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Quality and Safety evaluation of *Spirulina* (*Arthrospira*) biomass harvested from an Ethiopian Soda Lake, Lake Chitu for possible applications in human nutrition

A PhD Dissertation in Food Science and Nutrition

By

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Abbreviations/acronyms

Ala	Alanine
Amm	Ammoniac
AOAC	Association of Official Agricultural Chemists
Arg	Arginine
Asp	Aspartic acid
BGBB	Brilliant Green Bile Broth
BSA	Bovine Serum Albumin
BV	Biological Value
CFU	Colony Forming Units
Chl *a*	Chlorophyll *a*
CSA	Central Statistics Agency
Cys	Cysteine
DC	Digestibility Coefficient
DM	Dry matter
DNA	Deoxyribonucleic acid
DW	Dry weight
EA	Emulsifying activity
ECB	*Escherchia coli* broth
EHNRI	Ethiopian Health and Nutrition research Institute
ELISA	Enzyme-Linked Immunosorbent Assay
ES	Emulsion stability
FAME	Fatty Acid Methyl Esters
FAO	United Nations Food and Agriculture Organization
FC	Foaming capacity
FS	Foaming stability
GC	Gas chromatography
Glu	Glutamic acid
Gly	Glycine
His	Histidine

HPLC	High performance liquid chromatography
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IFAD	International Organization of Food and Agriculture Development
Ile	Isoleucine
LD	Lethal dose
Lys	Lysine
Met	Methionine
NB	Nutrient Broth
NMKL	Nordic committee on food Analysis
NPU	Net protein utilization
OAC	Oil absorption capacity
PBE	Phycobiliprotein rich crude extract
PER	Protein Efficiency Ratio
Phe	Phenylalanine
PUFA	Polyunsaturated fatty acid
RNA	Ribonucleic acid
Ser	Serine
SFA	Saturated Fatty Acid
Thr	Threonine
TSA	Trypton Soya Agar
Tyr	Tyrosine
UNU	United Nations University
USP	United States Pharmacopia
UV/VIS/NIR	Ultraviolet/ Visible/Near infrared
Val	Valine
VRBA	Violet Red Bile Agar
WAC	Water absorption capacity
WFP	World Food Program

Abstract

The cyanobacterium *Arthrospira* (*Spirulina*) is known for its high protein content and having various bioactive phytochemicals with potential health benefits. It is, therefore, produced worldwide either artificially or directly from natural lakes to be used as a dietary and/or protein supplement. *Spirulina* grows abundantly almost as a uni-algal population in an Ethiopian soda lake, Lake Chitu throughout the year. The objective of this study was to evaluate the nutrient profile and safety of Lake Chitu's *Spirulina* for possible applications in human nutrition. A one year study (March 2012–January 2013) was undertaken where sampling was done at two months interval. Variations were observed in the nutrient profile of the biomass on seasonal basis and in general wet season samples had relatively higher protein (47.9–55.7%) and low carbohydrates contents (30.9–36.4%) compared to dry season samples. Higher amino acids content was recorded for a wet season sample (86.4g/100g protein) compared to a dry season sample (62.1g/100g protein). Variations were also observed in the fatty acid composition of Lake Chitu's *Spirulina* as relatively higher proportion of unsaturated fatty acids were recorded for a sample harvested in wet season (61.8%) compared to a dry season sample (58.2%). Similarly, higher contents of phytonutrients (pigments) (mg g^{-1}): chlorophyll *a* (8.2–10.3), phycobiliproteins (104.1–120.7), total carotenoids (3.17–4.31) and β-carotene (1.24–1.61) were recorded for wet season samples. The mineral content of the biomass also varied according to season. While Na and K were found to be higher in the dry season sample, other major (Ca, P, Mg) and trace (Mn, Fe, Cu, Zn and Se) minerals were found in higher concentrations in the wet season sample.

The safety of Lake Chitu's *Spirulina* was evaluated in terms of its microbial profile, heavy metals, pheophorbides, microcystin and nucleic acids content. Aerobic mesophilic count of the biomass ranged from 0.4 $x10^3$ to 130 $x10^3$ CFU/g for wet season and from 0.37 $x10^3$ to 15 $x10^3$ CFU/g for dry season samples. Counts of total coliforms, fecal coliforms, *E.coli*, *S. aureus*, yeasts and molds were < 10^2CFU/g for both wet and dry season samples. Heavy metal contents of the biomass (mg kg^{-1}) were: Pb (0.18), Cd (0.03), Hg (0.04) and As (18.8). Pheophorbides content of the biomass were 16.2mg/100g and 22.3mg/100g for wet and dry season samples

respectively. The nucleic acids content of the samples were 0.18 and 0.13% for wet and dry season samples respectively. Microcystins were not detected in both wet and dry season samples and short term feeding of freeze dried biomass (10 g/kg body weight) to mice didn't result any signs of toxicity or mortality.

Phycobiliprotein-rich crude extract (PBE) was prepared from *Spirulina* and was evaluated for its chemical composition, amino acid profile, *in vitro* protein digestibility and functional properties for possible food applications. Phycobiliprotein-rich crude extract had higher contents of proteins (60.1 vs 45.2%) and amino acids (45.6 vs 39.0g/100gsample) than the crude *Spirulina* powder. Similarly, *in vitro* protein digestibility value of PBE was higher (86%) than the crude *Spirulina* powder (59%) in a single enzyme assay. The extract had 50.5% protein solubility at pH 7 and 62.2% solubility at pH 12 although at this pH discoloration of the pigment was noted. PBE showed good emulsifying activity at neutral and basic pH regions and at higher protein concentrations the extract showed comparable emulsifying activity with bovine serum albumin. Similarly, the foaming capacity and stability of PBE were high and comparable with the commonly used foaming agent, egg albumin.

In conclusion, the nutrient profile of Lake Chitu's *Spirulina* is comparable with that reported for commercial *Spirulina*. Except arsenic which was found in higher concentrations, the biomass is safe in terms of other safety evaluation parameters and thus can potentially be used in human nutrition. In addition to using the whole biomass as a food supplement, high value products such as phycobiliproteins can be extracted from the crude biomass which can serve as a component in functional foods.

Key words: *Arthrospira*, biomass quality, functional property, Lake Chitu, safety, season, phycobiliproteins, *Spirulina*

Chapter 1: Introduction

A growing concern for the protein needs of the world's increasing human population has led to the search for a variety of unusual or non-conventional protein sources as potential alternatives of protein supply (Dillon and Phan, 1993; Spolaore *et al.*, 2006). These non-conventional protein sources include, leaf protein concentrate, fish protein concentrate, proteins from oil seeds and single cell proteins or biomass proteins (Adedayo *et al.*, 2011). Among biomass protein sources, the use of the cyanobacterium *Spirulina (Arthrospira)* has gained maximum attention by researchers due to a number of reasons. It contains about 45-70% protein on dry weight basis (Ciferri, 1983; Fox, 1996; Habib *et al.*, 2008) and the qualities of its proteins are excelllent since all the essential amino acids are represented (Becker, 2007). It is rich in minerals, vitamins and various phytochemicals that have potential health benefits (Ciferri and Tiboni, 1985; Otles and Pire, 2001; Belay, 2002; Jiménez *et al.*, 2003; Shimamatsu, 2004). Moreover, *Spirulina* is easy to digest due to the absence of cellulose in its cell wall and has low nucleic acid content compared to other microbial protein sources such as bacteria and yeasts (Dillon *et al.*, 1995; Sasson, 1997). In addition to its nutritional value, the health promoting effects of *Spirulina* biomass and its extracts have been described by various researchers (Belay *et al.*, 1993; Belay, 2002; Khan *et al.*, 2005; Karkos *et al.*, 2008; Rasool and Sabina, 2009; Deng and Chow, 2010) which includes reduction of blood cholesterol, immunomodulation, growth promotion of intestinal *Lactobacillus*, radiation protection, antioxidant/anti-inflammatory effects, diabetes management, anti-cancer effects, anti-viral effects, reduction of drug and heavy metal toxicity and weight management. The immunomodulatory and anti-viral properties of *Spirulina* are interesting in the fight against malnutrition, since malnourished children or individuals generally have weakened immune systems (Hug and von der Weid, 2011). Because of these special features, the United Nations Food and Agriculture Organization (FAO) designated *Spirulina* as an ideal food and dietary supplement for the 21st century (Pelizer *et al.*, 2003).

It has been indicated that, in poor settings, complementation of traditional meals with *Spirulina* could be a cost-effective approach to provide to the most vulnerable populations a basis of physical and mental health (Ardiet and von der Weid, 2011). Several clinical studies in developing countries demonstrated the efficacy of *Spirulina* in the nutritional rehabilitation of

malnourished children and vulnerable populations (e.g. HIV-positives) when it is supplemented to their traditional meals (Sachdeva, 2004; Simpore *et al.*, 2005; Simpore *et al.*, 2006; Yamani *et al.*, 2009; Azabji-Kenfack *et al.*, 2011). China and India have declared *Spirulina* as a National food (Fox, 1993) and several African countries such as Burkina Faso, Togo, Democratic Republic of Congo, Bangui, Senegal and Cameroon are benefiting a lot from this microalga through small scale or village level production (Fox, 1993; Hug and von der Weid, 2011).

Rates of malnutrition in Ethiopia are among the highest in the world. FAO/ WFP/ IFAD's 2012 report on the state of food insecurity in the world stated that 40.2 % of the population of Ethiopia is undernourished (FAO/WFP/IFAD, 2012). Malnutrition in Ethiopia is primarily associated with the poor quality of the food consumed as most Ethiopian foods are starch based with low nutrient density (Grando and Gormez, 2005). Haile Mariam (2009) indicated that protein energy malnutrition is a major cause of morbidity and premature death among children and infants in Ethiopia. Micronutrient malnutrition, especially among children and women of reproductive age is also very high (Hambidge, 2006) which is manifested as high prevalence of vitamin A deficiency (CSA and ICF International, 2012), and anemia (Haidar and Pobocik, 2009; Haidar, 2010; CSA and ICF International, 2012).

Spirulina grows in some Ethiopian soda lakes and in lakes such as Lake Chitu abundantly almost as a monospesific population throughout the year (Wood and Talling, 1988; Kebede, 1997). Most researches so far done on Lake Chitu's *Spirulina* mainly focused on its liminological aspects such as its growth behavior and light utilization efficiency (Kebede and Alghrein, 1996), its tolerance limits to salinity and ionic concentrations (Kebede, 1997), its biomass concentration (Wood and Talling, 1988; Kebede *et al.*, 1994) and its morphological variability in relation to environmental conditions (Ogato and Kifle, 2014). A study by Willen *et al.* (2011) indicated that *Spirulina* species from this lake as a non–toxin producer. However, published data on the food value of Lake Chitu's *Spirulina* biomass are lacking. The paucity of data on the chemical composition, nutritional quality and safety may affect the likely contribution of this natural resource as an alternative food or dietary supplement. In order to use this natural resource as a dietary or nutritional supplement as well as to initiate large scale or small scale commercial production, the quality of the biomass should be known. Therefore, the major aim of this study

was to investigate the biochemical composition, nutritional quality and safety of the biomass harvested from Lake Chitu for possible applications in human nutrition.

Specifically this study addressed the following specific objectives:

1. Investigate the potential of Lake Chitu's *Spirulina* to be used in human nutrition
2. Determine seasonal variation in the nutrient profile of Lake Chitu's *Spirulina*
3. Evaluate the safety of Lake Chitu's *Spirulina* on seasonal basis for possible use of the biomass as a human food
4. Determine the chemical composition and functional property of a protein preparation (phycobiliprotien-rich crude extract) obtained from Lake Chitu's *Spirulina*

This PhD thesis is organized as follows:

Chapter one included introduction, statement of the problem and objectives

Chapter two presents the literature review that covers an overview of the historical use of *Spirulina* as a human food supplement and the general characteristics of *Spirulina* which includes its morphology, taxonomy, ecology and chemical composition. This is followed by a brief presentation about food products with *Spirulina* addition, cultivation and production of *Spirulina* and the role of *Spirulina* in the nutrional rehabilitation of malnourished and vurerable populations. Safety issues in the use of *Spiurlina* as a human food supplement are also addressed. Finally, brief review about the past and the present status of *Spirulina* in Ethiopian soda lakes and the potential of *Spirulina* production in Ethiopia are presented.

Chapter three addresses the results from the preliminary study on the possibility of using Lake Chitu's *Spirulina* in human nutrition.

Chapter four offers the results of the investigation of seasonal variations in the nutrient profile of Lake Chitu's *Spirulina*. In this part, the variation in the proximate composition, amino acid profile, fatty acid profile, mineral and phytonutrient composition of Lake Chitu's *Spirulina* is presented.

Chapter five presents results on safety evaluation of Lake Chitu's *Spirulina* harvested during dry and wet seasons for possible applications in human nutrition. It is an overview of the safety

of Lake Chitu's *Spirulina* with respect to its microbial profile, heavy metals, nucleic acids, microcystin and pheophorbides content as well as results from an acute toxicity test on mice.

Chapter six presents results on the functional property of a protein preparation (phycobiliprotein -rich crude extract) prepared from Lake Chitu's *Spirulina*. It gives an overview on the chemical composition, *in vitro* protein digestibility and functional property of the extract (protein solublity, water and oil absorption capacity, bulk density, foamoability, emulsifying activity, etc) for possible food applications.

Chapter seven gives a comprehensive discussion on the findings of the studies.

Chapter eight presents conclusions, recommendations and perspectives of the whole study.

The reference section provides relevant refernces in the subject

Chapter 2: Literature review

2.1 Historical use of *Spirulina* as a human food supplement

The use of *Spirulina* as a human food supplement has a very ancient history (Vonshak, 1997). Early in 1524, Fray Toribio de Benavente reported the harvest of "tecuitlatl" (floating mats of *Spirulina)* by the Aztecs from lakes in Mexico. The natives (Aztecs) who live near *Spirulina* lakes harvest this microalga from the water using a fine mesh, sun-dry the biomass, and use it as a staple food, or as a protein supplement to their diet (Ciferri, 1983; Richmond, 1986). Later on, the discovery of sun dried cakes which were also eaten by the Kanembu people who live along the shores of Lake Chad in Central West Africa has led the identification of this organism as a cyanobacterium with interesting combination of essential nutrients. Harvesting and selling of *Spirulina* cakes called 'Dihe' is a common activity for the Kanembu tribes (Abdulquader *et al.*, 2000). Women harvest the biomass and filter it through a dum palm basket. The filtered biomass is then transferred in to a sand-filter basin and sliced into squares and left for five to six days in the sun to dry (Fig 2.1).

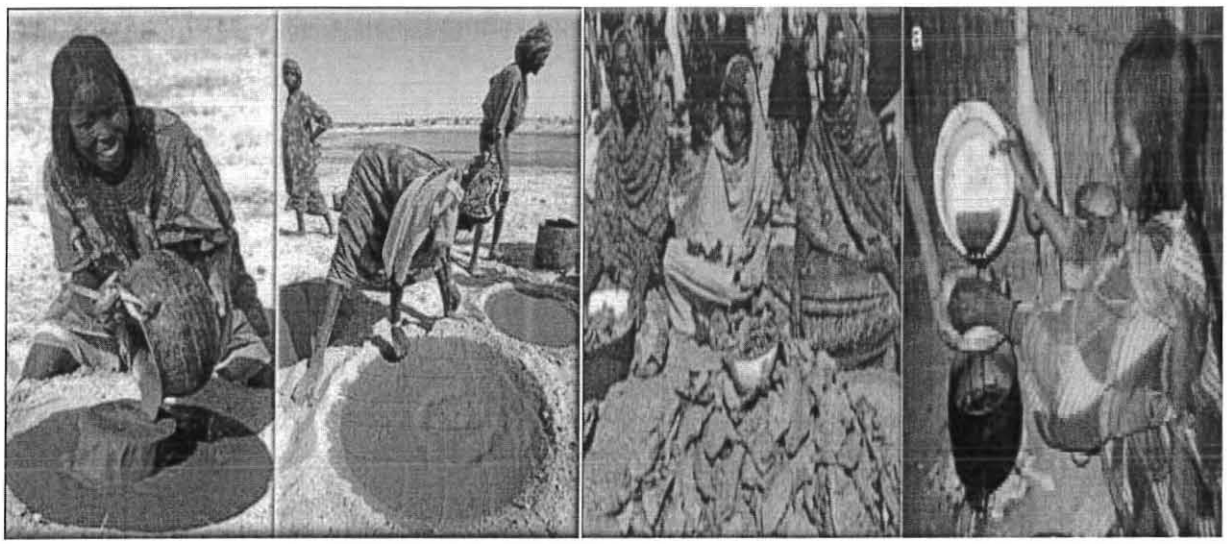


Fig 2.1. Harvesting *Spirulina*, drying it in a sand filter, and selling dried *Spirulina* cakes ('dihe') in local market by the Kanembu tribes and cooking of dihe to prepare 'la' sauce (Abdulquader *et al.*, 2000; Henrikson, 2011)

'Dihe' makes a large portion of the daily protein diet of the Kanembu tribe. It is crumbled and mixed with their local dishes (such as '*la*' sauce) and about 10-12 grams of *Spirulina* is consumed per person per meal (Ciferri, 1983; Abdulquader *et al.*, 2000). Selling *Spirulina* cakes contributes significantly to the local economy of Kanembu. From data collected at Lake Kossorom (Chad), Abdulqader *et al.* (2000) calculated an average harvest of 40 tones of *Spirulina* every year from this lake amounting more than US $100,000.

2.2 General characteristics of *Spirulina* (*Arthrospira*)

2.2.1 Taxonomy, morphology and ultrastructure

In earlier times, *Spirulina* was classified in the plant kingdom because of its richness in pigments that are also found in plants as well as its ability of photosynthesis. Nowadays, this species is classified in kingdom monera (bacteria) based on a new understanding with respect to its genetic make up, physiology and biochemical properties (Vonshak, 1997). *Spirulina* is usually used to describe two edible species of cyanobacteria, *Arthrospira platensis* and *Arthrospira maxima.* However, there is a well established difference between the genus *Spirulina* and *Arthrospira* (Belay, 2013). These two genera are different in their helicity and trichome size, cell wall structure and pore pattern, gas vesicles, thylakoid pattern, trichome motility and fragmentation, GC content as well as oligonucleotide cataloge of 16S rRNA (Tomaselli, 1997). These two genera are also different in their fatty acid profile as gamma linolenic acid is absent in species of the genus *Spirulina* (Muhling *et al.*, 2005). Thus, currently *Spirulina* is classified under Phylum Cyanobacteria, Order Oscillatoriales, Family Phormadiaceae and Genus *Arthrospira* (Belay, 2008). On the other hand, the commercial (common) name of the dried biomass of *Arthrospira* is *Spirulina* and most scientific reports about the genus are written in the name *Spirulina.* Therefore, in this thesis, these two species designations are used interchangeably.

Spirulina (*Arthrospira*) is a multicellular, helicoidal and filamentous cyanobacterium, which appears under a microscope as blue-green filament composed of cylindrical cells with 1 to 12 μm diameter (Ciferri, 1983; Richmond, 1990). The morphology of *Spirulina* (*Arthrospira*) varies in response to environmental changes or growth conditions in which the one which appeared tightly coiled may be changed into loosely coiled filament and vice versa (Cifferi, 1983; Vonshak, 1997;

Belay, 2013). Straight or nearly straight morphotypes of *Spirulina* have been also reported (Jeeji Bai and Seshadri, 1980; Jeeji Bai, 1985). A recent study by Ogato and Kifle (2014) indicated the ability of *Spirulina* to undergo morphological modifications in their natural habitats in response to environmental stresses which results in occurrence of various morphotypes of the same species. Some pictures of *Spirulina* (*Arthrospira*) isolated from Lake Chitu, Ethiopia are presented in Fig 2.2.

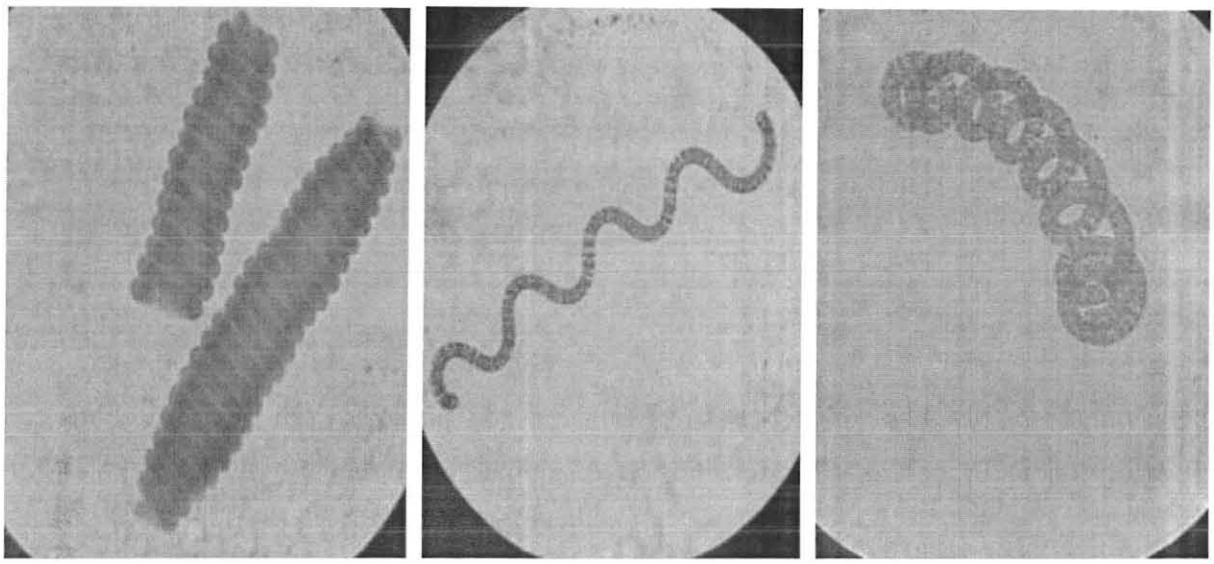


Fig 2.2.Microscopic view of *Spirulina* (different morphotypes) isolated from Lake Chitu, Ethiopia

The cell organization of *Spirulina* is similar with a gram-negative bacterium since it lacks membrane-bound organelles and has a cell wall that constitutes a weak envelope composed mostly of peptidoglycan and lipopolysaccharides. The inside of the cell is filled with a number of inclusions, such as thylakoid membranes (contains light harvesting pigments), carboxysomes, ribosomes, DNA fibrils, gas vacuoles, polyphosphate granules, polyglucan (carbohydrate storage) granules, and cyanophycin (nitrogen storage) granules (Ciferri, 1983; Ciferri and Tiboni, 1985; Belay, 2013). The presence of gas–filled vacuoles in the inter-thylakoid space of the cells together with the helical shape of the filaments give *Spirulina* its floating capacity (Ciferri, 1983).

2.2.2 Habitat and growth requirement

Spirulina is a ubiquitous organism and has been found in different environments such as marshes, soil, brackish water, sea water, fresh water and sewage water (Ciferri, 1983). The unique feature of *Spirulina* is its ability to colonize environments that are unsuitable for many organisms and it especially occurs naturally in massive populations in tropical and subtropical lakes with high carbonate/bicarbonate and pH levels (8.5-11.0) as well as high level of solar radiation (Cifferi, 1983; Richmond, 1990; Belov and Giles, 1997). Lake Texcoco in Mexico, Lake Chad in Central Africa and lakes along the Great Rift Valley in East Africa such as Lake Sonachi, Lake Nakuru, Lake Simbi and Lake Bogoria in Kenya and Lake Chitu in Ethiopia are places where massive growth of *Spirulina* is found (Cifferi, 1983; Wood and Talling, 1988; Kebede, 1997; Ballot *et al.*, 2004; Ballot *et al.*, 2005; Belay, 2008).

The main environmental factors affecting the growth of *Spirulina* are light, temperature, pH and nutrients such as nitrogen, phosphorous, pottasium and magnesium (Habib *et al.*, 2008). Light is considered as the most important environmental factor for growth and productivity of photosynthetic organisms. Temperature is also the most fundamental factor for all living organisms as it affects all metabolic activities. Moreover, temperature affects nutrient availability and uptake, as well as other physical properties of the cells' aqueous environment such as solubility of gases (Vonshak, 1997). Although there is strain variation, the optimal temperature for growth of *Spirulina* ranges from 35 to 37°C (Vonshak, 1997). This is the main reason that most *Spirulina* producing industries are located at tropical and sub-tropic areas of the world (Belay, 2013). pH determines the solubility of carbon dioxide and minerals in the growth medium and thus directly and indirectly influences the metabolism of algae (Becker, 1993). Different algae have different pH optima. In the case of *Spirulina*, high alkalinity is mandatory for its growth and bicarbonate is used to maintain the high pH. The optimum pH for the growth of most *Spirulina* strains ranges from 9.5 to 10.5 (Vonshak, 1997) but growth at pH values as low as 7.0 and as high as 11.3 has also been reported (Ciferri, 1983).

Species in the genus *Spirulina* (*Arthrospira*) are generally photoautotrophs, since they obtain carbon and energy from photosynthetic mechanisms (Ciferri, 1983; Vonshak, 1997). *Spirulina* is

endowed with a number of pigments which allow its cells to capture light in a wide range of wave lengths (Richmond, 1988). In contrast to green algae which mostly have chlorophyll b, cyanobacteria (blue green algae) typically contain chlorophyll *a* (Richmond, 1988). *Spirulina* has also carotenoids (yellow-green pigments) which assist the light harvesting process and protect the cells against single oxygen-induced damage (Mathew *et al.*, 1995). Another group of pigments which are found in *Spirulina* are phycobiliproteins. These pigments (chromoproteins) are the dominant pigments in *Spirulina* and comprise about 20% of the cellular protein (Glazer, 1985; Richmond, 1988). The main function of phycobiliproteins is to aid in the photosynthetic process (together with chlorophyll *a* and carotenoids), whereby they assist in the light harvest at wavelengths where chlorophyll absorbs poorly (Padyana *et al.*, 2001; Scheer and Zhao, 2008; Silveira *et al.*, 2008). These pigmented proteins were also reported to serve as nitrogen storage (Boussiba and Richmond, 1980; Richmond, 1988).

Although species in the genus cyanobacteria are considered as obligate photoautotrophs, it has, however, been established that these species also have mechanisms that enable them to utilize organic carbon substrates such as glucose for heterotrophic and mixotrophic growth (Marquez *et al.*, 1993; Marquez *et al.*, 1995; Chen and Zhang, 1997; Chojnacka, and Noworyta, 2004). Various studies showed a better growth of *Spirulina* on glucose supplemented medium (under mixotrophic conditions) than growth under photoautotrophic conditions. For instance, Chojnacka and Noworyta (2004) investigated the influence of growth parameters, light intensity and glucose concentration, on specific growth rate of *Spirulina* sp. in photoautotrophic, heterotrophic and mixotrophic batch modes. The highest specific growth rate (0.055 h^{-1}) was reached in mixotrophic culture.

2.3 Chemical composition

It has been indicated that the gross chemical composition of *Spirulina* can vary due to environmental factors such as light, nutrients, temperature and other factors such as genotype, the stage in growth cycle and the source and concentration of nitrogen used in the growth medium (Ciferri, 1983; Olguion *et al.*, 2001; Ruengjitchatchawalya *et al.*, 2002; Morais *et al.*, 2009). The interest in *Spirulina* by many researchers has been stimulated by its ideal chemical composition for nutritional applications (Richmond, 1988).

2.3.1 Macronutrient composition

Proteins

The primary characteristic of *Spirulina* that has attracted the attention of both scientists and industrialists is its high protein content (Ciferri, 1983; Hug and von der Weid, 2011; Melnic *et al.*, 2011). Belay (2013) reported a protein content of 67.5% for *Spirulina* powder produced in 2011 at Earthrise farms. Morais *et al.* (2009) also reported a protein content as high as 86 % for *Spirulina* produced in a pilot scale semicontinuous production in southern Brazil. In general, the protein content of *Spirulina* varies based on growth conditions and it is very high compared to other commonly used high protein containing plant sources such as soybeans (35%), peanuts (25%) or grains (8-10%) (Habib *et al.*, 2008)

The nutritional quality of a food protein is related to the content, proportion and availability of its amino acids as well as its digestibility by digestive enzymes to release its amino acids (Becker, 2007). Although variation exists in the amino acid profiles of *Spirulina* from different sources, its amino acid profile is superior to all plant proteins, including legumes (Ciferri, 1983). However, when compared to egg and milk which are well-balanced food proteins, *Spirulina* is somewhat deficient in the essential amino acids such as methionine, cysteine, lysine and tryptophan (Clement *et al.*, 1967; Dillon and Phan, 1993). But still, the amino acid pattern of *Spirulina* compares favorably with that of the reference pattern of a well-balanced protein, recommended by WHO/FAO (Table 2.1).

