 L pond were characterised through PCR amplification, clone library construction and sequencing of the 16S rRNA gene fragments.

In Chapter 5, the management of bacterial load in Spirulina cultures is the focus of the study. This study investigated the effect of process conditions, such as pH, temperature and salinity, on growth of Spirulina and associated bacteria in attempt to understand the impact of environmental and process conditions and cultures response under different conditions (Table 3.4).

Lastly, different methods of drying biomass were investigated for their effect on bacterial loads. Drying was carried out by oven-drying and freeze-drying. Samples of 25 g wet *Spirulina* biomass were oven dried at 45, 55 and 65°C and freeze dried overnight respectively. Another set of wet biomass samples was incubated at room temperature or 30°C for either 3 or 6 hours imitating what happens on an ordinary harvest day. These samples were freeze dried following incubation. Drying to constant was conducted. The bacterial analysis was done on the dried Spirulina powder. All tests were done in triplicate.

4 Results and Discussion I

Bacterial contaminants in Spirulina cultures: Their characteristics, identity and behaviour during outdoor cultivation

4.1 Introduction

As the health food market continues to grow and receive attention, Spirulina is among the functional foods in the limelight (Schilter *et al.,* 2003; Walker, 2004). The continued interest in Spirulina is due to its valuable nutritional properties such as its high protein content and the presence of essential amino acids and vitamins (Belay, 2002; Hosseini *et al.,* 2013). It is also reported to have therapeutic properties: antibacterial, anticancer, antiviral and antioxidant activities (Belay, 2002; Priyadarshani & Rath, 2012). Its popularity as a health food has brought about consumer concerns about the safety of the products (Schilter *et al.,* 2003) as it is commercially produced in outdoor open pond systems (Ciferri, 1983; Borowitzka, 1999; Spolaore *et al.,* 2006). Contamination of these open ponds with environmental contaminants has potential to result in Spirulina cultures which could pose a danger to consumers (Venkatraman & Mahadevaswamy, 1992; Venkatraman *et al.,* 1995; Sivakumar & Rajendran, 2013). Strict guidelines regarding the regulations governing microbiological standards for foodstuffs and related matters have been published by the Minister of Health in South Africa under the Governmental Notice no. R 692 of 16 May 1997. Very little information regarding the contaminants present within Spirulina cultivation ponds or the processed food grade powder is currently available in literature, making this study of great interest for Spirulina producers.

The primary tool used for determination of bacterial load in this study was the plate count method on selective agar. This was used owing to the specifications for product quality being given in terms of colony forming units, both total and specific pathogens. The identification of key culturable contaminants was performed by 16s rRNA sequencing of isolates. An alternative approach could have been to use a metagenomics approach to identify both culturable and non-culturable species. This was not used for two reasons, discussed here. The expense of a metagenomics approach does not allow for routine analysis in a production environment. While the metagenomics approach will allow non-culturable micro-organisms to be identified in

addition to the culturable ones, additional method development will be required for routine monitoring. This interesting approach is beyond the scope of the current project where rapidly transferable methodology was sought for the production facilities. Secondly, many metagenomics methods are not quantitative, owing to biases in DNA extraction. Further development is required for these approaches to replace the standard microbiological approach used in quality control and compliance with legislative requirements.

This chapter presents data regarding the bacterial loads and identification of bacteria associated with a Spirulina culture cultivated in an outdoor raceway pond and the food grade powder produced following the harvesting of this pond. The bacterial load present within outdoor raceway ponds, both a 50 000 L commercial scale and smaller experimental scale 80 L pond is presented. This sets the premise for the experimental work presented in Chapter 5, which investigated treatments and operating conditions to reduce the bacterial load associated with Spirulina cultures.

4.2 Identification of the Spirulina sp. used during this study

'Spirulina' is used when referring to the *Arthrospira* spp cultivated for the production of food grade Spirulina products. It is important to note that the majority of cyanobacterial species commercially cultivated for Spirulina production do not belong to the *Spirulina* genus, but within the *Arthrospira* genus of the same cyanobacterial family. A partial 16S ribosomal ribonucleic acid (rRNA) sequence of the cyanobacterium used in this study was obtained from Mr N van Wyk and subjected to the nucleotide BLAST (Basic Local Alignment Search Tool) to identify 16S rRNA sequences previously submitted to the NCBI (National Center for Biotechnology Information) database which showed a high degree of sequence similarity to those previously identified. These 16S rRNA sequences as well as other reference cyanobacterial 16S rRNA sequences and the outlier 16S rRNA sequence from the iron oxidising sp *Leptospirrilum ferriphilum*, were used to construct a phylogenetic tree, shown in Figure 4.1 to ascertain the relatedness of the studied species to other cyanobacteria. Sequence alignments were performed using a consensus 581 bp of 16S rRNA sequence spanning positions 256 to 837 of the 16S rRNA sequence of *Spirulina maxima* LB 2342 strain housed at the UTEX (University of Texas) Culture Collection of algae.

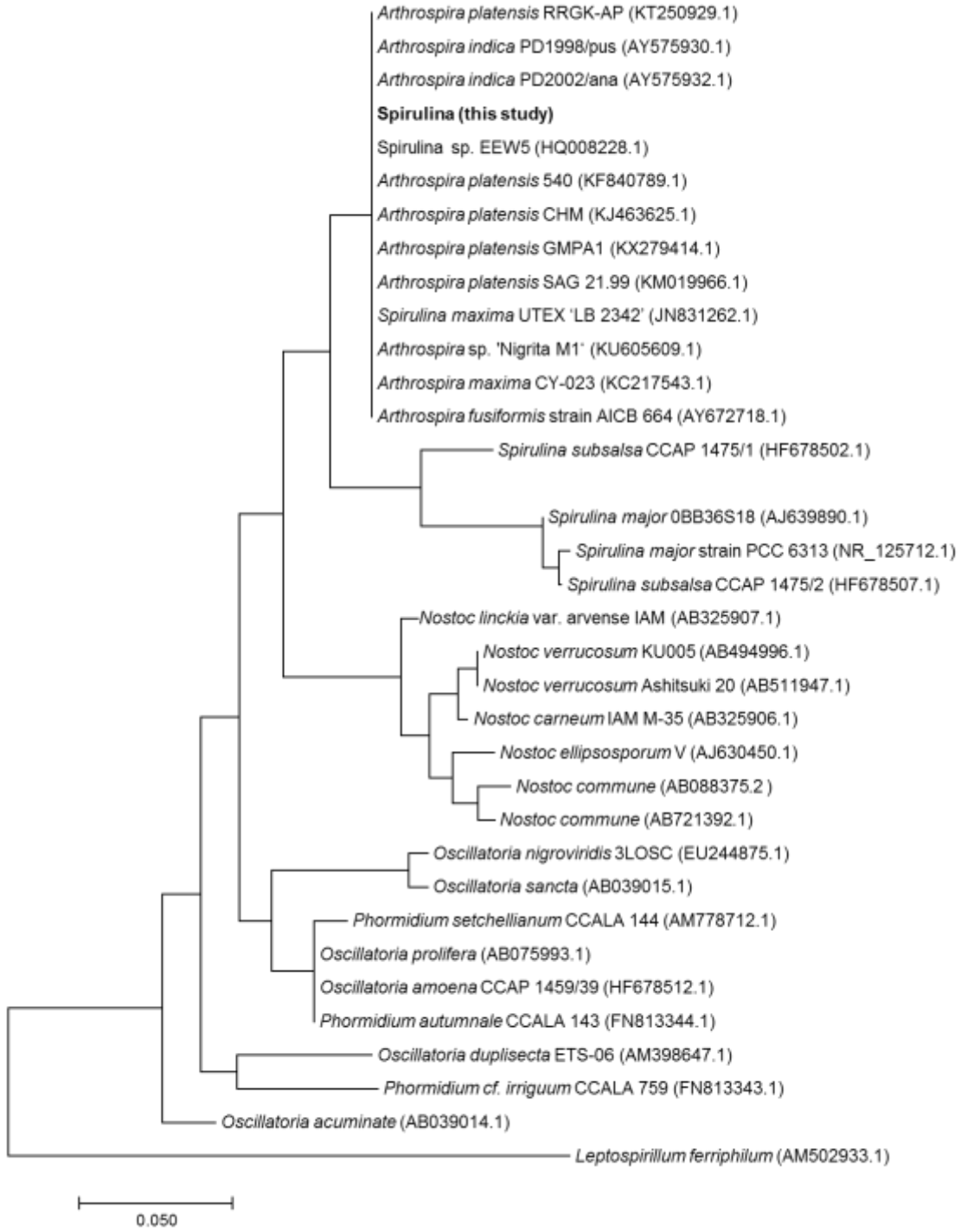


Figure 4.1: Molecular Phylogenetic analysis of the Spirulina species used in this study assessed by the Maximum Likelihood method. The evolutionary history was inferred based on the Tamura-Nei model and the tree with the highest log likelihood (-2727.3073) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 35 nucleotide sequences and there were a total of 565 positions in the final dataset with gaps and missing data removed. The iron

oxidising bacterium, *Leptospirillum ferriphilum*, was used as outlier. Evolutionary analyses were conducted in MEGA7.

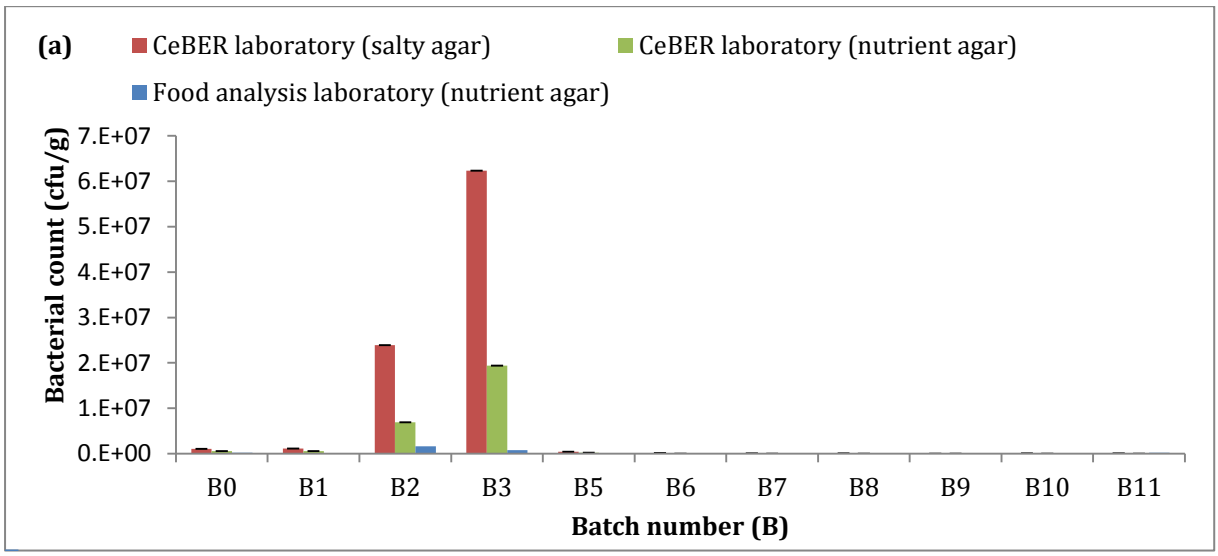
Phylogenetic relatedness suggests that the Spirulina species used in this study belongs to the *Arthrospira* genus, while species belonging to the true *Spirulina* genus resolved into the same clade as *Arthrospira*, but on their own branch (Figure 4.1). Unfortunately, the partial 16S rRNA sequence information used to calculate the phylogeny did not result in the identification of the closest related *Arthrospira* species to the study species. A deeper phylogenetic analysis with more molecular information would be required to calculate the closest related species and suggestions to achieve this are made below.

The phylogeny of the *Arthrospira* species used in this study can further be resolved by accessing the complete 16S rRNA sequence, as partial sequences do not allow many positions for calculation of phylogenetic relatedness. Inclusion of the 23S rRNA, 16S – 23S rRNA intergenic spacer region, *tRNA-Ile* and *rbc*L gene sequences may also increase the robustness of the phylogenetic analysis. Use of the *hoxH* gene encoding a NiFe-hydrogenase has also been demonstrated to be a suitable molecular marker for taxonomical studies of cyanobacterial systems (Zhang *et al.,* 2005) and may also be considered. Similarly an intergenic spacer region belonging to the *cpc* gene locus which encodes the phycocyanin protein which is a pigment association with photosystem II and is specific to the cyanobacterial family, has been successfully used to perform cyanobacterial systematics (Neilan *et al.,* 1995; Robertson *et al.,* 2001).

4.3 Quantifying bacterial load in processed Spirulina powder

Bacterial contaminants associated with dried Spirulina powder and Spirulina suspensions were assessed quantitatively to determine the point of origin of the contaminants, either introduced to the culture during open pond cultivation or during the downstream processing of the product. Standard plate counting methods were used to assess the bacterial load within each sample. For general bacterial contaminants nutrient agar as per ISO 4833 guidelines were applied whereas halophilic organisms, more likely to be present within the culture due to the culture medium composition, were accounted for by plating samples onto “salty” agar. The “salty” agar was supplemented with the medium components present in the BioDelta medium used for Spirulina cultivation (Section 3.2.3).

Bacterial load analyses of eleven batches of processed Spirulina powder were carried out. Figure 4.2 presents the comparison between the bacterial counts obtained by "salty" and nutrient agar plating (performed at the CeBER laboratory) and those obtained on nutrient agar plating recipe by both CeBER and SwiftSilliker food laboratory. In general the use of "salty" agar plating medium resulted in 2 to 4 times higher bacterial counts than the nutrient (conventional) agar plating recipe performed at CeBER laboratory. The highest bacterial loads recorded were 2.4 x 10^7 and 6.2 x 10^7 (colony forming units) cfu.g^{-1} processed powder in the salt enriched medium while 6.9 x 10^6 and 1.9 x 10^7 cfu.g^{-1} were recorded for B2 and B3 respectively in nutrient agar by CeBER laboratory respectively. Comparative bacterial loads for these samples measured by SwiftSilliker food laboratory using nutrient agar were 1.6 x 10^6 and 8.0 x 10^5 cfu.g^{-1} for B2 and B3 respectively (Figure 4.2a). The massive bacterial load in B3 was due to a long drying period. This batch did not dry properly overnight and had to be dried again the following day. The average bacterial load in B5 to B11 was 1.3 x 10^5 cfu.g^{-1} which was less than the specification for product compliance.



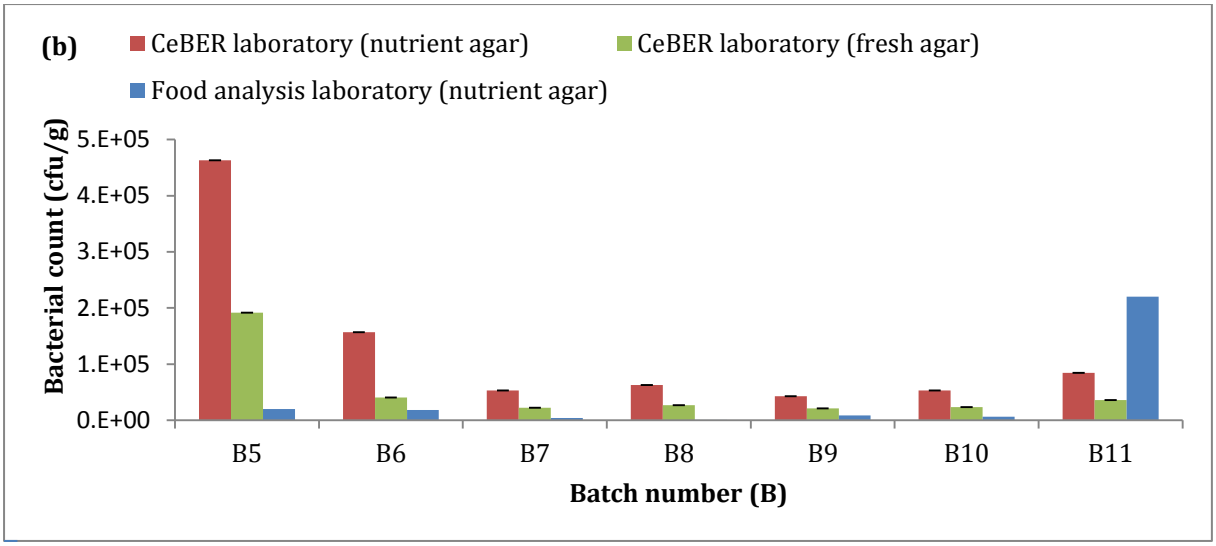


Figure 4.2: (a) Bacterial loads in commercially processed dried Spirulina powder samples plated on salty agar medium (CeBER laboratory) and nutrient agar medium (CeBER laboratory and SwiftSilliker food laboratory); (b) Bacterial loads of batches B5-B11 with axis scale changed to allow comparison.

The higher bacterial counts measured when plating on the salty agar is a result of the medium representing the culture conditions from which the microorganisms had been sampled. A greater number of bacteria are cultured using this medium as it supports the growth of the microorganisms present within the liquid culture with similar chemical composition used for the cultivation of Spirulina. It is widely accepted that a single medium cannot cater for or support the full diversity of the bacterial communities present within specific environmental samples (Orji et al. 2007; Ifeanyi et al. 2014). It is therefore important that a selective medium be used when investigating the bacterial communities present within a process adapted to a particular condition. In this study, a salt rich medium was used to match the culture conditions used for Spirulina production. Various studies have demonstrated an increase in the bacterial population cultured using enrichment media compared to standard media (Aspevall et al. 2002; Cassar & Cuschieri 2003; Ifeanyi et al. 2014; Orji et al. 2007).

4.4 Behaviour of contaminating bacteria during outdoor cultivation of Spirulina in a large scale raceway pond

In Section 4.3 bacterial contamination of processed Spirulina powder was shown. This suggested that contamination may occur in the cultivation stage and the contaminants may survive and multiply in the processing stages of the production line. Here the level

of contamination during Spirulina cultivation is assessed. The kinetics and relationship of the contaminants with Spirulina during cultivation, as well as their response to environmental changes within the cultivation media, are also investigated.

Results of the 50 000 L commercial scale raceway pond cultivation experiment are presented, together with the 80 L outdoor raceway pond cultivation experiment designed to give insight into the reproducibility of the 50 000 L raceway experiment without repeating the expensive commercial scale experiment. Comparison of Spirulina and bacterial contaminants was interrogated for trends to shed light on factors controlling contamination.

4.4.1 The effect of processing time and travelling conditions prior to analysis on bacterial loads in Spirulina culture samples

Samples collected from large scale Spirulina facilities would require transportation to a laboratory for testing. The effect that different sample handling techniques had on the bacterial loads measured in Spirulina cultures that were collected from a 50 000 L Spirulina raceway pond at the BioDelta farm, Franschhoek, and transported to the CeBER laboratories for processing was investigated. Large samples (in 500 mL bottle) were collected from the pond before (S1) and after (S2) harvesting of Spirulina biomass for processing to test the possible effect the harvesting technique may have. These samples were divided into 50 ml sub-samples which were treated as described Table 3.3. Samples collected and plated immediately at BioDelta (S1 no Inc) were used as controls as these would be indicative of the bacterial load in the pond at the time of sampling. The sterile buffer used to generate dilutions of the samples for plating was also plated at the farm and used as a negative control. The effect of time delay before plating and the cooling of samples before transport were investigated. All the plates were incubated for the same duration (72 hours) in a 35 ± 2°C controlled temperature room in the CeBER laboratories before counting.

Figure 4.3 illustrates the bacterial load in samples of Spirulina cultures after the various handling conditions. The grey bars show a repeat experiment conducted two days after the initial experiment (black bars). As such, the black bars should be compared and analysed as a unit or experiment and the grey bars as a different unit as the conditions within the pond would have changed between the sampling events; however, similar

trends in the bacterial counts with treatment condition were observed for the same treatments performed on the samples at the two different sampling times. The bacterial load measured in the pond before harvesting of Spirulina biomass (S1 no Inc) in Set 1 was 4.70×10^6 cfu.mL^{-1}. Incubation of this sample in a 50 ml polypropylene tube for 3 hours in the greenhouse housing the Spirulina pond (S1 3 hr Inc-farm), resulted in an increase in the bacterial load to 6.83×10^6 cfu.mL^{-1}. Generally, an increase in temperature increases the growth rate of bacteria (Ayaz & Gothalwal, 2014), and therefore this result was not unexpected. It was, therefore concluded that samples must be kept at relatively low temperatures to prevent increased bacterial growth before plating. Similar cell numbers were obtained when this sample was transported to the CeBER laboratory (S1 3 hr Inc-lab). This suggested that the effect of transporting samples for 1 hour at room temperature before plating was negligible, while the prolonged exposure to high ambient temperatures should be minimised (Figure 4.3). To test the effect of harvesting of the Spirulina biomass on a nylon screen over the production pond on the bacterial loads measured, a series of samples were taken and subjected to different treatments before bacterial loads were determined. Similar bacterial counts were measured for the sample taken after harvesting and plated on site (S2 no Inc) when compared to the pre-harvesting sample plated on site (S1_no Inc). An increase in the bacterial counts was observed when the same sample was plated upon return to the CeBER laboratories, approx. 1 hr later, (1.8 fold for the first test and 1.3 times for the repeat). Transportation at room temperature compared to transport in a cooler box at approx. 10 ± 2°C (with cooler bricks) had little impact on the bacterial counts, presumably because of the slow rate of cooling of samples. It was concluded that samples for bacterial counting should be taken after harvesting of the Spirulina biomass, to prevent prolonged exposure to high temperatures and that transportation to the laboratory for analysis can be done at room temperature permitted the sample is analysed as soon as it arrives.

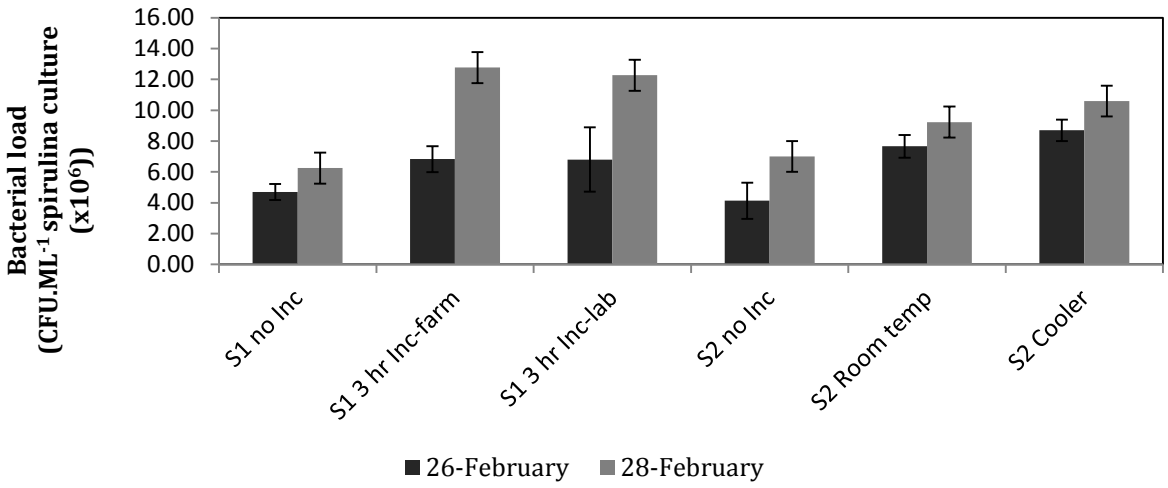


Figure 4.3: Bacterial load in Spirulina culture samples exposed to different incubation periods at different temperatures. Results shown here are averages of each treatment. Error bars indicate the standard deviation around the average bacterial load.

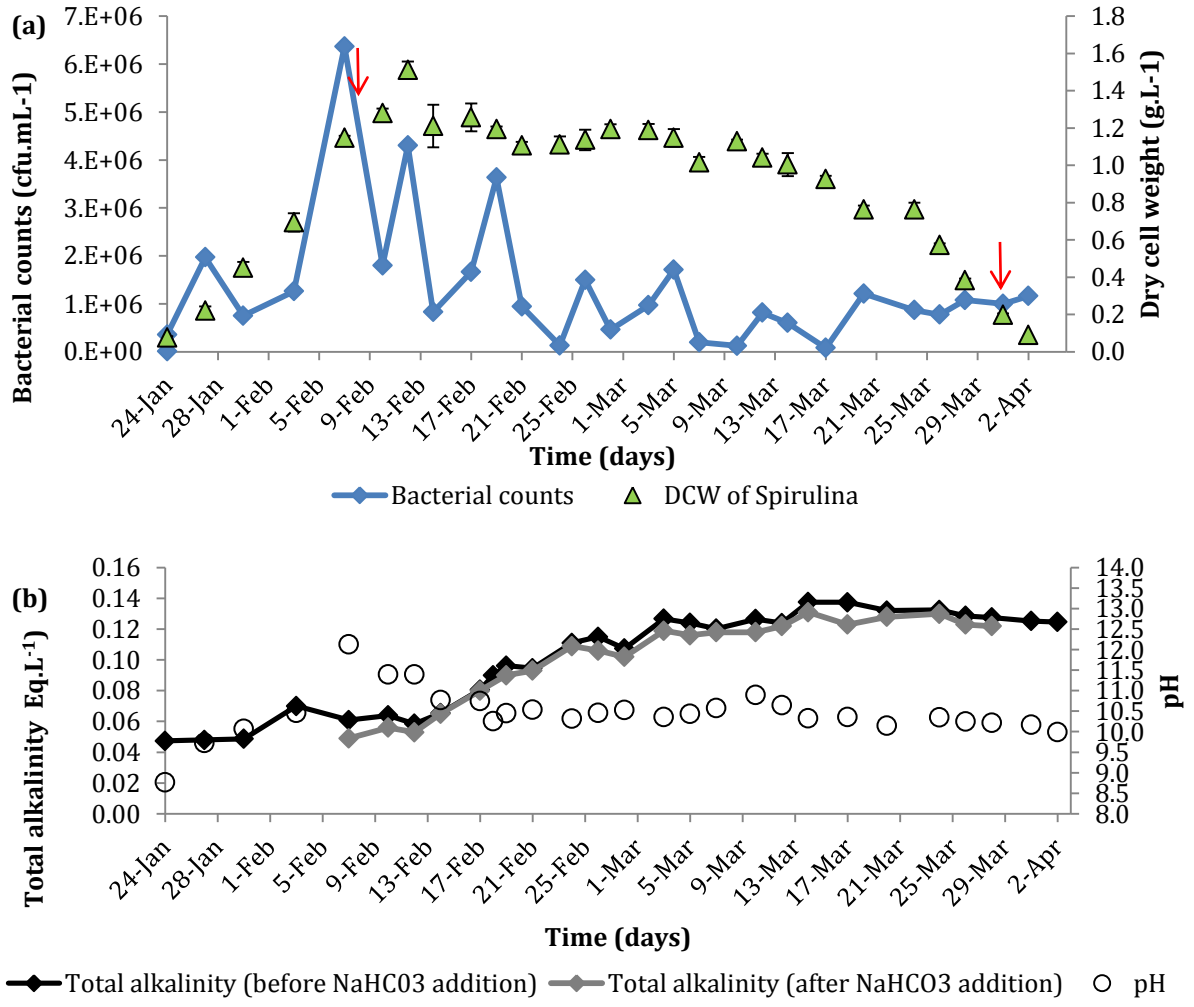
4.4.2 Outdoor 50 000 L and 80 L raceway ponds

Spirulina was cultivated in a 50 000 L outdoor raceway pond at the BioDelta farm between late January and early April 2014. The growth of Spirulina was monitored throughout the cultivation period and the bacterial loads in the Spirulina culture were monitored using the standard plate count method. Varying amounts of Spirulina biomass were harvested from February 10th (indicated by the red arrow) to March 28th in order to maintain a sufficiently dilute culture (approx. 1.2 – 1.5 g.L^{-1}) to ensure higher productivity. Harvesting was aborted from the end of March as the conditions became unfavourable for Spirulina cultivation.