Table 2.1. Amino acid profile of *Spirulina* as compared with conventional protein sources and the WHO/FAO reference pattern (g per 100 protein)

ource	Ile	Leu	Val	Lys	Phe	Tyr	Met	Cys	Trp	Thr	Ala	Arg	Asp	Glu	Gly	His	Pro	Ser
/HO/FAO	4.0	7.0	5.0	5.5	6.0		3.5		1.0									
gg	6.6	8.8	7.2	5.3	5.8	4.2	3.2	2.3	1.7	5.0		6.2	11.0	12.6	4.2	2.4	4.2	6.9
ybean	5.3	7.7	5.3	6.4	5.0	3.7	1.3	1.9	1.4	4.0	5.0	7.4	1.3	19.0	4.5	2.6	5.3	5.8
maxima	6.0	8.0	6.5	4.6	4.9	3.9	1.4	0.4	1.4	4.6	6.8	6.5	8.6	12.6	4.8	1.8	3.9	4.2
platensis	6.7	9.8	7.1	4.8	5.3	5.3	2.5	0.9	0.3	6.2	9.5	7.3	11.8	10.3	5.7	2.2	4.2	5.1

Source: (Becker, 2007)

In addition to evaluating a given food protein based on its amino acids, its nutritive value can be evaluated more accurately by determining its protein efficiency ratio (PER), biological value (BV), digestibility coefficient (DC) and net protein utilization (NPU) (Becker, 1993; Becker, 2007; Falquet, 1996; Zepka *et al.*, 2010) through animal feeding studies. The protein efficiency ratio (PER) refers to weight gain per unit of protein consumed by the test animal in short term feeding trials. The biological value (BV) of a protein is a measure of nitrogen retained for growth or maintenance and the digestibility coefficient (DC) indicates the quality of a protein. The net protein utilization (NPU) which is equivalent to BV×DC is a measure of both the digestibility of the protein and the biological value of the amino acids absorbed from the food. In this regard, numerous feeding experiments have been conducted to assess the nutritive value of both fresh and dry *Spirulina* strains either in animals such as rats or human beings (Table 2.2).

Table 2.2. Nutritional studies with *Spirulina* in rats, dried by different methods (at 10% protein level)

Protein source	PER	BV	NPU	Reference
Casein	2.5	94.4	90	Becker,1993
S. maxima (raw)		63.0	47.7	Clement *et al.*, 1967
S. maxima(spray dried)	2.2		57.0	Durand-Chastel,1980

Source: (Dillon and Phan, 1993)

The most common food crops used in areas where malnutrition occurs regularly are cereals such as rice, cassava, wheat, etc which are rich in energy but poor in their protein content. The nutritional quality of such type of crops can be improved by adding *Spirulina* (Dillon and Phan, 1993). Anusuya and Venkataraman (1983) studied the effect of cereal supplementation with *Spirulina* in rats, and the nutritional quality of cereals was improved when supplemented with *Spirulina* in various proportions (Table 2.3).

Table 2.3. Supplementary value of *Spirulina* when added to cereals

Diet	Protein efficiency ratio
Spirulina	1.90
Maize	1.23
Rice	2.20
Wheat	1.15
Rice+*Spirulina*(3:1)	2.35
Rice+*Spirulina*(1:1)	2.40
Wheat+*Spirulina*(3:1)	1.42
Wheat+*Spirulina*(1:1)	1.90
Maize+*Spirulina*(3:1)	1.80
Maize+*Spirulina*(1:1)	1.72
Maize+Oats+*Spirulina*(3:2:5)	1.90
Maize+Rice+*Spirulina*(2:2:1)	1.95

Source: (Anusuya and Venkataraman, 1983)

The nutritional quality of *Spirulina* was also studied in human subjects, in both children and adults. For instance, the nitrogen balance of *Spirulina* was evaluated in 10 children aged 5-10 months hospitalized for severe malnutrition (Proteus Inc., 1975 cited Henrikson, 1989). These children were given 2-3 grams of protein per kilogram of body weight in the form of *Spirulina*, cow's milk or soya for four days. Nitrogen absorption was 60% for *Spirulina* and 70% for soya. However, in the case of retention the proportions were reversed: 40% for *Spirulina* and 30% for soya. Thus, the relative retention of *Spirulina* was as high as that of cow's milk, indicating excellent protein utilization from *Spirulina* despite its average digestibility. The potential of *Spirulina* in improving the protein ration of 2-5 years old children was also investigated by mixing *Spirulina* with millet in different proportions (millet alone, millet-*Spirulina* (90/5) mix,

millet-*Spirulina*(90/10) mix, and millet-powdered creamed milk (90/10) mix (Delisle, 1990). It was found that at 10% level, *Spirulina* improved the proteinic value of the millet ration as efficiently as the same amount of powdered creamed milk. The apparent nitrogen digestibility of *Spirulina* was also studied in 5 malnourished adults, fed 80 to 90 grams of *Spirulina* per day. The absorption of nitrogen in these subjects was reached 90 % on average (Dillon and Phan, 1993).

Lipids

The lipid content of *Spirulina* ranges from 6 to 13% and its major lipids are monogalactosyldiacylglycerol, sulfoquinovosyldiacylglycerol and phosphatidylglycerol (Cohen, 1997) and each of these lipids account from 20 to 25% of the total lipid of *Spirulina*. On the other hand, digalactosyldiacylglycerol and triglycerides are minor components of *Spirulina* lipids accounting from 7 to 10 % and from 1 to 2% of the total lipid respectively (Cohen, 1997). Half of the lipids of *Spirulina* are fatty acids (Cohen, 1997) and *Spirulina* is particularly rich in γ-linolenic acid (6, 9, 12-octadecatrienoic acid) (Cohen, 1997; Otles and Pire, 2001). γ -linolenic acid is a precursor of chemical mediators of inflammatory and immune reactions (Falquet, 1996). This fatty acid is present only in the storage lipids of few seeds such as primerose, black currant and borrage, but at low level (from 10 to 20% of the total fatty acids)(Dubacq and Quoc, 1993). On the other hand, γ -linolenic acid constitutes up to 40% of *Spirulina*'s fatty acids which makes it one of the best known sources of this fatty acid (Ciferri, 1983; Belay, 2002). The therapeutic value of γ -linolenic acid in decreasing blood cholesterol has been reported by Ishikawa *et al.* (1989). γ -linolenic acid was also found important in treatment of atopic child eczema, to alleviate premenstrual syndrome and in immune system stimulation (Pascaud, 1993). Table2.4 shows the fatty acid profile of the two commercially important (edible) species of *Spirulina* (*S. maxima* and *S.platensis).*

Table 2.4. Principal fatty acids in two species of *Spirulina*, *S.maxima* and *S.platensis*

Fatty acids	*S. maxima* (% of total fatty acids)	*S.platensis* (% of total fatty acids)
Palmitic(16:0)	63	25.8
Palmitoletic(16:1)	2	3.8
Stearic(18:0)	1	1.7
Oleic(18:1)	4	16.6
Linoleic(18:2)	9	12
γ-linolenic(18:3)	13	40.1
α-linolenic(18:3)	traces	traces

Source: (Pascaud, 1993)

Carbohydrates

Carbohydrates constitute 15 to 25% of the dry weight of *Spirulina* (Falquet, 1996). Simple carbohydrates in *Spirulina* occur in small quantities and the only carbohydrate that occurs in sufficient quantities to be of interest is mesoinositol phosphate, which is an excellent source of organic phosphorus and inositol (350–850mg/kg dry matter) (Challem *et al.*, 1981). This inositol content is about eight times that of beef and several hundred times that of the vegetables with the highest levels (Falquet, 1996). A high molecular weight polysaccharide, with immunostimulatory activity has been isolated from *Spirulina* and is called "Immulina". This highly water-soluble polysaccharide represents between 0.5% and 2.0% (w/w) of the dry weight (Pugh *et al.,* 2001). A sulfated polysaccharide, calcium spirulan (Ca-SP) has been also isolated from *Spirulina platensis* by Hayashi *et al.* (1996) and reported to have anti-human immunodeficiency virus type 1 (HIV-1) and anti-herpes simplex virus type 1 (HSV-1) activities.

2.3.2 Micronutrients

Vitamins

Microalgae are considered as non-conventional sources of vitamins since they contain several water and lipid soluble vitamins (Kay, 1991). In terms of vitamins, *Spirulina* contains a valuable source of nearly all essential vitamins (e.g. A, B_1, B_2, B_6, B_{12}, E, nicotinate, biotin, folic acid and pantothenic acid) (Habib *et al.*, 2008) and it is especially a rich source of provitamin A (β-carotene) and vitamin B_{12} (Belay, 1997; Belay, 2002; Habib *et al.*, 2008; Hug and von der Weid, 2011; Bishop and Zubeck, 2012). Table 2.5 shows the concentrations of these vitamins in *Spirulina*.

Table 2.5. Vitamins and their concentrations in *Spirulina*

Vitamins	Concentrations (mg/100g dry weight)
Vitamin B_1(Thiamin)	1
Vitamin B_2(Riboflavin)	4.5
Vitamin B_3(Niacin)	14.9
Vitamin B_5(Panthothenic acid)	1.3
Vitamin B_8(Pyridoxine)	0.96
Vitamin B_9(Folic acid)	0.027
Vitamin B_{12}(Cobalamine)	0.16

Source: (Bishop and Zubeck, 2012)

Minerals

Like other nutrients, mineras are also essential nutrients for the functioning of certain physicochemical processes in the body which are essential to life (Soetan *et al.*, 2010). *Spirulina*

contains a number of minerals such as iron, magnesium, manganese, calcium, zinc and selenium (Guang *et al.*, 2007; Habib *et al.*, 2008). Zaretskaia *et al.* (2003) showed *Spirulina* as a suitable matrix for biotechnological incorporation of new food trace elements preparations (zinc, selenium, iron, manganese, chromium) since they found that the majority of trace elements incorporated in intercellular hydrophilic fractions. Various studies on iron bioavailability from *Spirulina* have shown that it is not only a good source of iron but more importantly the iron is highly bioavailable. Johnson and Shubert (1986a) reported that iron in *Spirulina* is 60% better absorbed than ferrous sulfate and other supplements. Kapoor and Mehta (1993) studied the effect of *Spirulina* on iron status of rats during pregnancy and lactation based on hemoglobin, packed cell volume, serum iron, total iron binding capacity and ferritin levels. Rats were fed 5 different kinds of diets (casein, *Spirulina*, wheat gluten, *Spirulina* + wheat gluten, *Spirulina* without additional vitamins and minerals) each providing 22 percent protein. Higher iron storage and hemoglobin contents were recorded in mice who fed diets containing *Spirulina* alone or in combination with wheat gluten. Puyfoulhoux *et al.* (2001) also investigated the bioavailability of iron from iron-fortified *Spirulina* as in comparison with beef, yeast, wheat flour, and iron sulfate plus ascorbic acid using in vitro digestion/Caco-2 cell culture system. Iron availability was assessed by ferritin formation in Caco-2 cells exposed to digests containing the same amount of iron. Their results showed a 27% higher ferritin formation from beef and Spirulina than from yeast and wheat flour. Moreover, the bioavailablity of iron from *Spirulina* was 6.5 times higher than that from meat.

2.3.3 Phytonutrient composition

Although the first impression of scientists on *Spirulina* was its high protein content, recently more interest has been developed on its pharmacological properties. A great deal of research has been therefore conducted on the potential health benefits (radiation protection, antioxidant effects, immunomodulation, diabetes management, anti-cancer effects, anti-inflammatory effects, etc) of *Spirulina.* Most of these health benefits or pharmacological properties of *Spirulina* are attributed to its pigments (phytonutrients) such as chlorophyll *a*, carotenoids and phycobiliproteins (Belay *et al.*, 1993; Belay, 2002; Belay, 2008)

Chlorophyll a

The chlorophyll *a* content of *Spirulina* varies from 0.8 to 1.5 % of its dry weight (Paoletti *et al.*, 1980 cited in Cohen, 1997). The typical chemical structure of chlorophyll *a* is shown in Fig 2.3. Nowadays, chlorophyll is gaining importance as a food additive (natural pigment) by food industries due to its strong green pigment and consumers demand for natural food colorants (Humphrey, 2004; Hosikian *et al.*, 2010).

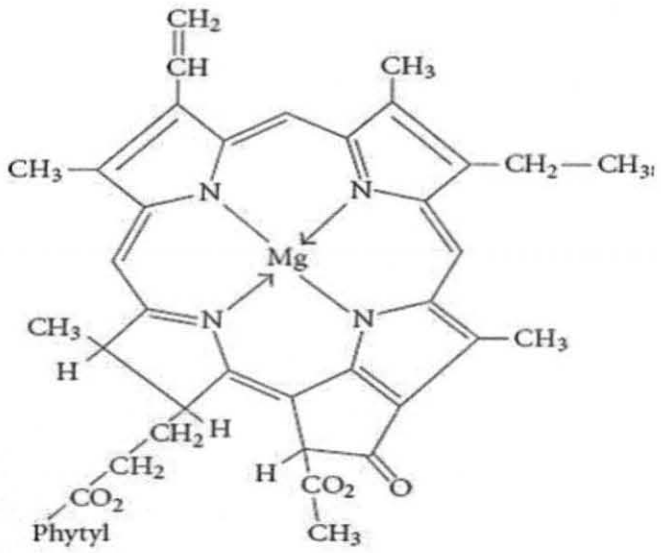


Fig 2.3. Chemical structure of chlorophyll *a*

Source: (Hosikian *et al.*, 2010)

Carotenoids

The consumption of a diet rich in carotenoids has been correlated with a lower risk for several diseases (Stahl and Sies, 2005). Compared to other natural sources, *Spirulina* produces large quantities of natural carotenoids directly in an edible state (Mathew *et al.*, 1995). Mixoxanthophyll and β-carotene are the major carotenoids in *Spirulina* with concentrations ranging from 0.2 to 0.4% of the dry weight (Ciferri, 1983).Carotenoids are strong antioxidants since they are capable of scavenging potentially harmful radicals, which are commonly associated with the induction of cancer (Stahl and Sies, 2005). β-carotene is one of the most effective carotenoids to counteract free radicals that alter cells causing cancer (Fedkovic *et al.*,

1993; Schwartz, *et al.*, 1990). It has been indicated that when β-carotene solution applied to oral cancer tumors in hamsters, it reduced both the number and sizes of tumors (Schwartz and Shklar, 1987; Schwartz, *et al.*, 1988). The bioavailability of total carotenes and β-carotene from *Spirulina* was investigated in apparently healthy preschool children in India and found to be comparable to those values reported for other plant sources like leafy vegetables and carrots (Annapurna *et al.*, 1991). A study on Chinese adults by Wang *et al.* (2008) has also shown that ingestion of 4.5 mg of β-carotene from *Spirulina* provides 1mg of vitamin A. The chemical structure of β-carotene is shown in figure 2.4.

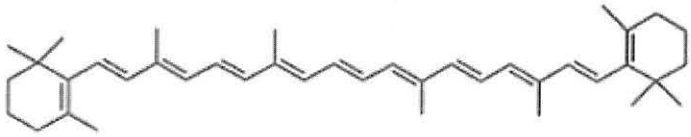


Fig 2.4. Chemiccal structure of β-carotene
Source: (Dufosse *et al.*, 2005)

Phycobiliproteins

Phycobiliproteins are among *Spirulina* proteins which have the highest market value (Cohen, 1997). *Spirulina* contains two biliproteins: C-phycocyanin and allophycocyanin. The chromophore is phycobilin, an open tetrapyrrole (Chopra and Bishnoi, 2008). The typical chemical structure of phycocyanin is presented in Fig 2.5.

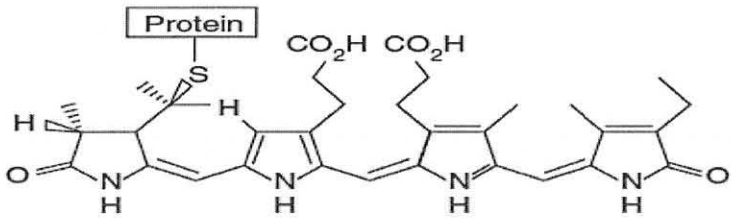


Fig 2.5. Chemical structure of phycocyanin bilin chromophore
Source: (Chopra and Bishnoi, 2008)

A Japanese company, Dainippon Ink & Chemicals, has already commercialized phycocyanin from *Spirulina* under the name of 'Lina blue' which is used as a colorant for candy, ice cream, dairy products and soft drinks (Cohen, 1997). Besides food coloring, phycobiliproteins have also

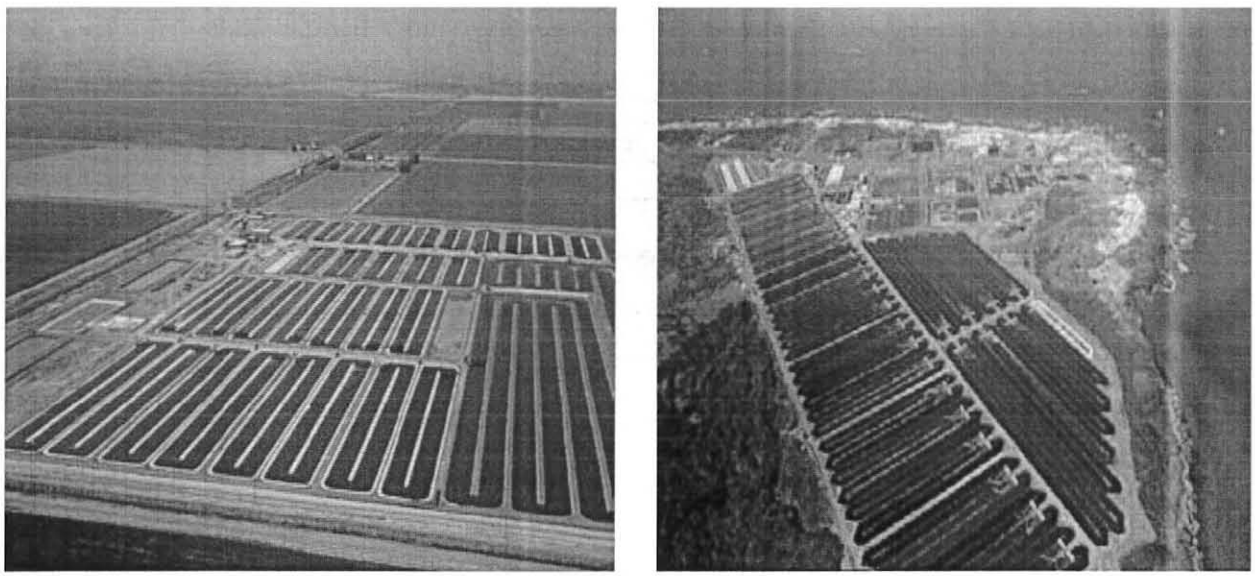
commercial applications in cosmetics as well as in biomedical research due to their fluorescent properties (Cohen, 1997). The phycobiliproteins of *Spirulina* especially C-phycocyanin has been also shown to have a number of pharmacological and therapeutic effects such as antioxidant, anti-inflammatory, hepatoprotecive, neurprotective and anticancer effects. As an antioxidant, phycocyanin was able to scavenge alkoxyl, hydroxyl and peroxyl radicals and to react with peroxinitrite (ONOO-), hypochlorous acid (HOCl) (Bhat and Madyastha, 2000, 2001; Romay and Gonzalez, 2000) as well as inhibition of NADPH oxidase activity (McCarty, 2007). The antioxidant capacity of phycocyanin was attributed to the structural similarity of its chromophore, phycocyanobilin, with that of bilirubin which is a powerful scavenger of reactive oxygen species and inhibitor of NADPH oxidase activity (Bhat and Madyastha, 2001; McCarty, 2007). The anti-inflammatory effect of C- phycocyanin was claimed to be associated with its ability to scavenge free radicals and inhibit enzymes involved in the formation of inflammatory prostaglandins (Romay *et al.*, 1998; Reddy *et al.*, 2000). The hepatoprotective effect of phycocyanin was attributed to its ability to scavenge free radicals and inhibiting lipid peroxidation (Romay *et al.*, 1998; Bhat and Madyastha, 2000) as well as cyclooxygenase -2 activity (Reddy *et al.*, 2000). The cytotoxic activity of phycocyanin against squamous cell carcinoma of hamsters has been also reported by Schwartz *et al.* (1987). In addition to C-phycocyanin, purified allophycocyanin from *S. platensis* has also shown to have an antiviral activity against enterovirus-71(Shih *et al.*, 2003).

2.4 Cultivation and production of *Spirulina*

The discovery of *Spirulina* as a high and quality protein food crop with many health promoting effects has led to its commercial production in the late 1970's in Mexico at Lake Texcoco by a company known as Sosa Texcoco (Vonshak, 1997; Borowitzka, 1999). Since then *Spirulina* is produced in many parts of the world targeting two different objectives; developed countries produce *Spirulina* to use it as a health food especially targeting vegetarians and developing countries produce *Spirulina* under local conditions to use it as a major source of protein (Vonshak and Richmond, 1988). The United States, China and India are the major producers of *Spirulina* in the world with modest production in Thailand, Taiwan, Pakistan and Burma (Habib *et al.*, 2008). *Spirulina* is in general the most cultivated photosynthetic microalga to be used as a health food, feed supplement, and as a source of bioactive chemicals (Kim *et al.* 2007; Eriksen,

2008). From data obtained from websites of various companies, Belay (2013) estimated an annual production of 10,000 tons of *Spirulina* worldwide.

In large scale commercial production of *Spirulina*, there are two main cultivation systems. The first one is open air-system which includes shallow big ponds, tanks, circular ponds, and raceway ponds and the second type is use of photobioreactors (Borowitzka, 1999). Mass cultivation of *Spirulina* is usually carried out in raceway ponds which can be either open or under green house conditions (Belay, 1997; Habib *et al.*, 2008; Belay, 2013). The two main advantages of open culture systems are a small capital investment and the use of a free source of energy, sun light (Chaumont, 1993). There are only few companies which produce *Spirulina* in closed photobioreactors since this production system is costly (Belay, 2013). Some examples of open pond cultivation systems of *Spirulina* are presented in Fig 2.6.



Earthrise farms (California) Cyanotech (Hawaii)

Fig 2.6. Production of *Spirulina* in open raceway ponds under controlled conditions
Source: www.Earthrise.com and www.cyanotech.com

Although artificial mass culivation constitutes the major percentage of the production system of *Spirulina*, there are also some examples of harvest and use of *Spiruina* directly from natural lakes (Fig 2.7). For instance, indigenous Kanembu women have been harvesting *Spirulina* (dihé) from the alkaline ponds near Lake Chad for centuries. The first commercial production of

Spirulina was started in Mexico by harvesting *Spirulina* from Lake Texcoco. Similarly, *Spirulina* is being harvested from natural lakes in Mynamar (Thein, 1993) and Lake Chenghai, Yunan Province, China (Jiang *et al.*, 2008). According to Henrikson (2011), by seasonally harvesting and processing *Spirulina* from natural lakes, Myanmar Pharmaceutical Industries produces about 150 dry tons of *Spirulina* in 60 days from February to April each year.

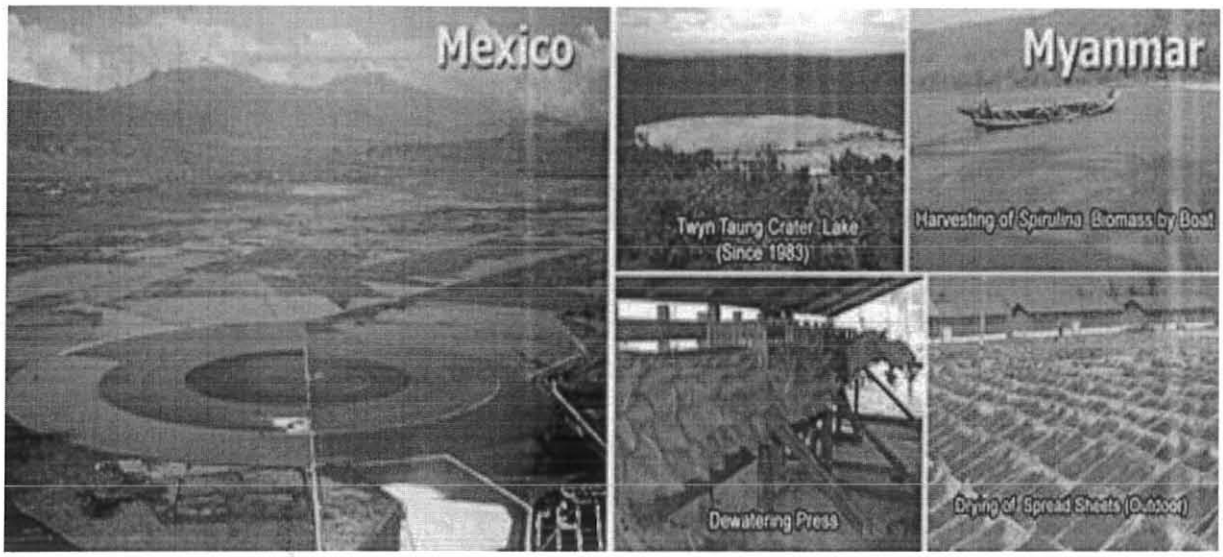


Fig 2.7. *Spirulina* Mexicana's Caracol in Lake Texcoco and lake harvesting, dewatering and drying *Spirulina* in Myanmar
Source: (Henrikson, 2011)

An interesting feature of *Spirulina* production is it lends itself to small scale or village level production using small tanks and cultivation pots (Figure 2.8).

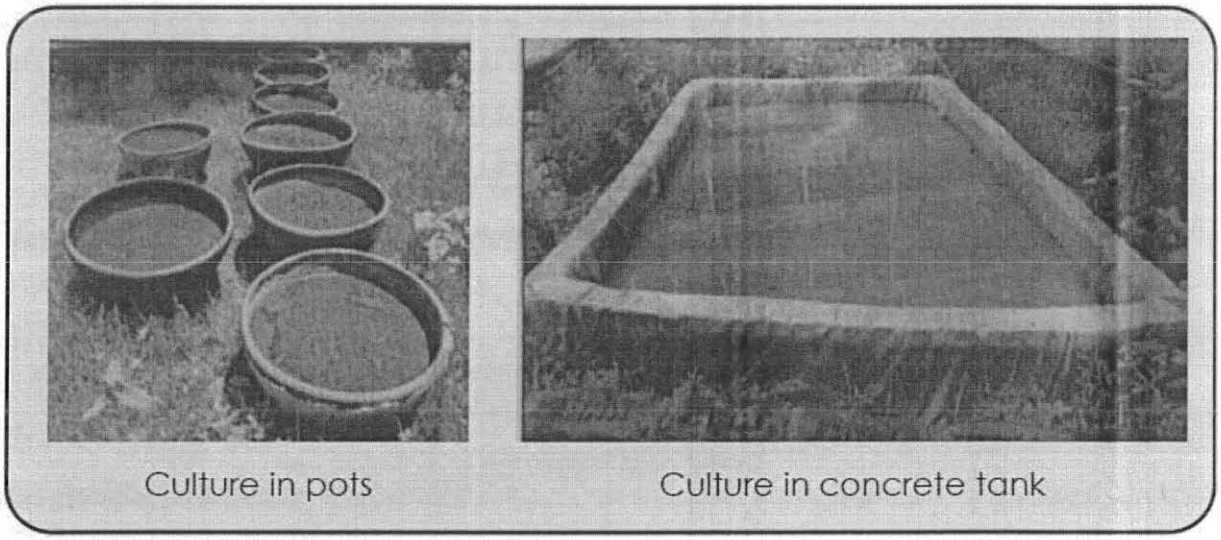


Fig 2.8. Forms of small-scale *Spirulina* cultivation in Bangladesh
Source: (Habib *et al.*, 2008)

In general, *Spirulina* production involves four major steps (Belay, 2013): (1) culturing (2) harvesting (3) drying and (4) packaging

Culturing or growing of Spirulina

Mass cultivation of *Spirulina* requires light, suitable temperature and pH, sufficient carbon and nutrient supply in the growth medium and mixing to expose all cells to light (Masojidek and Torzillo, 2008). During artificial outdoor production of *Spirulina*, the carbon source (sodium carbonate and bicarbonate) accounts for a major share of the costs (Vonshak and Richmond, 1988). In tropical countries, especially developing countries several researches have been therefore done to minimize production costs by finding cheap carbon sources. As a result, many growth media were developed using sewage water (Saxena *et al.*, 1982; Cafiizares-Villanueva *et al.*, 1995), sea water (Mary Leema, *et al.*, 2010), industrial effluents (Tanticharoen *et al.*, 1993), clean water/CFTRImedium (Venkataraman and Becker, 1985), lagoon water (Jimenz *et al.*, 2003), underground water (Choong *et al.*, 2007), cheaper and locally available commercial fertilizers(chemicals) (Madkour *et al.*, 2012), etc.

Harvesting

The filamentous nature of *Spirulina* is one of the main reasons for the success in mass culture of this organism since it eases the harvesting process (Borowitzka, 1988). In addition to its filamentous nature, *Spirulina* has a granular cytoplasm containing gas vacuoles (Richmond, 1986), and thus capable of autofloculation. Various methods of harvest have been employed by *Spirulina* producers including direct screen filtration and flotation and skimming of the biomass (Belay, 2013).

Drying

A number of drying methods are used by *Spirulina* producers depending on the quality and the quantity of biomass to be dried. These drying methods include drum drying, spray drying, sun drying, solar drying, cross-flow drying, vacuum-shelf drying, and freeze drying (Belay, 2013). Drying may constitute up to 30% of the production costs of *Spirulina* (Vonshak and Richmond, 1988). Although sun drying is the cheapest method, the process is slow which requires a number of days which in turn results in an unpleasant odour, degradation of biomass, and a higher bacterial count making the biomass unsuitable for human consumption (Richmond, 1988). As a result, solar driers that can dry the biomass within 4 to 5 hours were developed by researchers (Venkataraman *et al.,* 1980; Prakash *et al.* 1997) and thus the quality of the biomass could be improved. The most common drying method used by many commercial producers around the world is spray drying (Belay, 2013). In spray drying the biomass is exposed to heat for only seconds and thus guarantees the adequate preservation of heat-sensitive nutrients, pigments, and enzymes (Belay, 2013).

Packaging

Packaging is an important step in *Spirulina* production process as appropriate packaging is required to prevent the degradations of many of the phytochemicals contained by *Spirulina* (Belay, 2013). Therefore, gas-barrier bags or containers are usually used for packing *Spirulina* powder to prevent the degradation of these phytochemicals.

2.5 Food products with *Spirulina* addition

Worldwide, *Spirulina* is marketed as a dietary food supplement, commonly in the form of tablets, capsules or liquids in health food stores and mass-market outlets (Yamaguchi, 1997; Belay, 2002). There is also a growing interest and thus market in the world for food products with *Spirulina* addition (Yamaguchi, 1997; Becker, 2004; Gouveia *et al.*, 2008; Habib *et al.*, 2008). These food products not only provide the essential nutrients that are required for normal physiological functioning, but also may serve as the medium by which human beings receive other components that may affect their health and thus considered as functional foods. Food products such as pasta (Spolaore *et al.*, 2006; Habib *et al.*, 2008; Fradique *et al.*, 2010; Danesi *et al.*, 2010; Lemes *et al.*, 2012), manioc based bakery products (Danesi *et al.*, 2010), biscuits and sweets (Heierli and von der Weid, 2007), gelled food products (Batista *et al.*, 2012), bread and vegetable dressing (Khan *et al.*, 2005), instant noodles for children (Xu, 1993) and beverages (Zeng and Liang, 1995) have been used as food vehicles to *Spirulina* addition. Possibility to produce functional dairy foods (probiotic fermented milks) by adding *Spirulina* has been also demonstrated (Varga *et al.*, 2002; Beheshtipour *et al.*, 2013). In these cases, *Spirulina* not only improved the nutritional quality of fermented milks but also enhanced the viability of probiotic microorganisms. Morist *et al.* (2001) showed the possibility of preparing a concentrated and pasteurized drink 'green juice' from fresh *Spirulina* biomass. Novel food tablet formulations from date fruit and *Spirulina* powder were also reported by Adiba *et al.* (2011). Recently, Joshi *et al.* (2014) reported successful incorporation of *Spirulina* to maize flour in the preparation of extruded snack foods from maize and *Spirulina.* In their study, they found that, addition of *Spirulina* (7.5%) in the maize flour blend resulted crisp to hard-textured extruded products containing the valuable carotenoids, protein and zinc. According to the authors, a serving of 100 g of *Spirulina*–maize flour blended extruded product would contribute to fulfill the recommended dietary allowance approximately, 10% of Zn, 25% of protein and also requirement of carotenoids.

2.6 *Spirulina* in nutritional rehabilitation of malnourished and vulnerable populations: insights from clinical studies

The efficacy of *Spirulina* in the nutritional rehabilitation of malnourished children and vulnerable populations (HIV-positive) has been tested in many clinical studies in different countries (as mentioned below) and almost all studies demonstrated very positive results.

In Burkina Faso, the impact of *Spirulina* supplemented to traditional meals on the nutritional status of two groups of children (84 HIV-infected and 86 HIV-negative) was assessed at the Centre Médical St Camille of Ouagadougou for 8 weeks. The results demonstrated the benefits of *Spirulina* in the treatment of child malnutrition, as well as its particularly positive impact on the nutritional rehabilitation of HIV- infected children (Simpore *et al.*, 2005). Another study in Ouagadougou, Burkina Faso compared the nutritional benefits of diets composed of *Spirulina* and/or of Misola (Simpore *et al.*, 2006) for 8 weeks. Misola is a nutritional complement for children widely used in Western Africa which is composed of millet, soya bean and peanut. The study was carried out on 550 malnourished children under 5 (455 with severe marasma, 57 marasma of medium severity and 38 kwashiorkor plus marasma). An improvement in body weight as a function of height and age was observed for all children, especially those whose diet was made of *Spirulina* and Misola. The authors concluded that misola, *Spirulina* added to traditional meals or misola with *Spirulina* are good diets for severely malnourished children. The best results were obtained when children consumed *Spirulina* and misola as it combines misola's large caloric intake with *Spirulina*'s high protein content.

In China, at Nanjing Children's Hospital, *Spirulina* was prescribed as part of a 'baby nourishing formula' with baked barely sprouts. Out of 30 children (aged 2 to 5 years) included in the study, 27 were recovered from bad appetite, night sweat, diarrhea and constipation within a short period of time (Henrikson, 1989).

A study in Cameroon assessed the impact of nutritional rehabilitation using *Spirulina platensis* versus soya beans on the nutritional status and immune response of malnourished HIV-infected adults for 12 weeks (Azabji-Kenfack *et al.*, 2011). This study showed a comparable efficacy of *Spirulina* and soya beans with regard to weight gain, but also a significant increase in immunological markers for the group treated with *Spirulina*, and not with the group treated with soya beans.

Being rich in micronutrients, *Spirulina* was also tested for its potential to combat diseases due to micronutrient deficiencies. For instance, a one year feeding study in India on 5000 pre-school children showed that a *Spirulina*-supplemented diet reduced the occurrence of "Bitot's spot", a symptom of vitamin A deficiency, from 80% to 10%. The children were given 1 gram of *Spirulina* per day for 150 days (Seshadri, 1993). Selmi *et al.* (2011) tested the potential of *Spirulina* to ameliorate anemia and immunosenescence in adults with a history of anemia in a 12 week supplementation. The result showed the potential of *Spirulina* to counteract anemia and immunosenescence in older subjects.

A number of success stories on the efficacy of *Spirulina* in the nutritional rehabilitation of malnourished children are described in Dillion and Phan (1993) and Fox (1996).

2.7 *Spirulina* as a human food: safety issues

Although the chemical composition of *Spirulina* gives it interesting qualities which can be applied in human and animal nutrition, prior to its use, it must be analyzed for the presence of toxic compounds (Rebolloso Fuentes *et al.*, 2000; Rebolloso Fuentes *et al.*, 2001; Belay, 2008). In this regard, recommendations have been published by different producers around the world. In general, quality control for food grade *Spirulina* or microalgae includes microbiological tests, test for heavy metals, algal toxins, pesticides, nucleic acid contents and other extraneous materials (insect fragments, rodent hair, and feather fragments)(Belay, 1997).

2.7.1 Microbiological safety

Ensuring the safety of food products depends on minimizing the initial contamination with pathogenic microorganisms and inhibiting their development during handling and storage. Pathogenic microorganisms can render foods harmful to humans in a variety of ways. Foods may serve as vehicle of introduction of infectious microorganisms into the gastrointestinal tract, e.g., *Salmonella* and *Shigella* (IOM, 1985). Multiplication of certain microorganisms in foods prior to consumption may also result in production of toxins e.g. *Clostridium botulinum, S.aureus* and *B. cereus* (Jay, 1996).

To estimate food sanitary quality, the classic approach is based on the search for not only pathogenic microorganisms but also indicator microorganisms (Leclercq *et al.*, 2002). An indicator organism is a microorganism that indicates that a food has been exposed to conditions that pose an increased risk, that the food may have been contaminated with a pathogen or held under conditions conducive to pathogen growth (Buchanan, 2000).

The aerobic mesophilic count is among the more popularly used non pathogenic microbiological indicators of food quality (Vandereit, 1985; FEHD, 2001; FSAI, 2001). It is generally used for descriptive evaluation of microorganisms on non selective media under mesophilic and aerobic conditions of incubation (FEHD, 2001; FSAI, 2001). It is generally believed that high aerobic plate counts in foods indicate greater risks of pathogens being present in consumable products, poor implementation of sanitation procedures or problems in process controls to which a test food item has been subjected (Miskimin *et al.,* 1976).

Coliforms are also one of the typical indicator organisms of food quality. Tests for the presence and number of coliforms are usually used to assess the rate of total contamination of foods and the hygienic standard of food manufacture. Increased counts of coliform bacteria are indicative of failures in sanitation and very high counts can be dangerous to human health (Jay, 1996). This does not mean, however, that all foods that are free from coliforms are safe (Jay, 1996).

In addition to determining the counts of quality indicator microorganisms, it is also common to directly analyze potential pathogens in food items. Among these are *Escherichia coli*, *Salmonella* and *Staphylococcus aureus* (Jay, 1996).

Microbial contamination may be a concern during cultivation of *Spirulina* in open raceway ponds and natural lakes. Furthermore, handling of the biomass during processing may also introduce unwanted microorganisms (Vonshak, 1997; Belay, 2008). To safeguard the microbial quality and safety of their products commercial *Spirulina* producers take some precautionary measures. The growth media they usually use have high pH and salinity which are highly selective for the growth of *Spirulina* but inhibiting for the contamination of the culture by bacteria, protozoa and other algae (Walach *et al.*, 1987). Morist *et al.* (2001) indicated the practice of pasteurization to exclude faecal bacteria when *Spirulina* is harvested for human use from natural lakes. Most commercial *Spirulina* producers use aerobic plate counts and counts of total coliforms to evaluate the sanitary quality of their processing methods as well as their final products (US FDA, 1998 cited in Belay, 2008).

2.7.2 Cyanotoxins

Many genera of cyanobacteria produce toxic bioactive chemicals (cyanotoxins) under certain environmental conditions which can be of hepatotoxins, neurotoxins and lipopolysaccharide endotoxins (Chorus and Bartram, 1999). These toxins are usually deleterious or even fatal for consumers (Azevedo *et al.*, 2002; Ballot *et al.*, 2002). The most common cyanobacterial genera which produce these toxins are *Anabaena, Aphanizomenon, Cylindrospermopsis, Lyngbya, Microcystis, Nodularia, Nostoc* and *Planktothrix* (Mussagy *et al.*, 2006).

Among the cyanobacterial toxins, microcystins have been shown to cause liver damage by inhibiting a class of enzymes called protein phosphatases (Jiang *et al.*, 2008; Butler *et al.*, 2009). The function of this enzyme is to remove phosphate from a protein, a common step in many biochemical pathways. Inhibition of this enzyme by microcystins leads to accumulation of phosphorylated proteins which then results in liver damage (Butler *et al.*, 2009).

Microcystins are cyclic peptides formed from seven amino acids (monocyclic heptapeptides)

(Fig 2.9) with more than 80 structural variants (analogs) depending on the types of amino acid bonds occurring in the peptide (Codd *et al.*, 1999; Sivonen and Jones, 1999; Botha *et al.*, 2004; Jiang *et al.* 2008; Butler *et al.*, 2009).

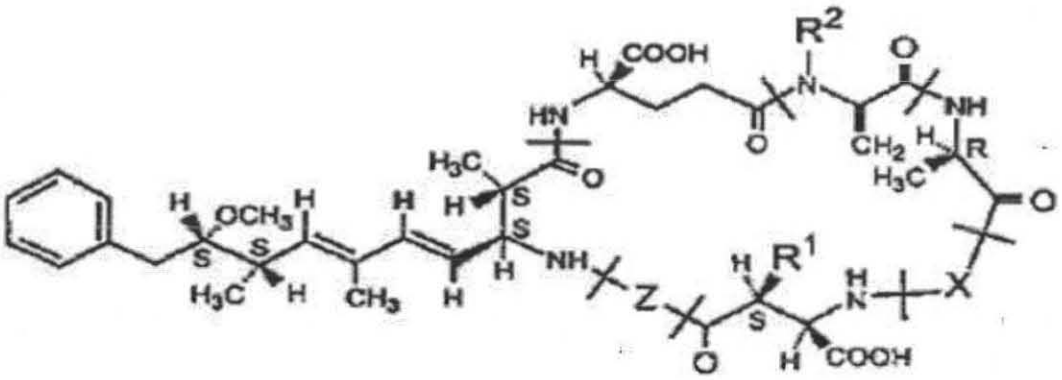


Fig 2.9. General structure of microcystins
Source: (Butler *et al.*, 2009)

Several studies indicated *Spirulina* (*Arthrospira*) as a non–toxic or microcystin-free genus (Ciferri, 1983; Jassby, 1988; Samuels *et al.*, 2002). For instance, Mussagy *et al.* (2006) have investigated toxin production in *Arthrospira fusiformis* isolated from African waters (two strains isolated from wastewater ponds in Mozambique and one from Lake Nakuru, Kenya) using ELISA and HPLC methods. None of the strains produced microcystins and anatoxin-a when cultivated in different light intensities and salinities. Yang *et al.* (2011) also evaluated long term effects of *Spirulina plantensis* consumption using *in vitro* and *in vivo* toxicity studies in rats. In their study, no detectable levels of microcystins were produced by *S. platensis* and cell viability remained 70–80% when HepG2 cells were incubated with 0–500 µg/ml of hexane, chloroform, methanol and water-extractable fractions of *Spirulina plantensis*. One study from Canada also reported the absence of microcystins in blue-green algal products containing only *Spirulina* (Health Canada, 1999). Similarly, microcystins or anatoxins were not detected in *A. fusiformis* isolated from Lake Nakuru (Ballot *et al.*, 2004) and Lake Simbi (Ballot *et al.*, 2005) as well as when the species were cultivated in laboratory conditions.

Contrary to the above reports, there are some reports about toxin production by the genus *Spirulina* (*Arthrospira).* For instance, Ballot *et al.* (2004) and Ballot *et al.* (2005) reported

cyanotoxin-producing strains of *A. fusiformis* from strains isolated from the Kenyan alkaline Lakes Bogoria and Sonachi respectively. These strains produced both microcystins and anatoxin-a (cyanobacterial neurotoxin). One case of hepatotoxicity associated with *Spirulina* consumption in a patient who had taken *Spirulina* for 2 weeks as a dietary supplement was reported by Iwasa *et al.* (2002) from Japan. Gilroy *et al.* (2000) also reported the presence of microcytins up to 2.12 μg g^{-1} DW when *Spirulina*-based human dietary supplements were evaluated by a combined HPLC-ELISA method

Mussagy *et al.* (2006) indicated the presence of both cyanotoxin-producing and non-producing strains within the genus *Arthrospira* and the toxicity of cyanobacteria is also strain-specific. Thus, strains of a given species may be non-toxic or toxin producers. Furthermore, it is also hypothesized that the difference between those strains of cyanobacteria that produce microcystin and those that do not lies primarily in the presence or absence of the microcystin synthethase gene cluster (Mussagy *et al.*, 2006). These genes were detected in microcystin-producing strains of *Microcystis*, *Anabaena, Plankothrix*, *Nostoc* and *Nodularia* (Neilan *et al.*, 1999). The Oregon Health Division and the Oregon Department of Agriculture has established a regulatory limit of 1 μg/g for microcystins in blue green algae containing products (Gilroy *et al.*, 2000).

2.7.3 Heavy metals

The term heavy metal is usually used as a group name for stable metals and metalloids such as cadmium, copper, iron, lead, mercury, arsenic and zinc which are usually metals associated with contamination and potential toxicity (Duffus, 2002; Anderson, 2003). Microalgae are known accumulators of heavy metals (Kay, 1991). This ability is due to the presence of pores in their cell walls that allow free passage of molecules and ions (Hope and Walker, 1975). In addition to pores, algal cell walls contain an array of ligands with functional groups able to bind various metallic species. Thus, the uptake of heavy metals through the production of intracellular chelators (phytochelatins) as response to different metal concentrations is a typical phenomenon in algal cells (Knauer *et al.*, 1998).

The ability of *Spirulina* to accumulate heavy metals has been reported from different studies (Johnson and Shubert, 1986b; Zhou *et al.*, 1998; Hernandez and Olguin, 2002; Disyawongs,

2002; Sabbagh, 2006). The cell wall of *Spirulina* consists of polysaccharides, proteins and lipids which have lots of negative carboxyl and phosphate groups. These carboxyl and phosphate groups are the dominant binding sites of toxic heavy metals cations (Vonshak, 1997). The biosorption property of *Spirulina* has been used for removing metals such as lead (Chen and Pan, 2005; Gong *et al.*, 2005), cadmium (Rangsayator *et al.*, 2002; Ahmad *et al.*, 2010), chromium (Jagiello *et al.*, 2006), copper (Ahmad *et al.*, 2010), mercury (Cain *et al.*, 2008), arsenic (Habib *et al.*, 2008; Ahmad *et al.*, 2010) and Zinc (Ahmad *et al.*, 2010) from media, solutions or waste waters containing these metals. Therefore, the only way to control the amount of metals taken up by microalgae or *Spirulina* intended for human consumption is to control the amount of heavy metals in the growth medium (Becker and Venkataraman, 1980).

Heavy metals can cause a number of health hazards when they are consumed by humans or animals. For instance, lead attacks the brain and the peripheral nervous system, bone marrow, kidney and liver. It also alters renal tubular function resulting in a variety of renal related disorders. The toxicity of lead was attributed to its ability to combine with sulfhydrl groups on proteins (Hughes, 1996).

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Exposure to cadmium in prolonged or acute doses may result in renal tubular dysfunction (Robertson, 1990). Cadmium is bound by high-molecular proteins (e.g. albumin) and non-protein sulfhydryl groups (e.g. metalthionein) and is accumulated by kidneys and liver (Robertson, 1990).

It has been indicated that ionic or partially covalent inorganic salts and many of the organic alkyl and aryl derivatives of mercury are highly toxic (Zalups and Lash, 1996; Karunasagar *et al.*, 2003). The extreme toxicity of mercury is due to its ability to cross biological membranes, its high affinity to thiol and amino groups in essential enzymes which results in damage to membranes as they pass through them, and inactivation of periplasmic and cytoplasmic enzymes (Kerper *et al.*, 1992). In addition to causing these health hazards, mercury compounds were found to be genotoxic (Akiyama *et al.*, 2001).

Arsenic is an environmental contaminant and can arise from natural sources such as rocks and sediments and also as a result of anthropogenic activities such as coal burning, copper smelting and the processing of mineral ores. Arsenic exists in different chemical forms, or species. These are either as 'free' inorganic arsenic species such as As (III) or As (V), As(-III) or as arsenic present in organic molecules such as arsenobetaine and arseno-sugars (Francesconi and Kuehnelt, 2004). Arsenic is genotoxic and is a known human carcinogen associated especially with liver, bladder, lung and skin cancer (Smith *et al.*, 1992). The toxicity of arsenic depends on its speciation and generally inorganic arsenic species are more toxic than organic forms (Goessler and Kuehnett, 2002; Ng, 2005). Among the inorganic species, As (III) is more toxic (Rose *et al.*, 2007) due to its high affinity for the sulfhydryl groups of biomolecules such as glutathione and lipoic acid and the cysteinyl residues of many enzymes (Aposhian and Aposhian, 2006).

2.7.4 Pheophorbides

Degradation of chlorophyll is a naturally occurring process and its degradation products are diagnostic indicators of the physiological condition, degradation and grazing processes of phytoplankton (Welschmeyer *et al.*, 1984). Photosynthetically active cells contain only minor concentrations of chlorophyll degradation products, whereas during senescence and death their fraction increases (Gors *et al.*, 2009). Degradation processes also occur at high irradiation or due to microbial activity (Brown *et al.*, 1991). Furthermore, rapid degradation of pigments occurs when the biomass is exposed to unfavourable and extreme light or temperature conditions, oxygen/air, acidic or basic conditions during processing or storage as these conditions accelerate their oxidation (Jeffrey *et al.*, 1997; Cubas *et al.*, 2008). Pheophorbides are formed through the step-wise loss of the phytol residue *via* chlorophyllase followed by the loss of the central magnesium atom *via* magnesium dechelatase (Hortensteiner, 1999; Hortensteiner *et al.*, 2000).

Chlorophyll degradation products usually cause skin irritations and inflammation of skin when the skin is exposed to light (Abrams, 1996; Jassby, 1988; Becker, 2004; Hwang *et al.*, 2005). The mechanism for pheophorbide-associated phototoxicity was described by Jitsukawa *et al.* (1984) and Jassby (1988). According to these authors, ingested pheophorbides enter the circulatory

system and distribute throughout the body and some of them deposit in the skin. Phototoxicity occurs when these deposited pheophorbides are exposed to light. Light causes the generation of oxygen and oxidation of fatty acids of nearby cell membranes are subsequently oxidized which leads to cell rupture, damage to dermal capillaries and escalating inflammation.

Some cases of illness associated with consumption of food products containing chlorophyll degradation substances were reported. One case was reported from Japan in 1977 as consequence of *Chlorella* consumption (Becker, 2004). Patients consumed *Chlorella* tablet which contained pheophorbide a and its ester in higher quantities (up to 8.2 mg/g) (Jitsukawa *et al.,* 1984). Another case of food poisoning was reported from Taiwan in 2001 associated with consumption of a dried purple laver product (nori) (Hwang *et al.*, 2005). The laver product contained the causative agents pyropheophorbide a and pheophorbide a with a value of 5460-5624 μg/g and 851-906 μg/g respectively (Hwang *et al.,* 2005). The LD_{50} values of pheophorbide a were 60 and 16mg/kg, respectively when rats were exposed to 20,000 Lux for 1 and 2 hrs (Endo *et al.*, 1982). This indicated that the toxicity of pheophorbide a depends on light intensity. The Environmental Health Bureau, Ministry of Health and Welfare, Japan declared that the minimum dose of pheophorbide to induce photosensitivity after ingestion of *Chlorella* was 25mg/man/day, and the maximum allowable level of pheophorbide a was set at 1000μg/g (Uchiyama *et al.*, 1991). Gors *et al.* (2009) in their study on the quality of commercial *Chlorella* products detected one type chlorophyll degradation product, pheophytin, in some of the samples in large concentrations.

2.7.5 Nucleic acid toxicity

A potential problem for the use of microalgae as a food source is their nucleic acid content. This is because of the production of uric acid during the metabolism of their purine moiety. Since uric acid is slightly soluble in human body fluids, there is a risk that salts may be deposited in the renal tract or joints which leads to renal disease or gout (Komaki *et al.*, 1998; Morist *et al.*, 2001). The nucleic acid content of microalgae has been reported to be in the range of 4-6% (Litchfield, 1979 cited in Maart, 1992). Physiological stresses such as nausea and vomiting associated with nucleic acids have been reported from human feeding trials with different

microalgae (Dam *et al.*, 1965 cited in Maart, 1992). Due to this, the maximum daily intake of dried microalgal biomass was suggested to be 30 g/day (Becker, 1980).

2.7.6 Safety studies of *Spirulina* on animal models (bioassays)

Any form of single cell or biomass protein intended for human use should be subjected to detailed toxicological investigations (Becker, 1995). Feeding studies employing different test animals (mice, rats, rodents, etc), and consequent pathological evaluations are usually the final steps in the evaluation of single cell (biomass) protein sources. In relation to this, a number of animal studies have been done to evaluate the safety of different blue green algae intended for human consumption where some were found toxic and others were not (Becker, 1995).

Recognition of *Spirulina* as a potential crop to fight against malnutrition in developing countries had led to safety studies on animals (usually rats and mice) since the early 1980s (Chamorro, 1980) on different *Spirulina* species/strains which were processed in various ways(fresh biomass, oven dried biomass, spray dried biomass, etc). The tests involved acute, sub chronic, chronic toxicity tests as well as mutagenic, teratogenic, carcinogenic and multiple generation effects.

Acute toxicity studies

Acute toxicity study refers to short-term feeding of a single dose or multiple doses of a test sample within 24 hrs to the test animal (Altug, 2003). The animal is then observed for the toxic effect of the administered sample usually for 7 or 14 days. The effect of *Spirulina* during short term feeding has been evaluated by different researchers by administering different doses of *Spirulina*. Krishnakumari *et al.* (1981) studied the acute effect of *Spirulina* by feeding albino rats 800 mg/kg body weight orally. The treatment didn't show changes in their body or organs weight or histology of their tissues. Hutadilok-Towatana *et al.* (2008) also studied the effect of *Spirulina* in short term treatments by exposing mice to very high single doses. In their experiment, male Swiss mice were given 30 and 10 g/kg body weight of fresh and dried *S. platensis*, respectively and were observed for 7 days. There were no obvious differences between the experimental and

the control groups. The gross examinations of their internal organs also revealed no pathological abnormalities.

Sub-chronic toxicity studies

Sub-chronic toxicity study is a type of toxicity study which usually involves feeding of a test sample either as single or multiple doses over a significant fraction (10%) of the life span of a test animal (Elias, 1980). Several sub-chronic toxicity studies have been undertaken to evaluate the safety of *Spirulina*. Chamorro (1980) evaluated the safety of *Spirulina* on Wistar rats feeding the rats for a period of 13 weeks a diet containing 10, 20 and 30% *Spirulina* replacing soya. At the end of the study period, rats were evaluated for various parameters and compared with the two control groups used in the study which were given soy-based diets and commercial diets. Addition of *Spirulina* to the diets at all levels did not bring any changes for all parameters studied (weight, behavior, appearance, food consumption, blood chemistry as well as macroscopic and histopathological examinations). Another sub-chronic toxicity study on mice was also conducted by Salazar *et al.* (1998) using *S.maxima*. Groups of 10 mice of each sex were fed *S. maxima* in the diet at concentrations of 10, 20 or 30% for 13 weeks. The addition of *Spirulina* on the diets didn't bring any effect on the behavior, food and water intake, growth or survival, hematology and clinical chemistry as well as post-mortem examination of the organs. Hutadilok-Towatana *et al.* (2008) also performed a sub-chronic study using commercial *Spirulina platensis*. They performed two separate experiments to evaluate the effect of long term feeding of fresh and oven dried *S. platensis* on rats. In each experiment, four groups of six Sprague-Dawley male and female rats were given water suspensions of fresh algae at 300, 600 and 1,200 mg/kg body weight or dried alga at various doses 30, 60 and 120 mg/kg body weight for dried algae treatment. The algae were orally administered (5 ml/kg body weight) daily for 12 weeks. In all instances, the consumption of algae showed no effect on behavior, food and water intake, growth or health status of the animals during the course of their investigatin. The values in clinical chemistry monitored throughout the study period did not reveal significant differences between the control and treated groups. The authors concluded that short-term and long-term consumptions of *S. platensis*, up to high feeding levels, did not produce any adverse effects in experimental animals.

Chronic toxicity studies

Chronic toxicity study refers to feeding of a test sample over the entire life-span of the test animal or at least a major fraction of the life span of the animal (Lu and Kacew, 2002). Chronic toxicity studies to evaluate the effect of long time *Spirulina* administration on hematological parameters, kidney function, clinical chemistry, and weight and histopathology of certain organs were also performed by different researchers. In all studies, *Spirulina* didn't bring any effect on the physiological or biological processes of study animals. For example, Chamorro (1980) studied the effect of *Spirulina* supplementation on rat diets at 10, 20 and 30% levels for a period of 84 weeks. Two groups were used as control groups in which one of the groups fed soy-based diet and the other a commercial diet. In all the levels tested, *Spirulina* did not produce any toxic effects in any of the parameters studied - weight gain, hematology, liver and kidney function, serum chemistry, relative organ weights, or histopathological parameters. A six month chronic toxicity study was also performed on C57BL/6J mice using *Spirulina plantensis* in the diet at 2.5 and 5% levels (Yang *et al.,* 2011). Throughout the study period, there were no signs of illness or behavioral changes in the mice, nor were there differences in body weight gain and liver histopathology compared to the control groups.

Other toxicity studies

In addition to short term and long term toxicity evaluations, *Spirulina* was also evaluated for its effect on reproduction and lactation (multigenerational study) by various researchers by incorporating *Spirulina* to diets at various levels (usually 10, 20 and 30%). All the investigators reported the absence of adverse effect on any measure of reproductive performance such as male and female fertility, duration of gestation and lactation associated with *Spirulina* (Chamorro, 1980; Salazar *et al.,* 1996). The teratogenecity effect (embryonic resorptions or fetal malformations) of *Spirulina* was also studied by researchers on different animals; Wistar rats, CD-1 mice, and Dorado hamsters (Chamorro, 1980; Chamorro and Salazar, 1989; Salazar *et al.*, 1996; Chamorro *et al.*, 1997). In all studies, the authors concluded that *Spirulina* does not cause gestational changes indicated by malformations, anomalies, or resorptions.

2.8 *Spirulina* (*Arthrospira*) in Ethiopian soda lakes: past and present status

Soda lakes are the most stable alkaline environments characterized by high Na^{+}, low Mg^{2+} and Ca^{2+} geology, high carbonate concentrations and typically with a pH between 9 to 11(Grant, 2004; Grant, 2006; Klemperer and Cash, 2007). In some cases salts, such as sodium chloride may also found in high concentrations leading these lakes to be alkaline-saline lakes (Grant, 2004). Most soda lakes in the world are mainly confined to subtropical latitudes typically in arid and semi-arid areas and in connection to tectonic rifts like the East African Rift Valley (Grant, 2004). These lakes are the most productive ecosystems on earth often inhabited by cyanobacteria (Talling *et al.*, 1973; Vareschi, 1982; Kebede and Ahlgren, 1996). Cyanobacterial dominance in these lakes is attributed to the large reserve of CO_2 for photosynthetic activity in photosynthetic zones (Talling *et al.*, 1973). These lakes could serve as a source of harvestable and cultivable algal resources such as *Arthrospira* (Fox, 1996).