Figure 4.4 (a) shows the growth profile of Spirulina and bacteria over a 67 day period in a 50 000 L pond at BioDelta farm. In Figure 4.4 (b) the change of pH with the addition of $NaHCO_3$ and HCl is given and in Figure 4.4 (c) daily temperature measurements. The Spirulina dry cell weights and bacterial counts were taken before biomass harvesting. The culture was started at a biomass concentration of 0.08 g.L^{-1} (day 0) (as indicated by dry cell weight) and the maximum yield reached 1.28 g.L^{-1} on February 10th (day 16) before the first harvest. During this period, the average specific growth rate of Spirulina was 0.22 day^{-1} and the productivity 0.08 g.L^{-1}.d^{-1}. The highest biomass concentration obtained was 1.51 g.L^{-1} on February 12th. On April 2nd (day 67), the pond was stopped due to lack of growth of Spirulina. After inoculation of Spirulina into the pond, the

bacterial load increased along with the growth of Spirulina. A decrease in bacterial load (from 1.97 × 10^6 to 7.53 × 10^5 cfu.mL^{-1}) was observed on the 30th of January (day 6) during the early exponential phase of Spirulina which was followed by 1.26 × 10^6 cfu.mL^{-1} on February 3rd (day 9). The highest bacterial load of 6.3 × 10^6 cfu.mL^{-1} was seen just prior to the highest dry cell weight of Spirulina and the first harvest (February 7th). The decrease in bacterial load on February 10th might have been due to harvesting of Spirulina biomass. While, the data showed a general increase of bacterial load with Spirulina growth, variations in bacterial counts started manifesting after the start of the continuous harvesting campaign. Following some fluctuation, the system settled at 1.2-1.3 g.L^{-1} Spirulina and a bacterial count of <1 × 10^6 cfu.mL^{-1} during regular harvesting.

The 50 000 L experimental procedure was repeated in a pilot scale raceway pond (80 L). These data are presented in Figure 4.5 (a), (b) and (c). Similar trends to the 50 000 L experiment were observed for growth of Spirulina and bacterial load.



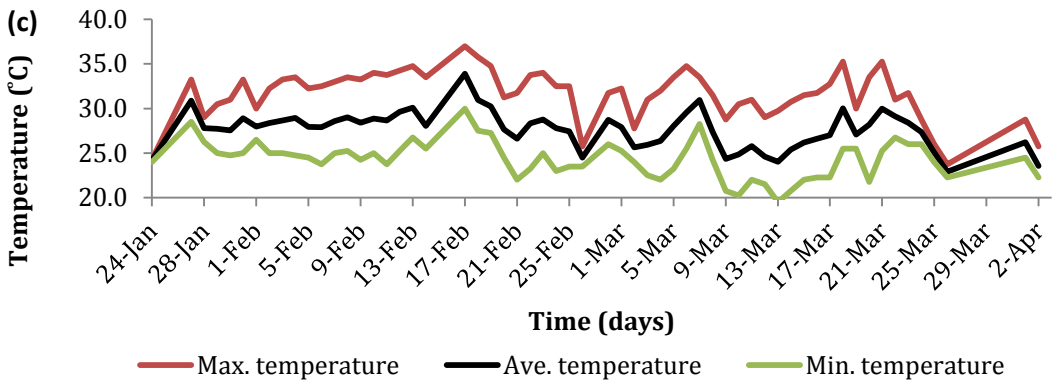
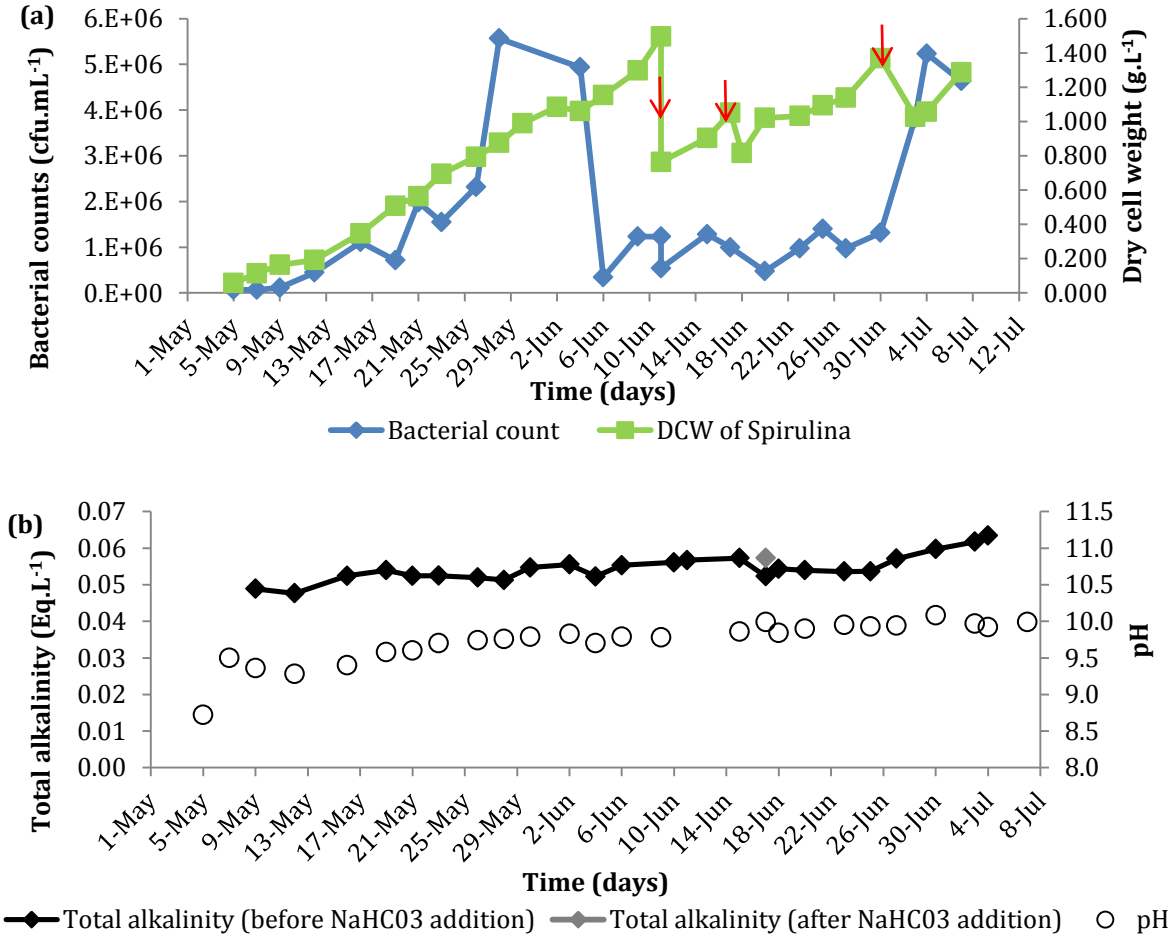


Figure 4.4: (a) Growth profile of Spirulina and bacteria in a 50 000 L pond at BioDelta farm. Error bars indicate standard deviation from the average biomass; (b) Total alkalinity measurements and pH, and (c) Daily temperature profiles during Spirulina cultivation experiment. All samples analysed were taken before harvesting, exept for the total alkalinity measurements after the addition of $NaHCO_3$. The red arrows indicate the beginning and end of biomass harvesting.



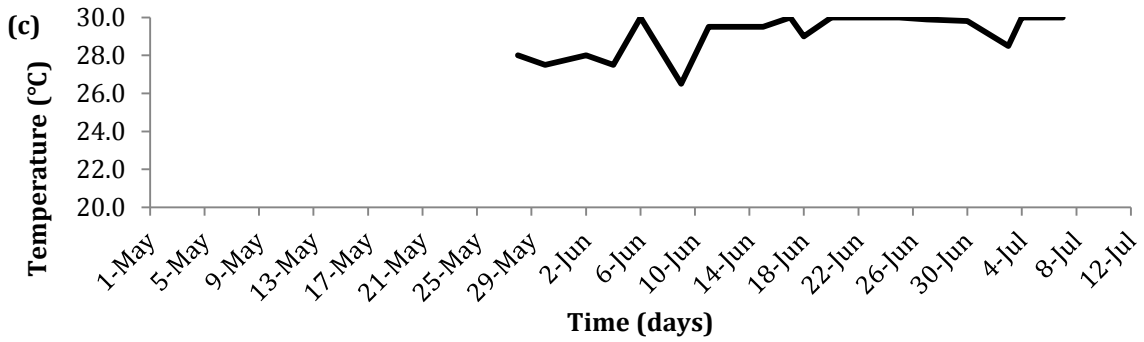


Figure 4.5: (a) Growth profile of Spirulina and bacteria in an 80 L pond at UCT greenhouse. Error bars indicate standard deviation from the average biomass; (b) Total alkalinity (before and after addition of $NaHCO_3$) and pH, and (C) Temperature inside the culture. The red arrows indicate days on which biomass was harvested.

In both experiments, increase in Spirulina biomass to over 1 $g.L^{-1}$ was accompanied by increase of bacterial load to some 6×10^6 $cfu.mL^{-1}$ and raise of pH before any harvesting and pH adjustment took place. The results suggested that the metabolic activities of the Spirulina during cultivation were beneficial to the bacteria, either by:

i) Creating environmental conditions (pH, chemical properties of the medium, light, provision of oxygen) that are favourable to bacterial growth, or
ii) Secreting metabolites that are beneficial to bacterial growth.

For both experiments, the bacterial load fell before the first harvest. This suggested further that:

iii) Whatever favourable condition the Spirulina creates for the bacteria has a terminal point at which its benefit is reduced, or
iv) Other factors start to affect the bacteria negatively.

The potential effects of environmental factors and Spirulina metabolites (i) and (ii) are discussed below.

Effect of environmental conditions on bacterial load

For the 50 000 L experiment, the sharp drop in bacterial counts prior to the first harvest (February 7th) coincided with pH adjustment by addition of HCl. About 30 L HCl and 50 kg $NaHCO_3$ were added, resulting in a decrease in pH from 12.13 to below 11.4. This suggested that addition of H^+ or Cl^- resulted in the reduction of bacterial load. The pH was not measured directly after addition of the HCl. It is expected that the pH on

February 7th after adjustment was below pH 11.4 (pH on February 10th) since the growth of Spirulina results in increasing pH due to uptake of CO_2 and release of OH^- into the medium by the culture (Richmond & Grobbelaar, 1986). The pH after adjustment may have been low enough to incapacitate some of the alkaliphilic bacteria identified in the Spirulina cultures (Section 4.5) such as the member of the *Alkalimonas* genus and the *Halomonas* genus. These communities of contaminants may have benefitted from increasing pH. In the 80 L experiment, the pH increased rapidly to 9.5 the following day, continuing to rise with concomitant growth of Spirulina in the range pH 9.4 to 10.3. Here, the pH was more stable, requiring little adjustment. This suggested different driver for the increasing bacterial load. As the role of pH on the increase and eventual drop in bacterial load was inconclusive from the data presented in Figure 4.4 and Figure 4.5, pH was investigated further as a potential control for bacterial load. This was done since pH influences both growth rate and bacterial survival.

Cultivation of Spirulina in the 80 L raceway was done in winter and a heater was used to help keep the temperatures high enough for growth. The minimum temperature recorded was 26.5 and 30°C as the maximum which was higher than the 16°C ± 2°C experienced by the culture before the heater was installed. The temperature of the culture stayed relatively constant with slight variations due to environmental temperature. Although the optimum temperature for Spirulina growth is between 35-38°C (Vonshak, 1997a), a good growth was seen in this case.

In both the 50 000 L and 80 L outdoor raceway pond experiments, the alkalinity trended upwards, except for times where $NaHCO_3$ (and HCl in the case of the 50 000 L experiment) was added. However, this finding is in disagreement with findings by Cheng (2009) who reported that the addition of $NaHC0_3$ increases the alkalinity of the medium. The alkalinity in the media increased with increase in Spirulina biomass due to the gradual CO_2 uptake by Spirulina cells. Changes in pH are more likely to affect the alkalinity of poorly buffered medium or solutions because of the close association of alkalinity and pH (Murphy, 2016). Furthermore, the addition of $NaHCO_3$ into the medium as a way to lower the pH ultimately affects the alkalinity, decreasing it. According to Grant et al. (1990) and Belkin & Boussiba (1971) high alkalinity and pH (Ciferri, 1983) are required for the growth of Spirulina and control of contamination by invasive species. This suggested that alkalinity and pH can be manipulated to favour

growth of Spirulina and inhibit growth of contaminating bacteria. For this reason, the effect of pH on Spirulina growth and bacterial load was investigated further (Section 5.5).

Total salinity refers to the content on salts in the medium. Although, not measured, salinity is expected to increase with addition of salts such as $NaHCO_3$. In Section 4.2 it was discussed that the addition of salts to standard agar plating medium resulted in increased bacterial counts being observed, suggesting that the bacterial contaminants favoured growth under saline conditions. Further, halophiles (salt-loving bacteria) such as members of the *Halomonas* genus were identified as contaminants (Section 4.5). Salt concentration negatively affects other non-halophilic bacteria such as members of the *Pseudomonas* genus identified in Section 4.5. For this reason, the effect of salinity on Spirulina growth and bacterial load was investigated in Section 5.4.

Effect of Spirulina metabolites on bacterial load

Photosynthetic Spirulina fixes CO_2 into biomass, releasing oxygen as a by-product. This would be favourable for aerobic microorganisms such as the members of the *Pseudomonas* and *Micrococcus* genera isolated in this study as increasing Spirulina biomass would increase the capacity for supply of O_2. A decrease in the rate of photosynthesis would reduce the supply of oxygen, potentially limiting these contaminating bacteria.

As discussed in Section 2.5.4 of the literature review and suggested by Figure 4.6, Spirulina releases exopolysaccharides (EPS) during its growth. It is possible that the heterotrophic bacteria may feed off these organics as reported in *Chlorella* cultures (Watanabe *et al.,* 2005). During the investigation (data not included), the formation of EPS also showed oscillations which might have been due to:

(a) At the beginning of the cultivation process when bacteria are in small amounts, Spirulina produces EPS that is then used by bacteria for growth.
(b) As the bacterial concentration increases, it increases at a rate that surpasses that of Spirulina and EPS production, resulting in the extracellular organic matter being depleted which then leads to a decline in bacterial counts as the bacteria run out of food.

(c) As the EPS accumulates, bacteria feeds off the EPS produced again and as a result, an increase in bacterial load is observed.

(d) The accumulation of these organics as a result of the nature of Spirulina and Spirulina cell lysis may have a negative effect on the Spirulina itself. Therefore Spirulina benefits from the removal of the EPS by bacteria.

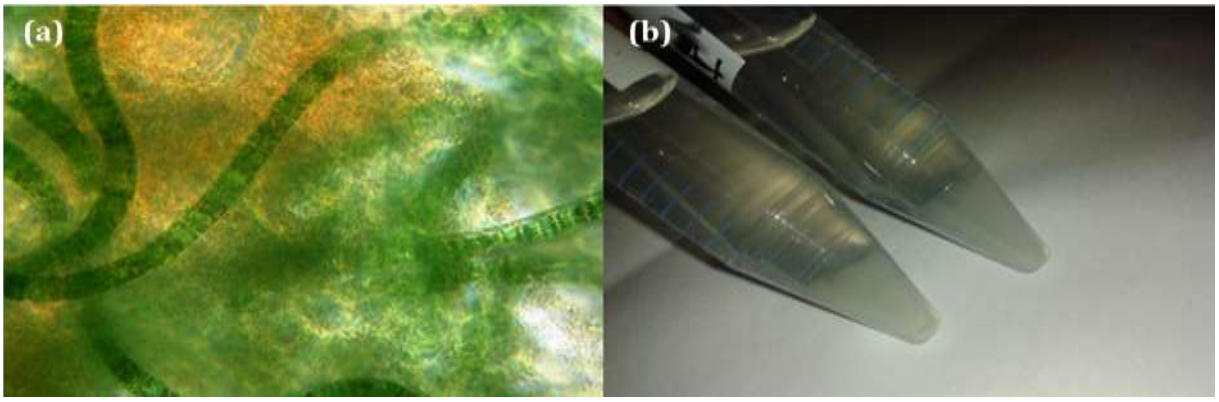


Figure 4.6: Exopolysaccharides (a) in Spirulina cultures and (b) from Spirulina filtrate (precipitated by ethanol).

The fluctuations of bacterial load were noted in both reactors. Among the many varying interactions taking place within Spirulina cultures, the relationship seemed to be beneficial for Spirulina and bacteria. The dynamics between Spirulina and bacteria are quite complex and more studies are needed for further understanding.

4.5 Characterisation and identification of the bacterial contaminants

Pure cultures of the bacterial contaminants associated with Spirulina cultivation were isolated from "salty" agar plates from the Spirulina powder samples (Section 4.3) and the outdoor experiments presented in Section 4.4. Isolates were chosen from the agar plates based on differences in morphology and pigmentation. Five different colony types, were observed and isolated from plates inoculated with samples of the processed powder, while, six colony types were isolated from the 50 000 L pond samples. All isolates were sub-cultured onto fresh agar plates to obtain pure cultures. Examples of the colony morphologies and pigmentation observed are shown in Figure 4.7.

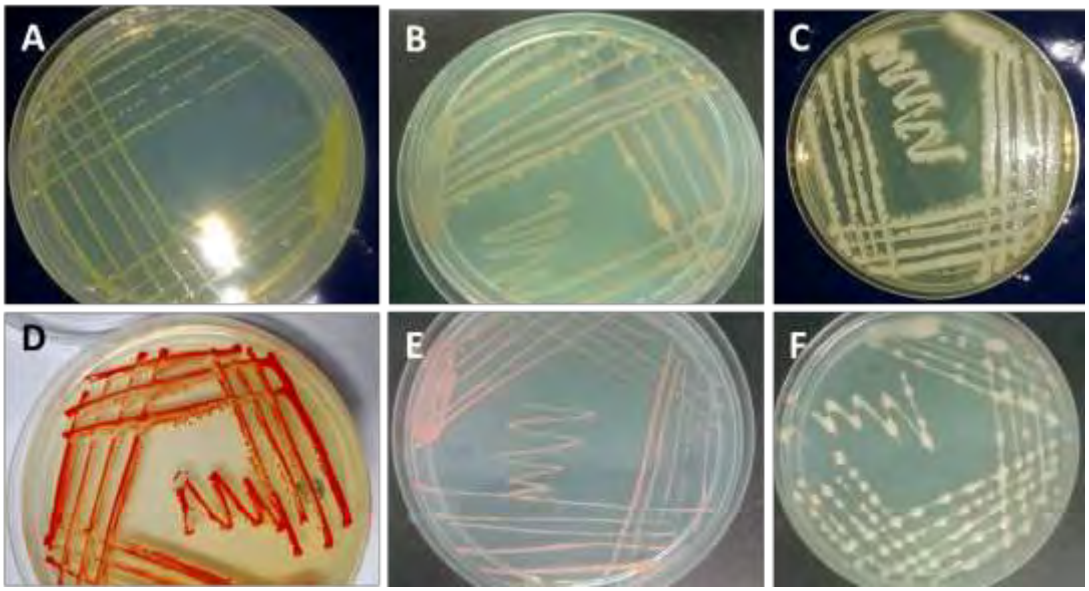


Figure 4.7: Morphological characteristics of isolates from Spirulina processed powder and liquid cultures from the 500 000L pond. A: B11, B: B9, C: B8, D: B7, E: B4, F: B1.

Genomic DNA (gDNA) was extracted from each of the isolates and the 16S rRNA gene amplified by PCR. The universal bacterial primers 27F (forward primer) and 1492R (reverse primer) were utilised for this purpose. Figure 4.8 shows the successful amplification of the 16S rRNA genes, yielding approx. 1 500 bp products, from the gDNA of the 11 isolates. Cloning of the 16S rRNA genes were performed using the pJET1.2 cloning system, prior to sequencing of the genes.

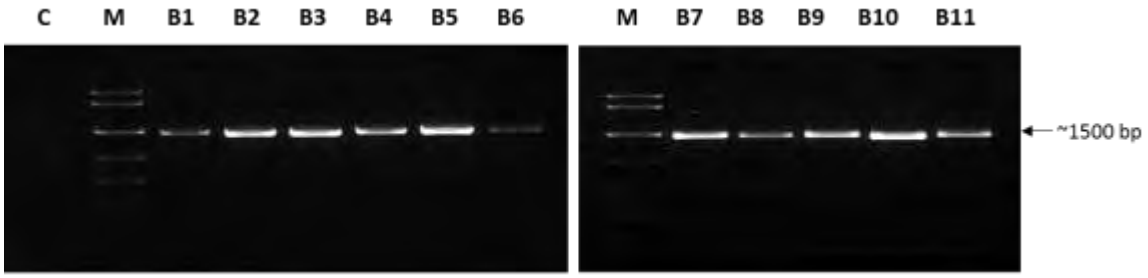


Figure 4.8: Agarose gel electrophoresis of the 16S rRNA gene products amplified from the bacterial isolates. Lanes are labeled as follow: C – no template control; M – Universal DNA ladder (Kapa Biosystems); B1 to B11 – PCR amplicons from the 11 baterial isolates associated with Spirulina samples.

The 16S rRNA sequences obtained were subjected to nucleotide BLAST (BLASTn) analyses (http://www.ncbi.nlm.nih.gov/BLAST) according to Altschul et al. (1997) and the top hits achieved following the BLAST analyses are given in Table 4.1.

Most of these bacterial isolates shared >99% sequence similarity to their closest relatives (Table 4.1). The Expect or E-value refers to a parameter that describes the number of hits one can "expect" to see by chance when searching a database of a particular size. The E-value scores equalled 0 in all cases, indicating that the match is significant.

Table 4.1: Similarity values for the closest relatives of the 16S rRNA gene sequences of bacterial isolates associated with *Spirulina* cultures and commercially processed powder.

Isolate	Closest related species	% Sequence similarity	E-value	Accession number
B1	Uncultured *Halomonas* sp clone SL52	99	0.0	JX240476.1
B2	Uncultured *Mesorhizobium* sp clone 3-F	99	0.0	EU305587.1
B3	Uncultured bacterium clone 6-11	99	0.0	JQ923679.1
B4	Uncultured bacterium from soil	99	0.0	KF912987.1
B5	*Idiomarina* sp	96	0.0	FJ170017.1
B6	*Alkaliminas delamerensis* strain 1E1	98	0.0	NR044879.1
B7	*Cecembia lonaresis* strain LW9	97	0.0	NR116971.1
B8	*Pseudomonas mendocina*	100	0.0	CP000680.1
B9	*Paenibacillus camelliae* strain G3-5-20	99	0.0	KC494322.1
B10	Uncultured bacterium clone 6-11	99	0.0	JQ923679.1
B11	*Micrococcus* sp B5W22-1	99	0.0	EF114312.3

Isolate B3 and B10 were identified as the same species based on the 16S rRNA gene sequences obtained for these. The closest related sequences identified by BLASTn analyses were identified as 'uncultured' spp. and therefore a phylogenetic analysis of sequences including sequences with high similarity identified by BLAST, were performed to test the evolutionary relatedness of the spp.

The 16S rRNA gene sequence of the Spirulina sp. used in this study and as outlier *Ferroplasma acidiphilum*, an iron oxidising archaeal species, was also included. The information extracted was used to construct a phylogenetic tree (Figure 4.9). The evolutionary history was inferred using the Neighbour-Joining method (Saitou & Nei, 1987). To determine the accuracy tree, the bootstrapping method (1000 replicates) was

used (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2004) and are in the units of the number of base substitutions per site. The analysis involved 63 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1288 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.,* 2013).

As would be expected for bacteria co-cultured with Spirulina in salty medium, many of the organisms identified by 16S rRNA sequencing were tolerant to saline and alkaline environments. Isolate B1 was found to be closely related to spp. from the *Halomonas* genus. *Halomonas* spp., commonly referred to as *Halomonads,* are mesophilic rod-shaped, gram-negative halophytes which can successfully grow in environments with 5-25% NaCl (LeFevre & Round, 1919; Quesada *et al.,* 1985; Kushner & Kamekura, 1988; Ventosa *et al.,* 1998; Mata *et al.,* 2002; Ghozlan *et al.,* 2006) and pH levels between 9 and 11 (Horikoshi, 1999). They are widely distributed in lakes and salterns (Luque *et al.,* 2012). The condition in which the genus is present is similar to that of Spirulina cultivation. Only three members of the *Halomonas* genus have been described as pathogenic: *H. johnsoniae, H. stevensii* and *H. hamiltonii* and where isolated from patients undergoing dialysis (Stevens *et al.,* 2009). These bacteria are capable of reducing nitrates to nitrites, and some *Halomonas* species are also known to produce siderophores (Vraspir & Butler, 2009; Vraspir, Holt & Butler, 2011; Baggesen, 2014) which are molecules that facilitate iron uptake and help promote growth of Spirulina. Keshtacher-Liebso et al. (1995) reported improved growth of *Dunaliella bardawil* cultured with bacteria compared to cultures grown axenically. Isolate B2 was found to be closely related to *Mesorhizobium* spp., commonly found in the soil. Some of the member of this genus, such as *M. loti* is capable of fixing nitrogen from the environment (Kaneko *et al.,* 2000) and has also previously been isolated from aqueous environments (Mwirichia *et al.,* 2011). Isolate B4 was also closely related an uncultured bacterium isolated from a soil sample (He *et al.,* 2016). This uncultured bacterium was found to be able to reduce chromium and its 16S rRNA gene sequence phylogeny indicates that it is related to *Indibacter alkaliphilus*.

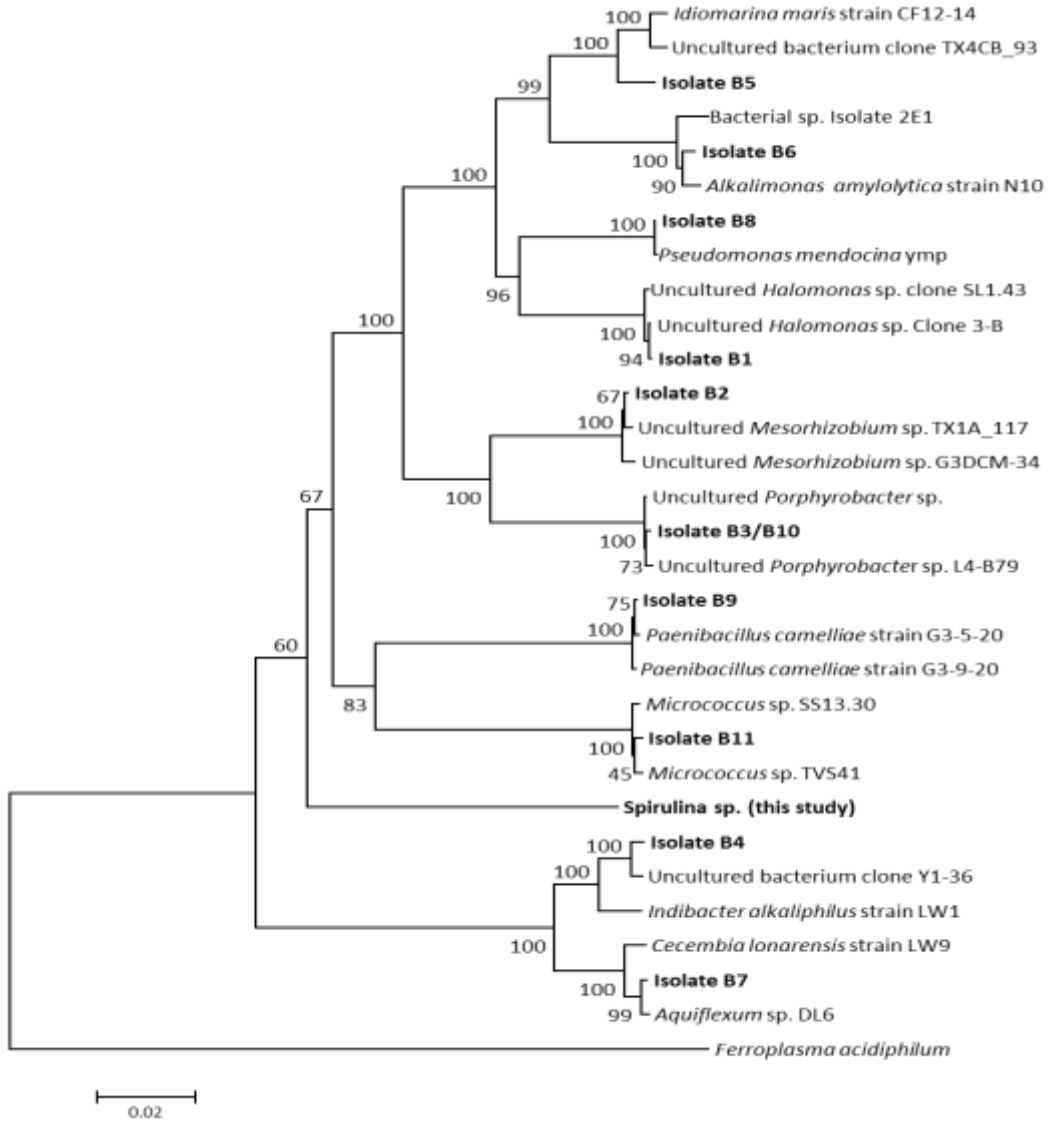


Figure 4.9: Phylogenetic analysis of the 16S rRNA sequences from the bacterial contaminants isolated from processed Spirulina powder and Spirulina cultures. A neighbour-joining phylogenetic tree was constructed using Mega 6 (Tamura *et al.,* 2013) and evolutionary distances were calculated using the Maximum Composite Likelihood method (Tamura *et al.,* 2004). The scale bar indicates branch lengths as the number of base substitutions per site.

Isolate B3, identified as the same organism as B10 by molecular analysis, was closely associated with a bacterium isolated from an environmental sample of tap water biofilm (Lin *et al.,* 2013). The most closely related species to this organism were uncultured members of the *Phorphyrobacter* genus. The isolate B5 was phylogenetically related to spp. from the genus *Idiomarina. Idiomarina maris* was first isolated from hypersaline water samples (Ivanova *et al.,* 2000) and it has also been isolated from the soda lake,

Lake Elmenteita, Kenya (Mwirichia *et al.,* 2010). This bacterium thrives at a temperature range of 35-40°C, pH 9 and 6-9% NaCl (Muruga & Anyango, 2013). *Idiomarina* spp. are Gram negative, protease-producing bacteria (Zhou *et al.,* 2009). González-Muñoz et al. (2008) investigated Ca-Mg kutnahorite and struvite production by *Idiomarina* species and demonstrated their biomineralization capability.

Isolate B6 showed close similarities with *Alkalimonas delamerensis* which is an alkaliphilic bacterium isolated from Lake Elmenteita (Mwirichia *et al.,* 2010). The genus *Alkalimonas* was first isolated from Lake Elmenteita. *Alkalimonas delamerensis* grows optimally at pH 10-10.5, 37°C and 3% NaCl. It is also a nitrate reducing bacterium (Ma *et al.,* 2004).

Isolate B7 is closely related to the red alkaliphilic *Aquiflexum* sp. DL6 and *Cecembia lonaresis.* is a gram negative aerobic and rod shaped bacterium isolated from water samples collected from Lonar This *Aquiflexum* sp. was originally isolated from and *Cecembia lonaresis* from a lake situated in the Buldhana district, Maharashtra, India (Kumar *et al.,* 2012; Shivaji *et al.,* 2012). The bacterium presented in the form of red colonies which are associated with the production of lycopene (carotenoid pigment) (Garcia-Castellanos *et al.,* 2004) recognized for its health benefits (Böhm *et al.,* 2012).

Pseudomonas mendocina is a Gram negative non-halophilic bacillus (Palleroni *et al.,* 1970) isolated from soil and water samples (Del *et al.,* 1992). It is an opportunistic pathogen (i.e only acts in certain circumstances such as lowered host resistance), causing nosocomial infections in immune compromised patients (Johansen *et al.,* 2001) but generally not harmful. The *Pseudomonas* genus is commonly known as a growth enhancer of algal cultures wherein photosynthetic oxygen is removed from *S. bicellularis* (Mouget *et al.,* 1995) and chlorophyll content increased in *Chlorella* (Watanabe *et al.,* 2005). Another member of the *Pseudomonas* family that has been found quite often in pharmaceutical products (Jimenez, 2007) and hydrocortisone ointment used for eye infection (Kallings *et al.,* 1966) is *P. aeruginosa.* Most importantly, *Pseudomonas mendocina* is also capable of degrading toluene (Ramos-Gonzalez *et al.,* 2003). Its optimal growth is reportedly at pH 6-7 and 20-30°C (Kao *et al.,* 2005).

Paenibacillus camelliae which is closely associated with isolate B9 was first isolated from a fermented green tea. The *Paenibacillus* genus represents a group of facultative

anaerobic, endospore-forming bacilli (Ash *et al.,* 1993). It is a gram positive bacterium that grows at temperatures between 15°C and 42°C, pH 6.0-10.2 and ≤ 3% NaCl (Oh *et al.,* 2008).

Isolate B11 is a round shaped Gram positive bacterium that showed 99% similarity with *Micrococcus*. *Micrococci* are generally strict aerobes and can reduce nitrate. Several *Micrococcus* species with visible pigments have been reported and are commonly found on the human skin as microflora (Smith *et al.,* 1999). Like the species we isolated in this study, *M. luteus* appeared yellow and grew in saline environments. *M. luteus* is commonly found in dust, soil, the air and as part of the human skin flora. The optimum growth temperature of *M. luteus* is 25°C-37°C and has recently been regarded as an opportunistic pathogen posing danger to immunocompromised patients ("Micrococcus luteus - encyclopedia article - Citizendium", 2015; "Micrococcus luteus - encyclopedia article - Citizendium", 2015).

Cyanobacteria cultures provide heterotrophic bacteria with EPS and other organic metabolites to grow and multiply (Hube *et al.,* 2009). They also provide them with photosynthetic products and oxygen which the heterotrophic bacteria use to degrade the organics that are released into the surrounding environment (De Philippis & Vincenzini, 1998; Paerl & Galluci, 1988). The presence of bacteria within photosynthetic cyanobacterial or microalgal cultures may give rise to beneficial or inhibitory metabolites (Salomon *et al.,* 2003). For example, a study by Qu et al. (2014) who demonstrated an improved growth of *Chlorella* when co-cultured with $< 5 \times 10^6$ cells.mL^{-1} of *Pseudomonas* and the inhibitory effect of bacterial cells at higher concentrations ($\geq 10 \times 10^6$ and 20×10^6 cells.mL^{-1}).

To assess the quality of water and food products, the typical bacterial analysis centres on the detection of the presence of easy to culture coliforms, typical contaminants of faeces that, while not typically causing illness themselves, are indicative of the presence of human pathogens. These bacterial consortia multiply at 37°C and include *E.coli, Klebsiella, Citrobacter* and *Enterobacter* (Bej *et al.,* 1990). Their presence within the cultures indicates the undesirable presence of pathogens such as *Salmonella, Shigella* and *Staphylococcus* (Payment *et al.,* 2003). However, the high pH and alkalinity serve as a good barrier against bacterial and yeast contamination (Vermorel *et al.,* 1975).

Bacterial analysis done on several Spirulina samples from the ponds on commercial farms in Thailand, Mexico, Japan and Taiwan showed that coliforms were rarely present (Jassby, 1988). However, this in itself is a partial view of the bacterial dynamics and population. Consequently, after the drying process, the food products should be further examined. A recent study investigating molecular diversity of bacteria in commercially available Spirulina products found that most were affiliated to the genera *Pseudomonas, Flavobacterium, Vibrio, Aeromonas, Clostridium, Bacillus, Fusobacterium, Enterococcus* (Vardaka *et al.,* 2016). Hoekstra et al. (2011) who investigated *Clostridium* in commercially available powder also reported the presence of five *Clostridial* species.

The high pH of the medium in which Spirulina is grown makes it difficult for bacteria to survive most especially pathogens due to their inability to survive in pH above 9. The high salinity and alkalinity of the medium also act as barriers against most bacteria (Vermorel *et al.,* 1975). Pradhan *et al.,* (2012) investigated the antibacterial activity of Spirulina extract towards pathogens and found that it may have potential use as an antibacterial agent.

The diversity of bacteria isolated from Spirulina was similar to that of saline environments such as soda lakes. *Halomonas* species often isolated from soda lakes (Mwirichia *et al.,* 2010) was also in this and other Spirulina studies (Choi *et al.,* 2008; Kawata & Aiba, 2010). The majority of bacteria isolated were gram-negative and Gammaproteobacteria. This was in agreement with Grant *et al.,* (1994). Most of the bacteria identified in this study had positive effects or contributions to the Spirulina culture. Similar studies of bacterial contaminants have been reported (Section 2.5.4). The characteristics of some isolates described by Sudha et al. (2011) were similar to isolates in this study. Bacteria isolated from different cyanobacterial species (*Oscillatoria brevis* and *Nodularia harveyana*) in a study by Hube et al. (2009) were shown to be different from this study by analysis of their 16S rRNA sequences. This showed that although the cyanobacteria and bacteria naturally live in symbiotic relationships (Imase *et al.,* 2008), different bacteria are present with different cyanobacterial species (Fukami *et al.,* 1991).

4.6 Conclusion

In this chapter, the presence of contaminating bacteria in both the liquid-based Spirulina culture and processed dried Spirulina was investigated. The methodology of monitoring bacterial load was refined to suit the purpose of this study. Thereafter, the nature of the contaminating bacteria was assessed and their behaviour during cultivation of Spirulina was monitored. From the observations presented, conclusions were drawn with respect to both the methodology used and the nature and abundance of the contaminating bacteria during the cultivation period and subsequent processing.

In the current and other studies (Torsvik et al. 1990a; Torsvik et al. 1990b), it has been shown that conventional agar plating techniques without modification resulted in underestimation of both the bacterial load and diversity. In this study, the addition of salts present in the cultivation media used in the pond to the agar media enhanced the capture of bacterial diversity. Morphological analysis and sequencing of 16s rRNA from isolates following PCR was used to characterise bacterial diversity.

The presence of contaminating bacteria was confirmed by bacterial count analyses of samples of Spirulina powder and cultures. The majority of bacteria isolated were gram-negative and Gammaproteobacteria in agreement with Grant *et al.,* (1994). The diversity of bacteria isolated from the Spirulina was similar to that of saline environments such as soda lakes. The lack of pathogens present in the contaminating bacteria characterised supports the hypothesis that the hypersaline and alkaline environment restricts the growth of pathogens. The contaminating bacteria increased concomitantly with growth of Spirulina. Most of the bacteria identified in this study were aerobic. It was concluded that the aerobic bacteria consumed the oxygen produced by the Spirulina, thereby increasing the availability of carbon dioxide for photosynthesis. As such, most of the bacteria had a symbiotic relationship with Spirulina. Initially, the bacterial count increased by an order of magnitude as the Spirulina biomass concentration increased to 1 $g.L^{-1}$. With increasing pH and the onset of regular harvesting and pH control, the bacterial count decreased to 1×10^6 $cfu.mL^{-1}$. Possible reason for this change in bacterial numbers was put forward for further investigation. These include pH conditions, alkalinity, salinity and availability of organic metabolites.

5 Results and Discussion II
Impact of physicochemical factors on growth of Spirulina and associated bacteria

5.1 Introduction

The growth dynamics of Spirulina cultures and their associated bacterial content were assessed in Chapter 4. In this chapter, physicochemical factors affecting the bacterial load are investigated, which may lead to the development of management strategies to improve the quality of cultures and the final product. Spirulina product quality was partially determined by its composition and bacterial content. Physicochemical factors such as temperature, salinity and pH were proven to influence the growth and biochemical composition of Spirulina (Zarrouk, 1966; Vonshak *et al.,* 1983, 1996; Walach *et al.,* 1987; Vonshak & Richmond, 1988; Vonshak, 1997a; De Oliveira *et al.,* 1999; Kim, Hoh, *et al.,* 2013). Temperature is referred to as one of the most important determinants of Spirulina growth, influencing the maximum specific growth rate more than other factors (Hu, 2004; Guedes *et al.,* 2011). Furthermore, it affects the metabolism and composition of Spirulina (Tomaselli *et al.,* 1987; De Oliveira *et al.,* 1999).

Salinity affects the growth, productivity, metabolic activity, biochemical pathways and composition of Spirulina (Vonshak *et al.,* 1988; Sreevani *et al.,* 2011; Mutawie, 2015). Most studies involved in the investigation of the effect of salinity on Spirulina growth are focused on NaCl as the predominant salt in hypersaline environments, lakes, ponds and Spirulina cultivation medium (Williams *et al.,* 1979; Nissenbaum, 1980; Vonshak *et al.,* 1988; Rai & Abraham, 1993; Ventosa *et al.,* 1998; Munns, 2002; Sudhir *et al.,* 2005; Ayachi *et al.,* 2007; Dhiab *et al.,* 2007; Sreevani *et al.,* 2011; Priyadarshani *et al.,* 2012; Mutawie, 2015). In this study, salinity is investigated as the total concentration of dissolved inorganic ions present in the media with modifications made to all salts. Hence these studies must be compared with caution, i.e. comparison on the basis of ionic strength only is valid; however, other parameters e.g. Na^+ concentration, recognised as an inhibitor of many unicellular systems, may be different in this work.

The pH is a key factor in the production of Spirulina (Göksan *et al.,* 2007), affecting growth, protein and chlorophyll *a* content and metabolic activities (Pelizer *et al.,* 2002;

Pandey *et al.,* 2010). The control of pH is mandatory due to its physiological effects on Spirulina. It influences the availability of nutrients and CO_2 within the culture medium and the control it has over contamination by bacteria and other foreign species (Chen & Durbin, 1994). The pH of the medium increases during photosynthesis, where there is a continual uptake of carbon and OH^- accumulation during cultivation (Richmond & Grobbelaar, 1986; Grobbelaar, 2004). The pH is generally controlled during algal cultivation by the addition of HCl, CO_2 or $NaHC0_3$ to the medium (Chen & Durbin, 1994; Pelizer *et al.,* 2003).

From the bacterial analysis presented in Chapter 4, it is evident that bacteria co-exist both in indoor and outdoor cultures and the final products. The presence of bacteria may pose a danger to the health of consumers, depending on the bacterial composition. For this reason, it is important that the bacterial load in Spirulina cultures and within the products is managed. Control of the bacterial load could be enhanced by knowing their identity (as presented in Section 4.5), which provides information about their nature and role within the systems. Their response to altered conditions can also be informative. Moheimani (2005) found that bacteria and other contaminants can be reduced by altering the physicochemical factors.

Understanding the response of Spirulina and associated bacteria to physicochemical factors could be valuable in designing systems that minimize bacterial content and improve Spirulina product quality. The impact of the key factors of temperature, pH and salinity on the growth of Spirulina and associated bacteria were assessed. Bacterial load and dry cell weight were measured in airlift reactors and shake flasks (Section 3.5.5 and 3.5.6). The factors and ranges investigated are shown in Table 3.4.

During the harvesting process, biomass is rinsed to remove excess salts, dewatered and dried. Drying of the biomass reduces bacterial load significantly (Section 5.6) (Becker, 1994a). The quality of the products can deteriorate if the proper care is not taken during these steps.

5.2 Growth of Spirulina in 3.2 L airlift reactors (ALRs)

BioDelta medium was used as the growth medium for Spirulina. Cultivation of Spirulina in airlift reactors was initiated by addition of inoculum in the exponential phase to between 0.1 and 0.2 $g.L^{-1}$. Growth of Spirulina results in an increase in pH due to CO_2

consumption by the cells. An increase in pH beyond the optimum level negatively affects the physiology of the cells (Pelizer *et al.,* 2003). Downward adjustment of pH is generally done by the addition of buffers or acids ($NaHC0_3$ or HCl in this case) or by directly bubbling the medium with CO_2-enriched air. This section investigated the effect of the pH control agents HCl and $NaHCO_3$ on the growth of Spirulina culture and associated bacterial load. Table 5.1 shows the treatment conditions in R1 to R4.

Spirulina grew rapidly across all reactors over the first 5 days of cultivation. Thereafter the growth slowed in Reactors 1 and 2 (R1 and R2) where pH was adjusted with 1M HCl (Figure 5.1a). Although Spirulina growth occurred at a slightly lower rate between days 5 and 6, 8 and 9 in R3 and R4 (adjusted with $NaHCO_3$), it continued to grow until day 14 when the experiment was stopped. Maximum dry cell weights were obtained in R3 and R4 at 3.09 and 2.96 $g.L^{-1}$ respectively. R1 and R2 had a lower biomass concentration of 2.16 $g.L^{-1}$ on day 12 and exhibited a lower growth rate from day 6 (Figure 5.1(a)). R1 and R2 had thick rings of biofilm formed just above the water level, which was evidence of high bacterial count in the culture and might have served as an on-going bacterial inoculum. Cultures R3 and R4, adjusted with $NaHCO_3$, were more stable and had slightly higher productivity than those adjusted with HCl (R1 and R2), indicating NaHCO3 to be a better option for pH control.

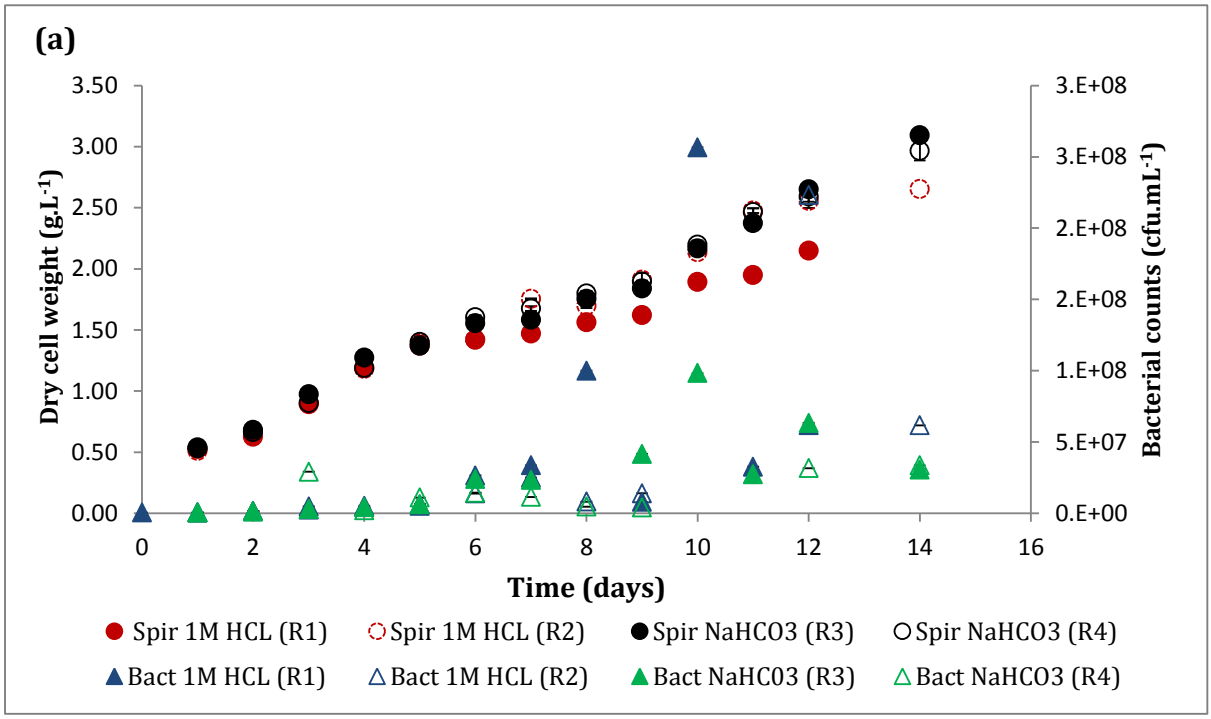
Table 5.1: Spirulina culture condition within the reactors

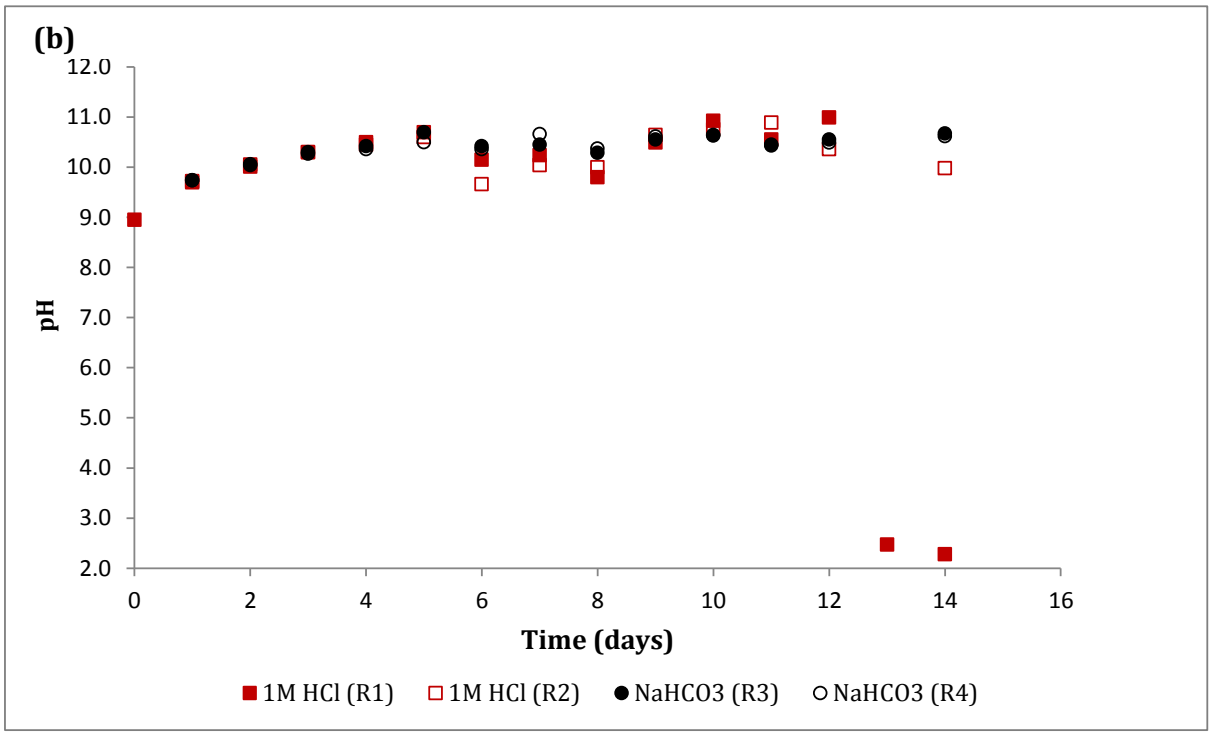
Reactors Code	Description of conditions
R1 and R2	pH of culture medium adjusted with 1M HCl
R3 and R4	pH of culture medium adjusted with $NaHCO_3$

The pH of the culture was monitored daily and increased gradually from pH 9.0 to pH 10.5, with little to no variations across all four reactors until day 5 and no pH adjustments were done over this period. The pH fluctuated more significantly in reactors adjusted by HCl than in reactors adjusted by $NaHCO_3$. This was because of the buffering capacity of the $NaHCO_3$. The HCL used in R1 was not able to restore the H^+ back to its pre-existing buffering point between day 12 and 14.

Chemical oxygen demand (COD) tests were carried out on the supernatant to determine the amount of organic compounds within the medium. The results did not show definite

trend across any of the reactors. The results showed no definite relationship between COD in solution and bacteria or Spirulina growth.





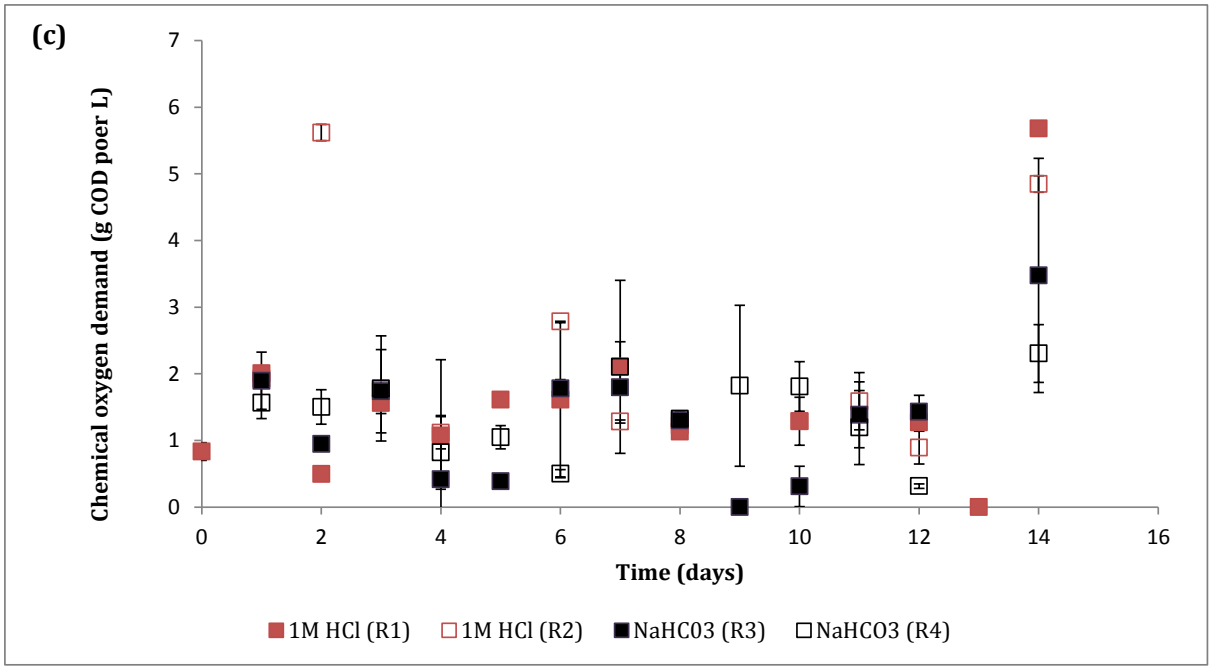


Figure 5.1: (a) Growth profile of *Spirulina* (measured as dry cell weight) and unicellular bacteria (measured as colony forming units) in ALRs (3.2 L) in the laboratory. Error bars indicate standard deviation from the average biomass; (b) Change of pH during Spirulina growth and (c) the chemical oxygen demand in the medium during cultivation to determine the amount of organics within the medium.