Ethiopia, being in the rift valley region is endowed with a number of soda lakes: Lake Aranguade, Lake Abijata, Lake Shala, Lake Chitu, Lake Hertale and Lake Beseka (Lake Metahara) (Grant, 2004). Most of these soda lakes at one time or another were /are able to support the mass growth of *Arthrospira* nearly as a pure culture. However, in recent years, this cyanobacterium (microalga) is found only in a few of these soda lakes due to changes in the chemistry of the lakes (Table 2.6).

2.8.1 *Spirulina* in Lake Arenguade

Lake Arenguade is an alkaline saline crater lake which lies at an altitude of 1900m and located in and around the town of Debre Zeit, about 50 km southeast of Addis Ababa. About 60 years back, *Spirulina* was reported to be found as a uni-algal population in this lake with a biomass concentration of 400–5000µg chl a L^{-1}(Wood and Talling, 1988) sustaining a large population of Lesser Flamingoes (*Phoenicoptrus minor*) (Talling *et al.*, 1973; Wood and Talling, 1988). Talling *et al.* (1973) reported a production rate of 30 g O_2 m^{-3} h^{-1} for this lake. The name Arenguade was so named (Arenguade means green in Amharic language) for this dense population of cyanobacterium observed in the lake (Kebede, 2002). According to Kebede (1997), cattle are encouraged to drink the lake water by the local people since they believe that

the *Spirulina* water has therapeutic effects and compensates for some lack in the diet of their cows. However, in recent years, many studies are indicating the change in the species composition of this lake, from monospecific to a habitat for various algal species. For instance, a study by Belachew *et al.* (2012) indicated the presence of another blue green alga, *Anabaenopsis elenkinii* Miller equally dominating the lake with genus *Arthrospira.* More recently, Lanzen *et al.* (2013) reported the presence of *Arthrospira* in Lake Arenguade only in trace amounts; instead the phytoplankton community of the lake was dominated by other cyanobacterial genera such as *Leptolyngbya* and *Anabaenopsis* and the *Pavlovaceae* family. Changes were not only observed in the species composition of this lake but also in other major liminological features. For instance, Belachew*et al.* (2012) reported a production rate of 2.02 g O_2 m^{-3} h^{-1} which was more than 90% reduction compared to that reported by Talling *et al.(* 1973) (30 g O_2 m^{-3} h^{-1}) in 1960's. Similarly the biomass concentration of the lake dropped from 2170 mg Chl a m^{-3}(Talling *et al.*, 1973) to 41.7–271mg Chl a m^{-3}(Belachew *et al.* 2012). This shift in the species composition (replacement of *Arthrospira* by other algal species) as well as reduction in production rate and biomass concentration was accompanied by the change in the chemistry of the lake. According to Prosser *et al.* (1968), the salinity of Lake Arenguade in the 1960's was 5.41g L^{-1}, recently; this number is 2.1–2.8g L^{-1} (Lanzen *et al.*, 2013) which is a reduction of about 50%. Changes in the ionic composition of the lake is also obvious as the Na^+ and K^+ contents of the lake reduced from 67meq $L^{-1}$and 8.1meq L^{-1} (Prosser *et al.*, 1968) to 54.5 meq L^{-1} and 5.82 meq L^{-1} respectively (Lanzen *et al.*, 2013). Klemperer and Cash (2007) attributed these changes as volume increase of the lake due to an increased rainfall; lowered evaporation and spring recharge to the lake. On the other hand, Belachew *et al.* (2012) explained these changes as results of several deep underwater seismic detonations that have been conducted in the lake in recent years which caused seepage of water from subterranean streams to the lake.

2.8.2 *Spirulina* in Lake Abijata

Lake Abijata is a shallow soda lake lying within the Ethiopian Rift Valley at an altitude of 1578m. It is a terminal lake in a closed drainage system with four interconnected lakes (Kebede and Willen, 1996). This lake, together with Lake Shala and Lake Chitu, has been enclosed in a national park since 1970 (Kebede *et al.*, 1994). In the 1960's *Arthrospira* was reported to be found in significant amounts (57μg chl *a* L^{-1}) in this lake (Talling and Talling,1965) together

with some *Oocystis* sp and the lake was also a place for vast flocks of Lesser Flamingo. In the 1990s, this cyanobacterium was also reported to be found co-dominating the lake with another blue green alga, *Anabaenopsis abijiate* (Kebede and Willen, 1996) with a high biomass concentration of 135μg chl *a* L^{-1} compared to that recorded in 1960s. However, a recent study by Lanzen *et al.* (2013) indicated the absence of any genera of cyanobacteria in the lake although these authors indicated the probability of filtering bias (pre-filtering of water samples) during sample preparation for molecular analysis.

Some studies (Kebede *et al.*, 1994; Ayenew, 2002) on Lake Abijata have indicated the retracting trend of the lake due to increased development schemes in the area since 1985 such as diversion of feeder rivers (Meki and Katar) to another lake (Lake Zwai) and pumping of water from the lake for soda ash extraction. Further to lowering the lake level, these development activities resulted in an increase in the salinity of the lake from 19.38g L^{-1}(Talling and Talling, 1965) to 26g L^{-1} (Kebede *et al.*, 1994). More recently, Lanzen *et al.* (2013) reported a salinity of 34g $L^{-1}$for this lake which shows an obvious increasing trend of salinity of the lake which may also result in changes in the phytoplankton community (Kebede *et al.*, 1994).

2.8.3 *Spirulina* in Lake Beseka

Lake Beseka formerly known as Lake Metahara is located in the tectonically active Main Ethiopian Rift about 150 km east of Addis Ababa and lies at an altitude of 1200m. In 1961, *Spirulina platensis* was reported as a dominant species of this lake (Wood and Talling, 1988). However, in the 1990s, this genus was reported absent by Kebede *et al.* (1994) instead the phytoplankton community of the lake was composed of a mixture of other green and blue-green algae. Recently, Lanzen *et al.* (2013) reported the absence of cyanobacteria from this lake.

Lake Beseka is expanding rapidly (Ayenew, 2004; Goerner *et al.*, 2008) as the surface area of the lake quadrupled from 11.1 km^2 in 1973 to 39.5 km^2 in 2002 (Goerner *et al.*, 2008). Several explanations have been given for the rapid expansion of this lake over recent decades. According to Ayenew (2004), the volume increase may be caused either by increased recharge from irrigation runoff from Metahara sugar cane plantations and the higher levels of the River Awash maintained by Koka Dam. Goerner *et al.* (2008) suggested that the expansion of the lake

is associated with increased discharge of the hot springs rather than change in local climate or diversion of surface water for irrigation. In general, the increase in volume of Lake Beseka resulted in changes in the chemistry and phytoplankton communities of the lake (Kebede *et al.*, 1994) with the total absence of cyanobacteria in the lake (Lanzen *et al.*, 2013). For instance, conductivity of the lake decreased from 74100 µs/cm (Talling and Talling, 1965) to 7441 µs/cm (Kebede *et al.*, 1994) which is an almost 100 % decline. Similarly, the salinity of the lake dropped to 2.9–3.1g L^{-1} (Lanzen *et al.*, 2013) from 56.3g L^{-1} reported by Talling and Talling (1965).

2.8.4 *Spirulina* in Lake Chitu

Lake Chitu is a small crater lake with a surface area of 0.8km^2 and a maximum depth of 21metres (Kebede *et al.*, 1994). It lies at an altitude of 1600m and is located in the vicinity of Lake Shala which is about 287 kms south of Addis Ababa. The lake has no inflowing rivers and obvious surface outlet and hot springs at the mouth of the lake feed it with water permanently (Kebede *et al.*, 1994).

Lake Chitu is presently the only soda lake in Ethiopia in which *Spirulina* grows almost as a uni-algal population (Kebede *et al.*, 1994; Kebede, 1997; Kebede *et al.*, 2002; Lanzen *et al.*, 2013) throughout the year (Figure 2.10). Sitotaw (2014) described this massive growth of *Spirulina* in the lake as '*Spirulina* soup'. In August 1966, Wood and Talling (1988) recorded a biomass concentration of 2600 µg chl *a* m^{-3} for this lake. Data taken in March 1991 by Kebede *et al.* (1994) showed a biomass concentration of 224µg chl *a* m^{-3}. The variation in the biomass concentration of the lake recorded by the authors might be due to differences in the sampling period as one is measured during the main rainy season (August) of the area and the other was taken at the beginning of the rainy season (March) which in turn has an effect on the chemistry of the lake. For instance, the salinity of the lake measured by Wood and Talling (1988) in August was 38g L^{-1} but measurement taken in March 1991 showed a value of 44.9g L^{-1} (Kebede *et al.*, 1994). Increment in salinity may reduce the growth rate (Kebede, 1997) and in turn the concentration of the biomass. Moreover, nutrient availability may be improved during wet season due to frequent mixing of the lake from winds and rains (Kebede, 2002; Zinabu, 2002)

which thus contributes for the presence of high phytoplankton biomass. Kebede *et al.* (1994) indicated nitrogen as a limiting nutrient in the lake with a value of less than 1 μg L^{-1} during their study period (March-May 1991). Recently, Ogato and Kifle (2014) indicated the presence of seasonal variations in the concentration of nitrogen containing compounds (NO_3^- and NH_3) in the lake. According to these authors the NO_3^- content of the lake varied from 0 to 9.5μg L^{-1} with a mean value of 1.5μg L^{-1} and a range from 0 to 84.0μg L^{-1} was reported for NH_3 with a mean value of 14.5μg L^{-1}.

A recent measurement by Lanzen *et al.* (2013) indicated a salinity of 58g L^{-1} for Lake Chitu which showed an increasing trend as it was 38g L^{-1} in 1966 (Wood and Talling (1988) and 44.9g L^{-1} in 1991(Kebede *et al.*, 1994). Kebede (1997) tested *Arthrospira* isolate from this lake to its tolerance to salinity stress by adding different concentrations (13–88 g L^{-1}) of $NaHCO_3$, NaCl or Na_2SO_4. The specific growth rate of the isolate declined with increasing salinity, but growth was maintained in all levels of salts. In fact, *Spirulina* was also shown to grow in external sea salt concentrations of up to 150 % (Warr *et al.*, 1985). The ability of *Spirulina* to withstand elevated salinities enables it to survive and grow in alkaline lakes and other similar waters (Warr *et al.*, 1985). *Spirulina* accumulates carbohydrates such as glucosyl glycerol as osmoprotectors (Warr *et al.*, 1985; Vonshak, 1997) to tolerate the high concentrations of salts in the growth media. Lake Chitu's *Spirulina* biomass was also evaluated for toxin production (Willen *et al.*, 2011). However, toxins were not detected in the biomass as well as in the water sample taken from the lake.

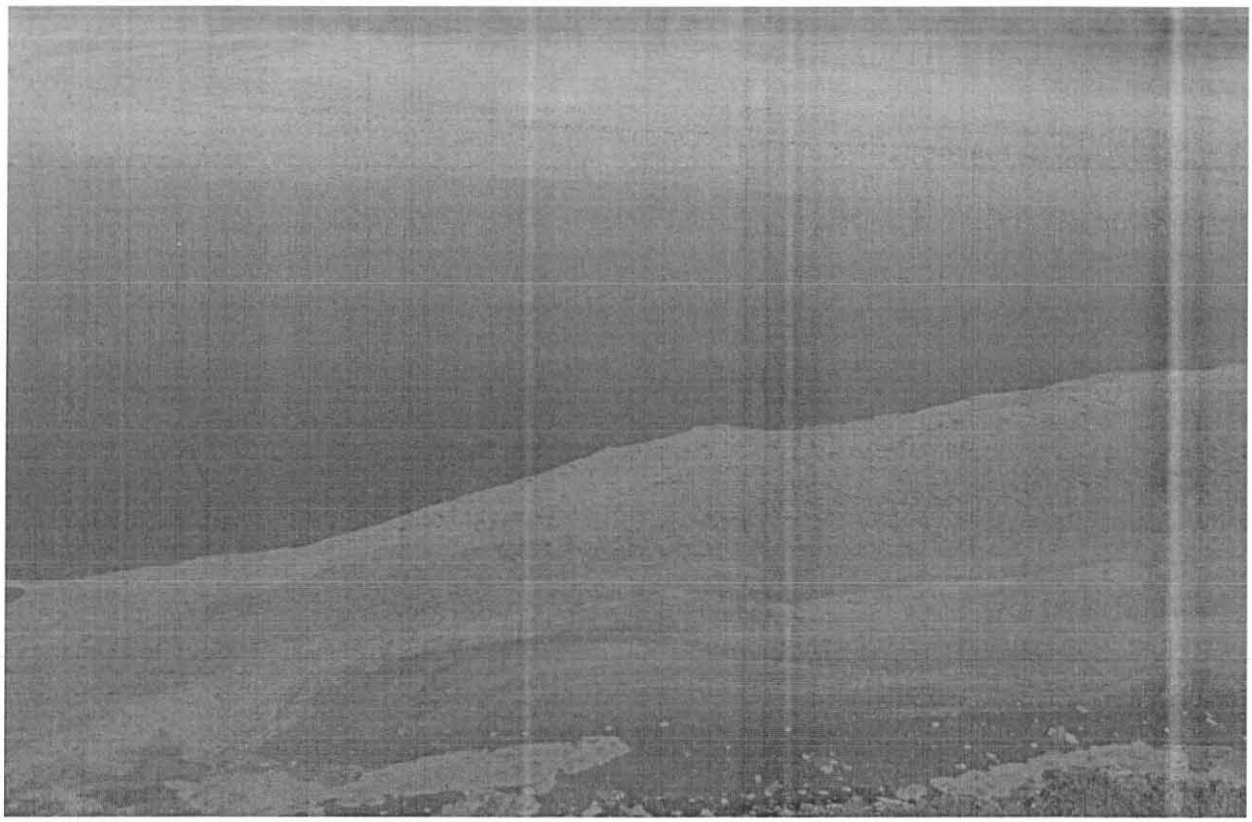


Fig2.10. Lake Chitu with massive growth of *Spirulina* (*Arthrospira)*

2.8.6 Spirulina in Lake Hora-Kilole (Lake Kilotes)

Lake Hora-Kilole (Lake Kilotes) is among the several of Bishoftu crater lakes located about 50 km south east of Addis Ababa. It is found at an altitude of 2000m with an area of 0.77km^2(Zinabu and Taylor, 1997). Lake Hora-Kilole was classified by Talling *et al.* (1973) as one of the soda lakes of Ethiopia supporting almost a monoculture of *Spirulina* (*Arthrospira*). However, a recent study by Rediat *et al* (2014) indicated the absence of *Spirulina* (*Arthrospira*) species in the lake . According to these authors, the phytoplankton community of the lake was dominated by seven other algal classes. The major changes in the physicochemical and phytoplankton structure of the lake was attributed to human intervention such as river diversion (diverting Mojo river to the lake for the purpose of large scale irrigation) and shoreline modification on the lake by local farmers to irrigate their crops which grow at the fertile soil found very near to the lake (Lemma, 1994 cited in Rediat, 2014).

Table 2.6. Changes in the chemical and phytoplankton composition of Ethiopian *Spirulina* lakes with time

Lakes	Data taken	pH	Salinity ($g\ L^{-1}$)	Conductivity (µS/cm)	Alkalinity (meq/l)	Na+ (meq/l)	K+ (meq/l)	*Spirulina*	References
Beseka (Metahara)	May, 61	9.9	56.3	74100	580	774	10.4	Dominant	Talling and Talling,1965
	May,91	9.4	5.3	7440		78.56	1.72	Other green and blue green algae	Kebede *et al.*,1994
	Jan,2003							Absent	Klemperer and Cash, 2007
	March,2011	9.6	2.9-3.1			69.78	60/	No cyanonbacteria	Lanzen *et al.*,2013
Arenguade	Apr,63	10.3	5.144			67	8.1	Dominant	Prosser *et al.*,1968
	Feb,64		5.541	6000	51.4	65.3	9.6	Dominant	Wood and Talling, 1988
	Jan,2003	9.64				61.73	5.20	Present	Klemperer and Cash, 2007
	Dec, 2008 - Oct, 2009.	9.62 - 9.84.			33-50.6			Present together with *Anabaenopsis elenkinii*	Belachew *et al.* 2012
	March,2011	9.7-9.9	2.1-2.8			54.5	5.82	Present in trace amounts	Lanzen *et al.*, 2013
Abijiata	May,61		20.1(19.38)	30000	210	277.2	8.5	Dominant	Talling and Talling,1965
	March,64	10.3	16.2	15800	166.4	222	6.5	Ooscystis spp	Wood and Talling,1988
	March,91	9.85	26.4	28130	349	416	9.72	Present with other filamentous blue green algae	Kebede *et al.*,1994
	Dec,2011	9.9	34.0			498.3	11.71	No cyanobacteria	Lanzen *et al.*, 2013
Chitu	Aug,66	9.8	38.3	28600	400	539	17.2	Dominant	Wood and Talling,1988
	May,71	10.2	34.8			608.7	10.5	Dominant	Baumann *et al.*,1975
	March,91	10.15	44.9	49100	573	864	31.2	Dominant	Kebede *et al.*,1994
	March,2011	10.4	58			801.3	1136/	Dominant	Lanzen *et al.*,2013

2.8.6 Potential of *Spirulina* production in Ethiopia

Although *Spirulina* is found as a uni-algal and harvestable population in Lake Chitu, harvesting and use of this natural resource as a food/dietary supplement is not yet practiced. This kind of harvesting system according to Fox (1996) can be operated by directly pumping the lake water onto harvesting screens and drying the algal biomass in the sun. However, harvesting *Spirulina* directly from lakes may have harmful effects for the ecosystem as there are animals such as birds which depend on the algal biomass maintained in the lakes (Fox, 1996). A more reasonable way of sustainable exploitation of these lakes is to build algae basins or ponds near the lakes which take filtered water from the lake and return their waste waters to the lake (Fox, 1996). Such type of production is cost effective as it minimizes media costs which usually take the higher share of production costs during artificial production of *Spirulina*.

Ethiopia is rich in the major nutrients (such as carbonates) required for *Spirulina* production in most rift valley lakes. A study by Endrie (2009) showed the possibility of producing *Spirulina* in outdoor cultivation technology using Trona (from Lake Chitu) as a substitute for the three major ingredients ($NaHCO_3$, Na_2CO_3 and NaCl) of the Zarrouk's standard medium. A recent report by Ogato *et al.* (2014) also showed a very good growth and biomass production of *Spirulina* using Lake Shalla and Lake Chitu waters when each of them were supplemented with 25 and 50% *Spirulina* medium (artificial medium).

There were initiatives to start commercial production of *Spirulnia* in Ethiopia by various stake holders. For instance, in 1995, microalgae-*Spirulina* project was initiated and some preliminary work was done under the former Ethiopian Science and Technology Commission with four affiliated institutions; Addis Ababa University, former Awassa College of Agriculture, Ethiopian Health and Nutrition Institute and former Food Research and Development Center (Document analysis). The objective of the project was to do a preliminary study on the potential productivity and the nutritional qualities of *Spirulina* from Ethiopian soda lakes (Document analysis). Another project on *Spirulina* was also proposed by two researchers at Addis Ababa University under a project name 'Preliminary studies on *Spirulina* production in Ethiopia' (Document analysis). However, all the projects were not implemented due to unknown reasons.

Chapter 3: Preliminary study on *Arthrospira* (*Spirulina*) biomass harvested from an Ethiopian soda lake, Lake Chitu for possible applications in human nutrition

3.1 Introduction

The cyanobacterium *Spirulina* (*Arthrospira*) is known for its high nutritional value with a protein content ranging from 45-71% on dry weight basis (Ortega *et al.*, 1993; Fox, 1996; Belay, 2008). It is also a host of various bioactive chemicals such as the rarely found γ-linolenic acid, pigments (carotenoids, phycobiliproteins, and chlorophyll *a*) as well as vitamins such as provitamin A and B_{12} (Belay, 2002; Habib *et al.*, 2008). The efficacy of *Spirulina* in the nutritional rehabilitation of malnourished individuals has been reported by various researchers (Simpore *et al.*, 2005; Yamani *et al.*, 2009; Azabji-Kenfack *et al.*, 2011). *Spirulina* and its extracts have also a range of pharmacological properties and therapeutic effects for various metabolic diseases such as cancer, diabetes, hyperlipidemia, etc (Belay, 2002; Khan *et al.*, 2005; Karkos *et al.*, 2008). It is, therefore, produced worldwide to be used as a dietary and/or protein supplement (Habib *et al.*, 2008; Belay, 2013).

Spirulina grows abundantly almost as a uni-algal population in an Ethiopian soda lake, Lake Chitu throughout the year (Wood and Talling, 1988; Kebede, 1997). The ability of the *Spirulina* species isolated from this lake to tolerate a wide range of salinity has been reported by Kebede (1997). This isolate was also reported to grow at a specific growth rate of 1.78 day^{-1} when cultured in modified Zarrouk's medium (Kebede and Ahlgren, 1996). *Spirulina* species from Lake Chitu was also reportd as a non-toxin producer (Willen *et al.*, 2011). This natural resource could be thus used as a protein source or food supplement in the country. The objective of this study was therefore to get background information about the chemical composition and safety of *Spirulina* biomass harvested from Lake Chitu for possible applications in human nutrition.

3.2 Materials and methods

3.2.1 Study area

The alkaline, saline crater Lake, Lake Chitu (7°23'N 38°24'E) is located in the Ethiopian rift valley adjacent to Lake Shala. Specifically, it is located about 287kms south of Addis Ababa (Fig 3.1). It has a surface area of 0.8km^2, a maximum depth of 21m and lies at an altitude of 1600m (Kebede *et al.*, 1994). The lake has no inflowing rivers and no obvious surface outlet and there are hot springs at the mouth of the lake which supply the lake permanently (Kebede *et al.*, 1994). Rainy seasons in the area usually occur between March and May (short rains) and from July to September (main rains) (Klemperer and Cash, 2007).

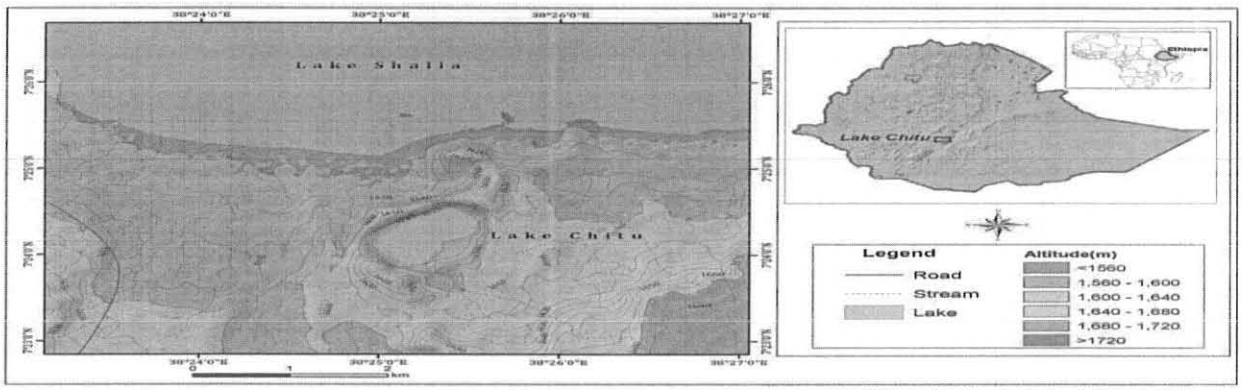


Fig 3.1 Location of the study site (Lake Chitu)

3.2.2. Sample collection and preparation

Spirulina biomass collection from Lake Chitu was carried out in March 2012. The biomass was harvested manually in plastic jars during morning hours. During harvesting, the algal biomass was allowed to pass through a 0.425mm sieve to remove insects, birds' hair and other large sized contaminants. The harvested biomass was immediately transported to the Center for Food Science and Nutrition laboratory of Addis Ababa University and filtered under vacuum by using a nylon cloth with 25μm openings. The biomass collected on the cloth was washed with distilled water to remove salts and debris attached to it. The washed biomass was freeze dried, grounded to powder and stored in brown bottles at -20˚C until analysis (Fig 3.2).

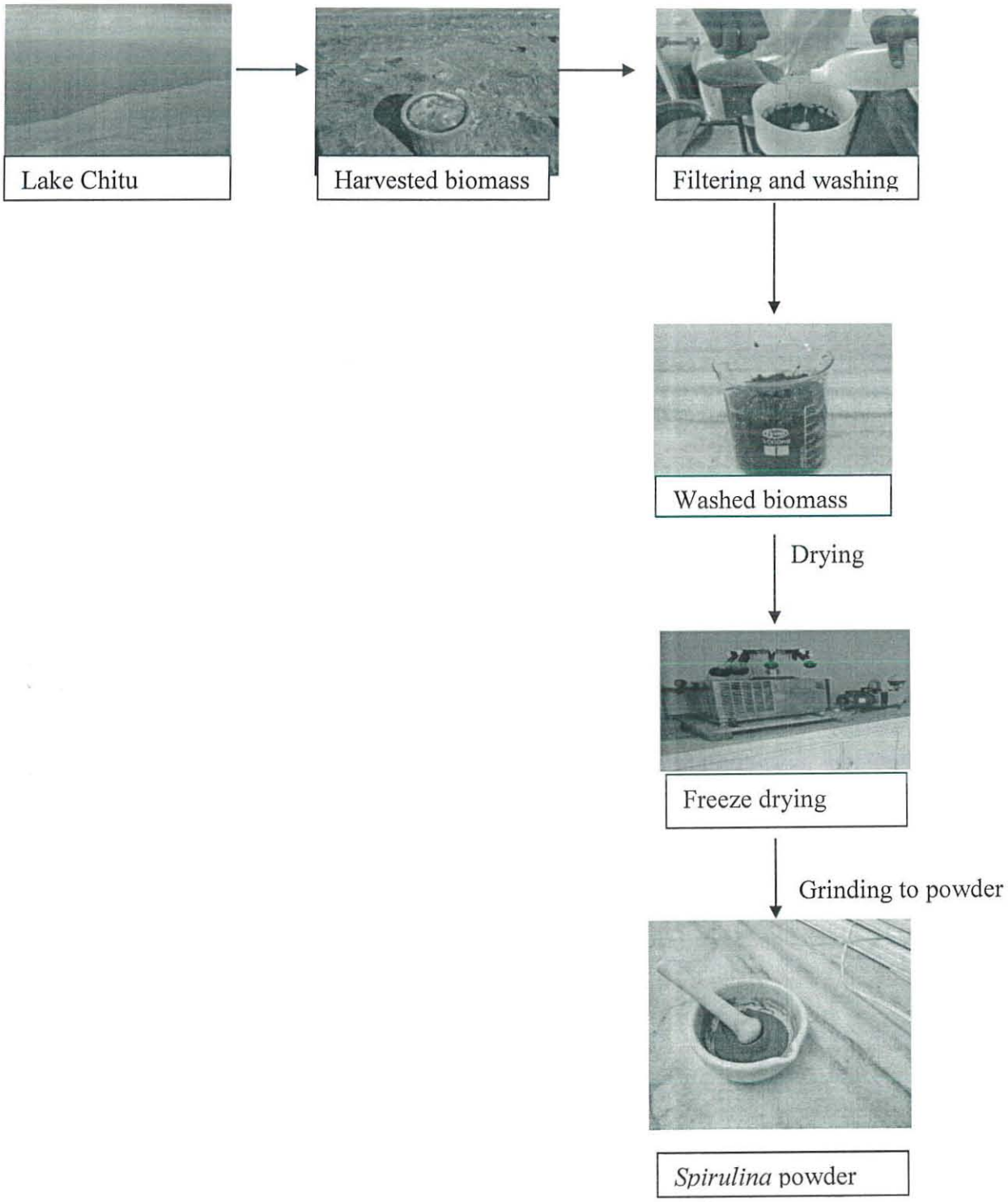


Fig 3.2. Flow diagram for sample (*Spirulina* powder) preparation

3.2.3 Measurements of physicochemical parameters

Physicochemical parameters of Lake Chitu were measured on site during sample collection. pH was measured with a pH-meter (Oakton 110, Eutech Instruments Pty. Ltd) and salinity was measured with a standard refractometer (0–100%, ATAGO Co. Ltd). Carbonate-bicarbonate alkalinity was determined according to Golterman *et al.* (1978) by titration of the water with 0.1N HCI to a pH of 4.5.

3.2.4 Biomass quality analysis

3.2.4.1 Chemical composition

The moisture content of the dried powder of Lake Chitu's *Spirulina* was determined according to AOAC (2000; 925.09). For the analysis, empty dishes and lids were dried using a drying oven for 1 hour at 100°C, transferred to desiccators, cooled for 30 minutes, and weighed. A representative 5g sample was then weighed and transferred to the dried and weighed dishes. The dishes and their contents were placed in the drying oven and dried at 105°C until no further weight loss was observed (approximately 5 hrs). The dishes were cooled in desiccators to room temperature and reweighed. The amount of moisture in the samples was then calculated as:

$$\text{Moisture (\%)} = \frac{W_2 - W_3}{W_2 - W_1} \times 100$$

Where: W_1= weight of the dish

W_2= weight of the dish and the sample before drying, and

W_3= weight of the dish and the sample after drying

The crude protein content of *Spirulina* powder was determined by employing the Kjeldahl method (AOAC, 2000; 979.09). A representative sample of 0.5 g dried *Spirulina* powder, 3 g of catalyst mixture (K_2SO_4–$CuSO_4$, 100:10) and 6 ml H_2SO_4 were added to a 250 ml borosilicate digest tube. The digestion was proceeding in a digestion block at 370°C for 4hrs until the contents of the tube become clear. After digestion, the tubes were cooled to room temperature until vapor production ceases, at which point they were connected to the distillation system. Meanwhile, 25 ml of an indicator solution (H_3BO_3, 2%) was placed in a 250 ml beaker. The distillation apparatus was programmed to add, in sequence, 30 ml water and 30ml of 35% NaOH, and an 8 min distillation was then initiated. The total amount of nitrogen was determined by titration of the H_3BO_3 excess with 0.1N HCl. Total protein was then determined by multiplying the amount of nitrogen with a factor of 6.25 (AOAC, 2000).

The ash content of Lake Chitu's *Spirulina* was determined following the procedure of AOAC (2000; 923.03). For the analysis, ashing dishes were first placed into a muffle furnace for 30min at 550 °C. The dishes were removed and cooled in desiccators for about 30 minutes to room temperature; each dish was weighed to the nearest g. A representative 2.5g of sample was added into each dish. The dishes were placed on a hot plate under a fume hood and the temperature was slowly increased until smoking ceases and the samples become thoroughly charred. The dishes were then placed inside the muffle furnace at 550°C until a gray ash was obtained. After ashing the dishes were removed from the muffle furnace and placed in desiccators for 1 hour to cool. Weight of total ash was calculated by difference and expressed as percentage of sample.

$$\text{Total ash (\%)} = \frac{W_2 - W}{W_1 - W} \times 100$$

Where: W= weight in grams of empty dish

W_1= weight of the dish plus the dried sample

W_2= weight in grams of the dish plus ash

Crude fiber was determined after digesting 1.5g of *Spirulina* powder by refluxing with 1.25% boiling sulfuric acid and 28% of boiling potassium hydroxide (AOAC, 2000; 962.09).

Digestion: The sample was placed into a 600ml beaker, and 200ml of 1.25% H_2SO_4 was added and boiled gently exactly for 30minutes placing a watch glass over the mouth of the beaker . During boiling, the level of the sample solution was kept constant with hot distilled water. After 30 minute boiling, 20ml of 28% KOH was added and boiled gently for a further 30minute, with occasional stirring.

Filtration: The bottom of a sintered glass crucible was covered with 10mm sand layer and wetted with a little amount of distilled water. The solution was poured from beaker into sintered glass crucible and then the vacuum pump was tuned on. The wall of the beaker was rinsed with hot distilled water several times; washings were transferred to crucible, and filtered.

Washing: The residue in the crucible was washed with hot distilled water and filtered (repeated twice). The residue was washed with 1% H_2SO_4 and filtered, and then washed with hot distilled

water and filtered and again washed with 1% NaOH and filtered. The residue was washed with hot distilled water and filtered; and again washed with 1% H_2SO_4 and filtered. Finally the residue was washed with water –free acetone.

Drying and combustion: The crucible with its content was dried for 2 hours in an electric drying oven at 130°C and cooled for 30min in the desiccatorsand then weighed. The crucible was transferred to a muffle furnace and incinerated for 30min at 550°C. The crucible was cooled in the desiccators and weighed. Then the fiber content was calculated as a residue after subtraction of the ash.

$$\text{Crude fiber g/100g} = \frac{W_1 - W_2}{W_3} \times 100$$

Where: W_1= weight of (crucible + sample) after drying

W_2= weight of (crucible + sample) after ashing

W_3= weight of sample

The chlorophyll *a* content of the sample was determined according to (AOAC, 1995: 940.03). Approximately 50mg of *Spirulina* was weighed in to a 35ml centrifuge tube and 5 grams of glass beads and 2.5ml of 85% acetone in water were added to it. The mixture was then vortexed for 5 minutes and after vortexing, 10ml of 85% acetone was added. After a brief vortex of the mixture, it was centrifuged at 3200 rpm for 5 minutes. The supernatant was collected in a 50ml volumetric flask. This step was repeated for about four times until the supernatant became clear. After complete extraction, the flask was filled up to volume with 85% acetone in water. The flask was then capped and gently inverted to mix the contents. The absorbance of the extract was then read with a Lambda 950 UV/VIS/NIR spectrophotometer (Perkin Elmer, and USA) at 666nm and 642nm against an 85% acetone/water blank. The amount (%) of chlorophyll *a* in the samples was then calculated with the following formula:

$$\text{Chlorophyll } a\ (\%) = \frac{[(9.93 \times \text{Abs}_{666}) - (0.0777 \times \text{Abs}_{642})] \times 0.05 \text{ liter} \times 100}{\text{Sample weight (mg)} \times \%\text{dry weight}}$$

The crude phycocyanin content of the sample was quantified according to AOAC (2008). About 0.5g of sample was weighed directly into a centrifuge tube and suspended in 25ml 100Mm Na-

Phosphate buffer (pH 6). The suspension was mixed with spatula to break up clumps and incubated for 16hrs at 30°C. After incubation, the sample was mixed again with spatula and centrifuged for 15minutes at 5000rpm at 4°C. The supernatant was filtered through a Whatman#41filter paper and 2ml filtrate was then pipette into a 50ml volumetric flask and filled up with 100Mm phosphate buffer (pH 6) in volumetric flask. The supernatant was analyzed spectrophotometrically for total crude phycocyanin content. The crude phycocyanin content of the sample was calculated according the following formula:

$$\% \text{ Total Crude Phycocyanin} = \frac{0.295 \times 100 \times D \times Abs_{618}}{W \text{ (mg)}}$$

3.2.4.2 Mineral analysis

Sodium and potassium contents of the biomass were determined by a flame photometer (Jenway PFP7, UK) after digesting a representative 2g sample with diluted (1:1) nitric acid (Osborne and Voogt, 1978). The phosphorous content of the biomass was determined using a colorimetric technique following the method of Fiske and SubbaRow (1925). For determination of the Calcium, Iron, and Zinc contents of the biomass, the ash obtained by incineration of 1g *Spirulina* powder at 450 °C was dissolved in a mixture of HNO_3 and HCl, diluted with deionized water and analyzed by Atomic Absorption Spectrophotometer (AA-6800, Shimadzu) (Osborne and Voogt, 1978).

3.2.4.3 Determination of *in vitro* protein digestibility

In vitro protein digestibility of Lake Chitu's *Spirulina* was determined according to the method described by Elkhalil *et al.* (2001) with some modifications. Twenty mg of *Spirulina* powder was digested in triplicate in 10 ml of trypsin (0.2 mg/ml in 100 Mm phosphate buffer, pH 8.0). The suspension was incubated at 37°C for 2 hours. Hydrolysis was stopped by adding 5 ml 50% trichloroacetic acid. The mixture was allowed to stand for 30 min at 4 °C and centrifuged at 4000rpm for 30 min using 80-2 Centrifuge (China). The nitrogen content of the supernatant was then determined by employing the Kjeldahl method. Digestibility was calculated as follows:

$$\text{Protein digestibility} = \frac{A}{B} \times 100$$

Where: A: total protein content (mg) in the supernatant.

B: total protein content (mg) in the sample.

3.2.4.4 Microbiological analysis

The freeze dried biomass was analyzed for aerobic mesophiles, total coliforms, fecal coliforms, *Escherchia.coli*, *Stphylococcus aureus*, yeasts and molds according to the Nordic Committee of Food Analysis (NMKL) methods.

Aerobic mesophilic count

Decimal dilution and pour-plate procedures were applied using Plate Count Agar medium. Inoculated plates were incubated at 30°C for 48 hrs and colony counts were done at 24 and 48 hrs (NMKL, 2006).

Total coliforms

Decimal dilution and pour plate procedures were performed using Trypton Soya Agar and Violet Red Bile Agar media. Culture media were incubated at 37 °C for 24 hrs. Confirmation was done by inoculating colonies into brilliant green bile broth tubes (NMKL, 2004).

Fecal coliforms and *Escherchia coli*

Decimal dilution and pour plate procedures were performed using Trypton Soya Agar and Violet Red Bile Agar media. Inoculated plates were incubated at 44 °C for 24 hrs. Confirmative test was done in *E. coli* and nutrient broths (NMKL, 2005).

S.aureus

Serial dilution and spread plate procedures were performed on pre-dried surfaces of Baird Parker Agar (supplemented with tellurite egg yolk emulsion) plates. The culture media were incubated at 37°C for 24-48hrs hours. Confirmation was done by performing coagulase test (NMKL, 2003).

Yeasts and molds

Serial dilution and spread plate procedures were applied on pre-dried surfaces of chloramphenicol supplemented Dichloran Rosebengal Agar plates. The culture media were incubated at 22°C for 5-7 days and colony counts were done at the 5^{th} and 7^{th} day (NMKL, 2005).

3.2.4.5 Acute toxicity test on mice

Adult male mice (Swiss mice, 4 week old, 25-30g) were used for the acute toxicity test. Mice of group A were treated as controls and mice of group B were administered fresh biomass (30g/kg body weight). The biomass was suspended in distilled water and the solution (5ml/kg body

weight) was administrated to mice groups in a single oral dose by intra gastric gavages using a feeding needle. The control group received an equal volume of distilled water as vehicle. Mice were observed thoroughly for onset of any immediate toxic signs (changes on skin and fur, eyes, behavior pattern and diarrhea) and also during the observation period of 14 days for any delayed acute effects. During the experiment, the animals were weighed and food intake was also monitored. At the end of the experiment, all animals were fasted overnight and sacrificed by decapitation to evaluate the gross internal abnormalities and organ coefficients (relative organ weight) and appearance of heart, gonads, spleen, lung, liver, spleen and kidneys. Organ coefficients were calculated according to Sagstad *et al.* (2008):

Organ coefficients (%) = Weight of organ (g) /Body weight (g) × 100

3.3 Results and discussion

3.3.1 Physicochemical properties of Lake Chitu

Table 3.1 shows the physicochemical properties of Lake Chitu measured during the sampling period (March 2012). The pH of the lake was 10.36 which is within the optimal pH range (9.5–10.5) that supports the growth of many *Spirulina* strains (Vonshak, 1997). The alkalinity of the lake was 637 meq L^{-1} (Table 3.1). Ballot *et al.* (2005) reported alkalinity value of more than 400 meq L^{-1} for a *Spirulina* dominated lake, Lake Sonachi in Kenya. *Spirulina* was also reported to be the dominant algal species in an Ethiopian soda lake, Lake Beseka, with an alkalinity of 580 meq L^{-1} (Talling and Talling, 1965) and in another Ethiopian soda lake, Lake Arenguade which had an alkalinity of 51.4 meq L^{-1}(Wood and Talling, 1988). It has been suggested that in natural environments, *Spirulina* species occur under a wide range of pH and alkalinity due to the ability of these species to acclimate such environments (Belay, 2013).

Table 3.1. Physicochemical properties of Lake Chitu measured in March 2012

Sampling date	pH	Alkalinity(meq L^{-1})	Salinity (g L^{-1})	References
March 2012	10.36	637	52	Present study

March 1991	10.15	573	44.9	Kebede *et al.*,1994
August 1966	9.8	400	38.3	Wood and Talling,1988

The salinity of the lake was recorded as 52g L^{-1} and was high compared to other *Spirulina* dominated lakes: 47g L^{-1} for Lake Bogoria, Kenya (Ballot *et al.*, 2004) and 14.35-23.2 g L^{-1} for Lake Chad, Central Africa (Albert *et al.*, 2012). *Spirulina* was also reported to be the dominant algal species in an Ethiopian soda lake, Lake Beseka (Lake Metahara), which had a salinity of 56.3g L^{-1}(Talling and Talling, 1965). The ability of *Spirulina* to tolerate a wide range of salinity was reported by several researchers (Kebede 1997; Vonshak 1997; Mussagy 2005). In general, as shown in table 1, all physicochemical properties of the lake showed an increasing trend with time which can be attributed to evaporative concentration.

3.3.2 Biomass quality

3.3.2.1 Chemical composition

The chemical composition of Lake Chitu's *Spirulina* is presented in Table 3.2. The moisture content of the powder was 6.7% which is within the range reported for commercial *Spirulina* (Table 3.2). The biomass had 43.0% protein which is comparable to the value found by Ogbonda *et al.* (2007) (39–46%), but lower than the usually reported protein values for this microalga which can be up to 71% (Table 3.2).

Table 3.2. Chemical composition of *Spirulina* harvested from Lake Chitu in March 2012

	Lake Chitu's *Spirulina* (Mean ±SD)	*Commercial *Spirulina*
Moisture (%)	6.7±0.07	4-7

Protein (%)	43.0±0.8	45-71
Crude fiber (%)	0.7±0.1	0.1-7.7
Ash (%)	13.1±0.08	6.4-13
Chlorophyll *a* (mg g^{-1})	6.7±0.07	6.1-12
Crude phycocyanin(mg g^{-1})	61.5±0.20	140-172

*Fox, 1996; Belay, 1997; 2008; Ortega *et al.*, 1993; Cohen, 1997

The crude fiber content of the biomass was 0.7% which is within the lower range of fiber contents reported for commercial *Spirulina* (0.1-7.7) (Table 3.2). The presence of low fiber in Lake Chitu's *Spirulina* suggests an easily digestible biomass for human use.

The ash value of microalgae represents the inorganic content of the biomass and includes both adsorbed and absorbed salts and minerals (Richmond, 1986). The ash content of Lake Chitu's *Spirulina* was 13.1 % and thus comparable with ash contents reported for commercial *Spirulina* (Table 3.2).

Lake Chitu's *Spirulina* was also analyzed for some phytonutrients (pigments) (Table 3.2) since the content of these phytochemicals is highly dependent on growth conditions. The chlorophyll *a* content of the biomass was 6.70 mg g^{-1}. This value is within ranges commonly reported for commercial *Spirulina* (Table 3.2). On the other hand, the phycocyanin content of the biomass was low (61.5mg g^{-1}) which may be attributed to environmental conditions in which the microalga was subjected to grow such as high light intensity and nutrient deficiency (Richmond and Boussiba, 1980).

3.3.2.2 Mineral composition

The mineral content of Lake Chitu's *Spirulina* is presented in Table 3.3. Sodium and K contents of the biomass were 2060 and 1931g/100g respectively. These minerals especially the potassium content was higher than that reported for commercial *Spirulina* (Table 3.3). The higher content

of Na and K might be due to the presence of high Na and K salts in the lake (Lanzen *et al.*, 2013). Lanzen *et al.* (2013) reported a sodium content of 18.43g L^{-1} and potassium content of 1.14 g L^{-1} for this lake for a water sample collected in March 2011. On the other hand, the biomass had low Ca (51.7g/100g) and Zn (0.65g/100g) contents compared to commercial *Spirulina*. The low Ca content of the biomass can be attributed to the high salinity and alkalinity of the lake which causes the removal of calcium from the solution by forming insoluble carbonate precipitates (Kebede *et al.*, 1994; Wood and Talling, 1988). Phosphorous (733.8 mg/100g) and iron (29.4mg/100g) contents of the biomass were within ranges reported for most commercial *Spirulina*.

Table 3.3. Mineral content of Lake Chitu's *Spirulina* harvested in March 2012

Minerals(g/100g DM)	Lake Chitu's *Spirulina* (Mean ±SD)	*Commercial *Spirulina*
Na	2060±0.61	282-2350
K	1931±0.07	233-1660
P	733.8±2.64	536-2307
Ca	51.7±0.42	110 -2610
Fe	29.4±1.99	22-117.6
Zn	0.65± 0.09	1.42-10.61

* Johnson and Shubert, 1986; Fox, 1996; Belay, 2008; Campanella *et al.*, 2008

3.3.2.3 *In vitro* protein digestibility by trypsin

One aspect of measuring the nutritional quality of a given food protein is its digestibility by digestive enzymes (Becker, 2007). Evaluation of Lake Chitu's *Spirulina* by using a single enzyme assay (trypsin) gave 59.3% digestibility. This digestibility is relatively high for a given protein as the sample was treated by a single enzyme which predicts that the action of multiple

enzymes which normally occurs in the human body will ensure higher digestibility. It has been reported that absence of cellulose in the cell wall of *Spirulina* facilitates cell lysis and release of its proteins (Vonshak, 1997).

3.3.2.4 Microbiological safety

As microbial safety is one aspect of quality control for food-grade *Spirulina* (Belay, 1997), Lake Chitu's *Spirulina* was evaluated for a range of microbial groups (Table 3.4). The aerobic mesophilic count of the biomass was 6.4 $x10^4$ CFU/g. This value is within the lower ranges of standards set by major *Spirulina* producers in the world (Table 3.4). It is also worth mentioning that, spray drying of the biomass, which is the common drying method used by most producers (Belay, 2013), would further decrease the microbial count of the biomass to a lower level. Counts of total coliforms, fecal coliforms, *E.coli*, *S.aureus*, yeasts and molds were also below 10^2CFU/g. The low aerobic mesophilic count together with the absence of pathogens or other safety indicator microorganisms in the freeze dried biomass was surprising as freeze drying mostly inactivates rather than killing microorganisms (Jay, 1996). These low counts of microbial groups can be thus explained by the high pH (pH $\geq$ 10.3) and salinity (>5%) (Table 3.1) prevailing in the lake which are inhibiting the growth of most pathogenic microorganisms. According to ICMSF (1980), most pathogenic microorganisms grow at pH minima of 4 and maxima of 10 with an optimum growth in pH between 6 and 7.5. Microscopic examination of the lake water also showed the absence of other algal contaminants in the lake which can be still attributed to the high pH value of the lake which favors the proliferation of *Spirulina* (Belay, 1997; Cohen, 1997; Kebede, 1997).

Table 3.4. Microbial profile of freeze dried *Spirulina* biomass harvested from Lake Chitu in March 2012

	Lake Chitu's *Spirulina*	*Commercial *Spirulina*

Aerobic mesophiles(10^3)	64	50-1000
Total coliforms	$<10^2$	Negative
Fecal coliforms	$<10^2$	Negative
E.coli	$<10^2$	Negative
S.aureus	$<10^2$	Negative
Yeasts	$<10^2$	Negative
Molds	$<10^2$	10^2 - $<10^3$

*Belay, 1997

3.3.2.4 Acute toxicity study

In the present study, mice fed with fresh *Spirulina* biomass (with 85% moisture content) at the dose of 30 g/kg body weight did not show any signs of toxicity during the 14 days observation period. There were no significant differences in the relative organ weights between the treated and control groups (Table 3.5). The gross examinations of their internal organs also revealed no pathological abnormalities. These results, thus, demonstrate the potential safety of the biomass for human consumption.

Table 3.5. Relative organ weights of mice fed fresh *Spirulina* biomass (30g/kg body weight) harvested from Lake Chitu

Groups	Relative organ weight(g/100g body weight)						
	Liver	Kidneys	Spleen	Heart	Lung	Brain	Gonads

Experimental	5.16±0.34	1.55±0.09	0.53±0.05	0.51±0.07	0.82±0.13	1.17±0.03	0.98±0.10
Control	5.06±0.77	1.52±0.08	0.56±0.31	0.54±0.08	0.81±0.10	1.12±0.11	0.98±0.08

Values are mean ± SD of five animals

3.4 Conclusions

Although the protein content of the biomass was low compared to commercial *Spirulina* it was still high compared to conventional high protein containing foods such as legumes with protein contents usually ranging from 20-35 %(Habib *et al.*, 2008). Furthermore, the digestibility value obtained from the single enzyme assay together with the low fiber content of the biomass is suggestive of the good digestibility of the biomass. The biomass was also safe in terms of pathogenic microorganisms and didn't bring any toxic effects to mice even at a very high dose. Therefore, Lake Chitu's *Spirulina* can be considered as a potential dietary supplement for humans. Since the chemical composition of *Spirulina* varies based on the prevailing growth conditions, seasonal variability in the chemical composition and safety of the biomass is worth investigating.

Chapter 4: Seasonal variation in the nutrient profile of Spirulina (Arthrospira) biomass harvested from an Ethiopian soda lake, Lake Chitu

4.1 Introduction

Spirulina (*Arthrospira*) is known for its excellent chemical composition as it contains high protein (up to 71%) along with high amounts of essential amino acids, essential fatty acids,

minerals (especially iron), vitamins (especially provitamin A) and various bioactive chemicals (pigments) with potential therapeutic effects (Cohen, 1997; Belay, 2002). For instance, its blue pigment, C-phycocyanin is claimed for having an antioxidant (Bhat and Madyastha, 2000, 2001; Romay and Gonzalez, 2000; Khan *et al.*, 2006; Patel *et al.*, 2006; MacCarty, 2007; Riss *et al.*, 2007; Guan *et al.*, 2009), anti-inflammatory (Romay *et al.*, 1998; Reddy *et al.*, 2000; Remirez *et al.*, 2002; Nemoto-Kawamura *et al.*, 2004; Shih *et al.*, 2009), hepatoprotective (Vadiraja *et al.*, 1998; Basu, *et al.*, 1999), neuroprotective (Rimbau *et al.*, 1999; Rimbau *et al.*, 2001; Bermejo-Bescós *et al.*, 2008) and anti-cancer (Schwartz *et al.*, 1987; Subhashini *et al.*, 2004) effects. Besides, this pigment has highest economic potential by being used as a natural pigment for food, feed, drug and cosmetics (Cohen, 1997). Carotenoids which are yellow-green pigments are also found in this microalga in large quantities (Mendiola *et al.*, 2005). A study on Chinese adults by Wang *et al.* (2008) has shown that ingestion of 4.5 mg of β-carotene from *Spirulina* provides 1 mg of vitamin A. Carotenoids are also strong antioxidants as they are capable of scavenging potentially harmful radicals, which are commonly associated with the induction of some types of cancers (Stahl and Sies, 2005). Consequently, this cyanobacetrium (microalga) is produced in many countries either from natural lakes or artificial pond cultures to be used as a health food or as a protein supplement (Belay, 2013; Vonshak and Richmond, 1988). From data obtained from websites of various companies, Belay (2013) estimated an annual production of 10,000 tons of *Spirulina* worldwide.

Spirulina grows abundantly almost as a unialgal population in an Ethiopian soda lake, Lake Chitu throughout the year (Wood and Talling, 1988; Kebede, 1997). A preliminary study reported elsewhere in this thesis indicated the possibility of using the biomass in human nutrition as the biomass had good chemical composition and safe in terms of microbial contamination. Moreover, short term feeding of the fresh biomass (30g/kg body weight) to mice didn't bring any signs of toxicity or mortality.

It has been reported that the chemical composition of microalgae can vary due to environmental factors such as temperature, salinity, light, and nutrient availability. Most of these environmental parameters vary according to season, and changes in these parameters can stimulate or inhibit the biosynthesis of several nutrients (Marinho-Soriano *et al.*, 2006). Therefore, the objective of this

study was to examine variation in the nutrient profile of Lake Chitu's *Spirulina* on seasonal basis.

4.2 Materials and Methods

4.2.1 Sampling and sample preparation

Spirulina biomass collection from Lake Chitu was carried out starting from March 2012 to January 2013 in two months interval. The samples were harvested manually in plastic jars during morning hours. During harvesting, the algal biomass was allowed to pass through a 0.425mm sieve to remove insects, birds' hair and other large sized contaminants. The harvested biomass samples were immediately transported to the Center for Food Science and Nutrition laboratory of Addis Ababa University and filtered under vacuum by using a nylon cloth with 25μm openings. The samples were washed with distilled water to remove salts and debris attached to them. The washed biomass samples were dried in two ways: oven dried (at 60°C) and freeze dried using a freeze drier. The dried biomass samples were then grounded to powder and stored in brown bottles at -20 ◦C until analysis.

4.2.2 Rainfall and temperature data

Rainfall and temperature data of the study area during the sampling period (March 2012-January 2013) were obtained from Ethiopian Meteorological Agency. Data recorded for the nearest meteorological station to the lake, Arsi Negele station was used for the study. A "rainfall coefficient" for each month was calculated which is the ratio between the monthly rainfall and one twelfth of the annual rainfall. A month is designated "wet" when the monthly rainfall coefficient is greater than or equal to 0.6 (60% of the rainfall module) (Gamachu, 1977 cited in Zinabu, 2002).

4.2.3 Physicochemical parameters

The pH of the lake was measured with a pH-meter (Oakton pH 110, Eutech Instruments Pty. Ltd., Singapore) and salinity was measured with a standard refractometer (0–100%, ATAGO Co. Ltd.). Carbonate-bicarbonate alkalinity was determined according to Golterman *et al.* (1978) by titration of the water with 1N HCI to a pH of 4.5.

Water samples were also collected during wet and dry months to determine some inorganic minerals of the lake. Minerals were determined according to the standard analytical methods described in APHA/AWWA/WEF(1999): Na^+ and K^+ by flame photometric method, Ca^{2+} by direct nitrous oxide-acetylene flame method, Mg^{2+}, Fe, Zn, Mn, and Cu by direct air-acetylene flame method.

4.2.4 Biomass nutrient profile analysis

4.2.4.1 Proximate composition

Spirulina samples were analyzed for their proximate composition using AOAC procedures. Moisture content was determined by drying a representative (5g) sample for 4hrs at 105°C (AOAC, 2000; 925.09). Crude protein content (N x6.25) was determined by employing the Kjeldahl method (AOAC, 2000; 979.09) from a 0.5g *Spirulina* powder. Fat content was quantified gravimetrically by exhaustively extracting 2g sample in diethyl ether (boiling point, 55°C) in a Soxhlet apparatus (AOAC, 2000; 4.5.01). The ether was evaporated from the extraction flask. The amount of fat was quantified gravimetrically and calculated from the difference in weight of the extraction flask before and after extraction as percentage. Briefly, the extraction flasks were cleaned, dried in a drying oven at 70°C for 1 hour, cooled in desiccators (with granular silica gel) for 30minutes, and then weighed. The bottom of the extraction thimble was covered with about 2cm layer of fat free cotton. A representative sample (2g) was added into the extraction thimbles, and then covered with about 2cm layer of fat free cotton. The thimbles containing the sample were placed into soxhlet extraction chamber. The cooling water was switched on, and 50ml of diethyl ether was added to the extraction flask through the condenser. The extraction was conducted for about 4 hours. The extraction flasks with their content were removed from the extraction chamber and placed in the drying oven at 70°C for 15minutes, cooled to room temperature in the desiccators for about 30minutes and reweighed.

$$W = W_2 - W_1$$

$$\text{Fat g/100g sample} = (W \times 100)/W_0$$

Where: W= weight of fat,

W_2= weight of extraction flask after extraction of fat,

W_1= weight of extraction flask before extraction (weight of flask)

W_0= weight of sample

Ash was determined by incineration of 2.5g sample in a muffle furnace at 550°C until a gray ash was obtained (AOAC, 2000; 923.03). Crude fiber was determined after digesting 1.5g of sample by refluxing with 1.25% boiling sulfuric acid and 28% of boiling potassium hydroxide (AOAC, 2000; 962.09). Carbohydrate content was determined as the weight difference using protein, lipid, fiber, moisture and ash content data (James, 1996).

Utilizable carbohydrates = 100 – (g of moisture + g of protein+ g of fat + g of ash + g of fiber).

Total energy was determined by calculation from fat, carbohydrate and protein contents using the Atwater's conversion factors; 16.7KJ/g (4kcal/g) for protein, 37.4KJ/g (9kcal/g) for fat and 16.7KJ/g (4kcal/g) for carbohydrates and expressed in kilocalories (Guyot *et al.*, 2007).

Energy (kcal) = 4 × (g of protein + g ofcarbohydrate) + 9 × (g of lipid).

4.2.4.2 Determination of amino acids

Amino acid composition of *Spirulina* samples collected on seasonal bases was determined according to AOAC (2005; Method 994.12). Samples (0.5g) were first oxidized with hydrogen peroxide/formic acid/phenol mixture in a fridge (4°C) for 16 hrs to preserve and convert cysteine and methionine into cysteic acid and methionine sulphone, respectively. After oxidation, performic acid was decomposed with sodium metabisulfite (0.84 g) prior to acid hydrolysis, using 6 M HCl-phenol solution. The hydrolysis was conducted in an oven for 24 hrs at 110°C, followed by the addition of 20 ml of norleucine standard solution. The hydrolysate was filtered into an evaporating flask and evaporated under vacuum at 40°C to 5.0 ml. The evaporate was adjusted to pH 2.2 using 2 M NaOH, made up to 50 ml with sodium citrate buffer, pH 2.2, and passed through a 0.22µm Millipore filters into a 1.0 ml amber vial. The analysis was performed using an ion exchange chromatography (Biochrom 20+ Amino Acid Analyser, Biochrom Ltd, Cambridge, UK). After reaction with ninhydrin, amino acids were detected at a wavelength of 570 nm. The amino acids were identified and quantified by comparing peak profiles of the proteins with amino acid profiles from external amino acid standards.