Due to the photosynthetic nature of microalgae and cyanobacteria, there is a need for regular supply of inorganic carbon for uptake as dissolved CO_2 or HCO_3^-. There are studies that investigated the influence of CO_2 addition on microalgae where cultures fed with more additional C achieved higher biomass than those not fed (Tin-Yao *et al.,* 2003; Chiu *et al.,* 2009). However, algal cultures are only able to tolerate CO_2 up to a certain level and concentrations above this can be detrimental. Further, CO_2 gas-liquid mass transfer is often rate-limiting. As an alternative, sodium bicarbonate can be added in its powdered form as its solubility overcomes the mass transfer limitations, hence it can be used instead of CO_2 gas (Richmond *et al.,* 1980; Hsueh *et al.,* 2007). HCl has been used to decrease pH, thus shifting the species equilibrium from carbonate which is not bioavailable, to bicarbonate or dissolved CO_2, but is not typically preferable due the inhibitory properties associated with chloride ions (Ben-Amotz, 1999).

5.3 Effect of temperature on Spirulina and associated bacterial growth

The inoculum used to initiate the shake flasks experiments was grown in BioDelta medium at room temperature (24 ± 2°C), on an orbital shaker agitated at a speed of 145

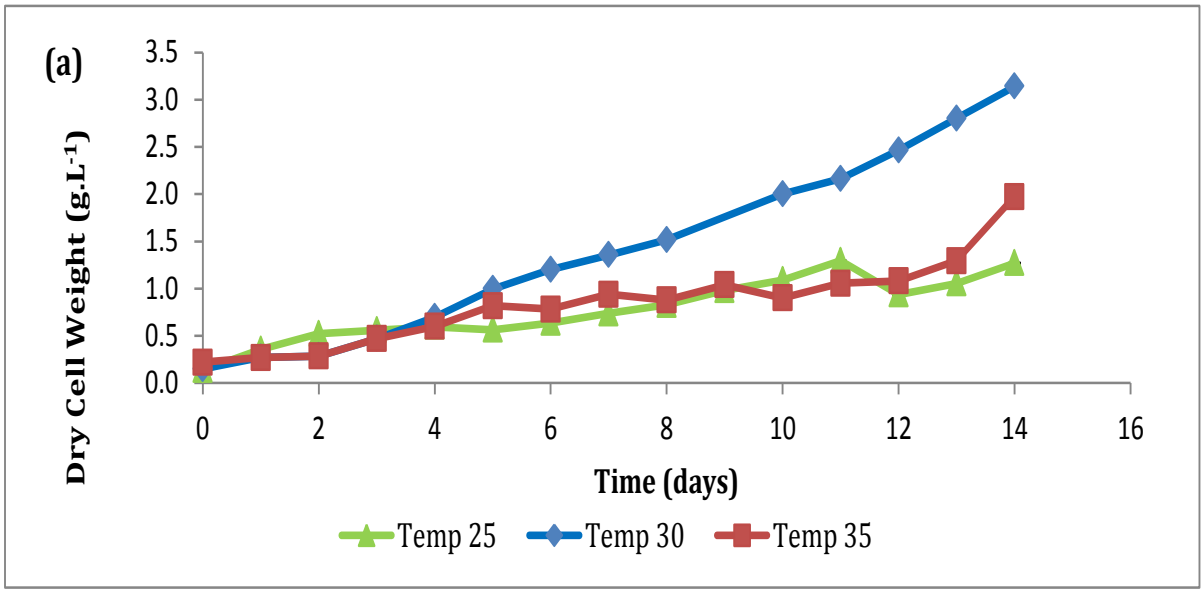
rpm. Cultures were initiated at an OD of 0.1. The pH and temperature at the start of the cultures was 9.27 ± 0.06 and 14 ppt respectively. Little lag phase was observed at each temperature tested: 25, 30 and 35°C. Among all the temperatures studied, the highest growth and a maximum cell concentration of 2.54 $g.L^{-1}$ was reached on day 14 for cultures grown at 30°C. At 25°C, the lowest level of growth was observed with a maximum biomass concentration of 1.29 $g.L^{-1}$ measured on day 11, followed by a decline in growth on day 12. The maximum cell concentration reached at 35°C was a dry cell weight of 1.97 $g.L^{-1}$ reached at day 14. The average specific growth rates at their exponential phase were 0.36 and 0.28 day^{-1} at 30°C and 35°C with productivities of 0.12, 0.19 and 0.14 $g.L^{-1}.day^{-1}$ for the 25, 30 and 35°C cultures respectively (Table 5.2). Spirulina grew actively at 30°C, until the end of the experiments while cultures at 25°C grew slower. Cultures at 35°C displayed slight fluctuations which could be attributed to adherence of cells to the walls of the flasks (Figure 5.3). Since the temperature was high, cells that were not in suspension but stuck on the hot glass suffered from thermal-inhibition, which ultimately led to death. Spirulina cells appeared to have reduced their pigmentation at 35°C.

Of the temperatures tested in this work (Figure 5.2a), the best one for growth of Spirulina was 30°C where the maximum productivity was recorded. These findings are in agreement with findings from the literature where several authors reported 30°C as the best temperature for Spirulina growth (Belay 1997; Jensen & Knutsen 1993; Danesi et al. 2002; Vonshak et al. 1996; Jain & Singh 2012). In contrast to this, Thirumala (2012) reported 35°C as the best temperature for Spirulina growth. Fu et al. (2007) also reported maximum growth together with increased chlorophyll at 35°C. However, Colla et al. (2007) and Ogbonda et al. (2007) achieved high biomass concentration of Spirulina at 30°C than 35°C.

Although this study focused on Spirulina growth and not any biochemical analysis, it is well known that temperature affects the amount of biomass generated, the morphology of the cells (from spiral to straight shaped filaments), biochemical reactions and the composition of Spirulina cells (Tomaselli *et al.,* 1987; Jensen & Knutsen, 1993; De Oliveira *et al.,* 1999). Ogbonda et al. (2007) found the highest lipid content and biomass concentration of Spirulina at 30°C during their investigation while keeping the pH at 9. Dejsungkranont et al. (2012) reported an increase in the protein, lipid and phenolic

content at 35°C over 30°C even though negative effects were noted on the biomass concentration, similarly to this study. Torzillo et al. (1991) investigated the effect of temperature on productivity and biomass loss of Spirulina taking place at night. They reported that cultures at 35°C showed higher growth than those at 25°C. In addition to this, these and other authors have reported that night-time biomass loss was not due to temperature alone but depended on the cell density and light irradiance at which they were grown (Vonshak *et al.,* 1982).

As previously mentioned, temperature is an important parameter for Spirulina growth. The optimum temperature range for maximum Spirulina growth is between 25 and 35°C (Tedesco & Duerr, 1989; Becker, 1993; Rafiqul *et al.,* 2003). Temperatures above the optimum reduce growth rates and protein synthesis while the lipid and carbohydrate content is increased (Konopka & Brock, 1978; Tedesco & Duerr, 1989; Kaixian *et al.,* 1995; Funteu *et al.,* 1997; Taha *et al.,* 2013). In addition to this, accumulation of non-chlorophyll carotenoids is usually seen under stress conditions such as at elevated temperatures (Tjahjono *et al.,* 1994; Liu & Lee, 2000; Tripathi *et al.,* 2002). Despite the adverse effect on biomass production at 35°C high temperatures are proven to be best for the production of valuable products such as lipids and carbohydrates (De Oliveira *et al.,* 1999). On the other hand, temperatures below 15°C, usually experienced at night or in the winter season, slow down the growth of Spirulina (Richmond *et al.,* 1990).



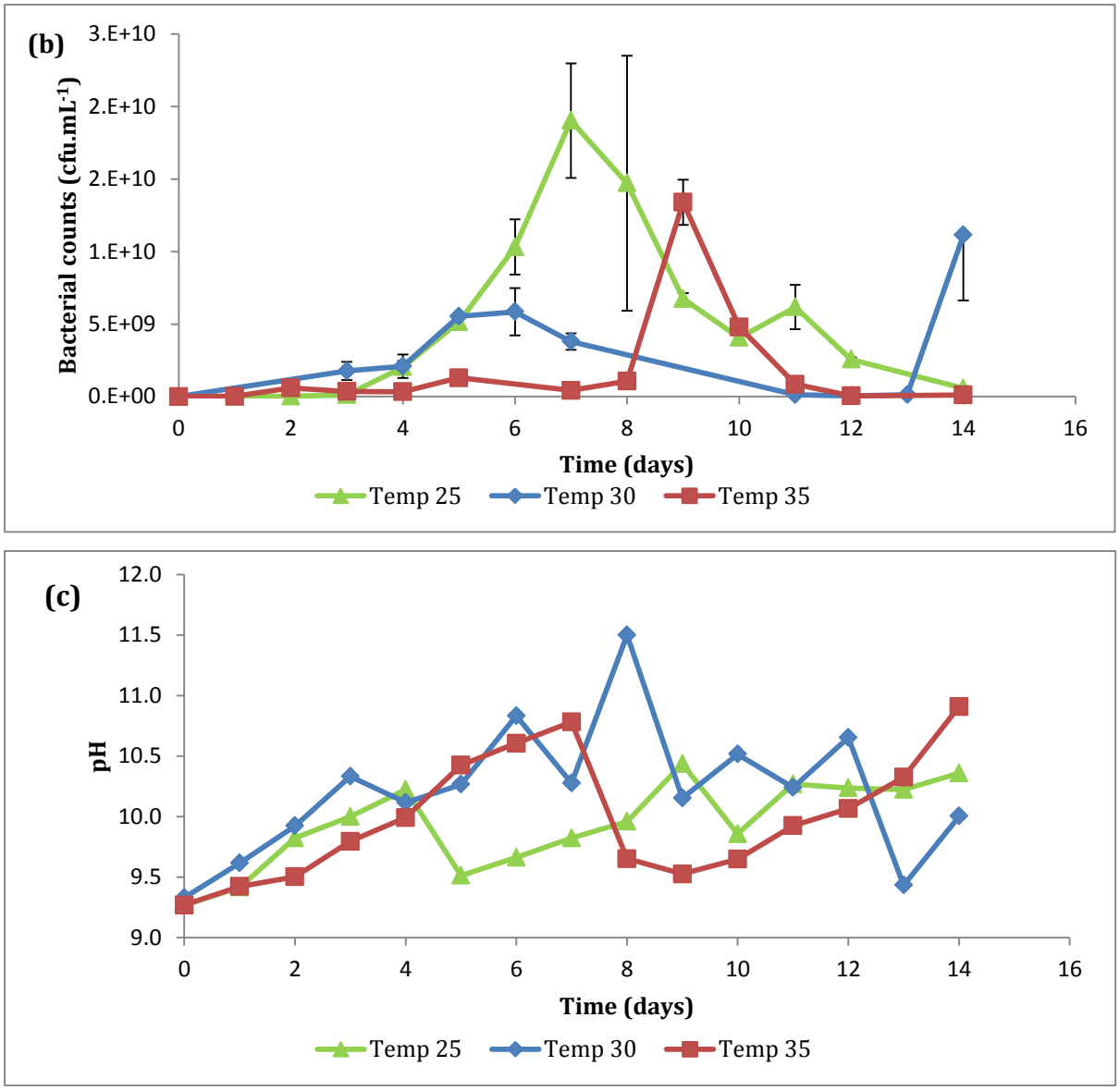


Figure 5.2: Growth profile of Spirulina (a) and bacteria (b) under varying temperature levels; 25, 30 and 35°C; (c) Change of pH with the addition of 2% CO_2 at different intervals during Spirulina growth cultivation. Error bars indicate standard deviation from the average biomass.

Figure 5.2(b) shows that the bacterial concentration did not differ much across all the temperatures until day 4. Following this, there was an increase in bacteria at 25 and 30°C which could have been due to the drop in pH for the former. Sudden decreases in bacterial load were recorded between the 7th and 10th day for both temperatures while an increase was seen at 35°C as the pH decreased below 10 due to prolonged sparging of CO_2. When the pH increased from 9.66 to 9.82, bacteria started to decrease (day 7 at 25°C). The highest bacterial loads, observed in cultures at 25°C (day 7) and 35°C (day 9), were 1.90 x 10^{10} and 1.34 x 10^{10} cfu.mL^{-1} respectively.

The pH of cultures was 9.27 ± 0.06 at the start of cultivation, and increased across all temperatures until day 4. When pH exceeded 10.3, 2% CO_2 was sparged in shake flasks until pH reached the desired level. Figure 5.2(c) demonstrates that the bacteria were more sensitive to high pH where the lowest bacterial load was observed. The pH of the culture at the lowest temperature ranged between 9.50 and 9.97 between days 4 and 9. During this period, the bacterial load showed a great increase, signifying that a pH below 10 can invite growth of pathogens (Jourdan, 2001) whereas the opposite is true (high pH decreases bacteria) as depicted between day 8 and 10 at 35°C. Similar increases in bacterial count following decrease of pH were observed for the 35°C culture at day 9 and the 30°C culture at day 13. The pH in 30°C culture was better maintained across the cultivation period and correlated with low bacterial loads throughout most of the run.

Table 5.2: Spirulina growth data at various temperatures; X_{max}; maximum biomass, μ_{max}: maximum specific growth rate, P_{max}: maximum productivity.

Variables	Temperature (°C)		
	25	30	35
X_{max} ($g.L^{-1}$)	1.29	2.35	1.97
μ_{max} (day^{-1})	0.18	0.36	0.28
P_{max} ($g.L^{-1}.day^{-1}$)	0.12	0.20	0.13
Max. bacterial count ($cfu.mL^{-1}$)	1.90×10^{10}	1.11×10^{10}	1.34×10^{10}
pH at max bacterial count	9.82	10.00	9.53

Spirulina cells forming a biofilm or ring on the sides, before mixing by hand

Dead (brown) cells in suspention after mixing by hand

Figure 5.3: Growth of Spirulina and the biofilm that forms in shake flasks at 35°C.

5.4 Effect of salinity on Spirulina and contaminating bacteria

Similarly to the other shake flask experiments, cultures were inoculated to achieve an OD of 0.1 from an exponentially-growing Spirulina inoculum cultivated in BioDelta medium. Slow increase in biomass concentration was observed across the various salinities. Following brief exponential growth, subsequent linear growth became apparent from day 5 and continued until day 9. During the initial period, the Spirulina cells increased with time and without any limiting factors. Following day 5, it is postulated that either light or CO_2 mass transfer for additional carbon supply were rate-limiting and the linear growth was correlated with the linear supply rate. According to the growth profiles depicted by Figure 5.4(a), cultures had a similar growth pattern across all salinity levels tested. The best growth of Spirulina occurred at salinity of 14 ppt (standard medium) and yielded the highest dry cell weight and average specific growth rate of 1.37 $g.L^{-1}$ and 0.25 day^{-1} respectively. Kouhgardi et al. (2015) investigated the effect of different salinities on Spirulina and reported that the best growth in terms of biomass concentration was at 15 ppt, which is close to the salinity of BioDelta medium. The maximum specific growth rates were determined to characterise the exponential growth of the cultures and the productivities were used to compare growth under limiting conditions. The maximum cell concentration and average specific growth rate for cultures at the highest salinity, 28 ppt was 1.37 $g.L^{-1}$ and 0.23 day^{-1}. Similarly, a maximum cell concentration of 1.37 $g.L^{-1}$ (13^{th} day) and average specific growth rate of 0.24 day^{-1} were recorded at 7 ppt. The productivities achieved across the different salinity tests were 0.094 ± 0.029 $g.L^{-1}.day^{-1}$, 0.122 ± 0.018 $g.L^{-1}.day^{-1}$ and 0.114 ± 0.018 $g.L^{-1}.day^{-1}$ for salinity at 7, 14 and 28 ppt respectively across the exponential phase.

Most cyanobacteria are able to survive in various saline environments (Blumwald & Tel-Or, 1982). According to several authors, high salinities (i.e. > 25 ppt) were growth inhibiting while no negative effects were seen at low salinities (Vonshak *et al.,* 1996; Kebede, 1997; Rafiqul *et al.,* 2003; Mahrouqi *et al.,* 2015). Despite the negative effects of high salinity, growth of undesired microorganisms such as competitors and predators were found to be inhibited under these conditions and, by manipulating the salinity of the medium, growth of the desired species could be enhanced above that of competing

contaminants (Chen, 1996). Under ideal conditions, the cellular osmotic composition is in balance with the surrounding medium. Under imbalanced circumstances, cells experience osmotic stress. This can lead to physiological changes which cause enhanced or reduced production of biochemical compounds and challenge the health of the Spirulina cells (Lee *et al.,* 2001; Shalaby *et al.,* 2010; Pade & Hagemann, 2015).

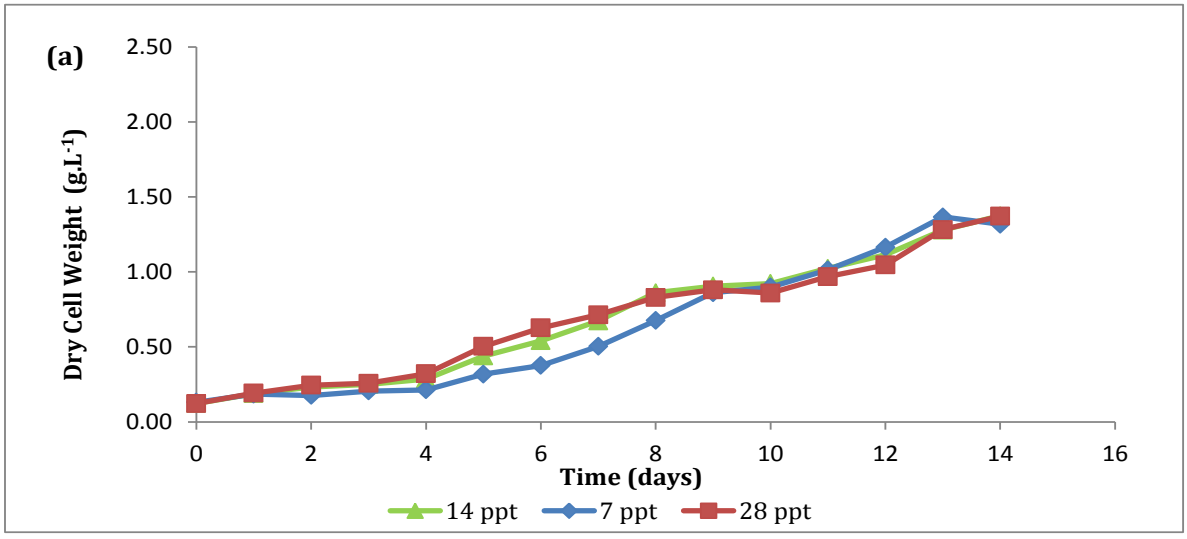
Lack or excess of salts in the culture medium may cause osmotic stress. Excess salts, leading to high salinities, stimulate the biosynthesis of carbohydrates while inhibiting fatty lipids and protein biosynthesis (Zeng & Vonshak 1998; Vonshak 1997; Kebede 1997; Shalaby et al. 2010; Ayachi et al. 2007; Priyadarshani et al. 2012). These findings are in agreement with other studies that investigated the physiological response of Spirulina to increased salt concentration (Vonshak *et al.,* 1988, 1996). High concentrations of inorganic ions also have a toxic effect on cellular metabolism. These studies demonstrated that an increase in salt concentration reduces growth, photosynthetic efficiency and phycobilin and chlorophyll content. According to Moradi & Ismail (2007), the reduced chlorophyll content at higher salinities was due to a decrease in photosynthetic rate because of osmotic stress. Spirulina growth is reported to be better at low salinities (<20 ppt) over high salinities (> 25 ppt) (Kouhgardi et al. 2015).

There are two mechanisms used by microorganisms to adapt to the changing salinities: the salt-in and salt-out mechanism. In environments with low salinity levels, large inorganic molecules accumulate within the cytoplasm of cells to ensure water uptake and turgor pressure. The salt-out mechanism which occurs in salt-stressed cells, help keep the internal ion concentration low by accumulating small organic molecules called 'compatible solutes' within the cells while actively exporting inorganic ions into the cytoplasm (Galinski & Trüper, 1994; Hagemann, 2011). Cyanobacteria use the latter to adapt to high salinities and keep osmotic balance (Pade & Hagemann, 2015). High salinities are useful where the main aim is to grow biomass for longer periods without nutrient limitation or depletion and to reduce bacterial contamination. Where rich bicarbonate medium is involved, a good buffering system is also maintained. All salinity treatments used in this study, including the lowest salinity level, showed no significant differences in biomass concentration, indicating a great possibility of saving on nutrient costs, especially in large scale production

The data for Spirulina growth at the varying salinity levels is given in Figure 5.4 and Table 5.3. The bacterial load did not differ significantly across the salinity tests until the 5th day. Bacterial load was highest at salinities of 14 and 28 ppt (1.31×10^9 and 7.53×10^8 cfu.mL^{-1} respectively on day 11) compared to 1.32×10^7 cfu.mL^{-1} at 7 ppt. The bacterial load at 28 ppt increased from day 5 (4.93×10^7 cfu.mL^{-1}) to day 7 (5.90×10^8 cfu.mL^{-1}). At the end of the run, the bacterial load had decreased in all cases. Generally, the lowest bacterial count is expected at the highest salinity, but that was not seen in these data. The salinity range may not have been sufficiently high or the bacteria present may have been salt tolerant, since acclimatised to a salt medium.

Table 5.3: Spirulina growth data at various salinity levels.

Variables	Salinity (ppt)		
	7	**14**	**28**
X_{max} (g.L^{-1})	1.37	1.37	1.37
u_{max} (day^{-1})	0.24	0.25	0.23
P_{max} (g.L^{-1}.day^{-1})	0.09	0.12	0.11
Max. Bacterial count (cfu.mL^{-1})	4.43×10^8	13.1×10^8	7.53×10^8
pH at max bacterial count	10.52	10.37	10.03



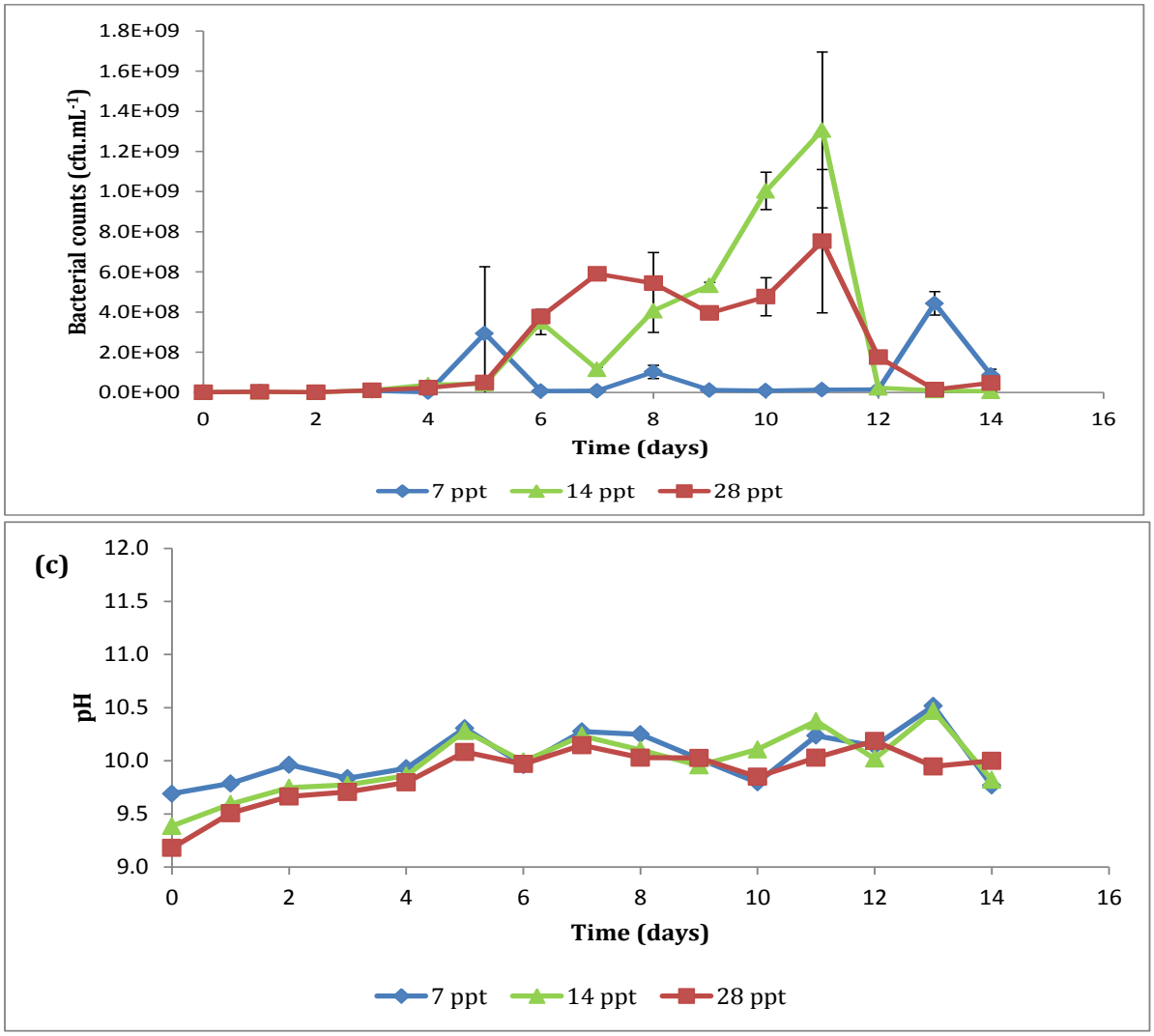


Figure 5.4: Growth profile of Spirulina (a) and bacteria (b) under varying salinity levels; (c) Change of pH with the addition of 2% CO_2 at different intervals during Spirulina growth cultivation. Error bars indicate standard deviation from the average biomass .

The pH of of the media differed right from the first day of Spirulina cultivation due to the difference in buffering capacity as a result of the varying amounts of bicarbonate ions ($NaHCO_3$) where the 7 ppt culture was poorer than in 14 and 28 ppt, with the latter showing the best buffering capacity. As expected, the starting pH at 7 ppt was pH 9.69 which was the highest, 14 ppt at 9.39 and 28 ppt at 9.18 being the lowest. During cultivation, the pH was maintaned between 10 and 10.3. When the pH increased above 10.3, it was brought down to pH 10 by sparging cultures with 2% CO_2 . The salinity levels tested did not show any major difference in terms of growth of Spirulina, except at the exponential phase where growth in the medium with the least salinity lagged behind for 4 days.