4.2.4.3 Determination of fatty acids

For fatty acid determination, lipids were extracted with chloroform–methanol–water (2:1:0.8) according to Bligh and Dyer method (Bligh and Dyer, 1959). The extracted lipid was dissolved in 2ml chloroform. 1 ml of the mixture was transferred to a new glass bottle and 100µl of internal standard (methyl pentadecanoate) was added to it. Fatty acids were transmethylated by treatment with trimethylsulfonium hydroxide (Sigma). 10 µl of fatty acid methyl esters (FAMEs) was injected into the capillary column (Phenonenex Zebron, ZB-FFAP, 30 m x 0.22 mm internal diameter) using a vaporising injector (split flow of 50 ml/min). The oven temperature was maintained at 120°C for 1 min, and then increased to 250°C (5°C/min) for 2 minutes. Hydrogen was used as a carrier gas at a flow rate of 0.5ml min^{-1}. Identification of fatty acids was made by comparing the relative retention times of FAME peaks of the samples with FAME standards. A standard library was also used through the "Thermo Scientific Xcalibur" software programme, to confirm identification by comparison of the mass spectrum.

The percentage of each fatty acid was calculated using the following formula:

$$\% \text{ Individual fatty acid} = \frac{\text{Individual fatty acid peak area}}{\text{Total fatty acid peak area}} \times 100$$

4.2.4.4 Determination of mineral content

For the determination of minerals, 0.2g *Spirulina* powder was weighed into a digestion vessel and 6ml of concentrated HNO_3 was added to the vessel. The digestion vessel was then placed in a microwave rotor and digested for 2hrs. When the digestion was complete, 4ml Milli-Q H2O was added to the digestion vessel and the sample was transferred to a universal sample bottle. 5 ml of Milli-Q H2O was then added to the digestion vessel and transferred to the universal bottle. This step was repeated again and the final volume of the solution was made up to 20ml. Mineral analysis of the diluted solutions was undertaken by ICP-MS (Thermo-Fisher Scientific Icap-Q; Thermo Fisher Scientific, Bremen, Germany). The instrument was run employing collision-cell technology with kinetic energy discrimination (CCT-KED) to remove polyatomic interferences; the collision cell gas was He. Samples were introduced from an autosampler (Cetac ASX-520) incorporating an ASXpress™ rapid uptake module through a PEEK nebulizer (Burgener Mira Mist). Internal standards were introduced to the sample stream on a separate line via the

ASXpress unit and included Sc (20 μg L^{-1}), Rh (10 μg L^{-1}), Ge (10 μg L^{-1}) and Ir (5 μg L^{-1}) in 2% trace analysis grade (Fisher Scientific, UK) HNO_3. External multi-element calibration standards (Claritas-PPT grade CLMS-2 from SPEX Certiprep Inc., Metuchen, NJ, USA) included Cu, Fe, Mn, Se and Zn, in the range 0 – 100 μg L^{-1} (0, 20, 40, 100 μg L^{-1}). A bespoke external multi-element calibration solution (PlasmaCAL, SCP Science, France) was used to create Ca, Mg, Na and K standards in the range 0-30 mg L^{-1}. Phosphorus calibration utilized an in-house KH_2PO_4 solution standard (10 mg L^{-1} P).

4.2.4.5 Determination of phytonutrients

The chlorophyll *a* content of the samples was determined according to (AOAC, 1995: 940.03). Approximately 50mg of *Spirulina* was weighed in to a 35ml centrifuge tube and 5 grams of glass beads and 2.5ml of 85% acetone in water were added to it. The mixture was then vortexed for 5 minutes and after vortexing, 10ml of 85% acetone was added. After a brief vortex of the mixture, it was centrifuged at 3200 rpm for 5 minutes. The supernatant was collected in a 50ml volumetric flask. This step was repeated for about four times until the supernatant became clear. After complete extraction, the flask was filled up to volume with 85% acetone in water. The flask was then capped and gently inverted to mix the contents. The absorbance of the extract was then read with a Lambda 950 UV/VIS/NIR spectrophotometer (Perkin Elmer, and USA) at 666nm and 642nm against an 85% acetone/water blank. The amount (%) of chlorophyll *a* in the samples was then calculated with the following formula:

$$\text{Chlorophyll } a\ (\%) = \frac{[(9.93 \times \text{Abs}_{666}) - (0.0777 \times \text{Abs}_{642})] \times 0.05 \text{ liter} \times 100}{\text{Sample weight (mg)} \times \%\text{dry weight}}$$

Total carotenoids content of *Spirulina* powder was analyzed according to the method developed by Cyanotech Corporation (2002). Approximately 30mg of *Spirulina* powder was directly weighed in to centrifuge tubes and 3 g of glass beads and 2.5ml of dimethyl sulphoxide (DMSO) was added to each tube. The tubes were tightly capped and vortexed briefly for 30 seconds. The tubes were then placed into a 45°C water bath for 20 minutes and removed from the water bath every 10min and vortexed for 30 seconds. After 20 minutes in the water bath, 5ml of methanol was added to each tube, vortexed and centrifuged at 4200rpm for 3 minutes. The supernatant was

taken out from each tube into a 25ml volumetric flask. Steps were repeated until all the supernatant was collected and the flask was filled up to volume with methanol (methanol extract). Exactly 2ml of the methanol extract was pipetted in to a 15ml centrifuge tube and 3ml of diethyl ether was added to it using a volumetric pipette. To this mixture, 0.5ml of saturated KOH in water was added and the mixture was left in the dark for 30minutes for saponification. After 30 minute incubation in the dark, 5ml of water was added to each sample (mixture), vortexed vigorously for 30 seconds and centrifuged at 4200rpm for 5minutes. At this step, the mixture had two layers, ether layer containing all the yellow pigment and aqueous layer containing the other pigments of *Spirulina* and with a pale blue green color. To the mixture containing the ether and aqueaous layer, 3ml of diethyl ether was added and the maximum absorbance of the ether layer was read with a Lambda 950 UV/VIS/NIR spectrophotometer (Perkin Elmer, and USA) at wave length between 450 to 453nm against a diethyl ether blank. The following formula was then used to calculate the total carotenoid content (percent) of the samples:

$$\text{Total carotenoids (\%)} = \frac{\text{Max Abs (450-453)} \times 25\text{ml} \times 1.5 \times 100}{259.2 \times \text{sample weight(mg)} \times \%\text{ dry weight}}$$

Where: Max Abs= maximum absorbance

$$\text{Percent dry weight} = \frac{\text{Weight of empty dish (g)+ dried powder(g)-Weight empty dish(g)}}{\text{Powder weight (not dried) (g)}}$$

β-carotene content of *Spirulina* powder was also analyzed according to the method developed by Cyanotech Corporation (2002). Approximately 30mg of *Spirulina* powder was directly weighed in to centrifuge tubes and 3 g of glass beads and 2.5ml of DMSO was added to each tube. The tubes were tightly capped and vortex briefly for 30 seconds. The tubes were then placed into a 45°C water bath for 20 minutes and every 10min the tubes were removed from the water bath and vortex for 30 seconds. After 20min in the water bath, 5ml of methanol was added to each tube, vortexed and centrifuged at 4200rpm for 3 minutes. The supernatant were taken out from each tube into a 25ml volumetric flask. Steps were repeated until all the supernatant was collected and the flask was filled up with methanol (methanol extract). Eight milliliter of the prepared methanol extract, 5ml of heptanes and 1.5ml of saturated potassium hydroxide were moved into a new test tube and left for saponification for 15min and lightly vortexed. The tubes

were centrifuged at 420 rpm for 3min. Pipette was used to remove the heptanes layer (upper layer) and put into a new 10ml volumetric flask. Approximately 1ml of fresh heptanes was gently added to the tube and washed the interphase. The upper layer was added into the 10ml volumetric flask. Three milliliter of fresh heptanes was added to the tube. The tube was capped and inverted 8 times to allow any remaining beta- carotene in the methanol enter the heptanes and separate from the methanol. The upper layer was pipette into the 10ml volumetric flask. The 10ml volumetric flask was brought up to volume with fresh heptanes. The volumetric flask was capped and inverted to mix up the volume. Approximately 5 ml of the heptanes extract was removed into a new centrifuge tube and an equal amount of distilled water was added and vortex vigorously for 5sec. The tubes were centrifuged for 3min at 4200rpm. On the spectrophotometer, the absorbance was read at 436 nm of the extract against a diethyl ether blank. The beta-carotene percentage was calculated by using the formula:

$$\beta\text{-carotene}(\%) = \frac{\text{Abs } 436 \times 25\text{ml} \times 1.25 \times 100 \times 0.84}{196 \times \text{sample wt (mg)} \times (\%) \text{ dry weight}}$$

Where: Abs =Absorbance

$$\text{Percent dry weight} = \frac{\text{Weight of empty dish (g)} + \text{dried powder(g)} - \text{Weight empty dish(g)}}{\text{Powder weight (not dried) (g)}}$$

The contents of phycobilproteins were quantified according to AOAC (2008). About 0.5g of sample was weighed directly into a centrifuge tube and suspended in 25ml 100Mm Na-Phosphate buffer (pH 6). The suspension was mixed with spatula to break up clumps and incubated for 16hrs at 30°C. After incubation, the sample was mixed again with spatula and centrifuged for 15minutes at 5000rpm at 4°C. The supernatant was filtered through a Whatman#41filter paper and 2ml filtrate was then pipette into a 50ml volumetric flask and filled up with 100Mm phosphate buffer (pH 6) in volumetric flask. The supernatant was analyzed spectrophotometrically for total crude phycocyanin, C-phycocyanin and allophycocyanin. The relative amounts of the phycobiliproteins were calculated according the following formulae:

$$\%\text{ Total Crude Phycocyanin} = \frac{0.295 \times 100 \times D \times Abs_{618}}{W\text{ (mg)}}$$

$$\%\text{ C- Phycocyanin} = \frac{0.162 \times Abs_{620} - 0.098 \times Abs_{650} \times D \times 100}{W\text{ (mg)}}$$

$$\% \text{ Allophycocyanin} = \frac{0.180 \times Abs_{650} - 0.042 \times Abs_{620} \times D \times 100}{W \text{ (mg)}}$$

Where: Abs = absorbance
D = Dilution factor = 625 = (25 x 50/2)
W = weight of sample (mg)
0.162, 0. 098, 0.180, 0.042, 0.295 = extinction coefficients

4.2.5 Statistical analysis

All data are expressed in terms of mean ± standard deviation. The effects of seasonal variation on the proximate and phytonutrients composition of Lake Chitu's *Spirulina* were analyzed by one-way ANOVA followed by post-hoc analysis with Tukey HSD test using the statistical program SPSS ver. 20. The effect of seasonal variation on the mineral composition of the biomass was analyzed using student's t-test. Significant levels for all analyses were set to $p < 0.05$.

4.3 Results and discussion

4.3.1 Rainfall and temperature regime

Rainfall and temperature pattern of the lake area during the study period (March 2012-January 2013) are shown in Fig 4.1a and b respectively. Based on the data, months from March to September were found to be wet (rainy) and months from November to January were found to be dry as they had rainfall coefficients of less than 0.6. Among the rainy months, the highest rainfall was recorded in September (138.8mm) and the lowest was recorded in July (43.9mm). The rainfall pattern was similar with that reported by Wood and Talling (1988) and Klemperer and Cash (2007) for the general climate of Ethiopian rift valley lakes where wet season ranges from March to September and dry season ranges from October to February.

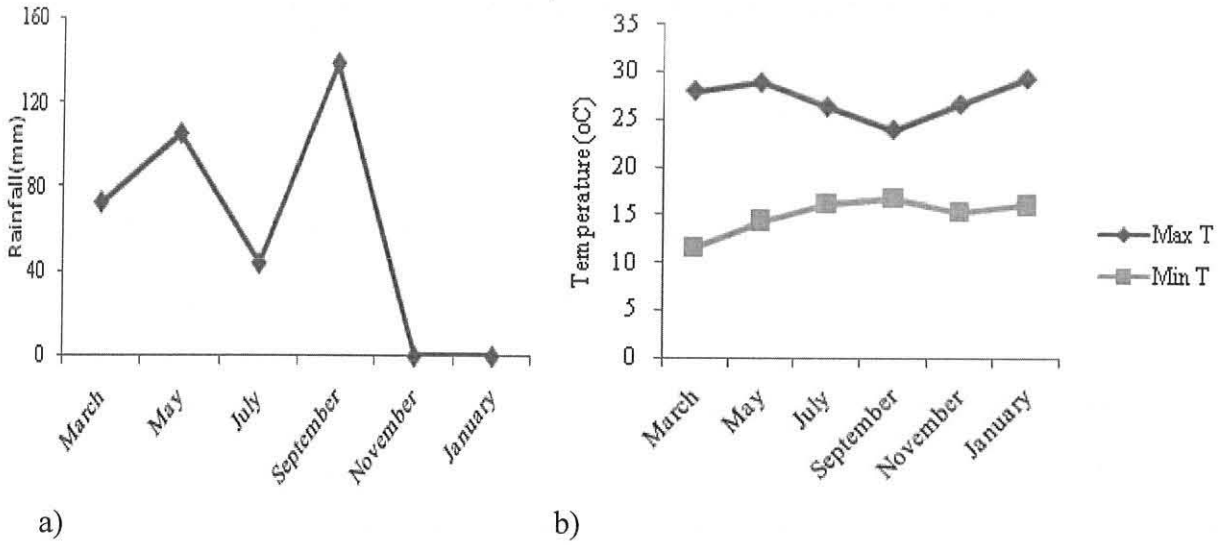


Fig 4.1. Rainfall (a) and Temperature (b) pattern of Lake Chitu area during the sampling period (March 2012-January 2013)

The maximum air temperature of the lake area during the study period varied from 24.0 to 28.9°C and from 26.7 to 29.4°C for wet and dry seasons respectively. The minimum air temperature varied from 11.6 to 16.8 °C in wet season and from 15.3 to 16.1°C in dry season. It has been reported that several *Spirulina* strains can grow between 12 and 43°C and the temperature optimum for growth is between 30 and 38 °C (Tomaselli *et al.*, 1993).

4.3.2 Physicochemical characteristics

The average pH value of the lake was 10.3±0.1 and in both wet and dry seasons the pH was within the optimal pH range (9.5–10.5) that support the growth of many *Spirulina* species (Vonshak, 1997). The alkalinity of the lake varied from 637 to 740 meq L^{-1} during wet and from 680 to 710 meq L^{-1} during dry season with a mean value of 681.4±44.5 meq L^{-1}. Similarly, the salinity of the lake oscillated from 53 to 60 g L^{-1} during wet and from 51 to 55g L^{-1} during dry season with a mean value of 55±3.34g L^{-1} (Table 4.1). Wood and Talling (1988) indicated that the high alkalinity and salinity of the lake is associated with the high evaporative concentration prevailing in the lake. Salinity values are usually expected to be lower in rainy seasons due to high precipitation and low evaporation. However, in this study high salinity values were also recorded in wet months. Similar findings were reported by Zinabu (2002) from a long term

limnological study of some Ethiopian rift valley lakes. Zinabu (2002) proposed the possible reason for these phenomena as exchange with sediments within the water bodies especially during frequent mixing which mostly occurs in rainy seasons.

Table 4.1. Physicochemical characteristics of Lake Chitu during the study period (March 2012-January 2013)

Physicochemical parameters	Mean ±SD	Range	
		Wet season	Dry season
pH	10.3±0.1	10.3 – 10.4	10.2 – 10.3
Salinity(g L^{-1})	55±3.34	53 – 60.1	51 – 55
Alkalinity(meq L^{-1})	681.4±44.5	637 – 740	680 – 710

Table 4.2 shows the inorganic mineral content of Lake Chitu during the study period. Sodium was found in high concentrations in both wet and dry season water samples with concentrations of 18065 and 18689 mg L^{-1}, respectively. Relatively, higher concentration of sodium was recorded during dry season compared to wet season. On the other hand, the potassium level of the lake did not show significant variation. Both sodium and potassium levels of Lake Chitu were high compared to the synthetic medium (Zarrouk's medium) which is usually used in artificial production of *Spirulina* (Table 4.2). The high concentration of these minerals in the lake is attributed to their large concentration in the trachytic and rhyolitic rocks of the Ethiopian rift (Klemper and Cash, 2007). Calcium was not detected in both wet and dry season collected water samples and magnesium was found relatively in higher concentrations in a wet season sample (1mg L^{-1}) compared to a dry season sample (0.18mg L^{-1}). Both Ca and Mg contents of the lake were very low compared to Zarrouk's medium. Their low concentrations could be explained by the high salinity and alkalinity of the lake which causes the removal of these ions from the solution as carbonate precipitates (Wood and Talling, 1988).

Table 4.2. Inorganic mineral contents of Lake Chitu as compared to Zarrouk's medium

Inorganic minerals	Lake Chitu		*Zarrouk's medium
(mgL^{-1})	Wet season	Dry season	
Na	18065	18689	3 800
K	1981	1200	125
Fe	0.22	0.18	0.4
Cu	0.034	0.022	
Zn	0.01	0.01	
Ca	n.d	n.d	13.3
Mg	1.00	0.18	7.0

*Vonshak, 1997
n.d: not detected

4.3.3 Biomass nutrient profile

4.3.3.1 Proximate composition

The various components of *Spirulina* may change depending on the method used for processing the biomass (Morist *et al.*, 2001). A number of drying methods are used by *Spirulina* producers depending on the quality and quantity of the final product required (Belay, 2013). Lake Chitu's *Spirulina* was processed in two ways, freeze dried and oven dried to evaluate the efficiency of the drying methods in maintaining the nutrient profile of the biomass. The mere purpose of this step was to select the suitable processing method of the biomass for evaluating the effect of seasonal variation on its nutrient profile. Table 4.3 shows comparison between oven dried and freeze dried *Spirulina* biomass with regard to their proximate and phytonutrients composition.

Table 4.3. Effect of drying method on the proximate and phytonutrients composition of Lake Chitu's *Spirulina*

Proximate components (%)	Oven dried biomass	Freeze dried biomass
Moisture	7.8±0.22[a]	6.4±0.34[b]
Crude protein	53.4±0.80[a]	55.7±0.58[b]
Crude fat	2.3±0.09[a]	2.5±0.11[a]
Crude fiber	0.7±0.04[a]	0.7±0.04[a]
Ash	11.4±0.13[a]	10.14±0.1[b]
Carbohydrates	32.2±0.78[a]	30.9±0.5[a]
Energy	363.0±0.79[a]	369.1±0.6[b]
Phytonutrients(mg g^{-1})		
Chlorophyll *a*	8.0±0.17[a]	8.2±0.17[a]
Total carotenoids	3.7±0.23[a]	4.3±0.1[b]
β-carotene	1.51±0.06[a]	1.6±0.03[a]
Crude phycocyanin	99.2±1.60[a]	104.1± 3.62[a]
C-phycocyanin	34.9±0.47[a]	38.0±2.52[a]
Allophycocyanin	21.9±0.60[a]	21.9±1.1[a]

In each raw different letters indicate significant differences ($p < 0.05$)

As shown in the table, some variations were noticed in the proximate composition between oven dried and freeze dried samples although most of the variations were not significant. Significant difference ($p<0.05$) was observed in the protein content of the samples as a loss was recorded in the oven dried biomass compared to the freeze dried one(53.4 vs 55.7%). With regard to phytonutrients, significant difference ($p<0.05$) was observed in the total carotenoids content of the biomass as the freeze dried biomass had higher value(4.3mg g^{-1}) than the oven dried one (3.7 mg g^{-1}). Only little variations were observed between the oven dried and the freeze dried biomass for other phytonutrients. In general, since most of the components were represented in higher quantities in the freeze dried biomass compared to the oven dried one, freeze drying was chosen as an appropriate drying method to evaluate the effect of seasonal variation on the nutrient profile of Lake Chitu's *Spirulina*. Freeze drying was also found by Morist *et al.* (2001)

as the best drying method in maintaining most of the nutrients contained in the fresh biomass compared to other processing methods such as spray drying. Here it is also interesting to note that, if properly done oven drying could be suitable for processing the biomass for human use. In fact, freeze drying is a slow as well as an expensive drying method to be used in large scale or for commercial purposes (Morist *et al.*, 2001).

Variation in the proximate composition of Lake Chitu's *Spirulina* on seasonal basis is presented in Table 4.4. The moisture content of the samples varied from 5.1 to 6.7% for wet season and from 5.8 to 6.1 % for dry season samples with a mean value of 5.9±0.50%. The moisture content of all the samples were within recommended value of such type of products (<7%) (Belay, 2013) which is important to reduce water activity and inhibit microbial growth in the product.

The protein content of Lake Chitu's *Spirulina* varied from 43.0 to 55.7% for wet and from 39.2 to 40.8% for dry season samples (Table 4.4). Significant variations ($p<0.05$) were observed in the protein content of the samples even in those harvested in the same season. For instance among wet months, lowest protein content (43.0%) was recorded for a biomass sample harvested in March and the highest protein content was recorded for a sample harvested in May (55.7%). On the other hand, no significant variations were observed in the protein content of samples harvested during dry season. In general, higher protein contents were recorded for wet season *Spirulina* samples compared to dry ones. It has been reported that increasing the nitrogen level in the growth medium leads to an increase in the protein content of microalgae (Fernandez-Reiriz *et al.*, 1989). The high protein content in wet season samples might be therefore related to nutrient availability in the lake during this season due to release of nutrients in the euphotic zone of the lake from mixing effects that result from cooling and winds associated with rains (Zinabu and Taylor, 1989). A one year study undertaken in a similar time period with the present study (February 2012 to January 2013) in the lake by Ogato and Kifle (2014) showed the variations in the concentration of possible nitrogen sources (NO_3^- and NH_3) of this microalga on seasonal basis. The authors reported a concentration range from 0 to 9.5μg L^{-1} for NO_3^- with a mean value of 1.5 μg L^{-1} and a range from 0 to 84.0 μg L^{-1} for NH_3 with a mean value of 14.5 μg L^{-1}. In general, the protein contents of wet season samples were within ranges (45–71%) reported for commercial *Spirulina* (Table 4.4).

Table 4.4. Variation in proximate composition (%, dry matter) and energy value (Kcal) of Lake Chitu's *Spirulina* on seasonal basis (mean ± SD)

	Lake Chitu's *Spirulina*						*Commercial *Spirulina*
	Wet season harvest				Dry season harvest		
	March	May	July	September	November	January	
Moisture	6.7±0.07^{d}	6.4±0.34^{a}	6.2±0.09^{a}	5.1±0.05^{c}	6.1±0.06^{ab}	5.8±0.12^{b}	4-7
Protein	43.0±0.8^{d}	55.7±0.58^{a}	48.2±0.2^{b}	47.9±0.11^{b}	39.2±0.36^{c}	40.8±1.62^{c}	45-71
CHO	34.8b±1.4^{b}	30.9±0.5^{a}	36.4±0.54^{bd}	34.5±0.1^{b}	41.3±0.44^{c}	38.0±1.69^{d}	15-20
Fat	1.4±0.3^{d}	2.5±0.11^{a}	2.6±0.19^{ab}	3.20±0.07^{c}	2.5±0.18^{b}	1.9±0.06^{d}	4.3-7.0
Fiber	0.70±0.1^{a}	0.7±0.04^{a}	0.8±0.08^{ab}	0.6±0.09^{a}	0.9±0.15^{b}	0.6±0.1^{a}	0.1-7.7
Ash	13.1±0.08^{c}	10.1±0.1^{a}	12.0±0.61^{b}	14.0±0.15^{c}	16.1±0.06^{d}	18.7±0.05^{e}	6.4-13
Energy	323.8±2.6^{a}	369.1±0.6^{b}	361.8±1.66^{c}	358.1±0.64^{d}	344.6±1.28^{e}	332.4±0.76^{f}	

In each raw different letters indicate significant differences ($p < 0.05$)

* Belay, 1997; 2008; Cohen, 1997; Fox, 1996; Ortega *et al.*, 1993

Changes in the carbohydrate content of the biomass were observed throughout the study period and varied from 30.9 to 36.4% for wet season and from 38.0 to 41.3% for dry season samples. Relatively higher carbohydrate contents were recorded in dry season samples compared to wet season samples. Cyanobacteria synthesize higher quantities of carbohydrates when they are exposed to grow in nitrogen deficient media (Fresnedo and Serra, 1992; DePhilippis *et al.*, 1993) or exposed to combined stress of nitrogen deficiency and high light intensity (Olguin *et al.*, 2001). In addition to these, *Spirulina* accumulates carbohydrates as osmoprotectors when it grows in brackish and saline waters (Reed *et al.*, 1984; Vonshak *et al.*, 1988; Zeng and Vonshak, 1998; Rosales *et al.*, 2005). Therefore, the relatively high carbohydrate content of the dry season samples might be due to combined stress from high salinity, high light intensity and nutrient deficiency (nitrogen limitation) (Kebede *et al.*, 1994; Ogato and Kifle, 2014) that prevail in the lake during this season. Compared to commercial *Spirulina* (*Spirulina* grown in artificial media) which usually have 13 to 20% carbohydrates (Table 4.4), the high amounts of carbohydrates can be explained by the variations in growth conditions. As mentioned above nitrogen deficiency

may contribute for the variation observed between commercial *Spirulina* and Lake Chitu's *Spirulina*. Besides, it is important to note that the salinity of Zarrouk's medium which is the basic media formulation used by commercial *Spirulina* producers has a salinity of 21.3g L^{-1}(Fox, 1996) which is extremely lower than the salinity of Lake Chitu which is >50 g L^{-1}(Table 4.1).

The crude fat content of Lake Chitu's *Spirulina* oscillated from 1.40 to 3.20% for wet season and from 1.9 to 2.5% for dry season samples. Significant differences were observed in the fat contents of the samples ($p<0.05$) and the contents were generally low compared to commercial *Spirulina* with crude fat levels ranging from 4.3 to 7 % (Table 4.4). However, the fat contents of Lake Chitu's *Spirulina* were comparable with other literature data: 3.0% (Becker and Venkataraman, 1984); 2-3 % (De la Noiie and De Pauw, 1988) and 2.5% (Cafiizares-Villanueva *et al.*, 1995). In addition to growth conditions, the crude fat content of microalgae might be influenced by the extraction method or the type of solvent used during analysis (Chaiklahan *et al.*, 2008, Li *et al.*, 2014).

The crude fiber content of Lake Chitu's *Spirulina* varied from 0.70 to 0.78% for wet season and from 0.6 to 0.9% for dry season samples. These values were within ranges reported for commercial *Spirulina* (0.1-7.7%) (Table 4.4). Higher crude fiber values were reported for *Spirulina* sp. isolated from an oil-polluted flame pit in Nigeria, 8.21–20.1% (Ogbonda *et al.*, 2007) and 7.55–7.78% for *Spirulina* sp. harvested from Lake Chad (Albert *et al.*, 2012).

The ash content of Lake Chitu's *Spirulina* varied from 10.1 to 13.9% for wet season and from 16.1 to 18.7% for dry season samples. Significant differences were observed ($p<0.05$) in the ash contents of the samples in which relatively higher contents were recorded in dry season samples compared to wet season samples. In general, the ash contents of most of the samples were higher compared to pure culture grown or commercial *Spirulina* with values usually ranging from 6.4 to 13 % (Table 4.4). The elevated ash content of the samples might be due to the high amount of carbonate salts present in the lake (Kebede, 1997; Ogato and Kifle, 2014). Additionally, it is possible that the washings with distilled water done before the samples were freeze dried may not have removed these salts completely. Since the lake is highly enriched with bicarbonate salts,

acid washing was necessary to eliminate or reduce them. Richmond (1988) indicated that when the biomass is not sufficiently washed with acid water, its ash content may reach as high as 25% due to adsorbed carbonates. According to Becker (1986), a high concentration of unused minerals results in a change in the proportion of the other major cellular constituents of microalgae. For instance, ash free protein contents of Lake Chitu's *Spirulina* would give values ranging from 49.5 to 62.1% for wet season and from 46.7 to 50.2% for dry season samples.

4.3.3.2 Amino acid composition

The amino acid profile of Lake Chitu's *Spirulina* is presented in Table 4.5. All essential and non-essential amino acids were represented in both wet and dry season samples although variations were observed in the contents of their amino acids. The wet season sample had higher contents of amino acids compared to the dry season sample (39g/100g sample vs 26.5g/100g sample). This could be associated with the difference in the protein content of the samples as higher contents were recorded in the wet season sample (47.9%) compared to the dry season sample (40.8%) (Table 4.4). Possible reason for the high protein content of the wet season sample compared to the dry season sample is explained elsewhere. Moreover, the difference in the ash contents of the samples may affect the amino acid contents of the samples as higher ash content was recorded for the dry season sample (18.7%) compared to the wet season sample (13.9%) (Table 4.4).

Table 4.5. Amino acid composition of Lake Chitu's *Spirulina*

Amino acids	Wet season harvest		Dry season harvest	
	g/100g protein	g/100g sample	g/100g protein	g/100g sample
Essential amino acids(EA)				
Ile	3.21	1.44	2.38	0.95
Leu	6.18	2.78	4.65	1.86
Lys	5.23	2.38	4.35	1.74
Met	2.85	1.28	2.10	0.84
Phe	3.66	1.65	2.70	1.08
Thr	4.27	1.92	3.24	1.30
Val	4.21	1.89	3.16	1.27
His	2.50	1.12	1.90	0.76
Non essential amino acids(NEAs)				
Cys	6.17	2.78	4.97	1.99
Tyr	2.72	1.22	2.11	0.84
Asp	6.92	3.12	5.20	2.08
Glu	15.30	6.88	11.80	4.72
Ser	4.46	2.00	3.35	1.34
Gly	4.22	1.90	3.17	1.34
Ala	4.01	1.84	3.13	1.25
Arg	5.08	2.29	3.85	1.54
Amm	5.44	2.49	4.06	1.63
Total amino acids	86.43	39.00	62.10	26.53

The essential amino acids isoleucine (Ile), leucine (Leu), methionine (Met), threonine (Thr), valine (Val), phenylalanine (Phe) and histidine (His) comprised 39.7% of the total amino acids for the wet season sample and 39.5% for the dry season sample (Table 4.6). In both wet and dry season samples, leucine (Leu) was the highest essential amino acid (Table 4.5). Misurcova *et al.* (2014) also reported the dominance of leucine among essential amino acids in their study on the amino acid composition of commercial algal products including *Spirulina*.

Table 4.6. Content of essential and non-essential amino acids of Lake Chitu's *Spirulina* from their respective total amino acids

Amino acids (%)	Lake Chitu's *Spirulina*	
	Wet season *Spirulina*	Dry season *Spirulina*
Essential amio acids(EA)	39.7	39.5
Non essential amino acids(NEA)	60.4	60.5
EA/NEA	0.66	0.65

Note: ammoniac is not considered in the calculation

The non-essential amino acid content (NEAs) of wet and dry season *Spirulina* samples comprised 60.4% and 60.5% of the total amino acids respectively (Table 4.6). In both samples, glutamic acid (Glu) was the highest amino acid taking a share about 18% of the total amino acids followed by aspartic acid (Asp). The glutamic acid content of Lake Chitu's *Spirulina* (especially the wet season sample) was higher compared to that reported for artificially grown or commercial *Spirulina* (Richmond, 1998; Campanella, 1999; Morist *et al.*, 2001; Becker, 2007; Belay, 2008; Morais *et al.*, 2009). Glutamic acid is one of the three constituents (together with alanine and glycine) of umami taste of seaweeds which plays a great role in their palatability (Yamaguchi and Ninomiya, 2000). The EA/NEA ratio of Lake Chitu's *Spirulina* was 0.66% for the wet season and 0.65% for the dry season sample (Table 4.6) and these values were comparable with that reported for other *Spirulina* strains: 0.50 – 0.65% (Ortega *et al.*, 1993) and 0.60 –0.70% (Fatma *et al.*, 1994).

The amino acid composition of Lake Chitu's *Spirulina* (specifically the wet season harvest) was also compared with amino acid levels recommended by FAO/WHO for an ideal protein and amino acid composition of artificially grown *Spirulina* (Table 4.7). Lake Chitu's *Spirulina* compared favorably with WHO/FAO reference protein for some essential amino acids (Met+Cys, Phe+Tyr, and Thr) but it is deficient in some essential amino acids such as isoleucine (20% deficiency), leucine (12% deficiency), lysine (5% deficiency) and valine (16% deficiency).

The artificially grown *Spirulina* species included for comparison in this study is also deficient in the essential sulphur amino acids (methionine and cysteine) as well as lysine.

Compared to artificially grown *Spirulina*, Lake Chitu's *Spirulina* was inferior in the levels of most of the amino acids. However, it is important to note that the quantities of sulphur containing amino acids (methionine and cysteine) were higher in Lake Chitu's *Spirulina* compared to the artificially grown *Spirulina* as well as values reported for commercial *Spirulina* (Richmond, 1998; Campanella *et al.*, 1999; Belay, 2008). Ciferri (1983) indicated about the existence of considerable variation in the amino acid profiles of *Spirulina* from different sources especially in the concentration of the essential sulphur amino acids.

Table 4.7. Comparison of amino acid pattern (g/100g protein) of Lake Chitu's *Spirulina* (wet season harvest) with artificially grown *Spirulina* and WHO/FAO reference protein

Amino acids	Lake Chitu's *Spirulina*	**Spirulina* (Artificially grown)	**WHO/FAO
Ile	3.21	6.7	4
Leu	6.18	9.8	7
Lys	5.23	4.8	5.5
Met	2.85	2.5	
Cys	6.17	0.9	
Met+Cys	9.02	3.4	3.5
Phe	3.66	5.3	
Phe+Tyr	6.38	10.6	6.1
Thr	4.27	6.2	4
Val	4.21	7.1	5
His	2.50	2.2	
Tyr	2.72	5.3	
Asp	6.92	10.3	
Glu	15.3	11.8	
Ser	4.46	5.1	
Gly	4.22	5.7	
Ala	4.01	9.5	
Arg	5.08	7.3	

* Richmond, 1998
** Becker, 2007

Commercial producers of *Spirulina* recommend doses up to 10 to 20g/day (Johnson and Shubert, 1986). Becker (1980) suggested a maximum daily intake of 30g/day dried algae for humans.

Assuming a daily intake of 20g Lake Chitu's *Spirulina* (wet season harvest), the contribution of its EAs to recommended dietary intake (RDI) for a 70kg adult human is illustrated in Table 4.8.

Table 4.8. Contribution of EAs of Lake Chitu's *Spirulina* (wet season *Spirulina*, 20g per day) to RDI (%) for a 70kg adult human

Amino acids	*RDIs ($mgkg^{-1}$per day)	Contribution of EAs to RDI (%)
Lys	30.0	22.7
Leu	39.0	20.4
Ile	20.0	20
Val	26.0	20.8
Thr	15.0	36.6
His	10.0	32.0
Phe	25.0	19.0
Trp	4.0	n.d
Met	10.0	36.6

*WHO/FAO/UNU, 2007

As is shown in the table, more than 20% of the RDI of Leu and Val as well as Lys and more than 30% of the RDI of Thr, Met and His can be met from 20 g of Lake Chitu's *Spirulina*. Lake Chitu's *Spirulina* may also provide between 19 to 20% of the RDI of Ile and Phe for an adult human.

4.3.3.3 Fatty acid composition

The fatty acid composition of Lake Chitu's *Spirulina* is shown in Table 4.9. Five fatty acids corresponding to the most abundant fatty acids reported for various *Spirulina* strains (Ortega *et al.*, 1993; Campanella *et al.*, 1999; Muhling *et al.*, 2005) were identified including another medium chain fatty acid (lauric acid) (Fig 4.2a and b) and expressed as weight percentage of total fatty acids (Table 8). Palmitic acid (16:0) was the dominant fatty acid in both wet and dry season samples followed by linoleic acid (18:2n6), γ- linoleic acid (18:3n6) and oleic acid (18:1n9). While lauric acid (12:0) was the least abundant fatty acid for a wet season sample, it

was found relatively in higher proportions in the dry season sample (12.7%). Lauric acid is a common fatty acid in coconut oil (Kushak *et al.*, 2000).

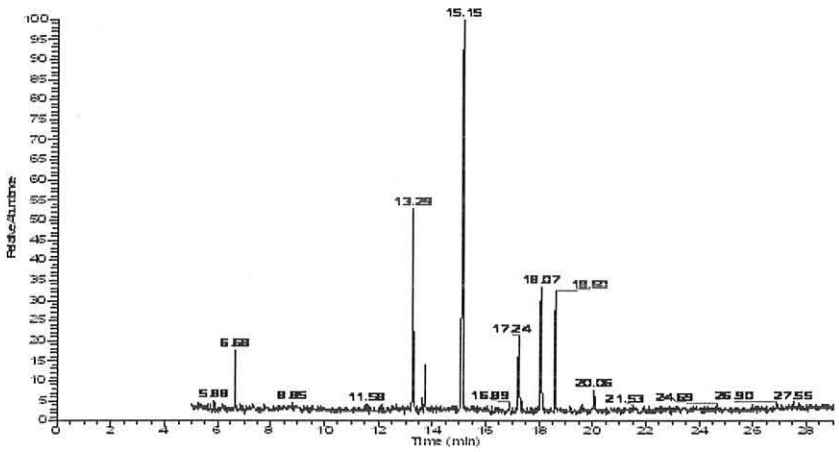
Table 4.9. Fatty acid composition of Lake Chitu's *Spirulina* harvested during wet and dry seasons

Fatty acid (%)	Lake Chitu's *Spirulina*	
	Wet season harvest	Dry season harvest
Palmitic (16:0)	31.7±2.88[a]	29.2±1.65[a]
Linoleic (18:2n6)	23.7±2.61[a]	25.6±4.45[a]
Γ-linolenic (18:3n6)	20.3±5.19[a]	16.0±0.51[a]
Oleic (18:1n9)	11.9±0.35[a]	10.3±4.06[a]
Palmitoleic (16:1n9)	5.9±0.87[a]	6.3±0.15[a]
Lauric (12:0)	4.8±3.56[a]	12.7±1.33[b]

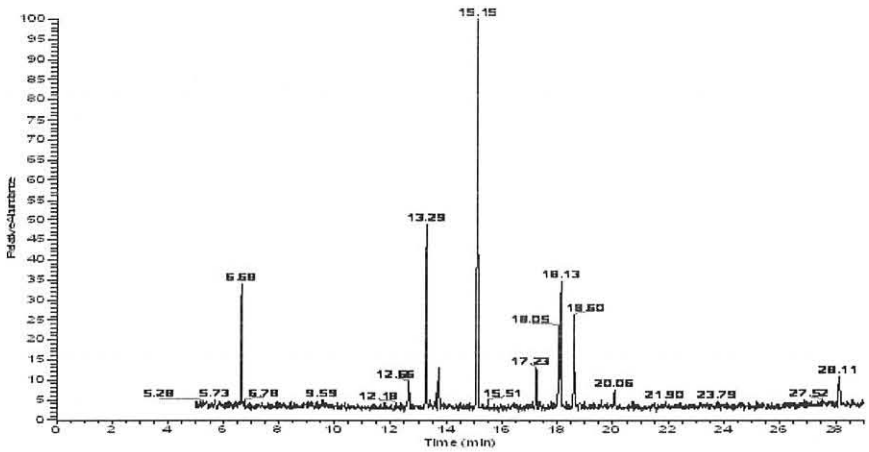
In each raw different letters indicate significant differences ($p < 0.05$)

The dominance of palmitic acid in Lake Chitu's *Spirulina*, 31.7% for the wet and 29.2% for the dry season sample, is in line with results reported for other artificially grown *Spirulina* sp. (Cohen *et al.*, 1987). The dominance of palmitic acid might be due the fact that the synthesis of fatty acids generally begins with saturated fatty acids in photosynthetic microorganisms (Morais *et al.*, 2009). The proportion of the essential fatty acid, linoleic acid, comprised 23.7% for wet and 25.6% for dry season samples. These values were much higher than that reported by Campanella *et al.* (1999) for commercial *Spirulina.* The contents of gamma linoleic acid (GLA) which is another essential fatty acid in *Spirulina* were 20.3 and 16.0% for wet and dry season samples respectively. Relatively higher contents of GLA were recorded for the wet season sample compared to the dry season sample. In general, the contents of GLA in Lake Chitu's *Spirulina* (both wet season and dry season samples) were comparable with that reported for other *Spirulina* strains (Otles and Pire, 2001), but much higher than that reported by Campanella *et al.* (1999) for commercial *Spirulina* in Italy. GLA is an important polyunsaturated fatty acid

(PUFA) and used as a dietary supplement for the treatment of various health problems such as arthritis, heart disease and obesity (Kay, 1991).



a)



b)

Fig 4.2. Fatty acid chromatogram of Lake Chitu's *Spirulina*: wet (a) and dry (b) season harvest

Table 4.10 shows the percentage contribution of saturated, monounsaturated and polyunsaturated fatty acids of the total fatty acids in Lake Chitu's *Spirulina*. Differences were observed in the proportion of these fatty acids where relatively higher contents of saturated fatty acids were recorded for a dry season sample compared to a wet season sample (41.9% vs 36.5%). Cohen *et al.* (1987) reported that the levels of saturated fatty acids increased in various *Spirulina* strains when cultivation temperature increased. Therefore, the higher proportion of saturated fatty acids in the dry season sample might be associated with the higher temperature (Fig 4.1b) prevailing in

the lake during this season. On the other hand, the wet season sample had higher contents of both mono (17.8 vs 16.6%) and polyunsaturated (44.0 vs 41.6%) fatty acids compared to the dry season sample. Although Lake Chitu's *Spirulina* had low fat content, the fatty acid profile of the biomass had some interesting characteristics. PUFAs (linoleic and GLA) contributed a high percentage of the total fatty acids in both wet and dry season samples. A higher content of polyunsaturated fatty acids (PUFA) increases the nutritional value of foods. According to Cuthbertson (1989), the recommended value for a human diet is a PUFA/SFA ratio greater than 0.45. The values for Lake Chitu's *Spirulina* for both wet season and dry season samples were above 0.9 (Table 4.10) and thus are of good quality interms of their fatty acid profile.

Table 4.10. Percentage contribution of saturated, monounsaturated and polyunsaturated fatty acids to the total fatty acids in Lake Chitu's *Spirulina*

Fatty acids (%)	Lake Chitu's *Spirulina*	
	Wet season harvest	Dry season harvest
Saturated (SFA)	36.5	41.9
Monounsaturated (MUFA)	17.8	16.6
Polyunsaturated (PUFA)	44.0	41.6
PUFA/SFA	1.22	0.99

4.3.3.4 Mineral composition

The ability of *Spirulina* to accumulate minerals is well documented (Kay, 1991; Zaretskaia, *et al.*, 2003) and the accumulation of essential minerals could be useful to provide essential minerals in the diet. The mineral content of Lake Chitu's *Spirulina* is shown in Table 4.11 and significant differences were observed in the mineral contents of wet and dry season samples ($p<0.05$). Sodium was the most abundant mineral in both wet and dry season samples with values of 3387mg and 5986mg/100g respectively. Next to Na, K was found in higher concentrations with values of 1564mg /100g for a wet season sample and 2044mg/100g for a dry season sample. In general, the dry season sample had higher concentrations of Na and K compared to the wet season sample which can be associated with the high concentration of these minerals in the lake during this season due to evaporative concentration (Table 4.2). It can be

also noted that higher ash contents were recorded in dry season samples compared to wet season samples (Table 4) which can be attributed to the presence of these minerals in the algae either as absorbed or adsorbed forms. Generally, the Na and K contents of Lake Chitu's *Spirulina* were found to be very high compared to that reported for commercial *Spirulina* (Table 4.11). These higher contents might be due to the high concentration of these minerals in the lake compared to the synthetic medium (Zarrouk's medium) usually used by artificial (commercial) *Spirulina* producers (Table 4.2). Very high levels of Na (8235.8mg/100g) and K (3434.0mg/100g) compared to our samples was reported by Choong *et al.* (2007) for *Spirulina* sp. cultivated using underground water.

Table 4.11. Major and trace minerals content (mg/100g dry matter) of *Spirulina* biomass harvested from Lake Chitu, during wet and dry seasons (mean ±SD)

Minerals	Lake Chitu's *Spirulina*		*Commercial *Spirulina*
	Wet season harvest	Dry season harvest	
Major			
Na	3387±330[a]	5986±507[b]	282-2350
K	1564±234[a]	2044±197[b]	233-1660
P	639±121[a]	514.5±64[b]	536-2307
Ca	55.0±7[a]	45.7±15[b]	110 -2610
Mg	112.1±18[a]	76.6±7[b]	67-545
Trace			
Mn	1.81±0.31[a]	1.30±0.03[b]	1.3-55.4
Fe	27.9±5.61[a]	18.5±1.91[b]	22-117.6
Cu	0.06±0.04[a]	0.04±0.05[b]	0.27-3.75
Zn	0.05±0.21[a]	0.04±0.29[b]	1.42-10.61
Se	0.03±0.01[a]	0.01±0.01[b]	0.025-0.65

In each raw different letters indicate significant differences ($p < 0.05$)

* Johnson and Shubert, 1986; Fox, 1996; Campanella *et al.*, 2008; Belay, 2008

In contrast to Na and K which were found in higher concentrations in the dry season sample, all the other major (P, Ca, Mg) and trace (Mn, Fe, Cu, Zn and Se) minerals analyzed in this study,

were found in higher concentrations in the wet season sample. Except Ca, Cu and Zn, the concentration of the other minerals (P, Mg, Mn, Fe and Se) in the wet season sample were also within ranges reported for commercial *Spirulina* (Table 4.11). The low Ca contents of Lake Chitu's *Spirulina* might be attributed to the high salinity and alkalinity of the lake which causes the removal of this mineral from the solution as insoluble carbonate precipitates (Wood and Talling, 1988; Kebede *et al.*, 1994) making it unavailable to the alga.

The contribution of Lake Chitu's *Spirulina* towards meeting the recommended mineral intakes was calculated by assuming a 20g daily intake of the biomass by adult human (Table 4.12). A consumer who eats 20g of Lake Chitu's *Spirulina* daily could receive from 8 to 11% of RDI of K, 9 to 12% of the RDI of P and 4 to 6% of the RDI of Mg. 20g of Lake Chitu's *Spirulina* could also provide from 24 to 36% of the RDI of Fe. The contributions of Lake Chitu's *Spirulina* towards meeting Fe requirements might be significant because of a relatively higher Fe bioavailability attributed to *Spirulina* compared to other food crops (Puyfoulhoux *et al.*, 2001). Iron deficiency is the major cause of the high prevalence of anemia in developing countries (Akhter *et al.*, 2005). On the other hand, Lake Chitu's *Spirulina* does not contribute significantly to the RDI of Ca, Cu, Zn and Se since the contribution of all these minerals was ≤1%. Jhonson and Shubert (1986) also indicated that *Spirulina* is in general a poor source of calcium.

Table 4.12. Percent contribution of minerals from Lake Chitu's *Spirulina* (based on intake of 20g/day) to Recommended Dietary Intake/day for adult males (19-50years old)

Minerals	*RDI(mg/day)	Wet season harvest	Dry season harvest
K	3500	8.90	11.68
P	1000	12.80	9.62
Ca	1000	1.10	0.92
Mg	350	6.40	4.37
Fe	15	36.50	24.7
Cu	2	0.60	0.40
Zn	15	0.06	0.05
Se	55	0.01	0.005

*Belitz *et al.*, 2009; CFIA, 2014

4.3.3.5 Phytonutrient (pigment) composition

Seasonal variations were observed in the phytonutrients composition of Lake Chitu's *Spirulina* during the study period (Fig 4.3a-d). Chlorophyll *a* contents of the biomass varied from 6.7 to 10.3 mg g^{-1} for wet season samples and from 6.5 to 7.13 mg g^{-1} for dry season samples (Fig 4.3a). The presence of high concentration of chlorophyll *a* in wet season samples might be associated with light limitation during this season and its relatively low concentration in dry season samples might be associated with nutrient limitation (Zinabu, 2002). Several studies also indicated that in lower light intensities, cyanobacteria produce higher amounts of chlorophyll *a* to optimize the light harvesting process (Vonshak *et al.*, 1982; Torzillo *et al.*, 1998; Danesi *et al.*, 2004; Rangel-Yagui *et al.*, 2004; Ravelonandro *et al.*, 2008; Danesi *et al.*, 2011). The chlorophyll *a* contents of both wet season and dry season samples were within standard ranges (6.1-12mg g^{-1}) reported for commercial *Spirulina* (Table 4.13).

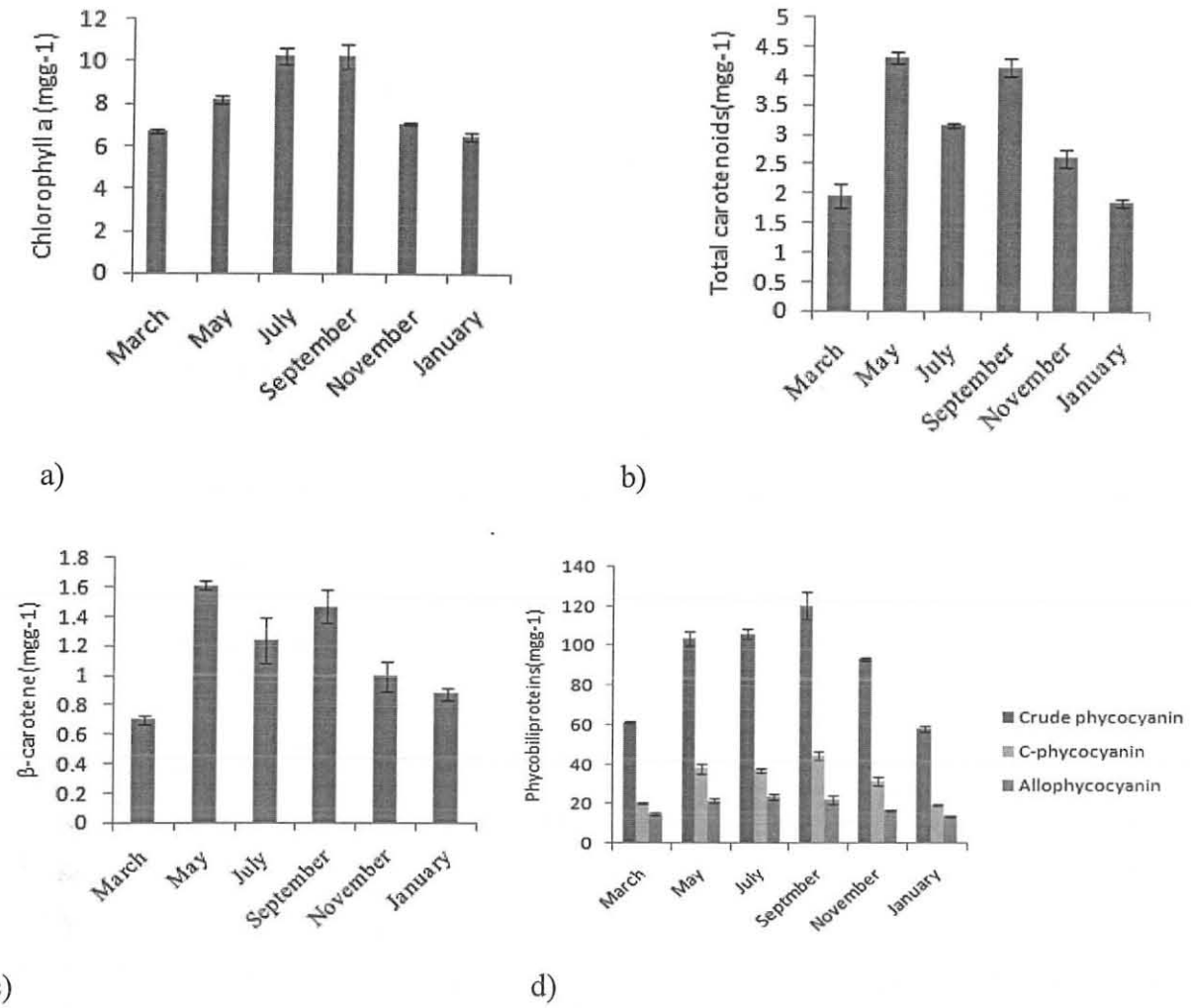


Fig 4.3. Seasonal variation in the phytonutrients composition of Lake Chitu's *Spirulina* (Mean± SD; n=3). Chlorophyll *a* (a) Total carotenoids (b) β-carotene (c) and Phycobiliproteins (d). Error bars represent the standard deviation of means

The total carotenoids content of Lake Chitu's *Spirulina* varied from 1.97 to 4.31 mg g^{-1} for wet season and from 1.86 to 2.63mg g^{-1} for dry season samples (Fig 4.3b). Higher values were recorded for wet season samples which can be explained by the fact that during this season the amount of light reaching to the cells might not be enough for photosynthesis thus high amounts of these pigments are synthesized to play an accessory role in light capturing (Kebede, 1997). The total carotenoid contents of wet season *Spirulina* were within ranges reported for commercial *Spirulina* (Table 4.13).

Table 4.13. Phytonutrients content of Lake Chitu's *Spirulina* as compared with commercial *Spirulina*

Phytonutrients (mg g^{-1})	Lake Chitu's *Spirulina*		*Commercial *Spirulina*
	Wet season harvest	Dry season harvest	
Chlorophyll *a*	6.7-10.3	6.5-7.13	6.1-12
Total carotenoids	1.97-4.31	1.86-2.63	2.9-5.04
β-carotene	0.7-1.61	0.88-1.00	1.5-1.9
Crude phycocyanin	61.5-120.7	58.3-93.6	140-172

*Fox 1996; Belay; 2008; 2013

The β-carotene content varied from 0.7 to 1.61mg g^{-1} and from 0.88 to 1.0 mg g^{-1} for wet and dry season harvested samples respectively (Fig 4.3c). Like the other pigments, higher values of β-carotene were recorded in wet season samples and values were a bit lower compared to that reported for commercial *Spirulina* (1.5-1.9 mgg^{-1}) (Table 4.13).

The crude phycocyanin content of Lake Chitu's *Spirulina* varied from 61.5 to 120.7mg g^{-1} for wet and from 58.3 to 98.7mg g^{-1} for dry season samples (Fig 4.3d). In cyanobacteria, phycobiliproteins serve both as a collector of light for photosynthesis and as a reserve of nitrogen (Richmond, 1988). El-Bakey (2003) reported variation in phycocyanin content of *Spirulina* from 17 mg g^{-1} when grown without nitrogen and 120 mg g^{-1} under higher nitrogen conditions. Higher contents of these chromoproteins in wet season samples compared to the dry season samples might be thus attributed to two reasons: low light (lead to synthesis of these pigments to optimize light capture) (Richmond, 1988) and high nutrient availability that prevail in the lake during this season due to mixing effects from rains and wind (Zinabu, 2002). Compared to commercial samples which are claimed to have values between 140–172mg g^{-1}(Table 4.13), the phycocyanin content of Lake Chitu's *Spirulina* was a bit inferior. In this study it was also noted that, in all the samples, the C-phycocyanin content was always higher than the allophycocyanin content. Yoshikawa and Belay (2008) and Su *et al.* (2012) also indicated allophycocyanin as a minor component of phycobiliproteins in cyanobacteria

4.4 Conclusions

The present study revealed pronounced seasonal variations in the nutrient profile of Lake Chitu's *Spirulina*. The protein, amino acids and phytonutrient contents of wet season samples were higher than dry season samples and were comparable with other *Spirulina* products commonly used in human nutrition. Except some minerals such as Ca, Cu and Zn, the mineral composition of Lake Chitu's *Spirulina* was within classical ranges reported for commercial *Spirulina*. Therefore, the finding favors the use of Lake Chitu's *Spirulina* as a potential food supplement for humans. However, in order to give a go-ahead of this naturally grown *Spirulina* for human use, detailed investigation of its safety is mandatory.

Chapter 5: Safety evaluation of *Spirulina* (*Arthrospira*) biomass harvested from an Ethiopian soda lake, Lake Chitu for possible use of the biomass as a human food

5.1 Introduction

The increasing shortage of protein in the world has led to the search for unconventional protein sources. The use of the cyanobacterium *Spirulina (Arthrospira)* has received considerable attention by scientists and industrialists as a potential source due to its high nutritional value (high protein content) and possible therapeutic effects (Fox, 1996; Belay, 2002; Habib *et al.*, 2008). *Spirulina* grows in an alkaline-saline lake, Lake Chitu throughout the year forming dense populations. Our previous studies (mentioned elsewhere in this thesis) on the nutrient profile and some safety aspects (microbial profile and acute toxicity test on mice) of the biomass showed the possibility of using the biomass as a human food/dietary supplement. In order to give a preliminary go-ahead of the biomass for human use, the biomass should be evaluated for its safety. Toxicological evaluation for food grade *Spirulina* or microalgae includes microbiological tests, test for heavy metals, algal toxins, pesticides, nucleic acid contents and other extraneous materials (insect fragments, rodent hair, and feather fragments)(Belay, 1997). Therefore, the objective of this study was to investigate the safety of *Spirulina* biomass harvested from the lake on seasonal bases.

5.2 Materials and methods

5.2.1 Sampling site and sampling

Spirulina biomass collection from Lake Chitu was carried out starting from March 2012 to January 2013 and sampling was done at two months interval. Samples were harvested from the lake manually in plastic jars during morning hours. During harvesting, the algal biomass was allowed to pass through a 0.425mm sieve to remove insects, birds' hair and other large sized contaminants. The harvested biomass samples were immediately transported to the Center for Food Science and Nutrition laboratory of Addis Ababa University and filtered under vacuum by using a nylon cloth with 25μm openings. The biomass samples collected on the cloth were washed with distilled water to remove salts and debris attached to them. The washed biomass

samples were freeze dried, grounded to powder and stored in brown bottles at -20°Cuntil analysis. In addition to biomass samples, water samples for determination of heavy metals were collected with acid washed polyethylene bottles and transported to the laboratory and stored at 4°C until analysis.

5.2.2 Safety evaluation

5.2.2.1 Microbiological analysis

NMKL methods (NMKL, 2003; 2004; 2005; 2006) were used for microbiological analysis of Lake Chitu's *Spirulina* samples collected on seasonal bases. For the analysis, 10g of *Spirulina* powder was transferred aseptically to a sterile Erlenmeyer flask and 90 ml of sterile 0.1% (w/v) bacteriological peptone water was added into it. This was mixed for 1-3 minutes with a shaker. Serial 10 fold dilutions were prepared by transferring 1 ml of the homogenized sample to 9 ml diluent. Nordic Committee of Food Analysis (NMKL) methods were used to determine the counts of each microbial group.