5.5 Effect of pH on *Spirulina* and contaminating bacteria

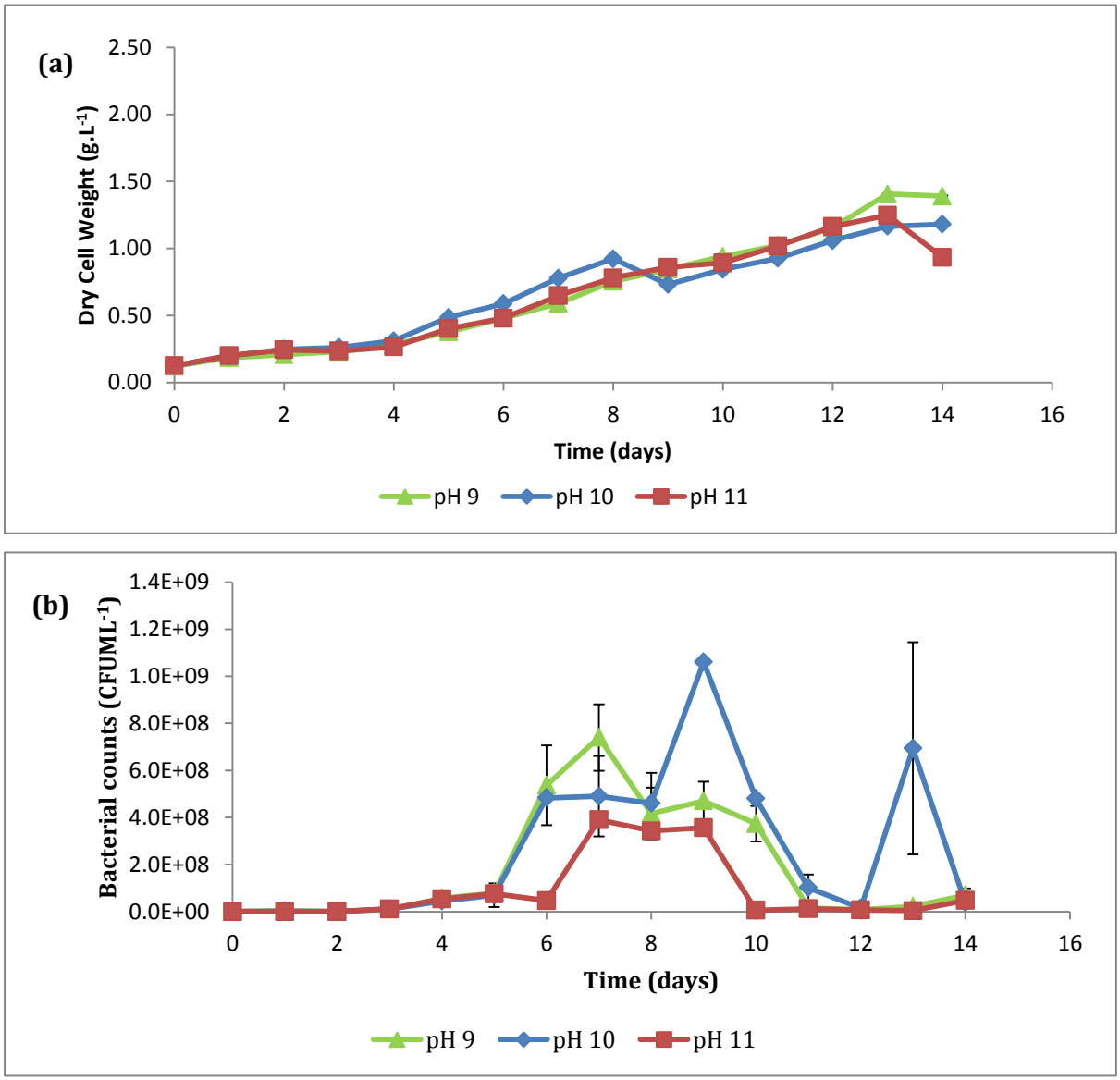
Shake flask cultures were set-up and inoculated in an identical manner and allowed to grow until they reached the pH of interest. This pH was then maintained by the addition of 2% CO_2. Figure 5.5 shows the effect of nominal pH in the range pH 9 to pH 11 on growths of Spirulina and contaminating bacteria where pHs of approximately pH 9.5, 9.8 – 10.2 and 10.6-11.4 were maintained. The growth of Spirulina followed a similar trend across all pH levels (Figure 5.5a). The highest maximum cell concentration of 1.41 $g.L^{-1}$ was obtained at pH 9 on day 13. From day 13, the pH 9 culture reached stationary phase while a decline was observed for the pH 11 culture, albeit for only a single data point. The dry cell weight at pH 10 was highest during the exponential phase from the 4^{th} to the 8^{th} day. For periods between day 4 and 8 and between day 9 and 11, productivity was similar. A decrease biomass concentration (therefore, productivity) was observed between day 8 and day 9. Similar average specific growth rates were observed at all pHs tested (pH 10 as 0.25 day^{-1} and pH 9 and 11 as 0.24 day^{-1})(Table 5.4). The pH 11 culture had the lowest final biomass concentration. The pH across the range pH 9 to 11 showed no significant effect on the growth of Spirulina.

Spirulina requires a high pH (between 9 and 11) to thrive, making it an alkaliphilic organism (Grant *et al.,* 1990). Pandey et al. (2010), who investigated the effect of pH and light intensity on the biomass production of Spirulina, reported pH 9 as the ideal pH for maximum growth, increased chlorophyll *a* and protein content. Belkin & Boussiba (1971) reported Spirulina's optimum pH as 9-9.5 which is in agreement with our findings. A high pH is not only suitable for the best Spirulina growth but it also minimises contamination by bacteria, pathogens and other algae (Ciferri 1983; Sornchai & Iamtham 2013; Supramaniyan & Jeeji Bai 1992; Oswald 1988).

Table 5.4: Spirulina growth data at various pH levels.

Variables	pH		
	9	10	11
X_{max} ($g.L^{-1}$)	1.41	1.18	0.93
u_{max} (day^{-1})	0.24	0.25	0.24
P_{max} ($g.L^{-1}.day^{-1}$)	0.11	0.08	0.11
Max. Bacterial count ($cfu.mL^{-1}$)	4.43×10^8	1.06×10^9	3.90×10^8
pH at max bacterial count	9.85	10.10	10.92

The bacterial load did not show any significant difference in the first five days of culture (Figure 5.5b). The highest bacterial counts at pH 9 and 10 were 4.43×10^8 and 1.06×10^9 cfu.mL^{-1} respectively which happened around the same time as the increase seen in salinity tests i.e. day 5 to 10. The later increase to 6.93×10^8 cfu.mL^{-1} on day 11 at pH 10 had a large error of $\pm 2.60 \times 10^8$. Bacterial concentration was lowest at pH 11. Overall, pH 11 kept the bacteria at low counts and Spirulina grew relatively well.



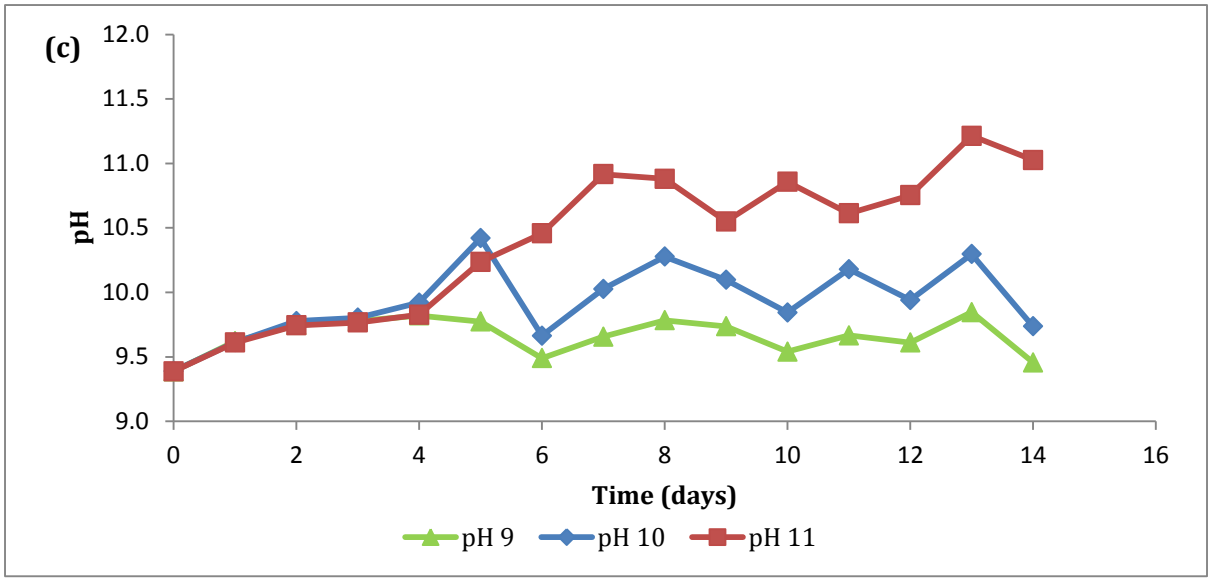


Figure 5.5: (a) Growth profile of Spirulina and bacteria under varying pH levels: pH 9, 10 and 11; (c) Maintenace of pH levels set with the addition of 2% CO_2 at different intervals during Spirulina growth cultivation. Error bars indicate standard deviation from the average biomass.

The pH of the cultures showed a similar trend until day 4 from which different pH levels were controlled (Figure 5.5c). While cultures maintained at pH 10 and 11 were left to reach their respective pH, pH 9 was reduced by the addition of 2% CO_2. For growth of Spirulina to occur, it requires CO_2 where the continual uptake of CO_2 from the medium causes the pH to rise. Although practical difficulty was experienced in keeping the pH exactly at pH 9, 10 and 11, they were all distinctly separated hence useful information was obtained.

Monitoring of pH is critical in microalgal and cyanobacterial culture. The pH affects the growth, biomass production and metabolism of Spirulina (Ogbonda *et al.,* 2007). Growing cultures at a very high or low pH keeps the medium selective, reducing chances of contamination and keeping contaminants at low densities (Goldman *et al.,* 1982; Ogbonda *et al.,* 2007).

5.6 Effect of drying on bacterial load in Spirulina biomass

Spirulina biomass meant for human consumption and animal feed is generally dried, to extend the shelf life and ensure reduced bacterial load. The effect of various drying methods on bacterial load was investigated. Samples of equal weight (25 g) of wet Spirulina biomass were oven dried at 45, 55 and 65°C and freeze dried overnight

respectively. The drying process was done until the biomass reached a constant mass. No test was done to ensure that the biochemical composition was not altered by the drying methods and temperature. However, it should be emphasised that drying methods and temperature are important in influencing the lipids, other important compounds and the quality of the final product (Tomaselli *et al.,* 1988; De Oliveira *et al.,* 1999; Ogbonda *et al.,* 2007).

Drying of biomass was able to reduce the amount of viable bacteria within the samples. Immediate freeze drying of samples reduced bacterial load from 40.5×10^6 cfu.g^{-1} of dry biomass to 1.2×10^6 cfu.g^{-1} of dry biomass while oven drying at 45 and 55°C reduced it to 1.35×10^6 and 1.36×10^6 cfu.g^{-1} of dry biomass respectively. Bacterial loads obtained during freeze-drying and oven-drying at 45 to 65°C were similar. Temperatures above 65°C are not recommended due to the effect on the product quality (protein denaturation and vitamin degradation).

Lower temperatures (45 and 55°C) could allow for bacteria to multiply before their death phase is reached during oven drying. This makes the time of drying and nature of the process important, placing emphasis on design of the drying ovens. Large scale freeze drying is exceedingly expensive and is not advisable. Spray drying, not investigated here, may be a good option. Incubation or storing of biomass, especially at elevated temperatures where growth is rapid, may increase the bacterial load and as a result have a negative effect on the quality of the final product. The samples incubated at room temperature for 3 or 6 h had a lower bacterial load than those incubated at 30°C for 3 or 6 h respectively. Bacterial load increased with increase in temperature and incubation period.

These results indicate that the harvested biomass should be dried as soon as possible and not be left standing in the sun or at room temperature (which may occur in a non-optimised commercial operation). A practice such as this helps avoid undesirable increases in bacterial load and deterioration of the quality of the product.

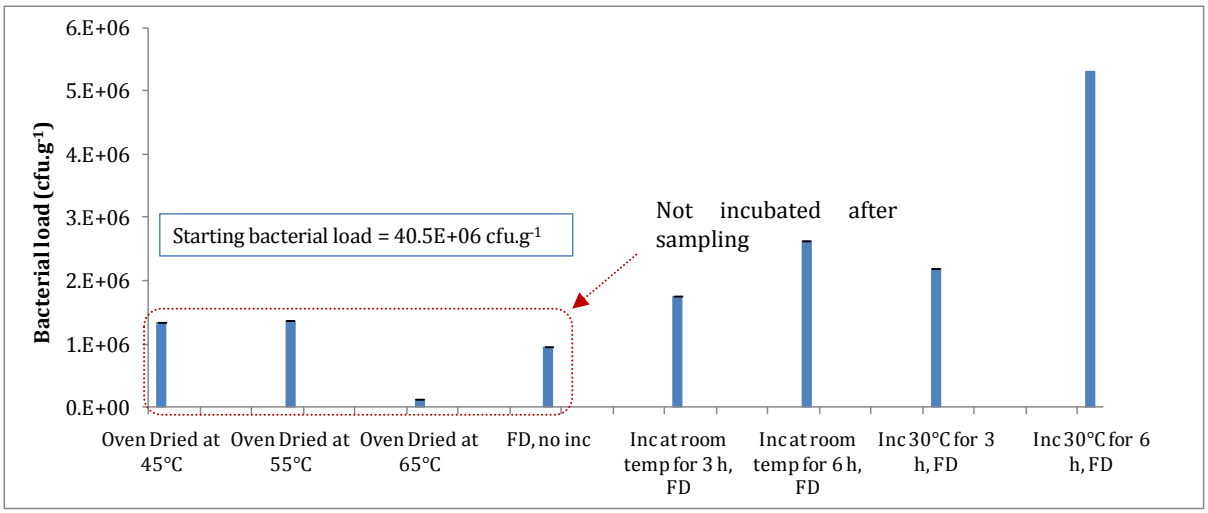


Figure 5.6: Bacterial load in dried Spirulina biomass. The results shown here are averages of tests done in triplicate with each test measured in triplicate. Error bars indicate standard deviation from the average bacterial load. FD: Freeze Dried.

5.7 Conclusion

An initial study investigated the effect of pH control by the addition of HCl and $NaHCO_3$. $NaHCO_3$ proved to have better buffering capacity than HCl. $NaHCO_3$ proved to be the best option and should be used going forward.

Analysis of the effect of physicochemical factors on the relative growth of Spirulina and bacterial growth showed that Spirulina grew best at 30°C and temperature in the range 25 to 35°C did not have a significant effect on bacterial load. Spirulina grew well across all salinities with no significant effect on Spirulina growth while bacterial load was low at low salinities. The various pH levels tested did not have a significant effect on Spirulina growth, however, a decrease in bacterial growth was seen at pH 11 compared to pH 9 and 10. Drying of Spirulina biomass reduces bacterial load in the final product. Freeze drying and oven drying at 45 and 55°C reduced the bacterial load almost equally. Oven drying at 65°C reduced the bacterial load even more significantly; however, further investigation into the resultant quality of the final product dried at 65°C is required. Biomass incubated at room temperature for several hours before drying showed a large increase in bacterial content. Harvested biomass should thus be dried as soon and as rapidly as possible following harvesting to prevent increase in contamination. Further, temperature should be minimised during any holding period.

6 Conclusions and Recommendations

6.1 Introduction

Arthrospira platensis is a filamentous cyanobacterium that grows in saline environments with warm climates at high pH. It is a source of multiple vitamins, growth factors and other nutraceutical compounds and has high protein content, hence is important in the nutraceutical industry. As a result, it is among species that are commercially exploited in the biotechnology industry. Commercial production of Spirulina is often carried out by cultivation in outdoor open systems, predominantly in large open ponds where they are susceptible to bacterial contamination. Subsequently, Spirulina biomass is harvested and dried. Spirulina biomass and final products derived from it that are targeted for human consumption and nutraceuticals need to be of food grade. Contamination by bacteria is undesirable and contamination by pathogens unacceptable.

This study comprised of two main components, namely: to determine the extent and nature of bacterial contamination and investigate approaches to controlling the bacterial contamination in Spirulina cultures and products. The conclusions drawn from the findings of the study are presented in Section 6.2 and Section 6.3 respectively. Future work and recommendations are presented in Section 6.4.

6.2 Bacterial contaminants in Spirulina cultures: Their characteristics, identity and behaviour during outdoor cultivation

6.2.1 Methodology for the quantification and identification of bacterial load in Spirulina processed powder

The effects of sample handling on bacterial count in Spirulina cultures were investigated. This was done with the aim of establishing appropriate methods for the quantification experiments. This study informed the standardisation of the handling methods such that relative bacterial data used to assess bacterial growth are not biased. It was established that exposure of samples to the sun or holding of the samples at ambient or slightly elevated temperature after sampling did affect the results of bacterial counts. The samples taken for bacterial load analysis in subsequent experiments were protected from exposure of the sun and cooled on transport following sampling.

The investigation of different media showed that the 'salty' medium is required to determine the dominant cell population present in the Spirulina culture while nutrient agar is important for the quantification of potential human pathogens. Further, these findings inform good practice in the production process. Bacterial analysis of the processed powder showed the highest and lowest bacterial loads as 6.2×10^7 cfu.g^{-1} and 4.3×10^4 cfu.g^{-1} respectively, which indicates that control of bacteria is possible. This confirmed that bacteria are present in the Spirulina culture and able to survive the production process but not in dangerous quantities. The high bacterial numbers obtained when using 'salty' medium suggests that these bacterial contaminants are halophiles and not pathogens. The nutrient agar cultivation supported no general pathogen threat with only one instance of a single opportunistic pathogen being found.

6.2.2 Identification of bacteria associated with outdoor cultivated Spirulina cultures

Identification of bacterial isolates revealed that bacteria are found in Spirulina cultures. The majority of bacteria in Spirulina powder and cultures were gram negative and aerobic. Their diversity was similar to that of saline environments and none were pathogenic. This was expected since the cultivation of Spirulina is carried out in 'salty' media. There may be more species present than identified because only clone libraries of the 16S rRNA gene fragments were used to identify the dominant species. It was thus concluded that non-halophiles are not a threat during outdoor cultivation of Spirulina. Further, halophiles found in the Spirulina cultures would not be able to grow inside the human body due to unfavourable conditions. The bacteria found in Spirulina cultures during pond culture therefore pose no danger to consumers (i.e. non-pathogenic to humans). It was concluded that any pathogenic bacteria found in Spirulina products would be from downstream processing of the biomass when in contact with the human skin or equipment that is not well maintained. These were not found in this processing facility. From all the findings outlined above, it was concluded further that salinity can be used to control the nature of the bacterial communities found in Spirulina cultures.

6.2.3 Behaviour of contaminating bacteria during outdoor raceway pond cultivation of Spirulina

Bacterial load and Spirulina growth (dry cell weight) analyses were conducted on a regular basis during outdoor cultivation of Spirulina. The increase in Spirulina cells was accompanied by increase of bacterial load in the early stage of pond growth, prior to maintenance of pH or harvesting of biomass. It can be concluded that the growth of Spirulina was not impeded by the presence of contaminating bacteria.

It was observed that while the bacterial load generally trends up during the cultivation of Spirulina, analyses of the bacterial counts also showed a cyclical nature. Night-day cycles have an effect on the photosynthetic activities of the Spirulina which affects other metabolic activities of Spirulina. It is known that the aerobic bacteria consume oxygen produced by Spirulina increasing the availability of carbon dioxide for photosynthesis in return. Furthermore, it has been shown that the metabolic activities of Spirulina can influence the pH and salinity of the growth medium (the growth environment of the bacteria). As a result, it was concluded that the cyclic nature of the bacterial load during Spirulina cultivation could be attributed to the variability in physicochemical conditions such as the availability of oxygen, temperature, pH and salinity. These results suggested that the nature of the relationship between Spirulina and bacteria during cultivation was symbiotic. Subsequent experiments sought to establish the source of bacterial contamination and the (growth) characteristics of the contaminants to provide insight into the potential control measures.

6.3 Impact of physicochemical factors on growth of Spirulina and associated bacteria

6.3.1 Growth of Spirulina in 3.2 L airlift reactors (ALRs): pH control

The influence of pH on carbon speciation in terms of dissolved CO_2, HCO_3^- and CO_3^{2-}, within culture reactors makes monitoring of pH essential during Spirulina cultivation period due to pH fluctuation**.** Further only the former two species are bioavailable to Spirulina. The effect of pH control agents (1M HCl and $NaHCO_3$) on the growth of Spirulina culture and associated bacteria was investigated. It was established that the growth of Spirulina was higher in $NaHCO_3$ buffered medium than in HCl buffered medium. Bacteria were better controlled in $NaHCO_3$ buffered medium than in the HCl

controlled medium. Therefore, it was concluded that $NaHCO_3$ was a better pH buffering agent for growth of Spirulina and inhibition of bacterial load than HCl.

6.3.2 Effect of temperature, salinity and pH on relative growth of Spirulina and bacteria

Temperature influences the production of Spirulina biomass. Of all the temperatures investigated (25°C, 30°C and 35°C), Spirulina grew best at 30°C with the highest biomass productivity. No significant effects of temperature on bacterial load were observed; effect of pH on bacterial growth overshadowed the changes due to temperature.

The effect of salinity was investigated by increasing the overall salt load in the medium in proportion to the media components and not by using NaCl as usually reported. The salinity levels for these experiments were chosen based on the expected requirements for Spirulina growth and the feasibility of bearing the extra cost of media comments and salts in the Spirulina cultivation process, recognising that this is the dominant operating cost (Griffiths and Harrison, personal communication). In addition, the load of higher salinities on effluent treatments adds further cost and environmental burden. The approach used here also reflected the increasing salinity of the medium with recycle and evaporation as well as addressing levels necessary to support biomass growth. The levels investigated ranged from half to twice that in the standard commercial medium. Spirulina grew well across all salinities (7, 14 and 28 parts per thousand (ppt)), with no significant effect on Spirulina growth. Bacterial loads were low at low salinities. The two higher salinities showed bacterial loads typical of the standard conditions. Increase in salinity of the media by addition of salts over the range studied did not increase the Spirulina growth or biomass concentration nor reduce the bacterial concentration, as expected. This finding suggested that these bacteria were halophiles and not pathogenic. Typical pathogens commonly found in food are not halophiles. The use of the lower salinity is thus preferred as it provided a reduction in the cost of the production in terms of media costs and waste treatment costs. Furthermore, bacterial load in Spirulina can be reduced by lowering the salinity of the growth medium down to 7 ppt without compromising the Spirulina biomass productivity. Therefore salinity cannot be used to remove these halophiles.

The pH levels of pH 9, 10 and11 tested did not show any significant effect on Spirulina growth. A significant effect of pH on the growth of bacteria was seen where bacterial load was significantly reduced at pH 11. This is supported by the data collected under different temperatures where bacterial load increased as pH decreased to pH 10 and below. The effect of pH on bacterial load was more noticeable than the effect of salinity and temperature. It was concluded that of all factors investigated, pH was the best in reducing the load of contaminants during cultivation of Spirulina without compromising biomass productivity.

6.3.3 Effect of drying on bacterial load in Spirulina biomass

The drying of Spirulina biomass follows the cultivation and harvesting steps in the production process. The effect of drying conditions on bacterial load associated with Spirulina cultivation was investigated to assess the impact of the drying process. It was discovered that the bacterial load obtained after freeze-drying and oven-drying Spirulina biomass at 45 and 55°C were not significantly different. Oven-drying at 65°C reduced the bacterial load significantly. However, it is well known that high temperature can denature proteins and degrade vitamins, hence the quality of the product dried at 65°C must be considered. It was concluded that freeze-drying is the most appropriate biomass drying method for the reduction of bacterial contaminants without compromising the nutraceutical value of the Spirulina biomass.

Storing or incubating samples at room temperature or 30°C for several hours before drying showed a large increase in bacterial load, with a greater increase with increased temperature. These findings implied that careful attention should be paid to the harvested biomass. Furthermore, harvested biomass should be dried immediately and not allowed to incubate prior to drying. These findings also suggest that the limited reduction in bacterial load during oven drying at 45 and 55°C may be the result of a prior increase in bacterial loading during the heating process. Further studies on the nature and rate of the drying process will be valuable.

6.4 Future work and Recommendations

Based on the findings and conclusions drawn from this study, the following recommendations were made:

- Samples collected for analyses of bacterial load should not be exposed to the sun and should be kept in a cool place and processed as quickly as possible prior to analysis in order to avoid bias introduced by sample handling.
- 'Salty' medium with similar salt levels to the Spirulina cultivation medium should be used when plating samples in analyses to identify bacterial communities present in Spirulina cultures representatively to ensure cultivation of halophilic bacterial communities that exist in the sample. Simultaneously, standard culture media should be used to ensure identification of pathogens.
- Where possible, Spirulina cultivation should be carried out at temperature of 30 °C for optimum biomass productivity.
- Cultivation media with salinity, based on media salts, around 7 ppt should be used as it favours reduced bacterial load and saves on production costs by requiring lower quantities of salts to be used while minimising wastewater treatment requirements.
- The pH of the cultivation medium should be used as the main manipulator of the load of bacterial contaminants during Spirulina cultivation as it has a significant effect on bacterial load. At pH 11, the bacterial load is significantly lower than at lower pHs.
- Where possible, freeze drying should be used as the most effective drying method of those investigated for further reducing bacterial load in the final product after harvesting. Where oven drying is used, a higher temperature of 65°C results in lower contamination than drying at temperatures closer to ambient e.g. 45°C; however, the nutraceutical value of the biomass may be compromised.

Considering both the findings and limited scope of the current study, the following aspects are recommended to be investigated further:

- Monitoring the diversity of the bacterial community present in the Spirulina cultures throughout the production process, in addition to the bacterial population, is expected to provide further insight into control of contamination. A metagenomics approach make be useful in this extended study.
- The effect of liberation of organic compounds from Spirulina cells during Spirulina cultivation, caused by overflow metabolism, hampered growth or cell

lysis, on bacterial contamination levels, in terms of bacterial load and community structure resulting, should be studied.

- Process conditions should be investigated very carefully to make sure that there are no conditions conducive for bacterial contaminants to thrive. This includes determining conditions under which structural integrity of Spirulina cells is not maintained to enable these to be avoided. Further, detailed monitoring of process conditions is essential to enable correlation of the effect of process conditions on bacterial contamination.
- The effect of salinity should also be investigated through carbonate and NaCl loading.
- The temperature and moisture profiles of drying conditions should be recorded and correlated to bacterial contamination. Further, extension of drying apparatus to include spray-drying and convection ovens should be considered.

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8 Appendix

8.1 Appendix A: Supplementary information for Chapter 3

8.1.1 Outdoor raceway pond (50 000 L) used for Spirulina production: dimensions and mixing study

The pond dimension and layout (geometry) of the 50 000 L outdoor raceway pond used for Spirulina production in this study is given below. The mixing time was calculated using NaCl and a conductivity probe.

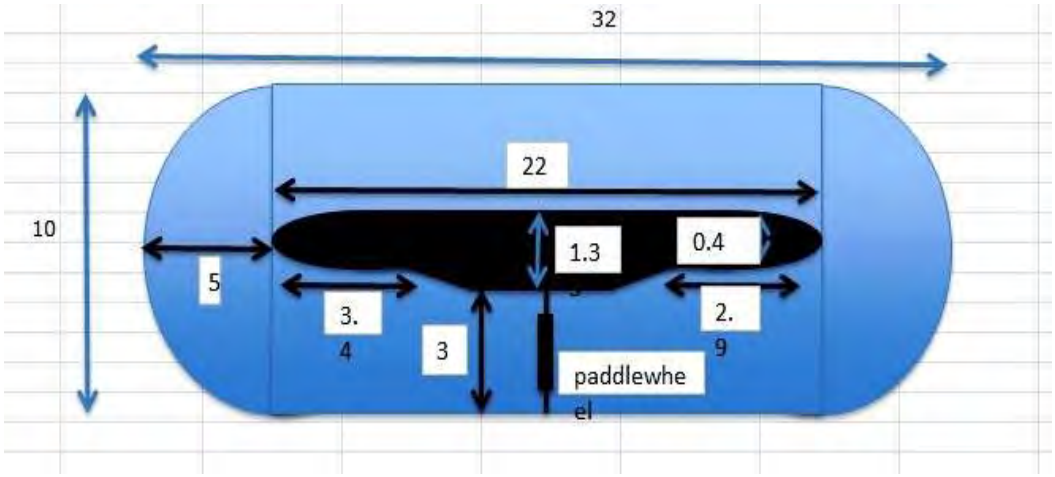

Figure 8.1: 50 000 L pond used to grow Spirulina

Calculation of linear velocity in 50 000 L pond (Melinda Griffiths, 2014)

Mixing time= 4 mins or 240s

Length of pond- length of island= (32-22) m =10 m (diameter of the full circle)

Diameter of the semi-circle= 5 m

Radius of the semi-circle= 2.5 m

$Cicumference = \pi d$ or

$Cicumference = 2\pi r$

=2x3.14x2.5

=15.7

Mixing velocity of the contents= 2 x 22 (length of island) + circumference

= (2 x 22 + 15.7) m

$= \frac{59.7\ m}{240s}$

=0.24875 $m.s^{-1}$ = 25 $cm.s^{-1}$

8.1.2 Total alkalinity test used during process monitoring

The method used to test total alkalinity was adopted from Snoeyink & Jenkins (1980). To determine the total alkalinity of samples, samples are titrated with sulphuric acid (H_2SO_4) or hydrochloric acid (HCl) to pH 4.5 and the volume of the acid used for the titration recorded.

The following reactions occur during titration:

At a pH above 8.3:

$$\boldsymbol{OH^- + H^+ \rightarrow H_2O} \qquad \textbf{Equation 8.1}$$

$$CO_3^{2-} + H^+ \rightarrow HCO_3^- \qquad \textbf{Equation 8.2}$$

At a pH range between 8.3 and 4.5:

$$HCO_3^- + H^+ \rightarrow H_2CO_3 \qquad \textbf{Equation 8.3}$$

Alkalinity is calculated from the moles of acid used to achieve titration to pH 8.3 and pH 4.5, based on the reactions given above. The procedure and calculation are given in Section 3.3.5.

8.1.3 Characterisation and identification of the bacterial contaminants associated with Spirulina cultures and processed powder

The 16S rRNA gene fragments from isolates were amplified to determine their identity based on highest similarity of the 16S rRNA gene sequences to those of known organisms. Genomic DNA (gDNA) was extracted from the 11 bacterial strains isolated from Spirulina pond cultures and processed powder using a method adapted from Krohn-Molt et al. (2013). The re-suspended DNA was stored at -20°C until further analysis.

In detail, isolates were inoculated into 10 mL salty agar medium without agar and cultivated at 37°C overnight (approx. 16 hours) with shaking (150 rpm). Cultures were centrifuged at 13 000 g for 10 min to harvest cells. Cell pellets were re-suspended in 0.5 mL extraction buffer (100 mM Tris-HCl, pH 8.0; 100 mM EDTA, pH 8.0; 1.5 M NaCl, 0.1% (v/v) Tween 20) supplemented with 0.5 mg lysozyme and 0.05 mg proteinase K. Samples were mixed by vortexing and incubated with shaking at 37°C overnight. Following incubation, 0.5 mL 20% (w/v) SDS was added, samples mixed and incubated

at 65°C for 90 min, followed by centrifugation at 6000 g, at room temperature for 10 min. The supernatant was removed to new tubes. The remaining pellet was dissolved in 0.5 mL extraction buffer, incubated at 65°C for 10 min and centrifuged as above. The supernatant was combined with earlier supernatant and mixed with 1mL of 50% (w/v) PEG 4000 and 1.6 M NaCl, incubated at room temperature for 2 hours and centrifuged for 20 min at 10 000 g. Pellets were re-suspended in 0.5 mL TE (10 mM Tris-HCl and 1 mM EDTA, pH 8.0), added 50 µl of 7.5 M potassium acetate and mixed carefully. The mixture was incubated on ice for 5 min and centrifuged at 16 000 g, at 4°C for 30 min. The supernatant was transferred to new tubes and an equal volume of phenol:choloroform:isoamylalcohol (25:24:1) was added, followed by centrifugation at 16000 g for 15 min. DNA (top aqueous phase) was removed, transferred to fresh tubes and precipitated by adding 0.7 volume isopropanol and 0.1 volume 3 M sodium acetate and incubating at -20°C overnight. The mixture was centrifuged at 16 000 g for 30 min at 4°C to obtain genomic DNA (gDNA). The gDNA was washed with 500 µl 70% Ethanol (v/v) and centrifuged as described above. DNA was suspended in TE buffer and stored at -20°C until further analysis

Amplification of the 16S rRNA gene fragments were performed using the universal bacterial primers 27F (forward primer) and 1492R (reverse primer). Primers used were synthesised at the Department of Molecular and Cell Biology, University of Cape Town. PCR reactions were performed in 25 µl reaction volumes. The PCR reaction mixture contained: 2x KAPA HIFI Readymix (Kapa Biosystems), 0.75 ul of 10 µM (µM 27F and 1492 R) primer each and 20 ng gDNA, extracted from the pure bacterial cultures, was used as template. The PCR conditions used for the amplification of the 16S rRNA gene fragments were: initial denaturing at 98°C for 5 min; 20 s denaturation at 98°C, 15 s annealing at 65°C, 90 s elongation at 72°C repeated 30 times; final elongation at 72°C for 5 min. PCRs were performed in a G-Storm GS1 Thermal cycler (Somerton Biotechnology Centre, United Kingdom). PCR products were analysed by agarose gel electrophoresis. In detail, PCR products were analysed on a 0.8% (w/v) agarose gel supplemented with 2 µl 10mg.mL^{-1} ethidium bromide after addition of 6x DNA loading dye (Kapa Biosystems). Electrophoresis was performed at 65 V for 45 min in 1xTAE prepared from a 50x TAE stock solution (2M Tris-base, 1M glacial acetic acid and 50 mM EDTA) buffer. Following electrophoresis, the agarose gel was visualised on a G Box

(SenGene) under UV light. A molecular weight size marker (KAPA universal ladder) from Kapa Biosystems was included on the agarose gel to determine the size of amplified fragments. PCR products were excised from the agarose gel using a clean scalpel blade and the DNA extracted from the agarose using a Nucleospin® PCR and gel extraction kit (Whitehead Scientific, Cape Town). Extracted DNA gene fragments were cloned into the PJET1.2/blunt cloning vector (Promega) and transformed into freshly prepared $CaCl_2$ competent cells. Frozen competent cells were thawed on ice and 5 µl of the ligation mix was added to 100 µl of the *E. coli* DH5α cells and incubated on ice for 30 min. The cells were heat shocked at 37°C for 1 min. Ψ broth (900µl) was added to the cells and incubated at 37°C for 30 min with shaking at 150 rpm. The transformed cells were plated onto LB medium agar plates supplemented with ampicillin (100mg.mL^{-1}) and incubated at 37°C overnight. The resulting colonies were screened for the presence of plasmid DNA with 16S rRNA inserts by performing colony PCR's. Five colonies were chosen from each plate and sub-cultured on to fresh agar plates. The gDNA of the resulting pure cultures was extracted and included in PCRs containing: 1x KAPA Taq Readymix, 0.2 µM pJET1.2 forward primer and 0.2 µM pJET1.2 reverse primer. The primer sequences of pJET1.2 forward and pJET1.2 reverse were 5'CGACTCACTATAGGGAGAGCGGC 3' and 5'AAGAACATCGATTTTCCATGGCAG 3' respectively. The PCR conditions applied were 10 min initial denaturation at 95°C, to allow cell lysis, followed by 35 cycles of 30 s denaturation at 95°C, 30 s annealing at 62°C, 90 s elongation at 72°C. A final elongation was performed at 72°C for 5 min. Colonies containing plasmids and 16S rRNA gene inserts were picked and used to inoculate 10 mL LB medium supplemented with 100 mg.mL^{-1} ampicillin. Cultures were incubated at 37°C overnight on a shaker (150 rpm). Overnight cultures, 10 mL, were centrifuged to obtain cell pellets and the plasmid DNA extracted using the GenElute™ Plasmid Miniprep Kit (Sigma Aldrich) as per manufacturer's instructions. To quantify the concentration of plasmid DNA extracted, a Nanodrop® 2000 (Thermo Fischer Scientific, USA) was used. DNA sequencing was performed by Inqaba Biotechnical Industries (Pty) Ltd.

The sequence results of isolates were edited and analysed using Chromas version 2.01 (Technelysium, Helensvale Queensland, Australia) and DNAMAN version 4.13 (LynnonBioSoft). Sequences were compared and aligned with the GenBank reference

sequences for known 16S rRNA sequences using the Basic Local Alignment Search Tool (BLAST) to identify sequences with the closest similarity to the isolated organisms. Alignments of the 16S rRNA fragments and several reference sequences were performed using the ClustalW (Thompson, Higgins & Gibson, 1994) version 2.1 multiple sequence alignment program. A phylogenetic tree showing the evolutionary relatedness of species was constructed using the Neighbour-Joining method in Mega 6 (Centre of Evolutionary Functional Genomics Biodesign Institute, Arizona State University, Arizona, USA).

8.1.4 Bacterial sequences used to construct the phylogenetic tree

Table 8.1 shows the similarities of the bacterial isolates, identified by 16S rRNA sequencing, and their closest relatives identified from the GenBank database following BLAST analysis.

Table 8.1: Similarity values for the closest relative of the 16S rRNA gene sequences of bacterial isolates identified from Spirulina cultures and commercially processed powder

Isolate B1	Genbank Accession numbers of closest similar sequences	% Cover	% Identity	E value
	JX240476.1	99	99	0.0
	JX240449.1	99	99	0.0
	EU305583.1	99	99	0.0
	AJ302088.1	99	99	0.0
	EU305592.1	99	99	0.0
Isolate B2				
	EU305587.1	99	99	0.0
	FJ152669.1	97	99	0.0
	EU037322.2	97	99	0.0
	JN178548.1	99	98	0.0
	HQ857675.1	99	98	0.0
Isolate B3				
	JQ923679.1	100	99	0.0
	JQ923713.1	100	99	0.0
	AY957902.1	100	99	0.0
	AJ223452.1	99	99	0.0
	KJ549090.1	100	99	0.0

Isolate B4				
	KF912987.1	100	99	0.0
	NR116922.1	99	96	0.0
	JF412423.1	91	96	0.0
	JF412416.1	91	96	0.0
	NR116971.1	99	93	0.0
Isolate B5				
	FJ170017.1	100	96	0.0
	FJ170028.1	100	96	0.0
	NR108223.1	100	96	0.0
	EF554894.1	98	96	0.0
	FJ152962.1	97	96	0.0
Isolate B6				
	NR044879.1	99	98	0.0
	NR041515.1	99	98	0.0
	NR041797.1	98	98	0.0
	KJ841885.1	99	97	0.0
	X92131.1	97	97	0.0
Isolate B7				
	NR116971.1	99	97	0.0
	JF812063.1	93	98	0.0
	FR687204.1	94	98	0.0
	HE806327.1	98	94	0.0
	NR108889.1	98	94	0.0
Isolate B8				
	CP000680.1	100	100	0.0
	KF928786.1	99	100	0.0
	NR074727.1	99	100	0.0
	FJ840535.1	99	100	0.0
	AY082368.1	99	100	0.0

Isolate B9				
	KC494322.1	100	99	0.0
	KC494326.1	100	99	0.0
	AB362824.1	100	98	0.0
	KC494305.1	100	98	0.0
	AB362827.1	100	98	0.0
Isolate B10				
	JQ923679.1	100	99	0.0
	JQ923713.1	100	99	0.0
	AY957902.1	100	99	0.0
	AF235395.1	100	99	0.0
	KC189653.1	100	99	0.0
Isolate B11				
	EF114312.3	100	99	0.0
	KC160768.1	100	99	0.0
	FJ457288.1	100	99	0.0
	GU726842.1	99	99	0.0
	KF142392.1	98	99	0.0

8.2 Appendix B: Supplementary information to Chapter 4

8.2.1 Quantifying bacterial load in processed Spirulina powder

The bacterial loads associated with processed Spirulina powder were analysed using both 'standard' plate counting techniques on nutrient agar and agar medium modified to contain the salts used during Spirulina cultivation ('salty' agar). The results achieved for these experiments are given in Table 8.2. Bacterial counts, using standard nutrient agar, was performed in the CeBER laboratories and by a commercial food safety laboratory (SwiftSilliker laboratory).

Table 8.2: Bacterial load in different batches of Spirulina powder following the harvesting of Spirulina biomass from a 500 000 L Spirulina pond and subsequent drying in an industrial sized oven. A subset of the Spirulina powder samples were plated on nutrient and "salty" agar while some were sent to SwiftSilliker laboratory for bacterial analysis. (Dil.: Dilution, Ave.: Average)

	Salt Agar						Nutrient Agar						
Batch	**Plate$_1$**	**Plate$_2$**	**Plate$_3$**	**Dil.**	**Ave.**	**CeBER Lab (cfu.g^{-1})**	**Plate$_1$**	**Plate$_2$**	**Plate$_3$**	**Dil.**	**Ave.**	**CeBER Lab (cfu.g^{-1})**	**Swift results (cfu.g^{-1})**
B_0	108	124	86	10	1060	1.1E+06	58	62	43	10	543	5.4E+05	2.4E+05
B_1	119	116	116	10	1170	1.2E+06	57	55	55	10	557	5.6E+05	1.6E+05
B_2	148	150	420	100	23933	2.4E+07	65	65	78	100	6933	6.9E+06	1.6E+06
B_3	48	40	99	1000	62333	6.2E+07	174	209	199	100	19400	1.9E+07	8.0E+05
B_5	44	52	43	10	463	4.6E+05	198	182	195	1	192	1.9E+05	2.0E+04
B_6	91	99	280	1	157	1.6E+05	40	43	38	1	40	4.0E+04	1.8E+04
B_7	46	58	54	1	53	5.3E+04	20	23	24	1	22	2.2E+04	4.0E+03
B_8	39	93	56	1	63	6.3E+04	19	34	27	1	27	2.7E+04	7.2E+02
B_9	54	44	30	1	43	4.3E+04	26	19	18	1	21	2.1E+04	8.5E+03
B_{10}	48	50	61	1	53	5.3E+04	21	22	27	1	23	2.3E+04	6.0E+03
B_{11}	80	87	86	1	84	8.4E+04	34	38	35	1	36	3.6E+04	2.2E+05

8.2.2 The effect of sample handling conditions prior to analysis on bacterial loads in Spirulina culture samples

The effect of post sampling handling of Spirulina samples on the effect of the bacterial counts achieved were investigated. Bacterial loads measured upon return to the CeBER laboratory was compared to those obtained when samples were plated at the site of Spirulina production at BioDelta. Care was taken to ensure sterile conditions during plating and the necessary controls were included to determine bacterial counts as a result of contamination. The results from two independent experiments are given in Table 8.3.

Table 8.3 (a) Bacterial load in Spirulina samples obtained from a 50 000 L Spirulina pond and introduced to different treatments simulating conditions prior to bacterial count plating in the laboratory; (b) repeat.

(a)

	Description: First run			Bacterial count (cfu.mL^{-1} x 10^6)			
Sample code	**Storage condition**	**Storage duration**	**Plated at:**	**Reading$_1$**	**Reading$_2$**	**Reading$_3$**	**Reading$_{Ave.}$**
S1 no Inc	-	0 hours	BioDelta	4.4	5.3	4.4	4.7
S1 3hr Inc-farm	Exposed to the sun	3 hours	BioDelta	6.4	7.8	6.3	6.8
S1 3hr Inc-lab	Exposed to the sun	3 hours in the sun, 1 hour in transit to CeBER	CeBER	5.8	9.2	5.4	6.8
S2 no Inc	-	0 hours	BioDelta	3.9	3.1	5.4	4.1
S2 room temp	Stored at room temperature	1 hour in transit to CeBER at room temperature	CeBER	7.4	8.5	7.1	7.6
S2 cooler	Stored in a cooler box	1 hour in transit to CeBER in a cooler box	CeBER	8.2	8.4	9.5	8.7

(b)

Sample code	Description: Second run			Bacterial count (cfu.mL^{-1} x 10^6)			
	Storage condition	Storage duration	Plated at:	Reading$_1$	Reading$_2$	Reading$_3$	Reading$_{Ave.}$
S1 no Inc	-	0 hours	BioDelta	6.0	6.5		6.2
S1 3hr Inc-farm	Exposed to the sun	3 hours	BioDelta	5.4	11.3	21.6	12.7
S1 3hr Inc-lab	Exposed to the sun	3 hours in the sun, 1 hour in transit to CeBER	CeBER	9.9	12.2	14.7	12.2
S2 no Inc	-	0 hours	BioDelta	7.0			7.0
S2 room temp	Stored at room temperature	1 hour in transit to CeBER at room temperature	CeBER	9.1	9.1	9.5	9.2
S2 cooler	Stored in a cooler box	1 hour in transit to CeBER in a cooler box	CeBER	10.0	11.2		10.6

Abbreviations used Table 8.3:

S1-S2: sample number 1 and 2

Inc: Incubation

Hr: hour

Lab: laboratory

Room temp: room temperature

8.2.3 Outdoor 50 000 L and 80 L raceway ponds: Spirulina growth data and bacterial contaminant levels

Table 8.4 and Table 8.5 gives the data obtained from the 50 000 L and 80 L outdoor raceway ponds used to study the behaviour of contaminating bacteria during the growth of Spirulina.

Table 8.4: (a) Optical density and dry cell weight measurements of Spirulina biomass; (b) Bacterial load, pH, total alkalinity and temperature measurements in the 50 000 L pond

(a)

		Optical Density (OD)					Dry Cell Weight (DCW)					
Date	Dilution	OD_1	OD_2	OD_3	Average OD	OD SD	DCW_1	DCW_2	DCW_3	Dilution	OD SD	Average DCW
24-01-14	1	0.066	0.063		0.065	0.00	0.078	0.074		1	0.00	0.076
27-01-14	1	0.192	0.183		0.188	0.01	0.226	0.215		1	0.01	0.221
30-01-14	1	0.406	0.371	0.372	0.383	0.02	0.478	0.436	0.438	1	0.02	0.451
03-02-14	1	0.561	0.614	0.595	0.590	0.03	0.660	0.722	0.700	1	0.03	0.694
07-02-14	1	0.928	1.008	0.989	0.975	0.04	1.092	1.186	1.164	1	0.05	1.147
10-02-14	2	0.548	0.545	0.539	1.088	0.00	0.645	0.641	0.634	2	0.01	1.280
12-02-14	2	0.645	0.631	0.651	1.285	0.01	0.759	0.742	0.766	2	0.01	1.511
14-02-14	2	0.513	0.496	0.534	1.029	0.02	0.604	0.584	0.628	2	0.02	1.210
17-02-14	2	0.496	0.518	0.589	1.069	0.05	0.584	0.609	0.693	2	0.06	1.257
19-02-14	2	0.540	0.477	0.504	1.014	0.03	0.635	0.561	0.593	2	0.04	1.193
21-02-14	2	0.477	0.468	0.465	0.940	0.01	0.561	0.551	0.547	2	0.01	1.106
24-02-14	2	0.481	0.465	0.471	0.945	0.01	0.566	0.547	0.554	2	0.01	1.111
26-02-14	2	0.489	0.498	0.462	0.966	0.02	0.575	0.586	0.544	2	0.02	1.136
28-02-14	2	0.494	0.534	0.493	1.014	0.02	0.581	0.628	0.580	2	0.03	1.193
03-03-14	2	0.503	0.495	0.518	1.011	0.01	0.592	0.582	0.609	2	0.01	1.189
05-03-14	2	0.49	0.5	0.473	0.975	0.01	0.576	0.588	0.556	2	0.02	1.147

07-03-14	2	0.452	0.43	0.412	0.863	0.02	0.532	0.506	0.485	2	0.02	1.015
10-03-14	2	0.493	0.467	0.479	0.959	0.01	0.580	0.549	0.564	2	0.02	1.129
12-03-14	2	0.438	0.446	0.443	0.885	0.00	0.515	0.525	0.521	2	0.00	1.041
14-03-14	2	0.421	0.437	0.422	0.853	0.01	0.495	0.514	0.496	2	0.01	1.004
17-03-14	2	0.395	0.367	0.419	0.787	0.03	0.465	0.432	0.493	2	0.03	0.926
20-03-14	2	0.32	0.32	0.333	0.649	0.01	0.376	0.376	0.392	2	0.01	0.763
24-03-14	2	0.33	0.328	0.314	0.648	0.01	0.388	0.386	0.369	2	0.01	0.762
26-03-14	2	0.23	0.261	0.238	0.486	0.02	0.271	0.307	0.280	2	0.02	0.572
28-03-14	2	0.161	0.16	0.168	0.326	0.00	0.189	0.188	0.198	2	0.01	0.384
31-03-14	1	0.158	0.173	0.172	0.168	0.01	0.186	0.204	0.202	1	0.01	0.197
02-04-14	1	0.071	0.085	0.073	0.076	0.01	0.084	0.100	0.086	1	0.01	0.090

(b)

	Bacterial counts (BC)										Temperature (°C)		
Date	**BC_1**	**BC_2**	**BC_3**	**Dilution**	**Average BC colony forming units (cfu)**	**BC SD**	**BC ($cfu.mL^{-1}$)**	**pH**	**Total alkalinity (TA) ($Eq.L^{-1}$)**	**TA w/ NaHCO3 added ($Eq.L^{-1}$)**	**Max**	**Min**	**Average**
24-01-14	66	58	83	10	6.90E+02	12.77	6.90E+03	8.77	0.047		24.25	24.00	24.19
27-01-14	158	250	183	1000	1.97E+05	47.57	1.97E+06	9.73	0.049		33.25	28.50	30.89
30-01-14	68	80	78	1000	7.53E+04	6.43	7.53E+05	10.07	0.049		31.00	24.75	27.53
03-02-14	127	130	122	1000	1.26E+05	4.04	1.26E+06	10.46	0.070		33.25	25.00	28.65
07-02-14	61	66	64	10000	6.37E+05	2.52	6.37E+06	12.13	0.061	0.049	33.00	25.00	28.58
10-02-14	190	150	200	1000	1.80E+05	26.46	1.80E+06	11.40	0.064	0.056	34.00	25.00	28.86
12-02-14	42	47	40	10000	4.30E+05	3.61	4.30E+06	11.40	0.059	0.053	34.25	25.25	29.64
14-02-14	53	108	88	1000	8.30E+04	27.84	8.30E+05	10.77	0.0654	0.065	33.50	25.50	28.04
17-02-14	197	135	168	1000	1.67E+05	31.02	1.67E+06	10.75	0.080	0.080	37.00	30.00	33.88
19-02-14	34	40	35	10000	3.63E+05	3.21	3.63E+06	10.45	0.090	0.090	34.75	27.25	30.23
21-02-14	75	100	109	1000	9.47E+04	17.62	9.47E+05	10.54	0.094	0.093	31.75	22.00	26.60
24-02-14	145	116	134	100	1.32E+04	14.64	1.32E+05	10.32	0.111	0.109	32.50	23.00	27.80
26-02-14	192	142	116	1000	1.50E+05	38.63	1.50E+06	10.46	0.114	0.106	25.75	23.50	24.50
28-02-14	38	54	46	1000	4.60E+04	8.00	4.60E+05	10.53	0.107	0.102	31.75	26.00	28.72
03-03-14	71	144	76	1000	9.70E+04	40.78	9.70E+05	10.36	0.126	0.119	31.00	22.50	25.91
05-03-14	150	164	200	1000	1.71E+05	25.79	1.71E+06	10.44	0.124	0.116	33.5	23.25	28.05

07-03-14	180	198	220	100	1.99E+04	20.03	1.99E+05	10.58	0.120	0.118	33.5	28.25	30.95
10-03-14	140	132	104	100	1.25E+04	18.90	1.25E+05	10.90	0.126	0.118	30.5	20.25	24.85
12-03-14	100	33	112	1000	8.17E+04	42.57	8.17E+05	10.65	0.124	0.122	29	21.5	24.58
14-03-14	64	56	60	1000	6.00E+04	4.00	6.00E+05	10.33	0.138	0.131	30.75	20.75	25.39
17-03-14	68	84	89	100	8.03E+03	10.97	8.03E+04	10.36	0.137	0.123	32.75	22.25	26.99
20-03-14	139	66	159	1000	1.21E+05	48.95	1.21E+06	10.15	0.132	0.128	33.5	21.75	28.18
24-03-14	82	84	95	1000	8.70E+04	7.00	8.70E+05	10.35	0.133	0.130	28.75	26	27.36
26-03-14	64	83	86	1000	7.77E+04	11.93	7.77E+05	10.25	0.129	0.123	23.75	22.25	22.90
28-03-14	118	98		1000	1.08E+05	14.14	1.08E+06	10.22	0.128	0.122			
31-03-14	100			1000	1.00E+05		1.00E+06	10.17	0.125				
02-04-14	85	129	135	1000	1.16E+05	27.30	1.16E+06	9.99	0.125		25.75	22.25	23.57

Table 8.5: (a) Optical density and dry cell weight measurements of Spirulina biomass; (b) bacterial load, pH, total alkalinity and temperature measurements in the 80 L pond

(a)

		Optical Density (OD)					Dry Cell Weight (DCW)				
Date	**Dilution**	**OD_1**	**OD_2**	**OD_3**	**Average OD**	**OD SD**	**DCW_1**	**DCW_2**	**DCW_3**	**OD SD**	**Average DCW**
05-05-14	1	0.051	0.05	0.046	0.049	0.00	0.060	0.059	0.054	0.00	0.058
07-05-14	1	0.098	0.089	0.104	0.097	0.01	0.115	0.105	0.122	0.01	0.114
09-05-14	1	0.143	0.144	0.13	0.139	0.01	0.168	0.169	0.153	0.01	0.164
12-05-14	1	0.163	0.166	0.161	0.163	0.00	0.192	0.195	0.189	0.00	0.192
16-05-14	1	0.294	0.297	0.296	0.296	0.00	0.346	0.349	0.348	0.00	0.348
19-05-14	1	0.436	0.442	0.419	0.432	0.01	0.513	0.520	0.493	0.01	0.509
21-05-14	1	0.488	0.474	0.478	0.480	0.01	0.574	0.558	0.562	0.01	0.565
23-05-14	1	0.592	0.59	0.589	0.590	0.00	0.696	0.694	0.693	0.00	0.695
26-05-14	1	0.659	0.682	0.683	0.675	0.01	0.775	0.802	0.804	0.02	0.794
28-05-14	1	0.756	0.738	0.737	0.744	0.01	0.889	0.868	0.867	0.01	0.875
30-05-14	1	0.832	0.849	0.841	0.841	0.01	0.979	0.999	0.989	0.01	0.989
02-06-14	1	0.929	0.909	0.93	0.923	0.01	1.093	1.069	1.094	0.01	1.085
04-06-14	1	0.91	0.896	0.898	0.901	0.01	1.071	1.054	1.056	0.01	1.060
06-06-14	1	0.977	0.975	0.989	0.980	0.01	1.149	1.147	1.164	0.01	1.153
09-06-14	2	0.552	0.57	0.532	1.103	0.02	0.649	0.671	0.626	0.02	1.297

11/6/2014 (before harvest)	2	0.648	0.631	0.629	1.272	0.01	0.762	0.742	0.740	0.01	1.496
11/6/2014 (after harvest)	1	0.643	0.65	0.656	0.650	0.01	0.756	0.765	0.772	0.01	0.764
15-06-14	1	0.748	0.783	0.772	0.768	0.02	0.880	0.921	0.908	0.02	0.903
17-06-14	1	0.913	0.895	0.872	0.893	0.02	1.074	1.053	1.026	0.02	1.051
18-06-14	1	0.713	0.693	0.677	0.694	0.02	0.839	0.815	0.796	0.02	0.817
20-06-14	1	0.867	0.864	0.872	0.868	0.00	1.020	1.016	1.026	0.00	1.021
23-06-14	1	0.866	0.883	0.884	0.878	0.01	1.019	1.039	1.040	0.01	1.033
25-06-14	1	0.916	0.928	0.945	0.930	0.01	1.078	1.092	1.112	0.02	1.094
27-06-14	1	0.96	0.976	0.968	0.968	0.01	1.129	1.148	1.139	0.01	1.139
30-06-14	2	0.569	0.599	0.576	1.163	0.02	0.669	0.705	0.678	0.02	1.368
03-07-14	1	0.876	0.883	0.862	0.874	0.01	1.031	1.039	1.014	0.01	1.028
04-07-14	1	0.901	0.903	0.89	0.898	0.01	1.060	1.062	1.047	0.01	1.056
07-07-14	2	0.535	0.547	0.559	1.094	0.01	0.629	0.644	0.658	0.01	1.287

(b)

	Bacterial Counts (BC)										
Date	**BC_1**	**BC_2**	**BC_3**	**Dilution**	**Average BC colony forming units (cfu)**	**BC SD**	**BC (cfu.mL^{-1})**	**pH**	**Total alkalinity (Eq.L^{-1})**	**TA w/ NaHCO3 added (Eq.L^{-1})**	**Temperature (°C)**
05-05-14	111	48	43	100	6.73E+03	37.90	6.73E+04	8.72			
07-05-14	60	62	84	100	6.87E+03	13.32	6.87E+04	9.5			
09-05-14	91	119	134	100	1.15E+04	21.83	1.15E+05	9.36	0.049		
12-05-14	36	53		1000	4.45E+04	12.02	4.45E+05	9.28	0.048		
16-05-14	135	123	75	1000	1.11E+05	31.75	1.11E+06	9.4	0.052		
19-05-14	71	70	73	1000	7.13E+04	1.53	7.13E+05	9.58	0.054		
21-05-14	212	236	147	1000	1.98E+05	46.05	1.98E+06	9.6	0.052		
23-05-14	160	158	147	1000	1.55E+05	7.00	1.55E+06	9.7	0.052		
26-05-14	274	190	tntc	1000	2.32E+05	59.40	2.32E+06	9.74	0.052		
28-05-14	49	62	56	10000	5.57E+05	6.51	5.57E+06	9.76	0.051		28
30-05-14				Data missing				9.79	0.055		27.5
02-06-14	tntc	tntc	tntc	10000				9.83	0.056		28
04-06-14	54	44	50	10000	4.93E+05	5.03	4.93E+06	9.7	0.052		27.5
06-06-14	32	36		1000	3.40E+04	2.83	3.40E+05	9.79	0.055		30
09-06-14	63	119	188	1000	1.23E+05	62.61	1.23E+06	9.78	0.034		26.5

11/6/2014 (before harvest)	119	127		1000	1.23E+05	5.66	1.23E+06	17.85			29.5
11/6/2014 (after harvest)	61	57	44	1000	5.40E+04	8.89	5.40E+05	17.85	0.056		29.5
15-06-14	121	144	120	1000	1.28E+05	13.58	1.28E+06	9.86	0.057		29.5
17-06-14	100	97	102	1000	9.97E+04	2.52	9.97E+05	9.99	0.052	0.057	30
18-06-14				Data missing				9.84	0.052		29
20-06-14	38	45	59	1000	4.73E+04	10.69	4.73E+05	9.90	0.054		30
23-06-14	91	100	101	1000	9.73E+04	5.51	9.73E+05	9.95	0.054		30
25-06-14	129	137	154	1000	1.40E+05	12.77	1.40E+06	9.93	0.054		30
27-06-14	105	87	100	1000	9.73E+04	9.29	9.73E+05	9.94	0.054		29.9
30-06-14	168	75	151	1000	1.31E+05	49.52	1.31E+06	10.08	0.057		29.8
03-07-14	36	38	41	10000	3.83E+05	2.52	3.83E+06	9.97	0.060		28.5
04-07-14	49	53	55	10000	5.23E+05	3.06	5.23E+06	9.92	0.062		30
07-07-14	43	46	50	10000	4.63E+05	3.51	4.63E+06	9.99	0.063		30

SD: Standard Deviation

The formulae used to calculate the growth rates and maximum productivities in Tables 5.2, 5.3 and 5.4 are given below: Equations adapted from Amara & Steinbüchel 2013.

$$\boldsymbol{Productivity}\ (\boldsymbol{P}) = \frac{(X_2 - X_1)}{(t_2 - t_1)} = g\ L^{-1} day^{-1}$$

$$\boldsymbol{Specific\ growth\ rate}\ (\mu) = \frac{\ln(\frac{X_2}{X_1})}{t_2 - t_1} = day^{-1}\ ;$$

where X_1 and X_2 are the biomass concentrations at the beginning (t_1) and the end (t_2) of a selected time interval between inoculation and maximum biomass production.

8.3 Appendix C: Supplementary information to Chapter 5

8.3.1 Growth of Spirulina in 3.2 L airlift reactors (ALRs)

The data generated during the cultivation of Spirulina in 3.2 L airlift reactors by controlling the pH with either 1 M HCl (Table 8.6 and Table 8.7) or 1 M NaHCO3 (Table 8 8 and Table 8 9) are given below. Each measurement was performed in triplicate and the experiment was repeated in a second reactor to generate an additional data set. The productivity of the Spirulina was

measured by optical density (OD) and converted to dry cell weight (DCW) using the biomass conversion factor (0.85) generated from a standard curve of OD plotted against DCW. pH, chemical oxygen demand (COD) of the supernatant of the culture and the bacterial load were determined daily.

Table 8.6: (a) Optical density, dry cell weight and pH measurements, (b) chemical oxygen demand and (c) bacterial load in reactor 1

(a) Reactor 1 (pH adjusted with 1 M HCL)

Date	Day	pH	Dilution	OD_1	OD_2	OD_3	Average OD	OD SD	DCW_1	DCW_2	DCW_3	OD SD	Average DCW
30-10-13	0	8.95	1	0.269	0.266	0.257	0.264	0.01	0.316	0.313	0.302	0.01	0.311
01-11-13	1	9.7	1	0.448	0.444	0.45	0.447	0.00	0.527	0.522	0.529	0.00	0.526
02-11-13	2	10.01	1	0.547	0.541	0.507	0.532	0.02	0.644	0.636	0.596	0.03	0.625
03-11-13	3	10.3	1	0.756	0.744	0.775	0.758	0.02	0.889	0.875	0.912	0.02	0.892
04-11-13	4	10.5	2	0.505	0.499	0.514	1.012	0.01	0.594	0.587	0.605	0.01	1.191
05-11-13	5	10.7	2	0.591	0.586	0.572	1.166	0.01	0.695	0.689	0.673	0.01	1.372
06-11-13	6	10.15	2	0.591	0.614	0.604	1.206	0.01	0.695	0.722	0.711	0.01	1.419
07-11-13	7	10.24	2	0.617	0.626	0.633	1.251	0.01	0.726	0.736	0.745	0.01	1.471
08-11-13	8	9.8	2	0.653	0.663	0.677	1.329	0.01	0.768	0.780	0.796	0.01	1.563
09-11-13	9	10.49	2	0.690	0.683	0.696	1.379	0.01	0.812	0.804	0.819	0.01	1.623
10-11-13	10	10.923	2	0.799	0.804	0.81	1.609	0.01	0.940	0.946	0.953	0.01	1.893
11-11-13	11	10.55	2	0.834	0.826	0.825	1.657	0.00	0.981	0.972	0.971	0.01	1.949
12-11-13	12	10.99	4	0.468	0.452	0.45	1.827	0.01	0.551	0.532	0.529	0.01	2.149
13-11-13	13	2.47	data missing						data missing				

SD: Standard Deviation; OD: Optical Density at 750 nm; CDW: Cell Dry Weight

(b) Chemical oxygen demand (g.L^{-1})

Date	Day	Dilution	OD_1	OD_2	OD_3	Average OD	OD SD	COD_1	COD_2	COD_3	COD SD	Average COD
30-10-13	0	1	0.091	0.119	0.166	0.125	0.04	0.607	0.793	1.107	0.25	0.836
01-11-13	1	1	0.287		0.315	0.301	0.02	1.913	0.000	2.100	1.16	1.338
02-11-13	2	1	0.074		0.076	0.075	0.00	0.493	0.000	0.507	0.29	0.333
03-11-13	3	1	0.225		0.244	0.235	0.01	1.500	0.000	1.627	0.90	1.042
04-11-13	4	1		0.178	0.144	0.161	0.02	0.000	1.187	0.960	0.63	0.716
05-11-13	5	1	0.121		0.363	0.242	0.17	0.807	0.000	2.420	1.23	1.076
06-11-13	6	1	0.227	0.243	0.255	0.242	0.01	1.513	1.620	1.700	0.09	1.611
07-11-13	7	1	0.49	0.316	0.143	0.316	0.17	3.267	2.107	0.953	1.16	2.109
08-11-13	8	1	0.219	0.182	0.109	0.170	0.06	1.460	1.213	0.727	0.37	1.133
09-11-13	9	1										
10-11-13	10	1	0.215	0.219	0.146	0.193	0.04	1.433	1.460	0.973	0.27	1.289
11-11-13	11	1					#DIV/0!	0.000	0.000	0.000	0.00	0.000
12-11-13	12	1	0.249	0.235	0.088	0.191	0.09	1.660	1.567	0.587	0.59	1.271
13-11-13	13	1					data missing					
14-11-13	14	1	0.852					5.680				5.680

SD: Standard Deviation; OD: Optical Density at 605 nm; COD: Chemical Oxygen Demand (g L^{-1})

(c) Bacterial load (cfu.mL^{-1})

Date	Day	BC_1	BC_2	BC_3	Dilution	Average BC (cfu)	BC SD	BC (cfu.mL^{-1})
30-10-13	0	46	47	48	1.00E+03	4.70E+04	1.00E+00	4.70E+05
01-11-13	1	45	52	60	1.00E+03	5.23E+04	7.51E+00	5.23E+05
02-11-13	2	105	150	170	1.00E+03	1.42E+05	3.33E+01	1.42E+06
03-11-13	3	250	185	300	1.00E+03	2.45E+05	5.77E+01	2.45E+06
04-11-13	4	50	46	32	1.00E+04	4.27E+05	9.45E+00	4.27E+06
05-11-13	5	60	73	56	1.00E+04	6.30E+05	8.89E+00	6.30E+06
06-11-13	6	250	274	270	1.00E+04	2.65E+06	1.29E+01	2.65E+07
07-11-13	7	31	40	30	1.00E+05	3.37E+06	5.51E+00	3.37E+07
08-11-13	8	90	80	130	1.00E+05	1.00E+07	2.65E+01	1.00E+08
09-11-13	9	50	80	110	1.00E+04	8.00E+05	3.00E+01	8.00E+06
10-11-13	10	250	265	255	1.00E+05	2.57E+07	7.64E+00	2.57E+08
11-11-13	11	33	35	30	1.00E+05	3.27E+06	2.52E+00	3.27E+07
12-11-13	12	50	70	65	1.00E+05	6.17E+06	1.04E+01	6.17E+07
13-11-13	13					data missing		
14-11-13	14							

SD: Standard Deviation; BC: Bacterial Count; cfu: colony forming units

Table 8.7: (a) Optical density, dry cell weight and pH measurements, (b) chemical oxygen demand and (c) bacterial load in reactor 2

(a) Reactor 2 (pH adjusted with 1M HCL)

Date	Day	pH	Dilution	OD_1	OD_2	OD_3	Average OD	OD SD	DCW_1	DCW_2	DCW_3	OD SD	Average DCW
30-10-13	0	8.95	1	0.269	0.266	0.257	0.264	0.01	0.316	0.313	0.302	0.01	0.311
01-11-13	1	9.72	1	0.444	0.44	0.42	0.435	0.01	0.522	0.518	0.494	0.02	0.511
02-11-13	2	10.05	1	0.591	0.588	0.538	0.572	0.03	0.695	0.692	0.633	0.04	0.673
03-11-13	3	10.3	1	0.757	0.791	0.751	0.766	0.02	0.891	0.931	0.884	0.03	0.902
04-11-13	4	10.45	2	0.499	0.514	0.494	1.005	0.01	0.587	0.605	0.581	0.01	1.182
05-11-13	5	10.6	2	0.563	0.61	0.602	1.183	0.03	0.662	0.718	0.708	0.03	1.392
06-11-13	6	9.66	2	0.605	0.598	0.609	1.208	0.01	0.712	0.704	0.716	0.01	1.421
07-11-13	7	10.04	2	0.744	0.746	0.747	1.491	0.00	0.875	0.878	0.879	0.00	1.755
08-11-13	8	10	2	0.73	0.723	0.711	1.443	0.01	0.859	0.851	0.836	0.01	1.697
09-11-13	9	10.64	2	0.844	0.800	0.793	1.625	0.03	0.993	0.941	0.933	0.03	1.911
10-11-13	10	10.76	2	0.905	0.907	0.91	1.815	0.00	1.065	1.067	1.071	0.00	2.135
11-11-13	11	10.89	4	0.521	0.529	0.529	2.105	0.00	0.613	0.622	0.622	0.01	2.477
12-11-13	12	10.36	4	0.555	0.547	0.527	2.172	0.01	0.653	0.644	0.620	0.02	2.555
13-11-13	13												
14-11-13	14	9.98	4	0.561	0.553	0.577	2.255	0.01	0.660	0.651	0.679	0.01	2.653

SD: Standard Deviation; OD: Optical Density at 750 nm; CDW: Cell Dry Weight

(b) Chemical oxygen demand (g.L^{-1})

Date	Day	Dilution	OD_1	OD_2	OD_3	Average OD	OD SD	COD_1	COD_2	COD_3	COD SD	Average COD
30-10-13	0	1	0.091	0.119	0.166	0.125	0.04	0.607	0.793	1.107	0.25	0.836
01-11-13	1	1	0.293		0.285	0.289	0.01	1.953		1.900	0.04	1.927
02-11-13	2	1	0.856		0.83	0.843	0.02	5.707		5.533	0.12	5.620
03-11-13	3	1	0.261		0.27	0.266	0.01	1.740		1.800	0.04	1.770
04-11-13	4	1		0.142	0.193	0.168	0.04		0.947	1.287	0.24	1.117
05-11-13	5	1										
06-11-13	6	1	0.418			0.418		2.787				2.787
07-11-13	7	1	0.19	0.195		0.193		1.267	1.300		0.02	1.283
08-11-13	8	1	0.163	0.205		0.184	0.03	1.087	1.367		0.20	1.227
09-11-13	9	1										
10-11-13	10	1	0.226	0.223	0.131	0.193	0.05	1.507	1.487	0.873	0.36	1.289
11-11-13	11	1	0.174	0.238	0.303	0.238	0.06	1.160	1.587	2.020	0.43	1.589
12-11-13	12	1	0.108		0.16	0.134	0.04	0.720	0.000	1.067	0.54	0.596
13-11-13	13	1										
14-11-13	14	1	0.706	0.738	0.738		0.02	4.707	4.920	4.920	0.12	4.849

SD: Standard Deviation; OD: Optical Density at 605 nm; COD: Chemical Oxygen Demand (g L^{-1})

(c) Bacterial load (cfu.mL^{-1})

Date	Day	BC_1	BC_2	BC_3	Dilution	Average BC (cfu)	BC SD	BC (cfu.mL^{-1})
30-10-13	0	46	47	48	1.00E+03	4.70E+04	1.00E+00	4.70E+05
01-11-13	1	50	54	45	1.00E+03	4.97E+04	4.51E+00	4.97E+05
02-11-13	2	85	220	140	1.00E+03	1.48E+05	6.79E+01	1.48E+06
03-11-13	3	37	52	56	1.00E+04	4.83E+05	1.00E+01	4.83E+06
04-11-13	4	54	54	50	1.00E+04	5.27E+05	2.31E+00	5.27E+06
05-11-13	5	34	30	92	1.00E+04	5.20E+05	3.47E+01	5.20E+06
06-11-13	6	132	100	185	1.00E+04	1.39E+06	4.29E+01	1.39E+07
07-11-13	7	240	250	260	1.00E+04	2.50E+06	1.00E+01	2.50E+07
08-11-13	8	83	82	82	1.00E+04	8.23E+05	5.77E-01	8.23E+06
09-11-13	9	80	290	50	1.00E+04	1.40E+06	1.31E+02	1.40E+07
10-11-13	10	tntc	tntc	tntc	1.00E+04	tntc	tntc	tntc
11-11-13	11	tntc	tntc	tntc	1.00E+04	tntc	tntc	tntc
12-11-13	12	200	260	210	1.00E+05	2.23E+07	3.21E+01	2.23E+08
13-11-13	13							
14-11-13	14	63	60	62	1.00E+05	6.17E+06	1.53E+00	6.17E+07

SD: Standard Deviation; BC: Bacterial Count; cfu: colony forming units; tntc: too numerous to count

Table 8.8: (a) Optical density, dry cell weight and pH measurements, (b) chemical oxygen demand and (c) bacterial load in reactor 3

(a) Reactor 3 (pH adjusted $NaHCO_3$)

Date	Day	pH	Dilution	OD_1	OD_2	OD_3	Av. OD	OD SD	DCW_1	DCW_2	DCW_3	OD SD	Ave.DCW
30-10-13	0	8.95	1	0.269	0.266	0.257	0.264	0.01	0.316	0.313	0.302	0.01	0.311
01-11-13	1	9.74	1	0.464	0.477	0.431	0.457	0.02	0.546	0.561	0.507	0.03	0.538
02-11-13	2	10.06	1	0.528	0.63	0.584	0.581	0.05	0.621	0.741	0.687	0.06	0.683
03-11-13	3	10.3	1	0.85	0.819	0.818	0.829	0.02	1.000	0.964	0.962	0.02	0.975
04-11-13	4	10.42	2	0.555	0.538	0.532	1.083	0.01	0.653	0.633	0.626	0.01	1.275
05-11-13	5	10.7	2	0.591	0.586	0.572	1.166	0.01	0.695	0.689	0.673	0.01	1.372
06-11-13	6	10.42	2	0.671	0.659	0.653	1.322	0.01	0.789	0.775	0.768	0.01	1.555
07-11-13	7	10.45	2	0.676	0.667	0.676	1.346	0.01	0.795	0.785	0.795	0.01	1.584
08-11-13	8	10.29	2	0.741	0.744	0.752	1.491	0.01	0.872	0.875	0.885	0.01	1.755
09-11-13	9	10.55	2	0.774	0.773	0.797	1.563	0.01	0.911	0.909	0.938	0.02	1.838
10-11-13	10	10.63	2	0.913	0.919	0.932	1.843	0.01	1.074	1.081	1.096	0.01	2.168
11-11-13	11	10.45	4	0.507	0.508	0.498	2.017	0.01	0.596	0.598	0.586	0.01	2.373
12-11-13	12	10.55	4	0.561	0.565	0.563	2.252	0.00	0.660	0.665	0.662	0.00	2.649
13-11-13	13												
14-11-13	14	10.67	4	0.671	0.654	0.647	2.629	0.01	0.789	0.769	0.761	0.01	3.093

SD: Standard Deviation; OD: Optical Density at 750 nm; CDW: Cell Dry Weight

(b) Chemical oxygen demand (g.L^{-1})

Date	Day	Dilution	OD_1	OD_2	OD_3	Av. OD	OD stddev	COD_1	COD_2	COD_3	COD stddev	Ave.COD
30-10-13	0	1	0.091	0.119	0.166	0.125	0.04	0.607	0.793	1.107	0.25	0.836
01-11-13	1	1	0.239		0.33	0.285	0.06	1.593	0.000	2.200	1.14	1.264
02-11-13	2	1	0.134		0.15	0.142	0.01	0.893	0.000	1.000	0.55	0.631
03-11-13	3	1	0.233	0.365	0.184	0.261	0.09	1.553	2.433	1.227	0.62	1.738
04-11-13	4	1		0.063	0.063	0.063	0.00	0.000	0.420	0.420	0.24	0.280
05-11-13	5	1	0.058	0.058		0.058	0.00	0.387	0.387		0.00	0.387
06-11-13	6	1	0.253	0.281		0.267	0.02	1.687	1.873		0.13	1.780
07-11-13	7	1	0.274	0.265		0.270	0.01	1.827	1.767		0.04	1.797
08-11-13	8	1	0.184	0.207		0.196	0.02	1.227	1.380		0.11	1.303
09-11-13	9	1										
10-11-13	10	1	0.049	0.091		0.070	0.03	0.327	0.607		0.20	0.467
11-11-13	11	1	0.174	0.293	0.157	0.208	0.07	1.160	1.953	1.047	0.49	1.387
12-11-13	12	1	0.257	0.187	0.2	0.215	0.04	1.713	1.247	1.333	0.25	1.431
13-11-13	13	1										
14-11-13	14	1	0.335	0.708		0.522	0.26	2.233	4.720		1.76	3.477

SD: Standard Deviation; OD: Optical Density at 605 nm; COD: Chemical Oxygen Demand (g L^{-1})

(c) Bacterial load (cfu.mL^{-1})

Date	Day	BC_1	BC_2	BC_3	Dilution	Ave.BC (cfu)	BC std. dev	BC (cfu.mL^{-1})
30-10-13	0	46	47	48	1.00E+03	4.70E+04	1.00E+00	4.70E+05
01-11-13	1	70	57	48	1.00E+03	5.83E+04	1.11E+01	5.83E+05
02-11-13	2	140	140	120	1.00E+03	1.33E+05	1.15E+01	1.33E+06
03-11-13	3	31	30	30	1.00E+04	3.03E+05	5.77E-01	3.03E+06
04-11-13	4	42	44		1.00E+04	4.30E+05	1.41E+00	4.30E+06
05-11-13	5	60	73	56	1.00E+04	6.30E+05	8.89E+00	6.30E+06
06-11-13	6	230	230	260	1.00E+04	2.40E+06	1.73E+01	2.40E+07
07-11-13	7	260	240	200	1.00E+04	2.33E+06	3.06E+01	2.33E+07
08-11-13	8	tntc	tntc	tntc	1.00E+04	tntc	tntc	tntc
09-11-13	9	30	35	60	1.00E+05	4.17E+06	1.61E+01	4.17E+07
10-11-13	10	90	105	100	1.00E+05	9.83E+06	7.64E+00	9.83E+07
11-11-13	11	20	32	30	1.00E+05	2.73E+06	6.43E+00	2.73E+07
12-11-13	12	65	60	65	1.00E+05	6.33E+06	2.89E+00	6.33E+07
13-11-13	13					data missing		
14-11-13	14	30	32	30	100000	3.07E+06	1.15E+00	3.07E+07

SD: Standard Deviation; BC: Bacterial Count; cfu: colony forming units; tntc: too numerous to count

Table 8.9: (a) Optical density, dry cell weight and pH measurements, (b) chemical oxygen demand and (c) bacterial load in reactor 4

(a) Reactor 4 (pH adjusted $NaHCO_3$)

Date	Day	pH	Dilution	OD_1	OD_2	OD_3	Av. OD	OD SD	DCW_1	DCW_2	DCW_3	OD SD	Ave.DCW
30-10-13	0	8.95	1	0.269	0.266	0.257	0.264	0.01	0.316	0.313	0.302	0.01	0.311
01-11-13	1	9.74	1	0.473	0.429	0.451	0.451	0.02	0.556	0.505	0.531	0.03	0.531
02-11-13	2	10.04	1	0.543	0.574	0.577	0.565	0.02	0.639	0.675	0.679	0.02	0.664
03-11-13	3	10.27	1	0.783	0.762	0.759	0.768	0.01	0.921	0.896	0.893	0.02	0.904
04-11-13	4	10.36	2	0.504	0.496	0.515	1.010	0.01	0.593	0.584	0.606	0.01	1.188
05-11-13	5	10.5	2	0.566	0.613	0.609	1.192	0.03	0.666	0.721	0.716	0.03	1.402
06-11-13	6	10.36	2	0.677	0.676	0.691	1.363	0.01	0.796	0.795	0.813	0.01	1.603
07-11-13	7	10.66	2	0.72	0.701	0.714	1.423	0.01	0.847	0.825	0.840	0.01	1.675
08-11-13	8	10.37	2	0.764	0.754	0.775	1.529	0.01	0.899	0.887	0.912	0.01	1.798
09-11-13	9	10.61	2	0.800	0.806	0.810	1.611	0.01	0.941	0.948	0.953	0.01	1.895
10-11-13	10	10.64	2	0.943	0.944	0.914	1.867	0.02	1.109	1.111	1.075	0.02	2.197
11-11-13	11	10.43	4	0.517	0.52	0.533	2.093	0.01	0.608	0.612	0.627	0.01	2.463
12-11-13	12	10.49	4	0.548	0.562	0.542	2.203	0.01		0.645	0.661	0.638	0.01	2.591
13-11-13	13												
14-11-13	14	10.62	4	0.616	0.652	0.622	2.520	0.02		0.725	0.767	0.732	0.02	2.965

SD: Standard Deviation; OD: Optical Density at 750 nm; CDW: Cell Dry Weight

(b) Chemical oxygen demand (g.L-1)

Date	Day	Dilution	OD_1	OD_2	OD_3	Av. OD	OD SD	COD_1	COD_2	COD_3	CODSD	Ave.COD
30-10-13	0	1	0.091	0.119	0.166	0.125	0.04	0.607	0.793	1.107	0.25	0.836
01-11-13	1	1	0.194	0.21	0.26	0.221	0.03	1.293	1.400	1.733	0.23	1.47556
02-11-13	2	1	0.237	0.182	0.257	0.225	0.04	1.580	1.213	1.713	0.26	1.502
03-11-13	3	1	0.381	0.145	0.276	0.267	0.12	2.540	0.967	1.840	0.79	1.782
04-11-13	4	1	0.218	0.063	0.089	0.123	0.08	1.453	0.420	0.593	0.55	0.822
05-11-13	5	1	0.139	0.176		0.158	0.03	0.927	1.173		0.17	1.050
06-11-13	6	1	0.082	0.069		0.076	0.01	0.547	0.460		0.06	0.503
07-11-13	7	1	0.17	0.241	0.537	0.316	0.19	1.133	1.607	3.580	1.30	2.107
08-11-13	8	1	0.196	0.205	0.196	0.199	0.01	1.307	1.367	1.307	0.03	1.327
09-11-13	9	1	0.227	0.120	0.473	0.273	0.18	1.513	0.800	3.153	1.21	
10-11-13	10	1	0.283	0.211	0.321	0.272	0.06	1.887	1.407	2.140	0.37	1.811
11-11-13	11	1	0.117	0.274	0.1465	0.179	0.08	0.780	1.827	0.977	0.56	1.194
12-11-13	12	1	0.048	0.042	0.052	0.047	0.01	0.320	0.280	0.347	0.03	0.316
13-11-13	13	1										
14-11-13	14	1	0.392	0.300		0.346	0.07	2.613	2.000		0.43	2.307

SD: Standard Deviation; OD: Optical Density at 605 nm; COD: Chemical Oxygen Demand (g L^{-1})

(c) Bacterial load (cfu.mL^{-1})

Date	Day	BC_1	BC_2	BC_3	Dilution	Ave.BC (cfu)	BC std. dev	BC (cfu.mL^{-1})
30-10-13	0	46	47	48	1.00E+03	4.70E+05	1.00E+00	4.70E+06
01-11-13	1	50	52	49	1.00E+03	5.03E+05	1.53E+00	5.03E+06
02-11-13	2	196	165	80	1.00E+03	1.47E+06	6.01E+01	1.47E+07
03-11-13	3	300	295	280	1.00E+04	2.92E+07	1.04E+01	2.92E+08
04-11-13	4	200			1.00E+03	2.00E+06	#DIV/0!	2.00E+07
05-11-13	5	115	130	85	1.00E+04	1.10E+07	2.29E+01	1.10E+08
06-11-13	6	183	120	130	1.00E+04	1.44E+07	3.39E+01	1.44E+08
07-11-13	7	130	95	120	1.00E+04	1.15E+07	1.80E+01	1.15E+08
08-11-13	8	50	43		1.00E+04	4.65E+06	4.95E+00	4.65E+07
09-11-13	9	46	50	23	1.00E+04	3.97E+06	1.46E+01	3.97E+07
10-11-13	10	tntc	tntc	tntc	1.00E+04	tntc	tntc	tntc
11-11-13	11	tntc	tntc	tntc	1.00E+04	tntc	tntc	tntc
12-11-13	12	30	35	30	1.00E+05	3.17E+07	2.89E+00	3.17E+08
13-11-13	13							
14-11-13	14	34	33	34	100000	3.37E+07	5.77E-01	3.37E+08

SD: Standard Deviation; BC: Bacterial Count; cfu: colony forming units; tntc: too numerous to count

8.4 Impact of physicochemical factors on growth of Spirulina and associated bacteria

Data generated from the study on the impact of temperature, salinity and pH on growth of Spirulina and associated bacteria which were carried out in shake flasks are given below.

Table 8.10: The effect of temperature on Spirulina growth and the bacterial counts of associated bacteria when Spirulina was cultivated at 25°C (a); 30°C (b) and 35°C (c).

(a) Temperature: 25°C

		Spirulina biomass concentration			Bacterial Counts (BC)					
Date	Day	OD 750 nm	DCW (g L^{-1})	pH	BC_1	BC_2	BC_3	Dilution Factor	Average BC	Overall cfu.mL^{-1}
10-11-14	0	0.138	0.162	9.27	249	249	260	1.00E+02	2.53E+04	2.53E+05
11-11-14	1	0.354	0.416	9.42	119	144	175	1.00E+03	1.46E+05	1.46E+06
12-11-14	2	0.521	0.613	9.82	192	202	181	1.00E+04	1.91E+06	1.91E+07
13-11-14	3	0.560	0.659	10.00	102	128	112	1.00E+05	1.14E+07	1.14E+08
14-11-14	4	0.595	0.700	10.22	171	250	190	1.00E+06	2.04E+08	2.04E+09
15-11-14	5	0.565	0.665	9.51	53	55	47	1.00E+07	5.15E+08	5.15E+09
16-11-14	6	0.637	0.749	9.66	122	84	105	1.00E+07	1.03E+09	1.03E+10
17-11-14	7	0.738	0.868	9.82	224	147	201	1.00E+07	1.90E+09	1.90E+10
18-11-14	8	0.828	0.974	9.96	247	116	80	1.00E+07	1.47E+09	1.47E+10
19-11-14	9	0.982	1.155	10.44	70	70	63	1.00E+07	6.75E+08	6.75E+09
20-11-14	10	1.091	1.283	9.86	38	44	40	1.00E+07	4.07E+08	4.07E+09
21-11-14	11	1.294	1.522	10.27	44	71	71	1.00E+07	6.17E+08	6.17E+09
22-11-14	12	0.937	1.103	10.24	250	248	272	1.00E+06	2.56E+08	2.56E+09
23-11-14	13	1.054	1.240	10.22						
24-11-14	14	1.269	1.493	10.36	68	47		1.00E+06	5.70E+07	5.70E+08

OD: Optical Density at 750 nm; CDW: Cell Dry Weight; cfu: colony forming units

(b) Temperature: 30°C

Date	Day	Spirulina biomass concentration		pH	Bacterial Counts (BC)					
		OD 750 nm	DCW (g L^{-1})		BC_1	BC_2	BC_3	Dilution Factor	Average BC	Overall cfu.mL^{-1}
10-11-14	0	0.125	0.148	9.33	202	192	196	1.00E+02	1.97E+04	1.97E+05
11-11-14	1			9.62						
12-11-14	2	0.320	0.376	9.92						
13-11-14	3	0.483	0.568	10.33	233	109	188	1.00E+06	1.77E+08	1.77E+09
14-11-14	4	0.701	0.825	10.12	187	140	298	1.00E+06	2.08E+08	2.08E+09
15-11-14	5	0.982	1.155	10.27	54	57	55	1.00E+07	5.53E+08	5.53E+09
16-11-14	6	1.015	1.194	10.83	70		47	1.00E+07	5.85E+08	5.85E+09
17-11-14	7	1.190	1.401	10.28	42		34	1.00E+07	3.80E+08	3.80E+09
18-11-14	8	1.367	1.608	11.50						
19-11-14	9			10.15						
20-11-14	10	1.828	2.151	10.52	67					
21-11-14	11	1.594	1.875	10.24	167	139	59	1.00E+05	1.22E+07	1.22E+08
22-11-14	12	1.923	2.263	10.65	45	49	44	1.00E+05	4.60E+06	4.60E+07
23-11-14	13	2.103	2.474	9.43	56	56	249	1.00E+05	1.20E+07	1.20E+08
24-11-14	14	2.353	2.768	10.00	155	65	114	1.00E+07	1.11E+09	1.11E+10

OD: Optical Density at 750 nm; CDW: Cell Dry Weight; cfu: colony forming units

(c) Temperature: 35°C

Date	Day	Spirulina biomass concentration		pH	Bacterial Counts (BC)					
		OD 750 nm	DCW (g L^{-1})		BC_1	BC_2	BC_3	Dilution Factor	Average BC	Overall cfu.mL^{-1}
10-11-14	0	0.219	0.258	9.27	58	63	56	1.00E+03	5.90E+04	5.90E+05
11-11-14	1			9.42	164	191	192	1.00E+04	1.82E+06	1.82E+07
12-11-14	2	0.286	0.336	9.50	56	60	63	1.00E+06	5.97E+07	5.97E+08
13-11-14	3	0.470	0.552	9.80	31	30	47	1.00E+06	3.60E+07	3.60E+08
14-11-14	4	0.599	0.705	9.99	33	37	30	1.00E+06	3.33E+07	3.33E+08
15-11-14	5	0.818	0.962	10.43	130	122	137	1.00E+06	1.30E+08	1.30E+09
16-11-14	6	0.782	0.919	10.61						
17-11-14	7	0.940	1.106	10.78	42	46	40	1.00E+06	4.27E+07	4.27E+08
18-11-14	8	0.878	1.032	9.65	104	109	105	1.00E+06	1.06E+08	1.06E+09
19-11-14	9			9.53	119	133	150	1.00E+07	1.34E+09	1.34E+10
20-11-14	10	0.901	1.060	9.65	52	47	45	1.00E+07	4.80E+08	4.80E+09
21-11-14	11	1.056	1.242	9.93	54	108	94	1.00E+06	8.53E+07	8.53E+08
22-11-14	12	1.080	1.271	10.07	36	64	42	1.00E+05	4.73E+06	4.73E+07
23-11-14	13	1.295	1.524	10.33				1.00E+05		
24-11-14	14	1.974	2.322	10.91	110	98	115	1.00E+05	1.07E+07	1.07E+08

OD: Optical Density at 750 nm; CDW: Cell Dry Weight; cfu: colony forming units

8.4.1 Effect of salinity on Spirulina and contaminating bacteria

Table 8.11: The effect of growth medium salinity on the growth of Spirulina and the bacterial load of associated bacteria at 7 ppt (a), 14 ppt (b) and 28 ppt (c).

(a) Salinity: 7 ppt

		Spirulina biomass concentration			Bacterial Counts (BC)					
Date	Day	OD 750 nm	DCW (g L^{-1})	pH	BC_1	BC_2	BC_3	Dilution Factor	Average BC	Overall cfu.mL^{-1}
19-09-14	0	0.131	0.154	9.69	64	66	69	1.00E+03	6.63E+04	6.63E+05
20-09-14	1	0.184	0.217	9.79	33	32	30	1.00E+04	3.17E+05	3.17E+06
21-09-14	2	0.176	0.207	9.96				data missing		
22-09-14	3	0.205	0.241	9.84	39	33	156	1.00E+04	7.60E+05	7.60E+06
23-09-14	4	0.213	0.251	9.93	13	4	0	1.00E+04	5.67E+04	5.67E+05
24-09-14	5	0.318	0.374	10.31	66	1	21	1.00E+06	2.93E+07	2.93E+08
25-09-14	6	0.376	0.442	9.96	58	88	48	1.00E+04	6.47E+05	6.47E+06
26-09-14	7	0.504	0.593	10.28	56	42	129	1.00E+04	7.57E+05	7.57E+06
27-09-14	8	0.676	0.795	10.25	65	110	130	1.00E+05	1.02E+07	1.02E+08
28-09-14	9	0.861	1.013	10.02	150	113	74	1.00E+04	1.12E+06	1.12E+07
29-09-14	10	0.899	1.058	9.80	78	104	75	1.00E+04	8.57E+05	8.57E+06
30-09-14	11	1.013	1.192	10.24	183	140	73	1.00E+04	1.32E+06	1.32E+07
01-10-14	12	1.164	1.370	10.14	205	170	63	1.00E+04	1.46E+06	1.46E+07
02-10-14	13	1.366	1.607	10.52	42	51	40	1.00E+06	4.43E+07	4.43E+08
03-10-14	14	1.317	1.549	9.77	67	119	75	1.00E+05	8.70E+06	8.70E+07

OD: Optical Density at 750 nm; CDW: Cell Dry Weight; cfu: colony forming units

(b) Salinity: 14 ppt

Date	Day	Spirulina biomass concentration		pH	Bacterial Counts (BC)					
		OD 750 nm	DCW (g L^{-1})		BC_1	BC_2	BC_3	Dilution Factor	Average BC	Overall cfu.mL^{-1}
19-09-14	0	0.124	0.145	9.39	34	36	36	1.00E+03	3.53E+05	3.53E+06
20-09-14	1	0.187	0.220	9.59	177	274	298	1.00E+03	2.50E+06	2.50E+07
21-09-14	2	0.231	0.272	9.75	30	36	39	1.00E+03	3.50E+05	3.50E+06
22-09-14	3	0.250	0.294	9.77	94	65	140	1.00E+04	9.97E+06	9.97E+07
23-09-14	4	0.285	0.335	9.86	32	32	51	1.00E+05	3.83E+07	3.83E+08
24-09-14	5	0.438	0.516	10.28	34	60	33	1.00E+05	4.23E+07	4.23E+08
25-09-14	6	0.541	0.636	9.99	30	42	33	1.00E+06	3.50E+08	3.50E+09
26-09-14	7	0.673	0.792	10.24	110	126	108	1.00E+05	1.15E+08	1.15E+09
27-09-14	8	0.861	1.013	10.10	33	53	36	1.00E+06	4.07E+08	4.07E+09
28-09-14	9	0.904	1.064	9.96	55	52	53	1.00E+06	5.33E+08	5.33E+09
29-09-14	10	0.923	1.085	10.11	103	108	90	1.00E+06	1.00E+09	1.00E+10
30-09-14	11	1.023	1.204	10.37	86	150	156	1.00E+06	1.31E+09	1.31E+10
01-10-14	12	1.114	1.311	10.02	105	280	274	1.00E+04	2.20E+07	2.20E+08
02-10-14	13	1.278	1.504	10.47	100	93	82	1.00E+04	9.17E+06	9.17E+07
03-10-14	14	1.374	1.617	9.82	44	62	50	1.00E+04	5.20E+06	5.20E+07

OD: Optical Density at 750 nm; CDW: Cell Dry Weight; cfu: colony forming units

(c) Salinity: 28 ppt

		Spirulina biomass concentration			Bacterial Counts (BC)					
Date	Day	OD 750 nm	DCW (g L^{-1})	pH	BC_1	BC_2	BC_3	Dilution Factor	Average BC	Overall cfu.mL^{-1}
19-09-14	0	0.122	0.144	9.18	34	36	36	1.00E+03	3.53E+04	3.53E+05
20-09-14	1	0.191	0.225	9.51	286	253	269	1.00E+03	2.69E+05	2.69E+06
21-09-14	2	0.244	0.287	9.66	200	215	224	1.00E+02	2.13E+04	2.13E+05
22-09-14	3	0.257	0.303	9.71	93	91	115	1.00E+04	9.97E+05	9.97E+06
23-09-14	4	0.321	0.377	9.80	220	218	250	1.00E+04	2.29E+06	2.29E+07
24-09-14	5	0.503	0.592	10.08	50	55	43	1.00E+05	4.93E+06	4.93E+07
25-09-14	6	0.627	0.737	9.97	35	37	41	1.00E+06	3.77E+07	3.77E+08
26-09-14	7	0.713	0.839	10.15			59	1.00E+06	5.90E+07	5.90E+08
27-09-14	8	0.829	0.976	10.03	37	60	66	1.00E+06	5.43E+07	5.43E+08
28-09-14	9	0.880	1.036	10.03	40	41	38	1.00E+06	3.95E+07	3.95E+08
29-09-14	10	0.859	1.011	9.85	57	38	48	1.00E+06	4.77E+07	4.77E+08
30-09-14	11	0.969	1.139	10.03	101		50	1.00E+06	7.53E+07	7.53E+08
01-10-14	12	1.046	1.231	10.19	180	169	177	1.00E+05	1.75E+07	1.75E+08
02-10-14	13	1.282	1.508	9.95	155	159	95	1.00E+04	1.36E+06	1.36E+07
03-10-14	14	1.371	1.911	10.00	55	41	44	1.00E+05	4.67E+06	4.67E+07

OD: Optical Density at 750 nm; CDW: Cell Dry Weight; cfu: colony forming units

8.4.2 Effect of pH on *Spirulina* and contaminating bacteria

Table 8.12: The effect of pH on Spirulina growth and the bacterial loads of bacteria associated with the Spirulina culture when cultivated at pH 9 (a), pH 10 (b) and pH 11(c)

(a) pH 9

		Spirulina biomass concentration			Bacterial Counts (BC)					
Date	Day	OD 750 nm	DCW (g L^{-1})	pH	BC_1	BC_2	BC_3	Dilution Factor	Average BC	Overall cfu.mL^{-1}
19-09-14	0	0.123	0.145	9.39	64	66	69	1.00E+03	6.63E+04	6.63E+05
20-09-14	1	0.185	0.218	9.62	33	32	30	1.00E+04	3.17E+05	3.17E+06
21-09-14	2	0.209	0.245	9.75				data missing		
22-09-14	3	0.231	0.272	9.78	39	33	156	1.00E+04	7.60E+05	7.60E+06
23-09-14	4	0.283	0.333	9.82	13	4	0	1.00E+04	5.67E+04	5.67E+05
24-09-14	5	0.378	0.445	9.77	66	1	21	1.00E+06	2.93E+07	2.93E+08
25-09-14	6	0.481	0.566	9.49	58	88	48	1.00E+04	6.47E+05	6.47E+06
26-09-14	7	0.591	0.696	9.66	56	42	129	1.00E+04	7.57E+05	7.57E+06
27-09-14	8	0.756	0.889	9.78	65	110	130	1.00E+05	1.02E+07	1.02E+08
28-09-14	9	0.846	0.995	9.74	150	113	74	1.00E+04	1.12E+06	1.12E+07
29-09-14	10	0.941	1.107	9.54	78	104	75	1.00E+04	8.57E+05	8.57E+06
30-09-14	11	1.023	1.204	9.67	183	140	73	1.00E+04	1.32E+06	1.32E+07
01-10-14	12	1.155	1.359	9.61	205	170	63	1.00E+04	1.46E+06	1.46E+07
02-10-14	13	1.406	1.654	9.85	42	51	40	1.00E+06	4.43E+07	4.43E+08
03-10-14	14	1.391	1.636	9.46	67	119	75	1.00E+05	8.70E+06	8.70E+07

OD: Optical Density at 750 nm; CDW: Cell Dry Weight; cfu: colony forming units

(b) pH 10

Date	Day	Spirulina biomass concentration OD 750 nm	DCW (g L^{-1})	pH	Bacterial Counts (BC) BC_1	BC_2	BC_3	Dilution Factor	Average BC	Overall cfu.mL^{-1}
19-09-14	0	0.125	0.147	9.39	34	36	36	1.00E+03	3.53E+04	3.53E+05
20-09-14	1	0.197	0.232	9.61		256		1.00E+03	2.56E+05	2.56E+06
21-09-14	2	0.249	0.293	9.78	36	42	67	1.00E+03	4.83E+04	4.83E+05
22-09-14	3	0.262	0.308	9.80	117	68	119	1.00E+04	1.01E+06	1.01E+07
23-09-14	4	0.311	0.366	9.92	43	42	48	1.00E+05	4.43E+06	4.43E+07
24-09-14	5	0.487	0.573	10.42	47	35	128	1.00E+05	7.00E+06	7.00E+07
25-09-14	6	0.589	0.693	9.66	49	49	47	1.00E+06	4.83E+07	4.83E+08
26-09-14	7	0.779	0.916	10.03	68	44	35	1.00E+06	4.90E+07	4.90E+08
27-09-14	8	0.923	1.086	10.28	59	33	46	1.00E+06	4.60E+07	4.60E+08
28-09-14	9	0.731	0.861	10.10	106	data missing		1.00E+06	1.06E+08	1.06E+09
29-09-14	10	0.845	0.994	9.84	48	data missing		1.00E+06	4.80E+07	4.80E+08
30-09-14	11	0.926	1.090	10.18	37	136	131	1.00E+05	1.01E+07	1.01E+08
01-10-14	12	1.060	1.247	9.94	218	67	140	1.00E+04	1.42E+06	1.42E+07
02-10-14	13	1.166	1.372	10.30	121	38	49	1.00E+06	6.93E+07	6.93E+08
03-10-14	14	1.181	1.390	9.74	46	30		1.00E+05	3.80E+06	3.80E+07

OD: Optical Density at 750 nm; CDW: Cell Dry Weight; cfu: colony forming units

(c) pH 11

Date	Day	Spirulina biomass concentration OD 750 nm	DCW (g L^{-1})	pH	Bacterial Counts (BC) BC_1	BC_2	BC_3	Dilution Factor	Average BC	Overall cfu.mL^{-1}
19-09-14	0	0.125	0.148	9.39	34	36	36	1.00E+03	3.53E+04	3.53E+05
20-09-14	1	0.202	0.238	9.61	30	32	data missing	1.00E+03	3.10E+04	3.10E+05
21-09-14	2	0.244	0.287	9.74	38	data missing	36	1.00E+03	3.70E+04	3.70E+05
22-09-14	3	0.236	0.278	9.77	148	129	68	1.00E+04	1.15E+06	1.15E+07
23-09-14	4	0.266	0.313	9.83	75	33		1.00E+05	5.40E+06	5.40E+07
24-09-14	5	0.403	0.474	10.24	123	69	36	1.00E+05	7.60E+06	7.60E+07
25-09-14	6	0.481	0.565	10.46	60	46	34	1.00E+05	4.67E+06	4.67E+07
26-09-14	7	0.648	0.762	10.92	42	36		1.00E+06	3.90E+07	3.90E+08
27-09-14	8	0.781	0.919	10.88	37	32	34	1.00E+06	3.43E+07	3.43E+08
28-09-14	9	0.860	1.012	10.55	42	30	35	1.00E+06	3.57E+07	3.57E+08
29-09-14	10	0.893	1.050	10.86	30	69	77	1.00E+04	5.87E+05	5.87E+06
30-09-14	11	1.019	1.198	10.61	151	161	58	1.00E+04	1.23E+06	1.23E+07
01-10-14	12	1.164	1.369	10.75	53	45	121	1.00E+04	7.30E+05	7.30E+06
02-10-14	13	1.248	1.468	11.21	34	40	59	1.00E+04	4.43E+05	4.43E+06
03-10-14	14	0.934	1.099	11.03	53	44	45	1.00E+05	4.73E+06	4.73E+07

OD: Optical Density at 750 nm; CDW: Cell Dry Weight; cfu: colony forming units

8.5 Effect of various drying methods on bacterial load of Spirulina powder

The effect of various drying methods on the bacterial load associated with Spirulina powder was investigated. Spirulina biomass was dried at various temperatures in a laboratory scale oven and the bacterial loads resulting from these drying methods were compared to those of the biomass subsequent to drying. Spirulina biomass was also freeze dried following incubation room temperature for 3 or 6 hrs, or incubation at 30°C for 3 or 6 hrs. The data generated from these studies are given below.

Table 8.13: (a) Bacterial load in Spirulina biomass before drying and following drying at various temperatures in a laboratory scale oven, Bacterial load in Spirulina powder, (b) freeze dried after incubation at room temperature or 30°C for 3 or 6 hrs

(a)

Treatment	Sample Number	Plate 1	Plate 2	Plate 3	Dilution Factor	Average Bacteria (cfu.g^{-1})	SD	Bacterial load (cfu.g^{-1})
Spirulina biomass following harvesting	1	90	74	97	100000	8.70E+06	11.79	**8.10E+06 (wet biomass)**
	2	98	96	120	100000	1.05E+07	13.31	
	3	72	39	43	100000	5.13E+06	18	
Oven Dried at 45°C		184	154	159	10000	1.66E+06	16.07	1.35E+06
Oven Dried at 45°C		87	93	96	10000	9.20E+05	4.58	
Oven Dried at 45°C		141	148	153	10000	1.47E+06	6.02	
Oven Dried at 55°C		148	148	141	10000	1.46E+06	4.04	1.36E+06
Oven Dried at 55°C		84	121	93	10000	9.93E+05	19.29	
Oven Dried at 55°C		160	165	162	10000	1.62E+06	2.51	

Oven Dried at 65°C	15	7	9	10000	1.03E+05	4.16	1.24E+05
Oven Dried at 65°C	14	17	12	10000	1.43E+05	2.51	
Oven Dried at 65°C	11	15	12	10000	1.27E+05	2.08	

(b)

	Freeze Drying $_1$ FD (no Incubation)						
	Plate$_1$	**Plate$_2$**	**Plate$_3$**	**Dil. F**	**Av. Bacteria**	**SD**	**Bacterial load (cfu)**
Freeze dried (FD), no incubation	56	52	49	10000	5.23E+05	3.51	9.61E+05
Freeze dried (FD), no incubation	115	95	102	10000	1.04E+06	10.14	
Freeze dried (FD), no incubation	140	125	131	10000	1.32E+06	7.55	
Incubated at room temp for 3 h, FD	170	230	223	10000	2.08E+06	32.8	1.76E+06
Incubated at room temp for 3 h, FD	162	151	128	10000	1.47E+06	17.34	
Incubated at room temp for 3 h, FD	173	168	181	10000	1.74E+06	6.55	
Incubated at room temp for 6 h, FD	237	249	221	10000	2.36E+06	14.04	2.63E+06
Incubated at room temp for 6 h, FD	298	284	279	10000	2.87E+06	9.84	
Incubated at room temp for 6 h, FD	265	266	271	10000	2.67E+06	3.21	
Incubated at 30°C for 3 h, FD	33	30	31	100000	3.13E+06	1.52	2.20E+06
Incubated at 30°C for 3 h, FD	152	140	135	10000	1.42E+06	8.73	
Incubated at 30°C for 3 h, FD	198	205	212	10000	2.05E+06	7	
Incubated at 30°C for 6 h, FD	63	66	71	100000	6.67E+06	4.04	5.32E+06
Incubated at 30°C for 3 h	53	60	64	100000	5.90E+06	5.56	
Incubated at 30°C for 3 h	33	36	33	100000	3.40E+06	1.73	

FD: freeze dried; SD: standard deviation

8.6 Ethics form

EBE Faculty: Assessment of Ethics in Research Projects

Any person planning to undertake research in the Faculty of Engineering and the Built Environment at the University of Cape Town is required to complete this form before collecting or analysing data. When completed it should be submitted to the supervisor (where applicable) and from there to the Head of Department. If any of the questions below have been answered YES, and the applicant is NOT a fourth year student, the Head should forward this form for approval by the Faculty EIR committee: submit to Ms Zakiya Chikte (Zakiya.chikte@uct.ac.za); New EBE Building, Ph 021 650 5739).
Please note – It is important to keep a signed copy of this form as students must include a copy of the completed form with the dissertation/thesis when it is submitted for examination.

Name of Principal Researcher/Student: Mothalekgomo Department: CHEM ENG

If a Student: Degree: M Sc Supervisor: Prof Sue Harrison

If a Research Contract indicate source of funding/sponsorship:

Research Project Title: Identification and quantification of bacteria associated cultivated Spirulina and impact of physicochemical factors

Overview of ethics issues in your research project:

Question 1: Is there a possibility that your research could cause harm to a third party (i.e. a person not involved in your project)?	YES	NO ✓
Question 2: Is your research making use of human subjects as sources of data? If your answer is YES, please complete Addendum 2.	YES	NO ✓
Question 3: Does your research involve the participation of or provision of services to communities? If your answer is YES, please complete Addendum 3.	YES	NO ✓
Question 4: If your research is sponsored, is there any potential for conflicts of interest? If your answer is YES, please complete Addendum 4.	YES	NO ✓

If you have answered YES to any of the above questions, please append a copy of your research proposal, as well as any interview schedules or questionnaires (Addendum 1) and please complete further addenda as appropriate.

I hereby undertake to carry out my research in such a way that

- there is no apparent legal objection to the nature or the method of research; and
- the research will not compromise staff or students or the other responsibilities of the University;
- the stated objective will be achieved, and the findings will have a high degree of validity;
- limitations and alternative interpretations will be considered;
- the findings could be subject to peer review and publicly available; and
- I will comply with the conventions of copyright and avoid any practice that would constitute plagiarism.

Signed by:

	Full name and signature	Date
Principal Researcher/Student: Mothalekgomo		17 Feb 2016

This application is approved by:

Supervisor (if applicable):		17/2/16
HOD (or delegated nominee): Final authority for all assessments with NO to all questions and for all undergraduate research.		2016/2/16
Chair : Faculty EIR Committee For applicants other than undergraduate students who have answered YES to any of the above questions.		

ADDENDUM 1:
Please append a copy of the research proposal here, as well as any interview schedules or questionnaires.

ADDENDUM 2: To be completed if you answered YES to Question 2

It is assumed that you have read the UCT Code for Research involving Human Subjects (available at http://web.uct.ac.za/depts/educate/download/ucicodeforresearchinvolvinghumansubjects.pdf) in order to be able to answer the questions in this addendum.

2.1 Does the research discriminate against participation by individuals, or differentiate between participants, on the grounds of gender, race or ethnic group, age range, religion, trauma, handicap, illness or any similar classification?	YES	NO ✓
2.2 Does the research require the participation of socially or physically vulnerable people (children, aged, disabled, etc) or legally restricted groups?	YES	NO ✓
2.3 Will you not be able to secure the informed consent of all participants in the research? (In the case of children, will you not be able to obtain the consent of their guardians or parents?)	YES	NO ✓
2.4 Will any confidential data be collected or will identifiable records of individuals be kept?	YES	NO ✓
2.5 In reporting on the research is there any possibility that you will not be able to keep the identities of the individuals involved anonymous?	YES	NO ✓
2.6 Are there any foreseeable risks of physical, psychological or social harm to participants that might occur in the course of the research?	YES	NO ✓
2.7 Does the research include making payments or giving gifts to any participants?	YES	NO ✓

If you have answered YES to any of these questions, please describe how you plan to address these issues (append to form):

ADDENDUM 3: To be completed if you answered YES to Question 3:

3.1 Is the community expected to make decisions for, during or based on the research?	YES	NO
3.2 At the end of the research will any economic or social process be terminated or left unsupported, or equipment or facilities used in the research be recovered from the participants or community?	YES	NO
3.3 Will any service be provided at a level below the generally accepted standards?	YES	NO

If you have answered YES to any of these questions, please describe how you plan to address these issues (append to form):

ADDENDUM 4: To be completed if you answered YES to Question 4

4.1 Is there any existing or potential conflict of interest between a research sponsor, academic supervisor, other researchers or participants?	YES	NO
4.2 Will information that reveals the identity of participants be supplied to a research sponsor, other than with the permission of the individuals?	YES	NO
4.3 Does the proposed research potentially conflict with the research of any other individual or group within the University?	YES	NO

If you have answered YES to any of these questions, please describe how you plan to address these issues(append to form)