Aerobic plate count

From appropriate dilutions, 0.1ml aliquots were spread plated in duplicates on pre-dried surfaces of Plate Count Agar plates. The culture media were incubated at 30-32°C for 48 hrs (NMKL, 2006)

Total coliforms

From appropriate dilutions, 1ml of aliquots were transferred to duplicate dishes and to the dishes molten Trypton Soya Agar (TSA) and Violet Red Bile Agar (VRBA) were added and mixed gently by moving the dishes in clockwise and anticlockwise directions. The dishes were incubated at 37°C for 24 hours. After incubation, typical colonies (purplish red colonies surrounded by reddish zone of precipitated bile) were counted. Confirmation was done by inoculating the colonies into Brilliant green bile broth (BGBB) tubes and incubating at 37°C for 48hrs. The fermentation of lactose and formation of gas in any of the tubes with in 48hrs, regardless of the amount, was considered as positive for coliform and the absence of gas formation with in this period was considered as negative for coliforms (NMKL, 2004).

Fecal coliforms

From appropriate dilutions, 1ml of aliquots were transferred to duplicate dishes and to the dishes molten TSA and VRBA were added and mixed gently by moving the dishes in clockwise and anticlockwise directions. The dishes were incubated at 44°C for 24 hrs. After incubation, red colonies with shining clearing edges were counted as fecal coliforms. For further confirmation, about 5 typical and 5 atypical colonies were inoculated into tubes containing *Escherchia coli* broths (ECB) and Nutrient Broths (NB). ECB tubes were incubated at 44°C for 24hrs and NB tubes were incubated for 48hrs in a water bath. The fermentation of lactose and formation of gas in any of ECB tubes with in 24hrs, regardless of the amount was considered as positive for fecal coliform and the absence of gas formation within this period was considered as negative for fecal coliforms. The presence of *E.coli* was confirmed by checking NB for indole test (NMKL, 2005).

Staphylococcus aureus

From appropriate dilutions, 0.1 ml aliquots were spread plated in duplicates on pre-dried surfaces of Baird parker agar (supplemented with tellurite egg yolk emulsion) plates. The culture media were incubated at 37°C for 24-48hrs hrs after which black and shiny colonies with narrow-white marigins and surrounded by the zones extending in the opaque medium were counted as *S. aureus*. Further confirmation of the colonies was done by performing coagulase test (NMKL, 2003).

Yeasts and molds

From appropriate dilutions, 0.1 ml aliquots were spread plated in duplicates on pre-dried surfaces of chloramphenicol supplemented Dichloran Rosebengal Agar plates. The culture media were incubated at 22 °C for 5-7 days after which smooth and non- hairy colonies were counted as yeast where as hairy colonies were counted as molds (NMKL, 2005).

5.2.2.2 Heavy metal analysis

5.2.2.2.1 Lake water

Heavy metals (Pb, Cd, Hg and As) content of water samples were determined using Graphite Furnace Atomic Absorption Spectrometry (AA analyst 600, Perkin Elmer) equipped with hollow cathode lamps. Samples were digested using HNO_3 and HCl. For standard preparations of the analytes, proper dilutions were made using 10% nitric acid from the stock solutions (atomic absorption standard solutions of As, Pb, Cd, and Hg (1000 mgL^{-1}) (SISCO research laboratories Pvt. Ltd, India). For the determination of cadmium, arsenic and mercury, standard concentrations were prepared in ppb (10, 30 and 60) and for the determination of lead, standard concentrations were prepared in ppm (0.5, 1 and 2). 20μL of sample and calibration solutions were used for measurements.

5.2.2.2.2 *Spirulina* powder

Heavy metals (As, Cd, Pb and Hg) of *Spirulina* powder were determined after acid digestion (using conc HNO_3) of the samples in a microwave digester. Diluted solutions were then analyzed by ICP-MS (Thermo-Fisher Scientific iCAP-Q; Thermo Fisher Scientific, Bremen, Germany). The instrument was run employing collision-cell technology with kinetic energy discrimination (CCT-KED) to remove polyatomic interferences; the collision cell gas was He. Samples were introduced from an autosampler (Cetac ASX-520) incorporating an ASXpress™ rapid uptake module through a PEEK nebulizer (Burgener Mira Mist). Internal standards were introduced to the sample stream on a separate line via the ASXpress unit and included Sc (20 $\mu g\ L^{-1}$), Rh (10 $\mu g\ L^{-1}$), Ge (10 $\mu g\ L^{-1}$) and Ir (5 $\mu g\ L^{-1}$) in 2% trace analysis grade (Fisher Scientific, UK) HNO_3. External calibration standards (Claritas-PPT grade CLMS-2 from SPEX Certiprep Inc., Metuchen, NJ, USA) for As, Cd, and Pb were prepared in the range 0 – 100 $\mu g\ L^{-1}$ (0, 20, 40, 100 $\mu g\ L^{-1}$). Mercury analysis was undertaken semi-quantitatively using sensitivity values from Cd and Pb calibrations. Sample processing was undertaken using Qtegra™ software (Thermo-Fisher Scientific) utilizing external cross-calibration between pulse-counting and analogue detector modes when required.

5.2.2.3 Determination of pheophorbides content

Total pheophorbide content of the biomass was determined by using an official method of Japan Health Food Association (JHFA), Environmental Food Number 99 (1981) Director of Environmental Health Bureau of the Ministry of Health and Welfare at Earthrise farms laboratory, California, USA. To extract the pigment complex, a 0.1 g of sample was weighed and M/15 phosphate buffer: acetone solution in the ratio of 7:3 was added to the sample. The mixture was then incubated in a shaker water bath (37°C, 75rpm) for 3 hours and acidified using 10% HCl to pH 2-5. The mixture was then centrifuged at 3000 rpm for 5 minutes and 5°C and the supernatant was put in a separatory funnel (250ml) to extract further with 20ml of 85% aqueous acetone solution. The mixture was centrifuged and the supernatant was transferred to the separatory funnel. The residue was further extracted by adding 10ml 85% acetone two times (at least four supernatatnts were collected during this extraction procedure). Further separation into an ether phase was performed by the addition of 30 ml of ethyl ether followed by adding 50ml of 5% sodium sulphate and discarding the water/acetone phase. After repeated additions and washings (at least four times), the ether layer was filtered and 20ml of 17% HCl added to separate the pigment into an acid phase. Anhydrous sodium sulphate and ethyl ether was then added to the acid phase and shaken to separate the pigment into the ethyl ether phase and left for 30 minutes. The ether layer was separated, filtered, made to volume and absorbance was measured in a 1 cm cell at 667 nm using ethyl ether as blank. The total pheophorbide content was estimated using the following formula:

$$\text{Total pheophorbides (mg/100g)} = \frac{\text{AsxD (25)}}{\text{Wx 70.2}} \times 100$$

Where: As = Absorbance

D = Dilution factor

W= weight of sample

5.2.2.4 Determination of microcystin content

The microcystin content of Lake Chitu's *Spirulina* was determined at the Earthrise farms laboratory, California, USA. For the determination microcystin, 0.5g of *Spirulina* powder was homogenized in an aliquot of methanol (100%) using an electric homogenizer. The homogenate was extracted and centrifuged (10,000 ×*g*, 10 min) twice overnight and for 3hrs, respectively. The supernatants were combined and evaporated to dryness with sterilized air. The remaining residue was re-suspended in phosphate buffer saline (PBS) and subjected to enzyme-linked immunosorbent assay (ELISA) detection according to the method of Carmichael and An (1999).

5.2.2.5 Nucleic acid determination

Extraction of DNA from *Spirulina* samples (20 mg for each sample) was done using GeneJET Plant Genomic DNA Purification Mini Kit (#K0791, Thermoscientific, EU) according to manufacturer's instructions using the plant genomic DNA purification main protocol. Similarly, RNA extraction from *Spirulina* samples (20mg for each sample) was done using GeneJET Plant Genomic RNA Purification Mini Kit (#K0801, Thermoscientific, EU) according to manufacturer's instructions using the plant RNA purification protocol. A Nanodrop 2000c UV/IV Spectrophotometer (Thermo scientific) was then used to quantify the amount of DNA and RNA from the respective extracts.

5.2.2.6 Acute toxicity test on mice

Adult Swiss male mice with weights ranging from 25-30g were employed for the acute toxicity test. Mice of group 1 were treated as controls. Mice of group 2 were administered *Spirulina* powder (10g/kg body weight) harvested during wet season and mice of group 3 were given *Spirulina* powder (10g/kg body weight) harvested during dry season. Both *Spirulina* powders were suspended in distilled water and the solution (5ml/kg body weight) was administrated to mice groups in a single oral dose by intra gastric gavages using a feeding needle. The control group received an equal volume of distilled water as vehicle. Mice were observed thoroughly during the observation period of 14 days for any delayed acute effects. Criteria evaluated for treatment effects during the 14-days observation period were mortality, general symptoms

(changes on skin and fur, eyes, behavior pattern, and diarrhea), body weight, gross necropsy and measurement of organ weights (organ coefficients). Organ coefficients were calculated according to Sagstad *et al.* (2008).

Organ coefficients = Weight of organ (g) /Body weight (g) × 100

5.3 Results and discussion

5.3.1 Microbiological safety

Microbiological safety is one of the basic requirements for the utilization of microalgal biomass as a human food (Morist *et al.*, 2001; Belay, 2008). In large scale cultivation, especially in open ponds as well as natural lakes, there is a greater susceptibility of the biomass to microbial contamination (Belay, 1997; 2008). In relation to this, Lake Chitu's *Spirulina* was tested for a range of quality and safety indicator microorganisms. The aerobic mesophilic counts of the samples ranged from 0.4 x 10^3 to 130 x 10^3 CFU/g for wet season and from 0.37 x10^3 to 15 x 10^3CFU/g for dry season samples respectively (Table 5.1). These counts are within the lower ranges of standards set by commercial producers (Table 5.1). Analyses of other microbial groups such as total coliforms, fecal coliforms, *E.coli*, *S. aureus*, yeasts and molds(except some samples) were < 10^2CFU/g for both dry and wet season samples (Table 5.1). What is surprising with respect to the microbial profile of Lake Chitu's *Spirulina* especially wet season samples was the absence of pathogens or in general the good microbial quality of the product since during this season there is a high possibility that flood from the surrounding environment may introduce microbial contaminants. The freeze drying treatment would also inactivate but does not usually kill most microorganisms (Jay, 1996). The absence of pathogens in these samples could be therefore associated with the alkaline pH (pH ≥10.2) as well as the high salinity (>5%) prevailing in the lake which are inhibiting for the growth of most pathogenic as well as non pathogenic microbial groups.

Table 5.1. Microbiological profile of *Spirulina* biomass harvested from Lake Chitu during dry and wet seasons

crobial groups(CFU/g)	Lake Chitu's *Spirulina*		*Commercial *Spirulina*
	Wet season harvest	Dry season harvest	
robic mesophiles ($x10^3$)	0.4–130	0.37–15	50-1000
tal coliforms	$<10^2$	$<10^2$	Negative
cal coliforms	$<10^2$	$<10^2$	Negative
coli	$<10^2$	$<10^2$	Negative
ureus	$<10^2$	$<10^2$	Negative
asts	$<10^2$	$<10^2$	Negative
olds	$<10^2$	$<10^2$ - $2x10^2$	10^2-$<10^3$

*(Belay, 1997)

5.3.2 Heavy metals content

One aspect of quality control for microalgae intended for human consumption is their heavy metal contents since they are known accumulators (Kay, 1991). In this case, heavy metals content of both the lake water and the biomass were determined to evaluate the content of these metals in the lake water and the extent of accumulation by the biomass. In the lake water, the heavy metals Pb and Hg were not detected in both water samples collected in wet and dry seasons (Table 5.2). On the other hand, Cd was detected at a concentration of 0.18 mg L^{-1} in the water sample collected in wet season but it was not detected in the dry season water sample. Arsenic was detected relatively in higher concentration in the wet season water sample (0.14 mg L^{-1}) compared to the dry season water sample (0.08 mg L^{-1}).

Table 5.2. Heavy metal contents of Lake Chitu's water

Seasons	Heavy metals(mg L^{-1})			
	Pb	Cd	Hg	As
Wet	n.d	0.18	n.d	0.14
Dry	n.d	n.d	n.d	0.08

n.d not detected

The heavy metals content of Lake Chitu's *Spirulina* biomass is presented in Table 5.3. Lead was detected at concentrations of 0.2 and 0.17mg kg^{-1} in wet and dry season samples respectively. The cadmium content of the biomass didn't vary seasonally since in both wet and dry season samples its concentration was similar, 0.03mg kg^{-1}.

Table 5.3. Heavy metals content of Lake Chitu's *Spirulina*

Heavy metals (mg kg^{-1})	Lake Chitu's *Spirulina*	
	Wet season harvest	Dry season harvest
Pb	0.20±0.001a	0.17±0.01a
Cd	0.03±0.006a	0.03±0.003a
Hg	0.05±0.003a	0.03±0.002b
As	17.21±0.29a	20.4±0.43b

In each raw different letters indicate significant differences ($p < 0.05$)

Arsenic was the most abundant heavy metal obtained in Lake Chitu's *Spirulina* and relatively a higher value was recorded for a dry season (20.4mg kg^{-1}) sample compared to the wet season sample (17.2mg kg^{-1}). On the other hand, relatively higher content of Hg was detected in wet season *Spirulina* (0.05mg kg^{-1}) compared to dry season *Spirulina* (0.03mg kg^{-1}). In general,

heavy metals content of the biomass samples were by far greater than that found in the lake water. It has been reported that microalge accumulate heavy metals at concentrations that are several orders of magnitude higher than that present in the growth media (Becker, 1995). Moreover, Becker (1995) indicated that under alkaline conditions and in the presence of phosphate and sulfate ions, dissolved heavy metals (especially cadmium and lead ions) in the medium produce slightly soluble compounds that precipitate or can float when attached to small particles. These particles are harvested together with algal biomass and subsequently found in algal material after processing. Therefore, the relatively high heavy metal content in the biomass compared to the lake water might be due to the attachment of the metals with salts adsorbed at the surface of the biomass. This reason seems plausible as the ash content of the samples was high due to insufficient washing of the biomass by acid water.

There is no international standard for heavy metals concentration in microalgal products (Becker, 2004). Rather some countries, industry associations and major algae producers in the world have established standards for algal products including *Spirulina*. Table 5.4 shows comparison of heavy metals content of Lake Chitu's *Spirulina* with that of standards set by major producers. Based on the data, Pb, Cd, and Hg contents of both wet and dry season *Spirulina* are within the standards set by the three major producers USA, India and Israel. However, the arsenic content of Lake Chitu's *Spirulina* was much higher than the standards set by all producers. The high arsenic content of the biomass might be due to insufficient washing of the biomass with acid water. From the viewpoint of safety evaluation, total arsenic content in foods has no toxicological value since many types of arsenic are not metabolized and do not bring a health risk(Le *et al.*, 2004; Almela *et al.*, 2006). It is therefore necessary to quantify inorganic arsenic levels such as As^{3+} and As^{5+}due to the known toxicity of inorganic forms (Goessler and Kuehnett, 2002; Ng, 2005). It has been reported that inorganic arsenic species (As^{3+} and As^{5+}) generally contribute less than 50 percent of the total arsenic measured in algae(Kohlmeyer *et al.*, 2002). For instance, in the study of arsenic content of sea weeds marketed in Spain (Almela *et al.*, 2006), the total arsenic content ranged from 15.9–45.2 mg kg^{-1} whereas the inorganic As contents were less than 0.5 mg kg^{-1} (0.070–0.487mg kg^{-1}).

Table 5.4. Heavy metals content of Lake Chitu's *Spirulina* in relation to standards set by major producers

Heavy metals	Lake Chitu's *Spirulina*		Standards set by major producers		
(mg/kg)	Wet season harvest	Dry season harvest	[1]USA	[2]India	[3]Israel
Pb	0.20±0.001	0.17±0.01	<1.0	2.5	0.4
Cd	0.03±0.006	0.03±0.003	<0.05	1	<0.1
Hg	0.05±0.003	0.03±0.002	<0.05	0.1	0.24
As	17.21±0.29	20.4±0.43	<1.0	1.1	1.1

[1]Belay, 1997; [2] Toress-Duran *et al.*, 1998; [3] Richmond, 1990

According to WHO recommendation, adults weighing 60kg should not take more than 3mg of lead, 0.5mg of cadmium, 0.3mg of mercury and 20mg of arsenic per week (Becker, 2004). The US Pharmacopeia (USP, 2012) in its latest revision of metal limit has also set the oral permitted daily exposure from drugs and dietary supplements to be: Pb (5 μg/day), Hg (1.5 μg/day), Cd (25 μg/day), and As (1.5 μg/day). Commercial suppliers of *Spirulina* recommend doses up to 10-20g/day (Johnson and Shubert, 1986). Becker (1980) suggests a maximum daily intake of 30 g/day dried algae for humans. Table 5.5 shows the heavy metal consumption pattern by an adult weighing 60kg either weekly (based on WHO recommendation) or daily (based on USP recommendation) with a maximum daily intake of 30g *Spirulina* from Lake Chitu. Average values of the heavy metal contents of wet season and dry season samples are considered for the calculation.

Table 5.5. Weekly and daily intake of heavy metals by a 60kg weighing adult consuming 30g of Lake Chitu's *Spirulina* daily

Heavy metals	Heavy metals in the powder(mg kg^{-1})	[1]Intake per week(mg)	[2]Daily intake(µg per day)
Pb	0.18	0.039(3)	5.4(5)
Cd`	0.03	0.006 (0.5)	1.173(25)
Hg	0.04	0.008(0.3)	1.2(1.5)
As	18.8	3.95(20)	564(1.5)

[1] Calculated based on WHO recommendation

[2] Calculated based on USP recommendation

*Numbers in brackets are recommended values

Except arsenic, which was much higher than the daily recommended intake by USP (2012), the other heavy metals were within recommended values by both organizations which to some extent indicate the apparent safety of the biomass if it is taken even in a maximum amount permitted to be consumed per day.

5.3.3 Pheophorbides content

Pheophorbides are one of the indices of quality control for food grade microalgae as they induce photosensitive dermatitis when taken above certain limits (Jitsukawa *et al.,* 1984; Jassby, 1988). When Lake Chitu's *Spirulina* was assayed for these degradation products, the concentrations were 16.2 and 22.3mg/100g for wet and dry season samples respectively (Table 5.6). These concentrations were lower than the limit of 120 mg/100g established by the Japanese Public Health Ministry in 1981 (Jassby, 1988; Becker, 1994). The low pheophorbides concentration in the samples generally shows that the harvested *Spirulina* cells were photosynthetically active cells or natural degradation of chlorophyll in the lake is minimal.

Table 5.6. Total pheophorbides content of Lake Chitu's *Spirulina*

Lake Chitu's *Spirulina*	Total pheophorbides(mg/100g)
Wet season harvest	16.2
Dry season harvest	22.3
*Standards	< 120

*Becker, 1994; Jassby, 1988

5.3.4 Microcystin content

The assessment of cyanobacterial toxins in the biomass is fundamental for quality control for food grade *Spirulina*. It has been indicated that naturally harvested blue-green algae may be contaminated with algal toxins from toxin-producing algal species (Cox *et al.*, 2005). Since our *Spirulina/Arthrospira* samples were harvested directly from nature (wild), they are prone to contamination by other cyanobacterial groups. Besides this, reports about the presence of toxin-producing species of *Arthrospira* in 2 *Arthrospira* dominated Kenyan lakes are a matter of concern (Ballot *et al.*, 2004; Ballot *et al.*, 2005). In relation to this, *Arthrospira* biomass samples collected from Lake Chitu on seasonal bases (wet and dry seasons) were evaluated for their microcystin content and in both samples microcystins were not detected(Table 5.7). Willén *et al.* (2011) also reported the absence of microcystins in water and biomass samples collected from this lake. Therefore, the result indicates that Lake Chitu's *Spirulina/Arthrospira* is non-toxin producer and contamination of the biomass by other toxin producing algal species is negligible or none as the lake is characterized by an almost uni-algal population (Wood and Talling, 1988).

Table 5.7 Microcyctin content of Lake Chitu's *Spirulina* as compared to *Spirulina* from other lakes

ource of *Arthrospira*	Microcystin (µg/g DW)	Remark	References
ake Chitu (Wet season harvest)	n.d	harvested from lake	Present study
ake Chitu (Dry season harvest)	n.d	harvested from lake	Present study
ake Chitu(sample collected in 2011)	n.d	harvested from lake	Willen *et al.*, 2011
ake Bogoria	155.00	harvested from lake	Ballot *et al.*, 2004
ake Bogoria	15.02	cultured	Ballot *et al.*, 2004
ake Sonachi	1.60 - 12.00	harvested from lake	Ballot *et al.*,2005
ake Sonachi	2.20	cultured	Ballot *et al.*, 2005

n.d - not detected

5.3.5 Nucleic acids content

Evaluation of nucleic acid content is one aspect in the use of single cell protein products as a human food. For this reason, Lake Chitu's *Spirulina* was evaluated for its nucleic acids content and values were 0.18 and 0.13% for wet and dry season samples respectively (Table 5.8). The wet season sample had relatively higher nucleic acids (mainly contributed from RNA) compared to the dry season sample. Becker (1980) indicated the existence of variations in the nucleic acid content of algae from batch-to batch. Since RNA is an essential component of protein synthesis, its concentration often reflects the rate of protein synthesis (Wagner *et al.*, 1998). The presence of relatively higher RNA concentration in the wet season sample might be therefore associated with high protein synthesis by the cells during this season. In fact, high protein content was recorded for *Spirulina* biomass harvested in wet season samples compared to dry season samples. The amount of DNA per cell is assumed not to vary with environmental condition (Bulow, 1987 cited in Wagner *et al.*, 1998). In their study on the effect of nitrogen source(air, NO_3^-, and NH_4) on the biochemical composition of the cyanobacterium *Nostoc paludosum,*

Vargas *et al.* (1998) recorded the highest RNA content in cells grown with NH_4 than that grown on air or NO_3^-. On the other hand, DNA content of the organism remained the same in all cultivation conditions.

In general, the nucleic acids content of Lake Chitu's *Spirulina* in both wet and dry season samples were very low compared to that reported for other *Spirulina* samples: 2.9-4.5% (Durand-Chastel, 1980); 4% (Aaronson *et al.*, 1980) and 6.4% (Morist *et al.*, 2001). This variation might be due to growth conditions as well as difference in the method of analysis used for extraction and quantification of these macromolecules. Since a high amount of uric acid (final product of nucleic acid metabolism) in humans may produce toxicity problems such as gout or kidney stones (Morist *et al.*, 2001), the low concentration of these acids in Lake Chitu's *Spirulina* is of interest.

Table 5.8. Nucleic acid content of *Spirulina* biomass harvested from Lake Chitu during dry and wet seasons

Nucleic acids (%)	Lake Chitu's *Spirulina*	
	Wet season harvest	Dry season harvest
RNA	0.08±0.03	0.04±0.02
DNA	0.09± 0.03	0.09±0.02
Total nucleic acids	0.18±0.01	0.13±0.02

5.3.6 Acute toxicity study

Mice fed on freeze dried biomass of Lake Chitu's *Spirulina* (10g/kg) did not develop any clinical signs of toxicity either immediately or during the post treatment irrespective of the high dose given to them. Feeding of the biomass did not cause any alteration in food intake and body weight gain (data not shown) of the mice. Necropsy at the end of the experiment (day 14) did not also show any macroscopic organ changes in both treated (groups 2 and 3) and control group

(group 1). Furthermore, the relative organ weights (Table 5.9) of the treated groups, were comparable to that of the control group.

Table 5.9. Relative organ weights of mice fed an acute dose of freeze dried *Spirulina* (10g/kg body weight) harvested from Lake Chitu during dry and wet seasons

oups	Relative organ weights(g/100g body weight)						
	Liver	Kidneys	Spleen	Heart	Lung	Brain	Gonads
ɔup 1	5.04±0.36	1.45±0.10	0.41±0.036	0.51±0.03	0.75±0.17	1.22±0.13	0.90±0.07
ɔup 2	4.99±0.54	1.44±0.12	0.42±0.05	0.52±0.06	0.78±0.09	1.18±0.11	0.90±0.09
ɔup 3	5.16±0.50	1.43±0.12	0.47±0.14	0.51±0.05	0.76±0.08	1.27±0.23	0.89±0.052

Values are mean ± SD of five animals

5.4 Conclusions

Lake Chitu's *Spirulina* was safe interms of microbial contamination as it has low mesophilic count and many of the safety indicator microorganisms were absent. The heavy metal contents of the biomass, except arsenic, were within standards set by major *Spirulina* producers in the world. The microcystin and pheophorbides concentration of the biomass were also within the safe levels recommended for such type of products. Furthermore, feeding of mice at a very high dose of 10g/kg body weight didn't bring any signs of toxicity and mortality to the animals. The results, thus, show the potential safety of Lake Chitu's *Spirulina* for human use. However, to declare the safety of the biomass for human use, long term (sub chronic and chronic) animal feeding studies and evaluation of the biomass for its inorganic arsenic content are necessary.

Chapter 6: Chemical composition and functional property of a phycobiliprotein –rich crude extract obtained from *Spirulina*

6.1 Introduction

The use of protein preparations as functional components in processed food products is becoming common since proteins are responsible for many of the properties of food products that influence consumer acceptance (Ogunwolu *et al.*, 2009). The major functional properties of proteins include solubility, foamability, emulsifying activity, gel formation as well as water and oil absorption capacity (Ahmedna *et al.*, 1999). Proteins with high oil and water absorption capacities are desirable for use in meats, sausage, breads and cakes, while proteins with high emulsifying and foaming capacity are used for salad dressing, sausage, bologna, soups, confectionery, frozen dessert and cakes (Ahmedna *et al.*, 1999). The functional properties of proteins usually depend on the molecular properties of proteins which can be further influenced by other parameters like pH and the presence of other molecules (Schwenzfeier *et al.*, 2013a). Functional properties of proteins may also vary based on their source, composition, method of preparation/extraction, prevailing environment, etc (Adedayo *et al.*, 2011). Algal proteins, both in the form of whole algae and as protein concentrates and/or isolates, are among the proteins that are being used in some human foods. These proteins are usually marketed as health foods or functional foods (Becker, 2007). There is also a growing interest to use algal proteins as functional components in processed foods since many of them showed promising results. For instance, Guil-Guerrero *et al.* (2004) studied the functional properties of defatted biomass of three microalgal species, *Porphyridium cruentum*, *Nannochloropsis* sp. and *Phaeodactylum tricornutum* and found comparable functional properties with soya bean flour. Recently, a soluble protein isolate from the microalga *Tetraselmis* sp. was isolated by Schwenzfeier *et al.* (2011) and indicated for having superior techno-functional properties compared with common plant protein isolates used by food industries (Schwenzfeier *et al.*, 2013a; Schwenzfeier *et al.*, 2013b)

The microalga *Spirulina* is known for its high protein content, 45-71% (Ortega *et al.*, 1993; Fox, 199) and various phytochemicals (pigments) such as chlorophyll *a*, carotenoids and phycobiliproteins with potential therapeutic effects (Belay *et al.*, 1993; Belay, 2002). Among the various phytochemicals of *Spirulina*, phycobiliproteins have got a wide range of commercial applications in food coloring, cosmetics and biomedical research replacing synthetic pigments (Cohen, 1997).

Phycobiliproteins are proteins which have covalently attached linear tetrapyrrole chromophoric groups which are called bilins or phycobilins (Cohen, 1997). The phycobiliproteins in *Spirulina*, C-phycocyanin and allophycocyanin, comprise quantitatively about 20% of *Spirulina* protein (Richmond, 1989). C- phycocyanin has been indicated as a safe pigment for human use (Naidu *et al.*, 1999) and claimed for having various health benefits such as antioxidant, anti-inflammatory, anticancer, antiaging and antimutagenic properties (Romay *et al.*, 1998; Bhat and Madyastha, 2000; Romay *et al.*, 2000). Allophycocyanin has also shown to have an antiviral activity against enterovirus-71(Shih *et al.*, 2003). Thus, these natural bioactive proteins could be used as nutraceutical ingredients by food industries (Plaza *et al.*, 2007). Moreover, being proteins, investigating their techno-functional properties in processed foods may also maximize their use. The objective of this study was therefore to evaluate the chemical composition, *in vitro* protein digestibility and functionality of a phycobiliprotein-rich crude extract obtained from *Spirulina* for possible food applications.

6.2 Materials and methods

6.2.1 Preparation of *Spirulina* powder

Spirulina biomass was harvested from Lake Chitu manually in plastic jars. During harvesting, the algal biomass was allowed to pass through a 0.425mm sieve to remove insects, birds' hair and other large sized contaminants. The harvested biomass was immediately transported to the Center for Food Science and Nutrition laboratory of Addis Ababa University and filtered under vacuum by using a nylon cloth with 25μm openings. The biomass collected on the cloth was washed with distilled water to remove salts and debris attached to it. The washed biomass was oven dried at 60°C, grounded to powder and stored in brown bottles at -20°C until use.

6.2.2 Preparation of a phycobiliprotein–rich crude extract (PBE)

Phycobiliprotein- rich crude extract was extracted and recovered from the crude *Spirulina* powder following the method of Chronakis (2001) with some modifications. The first step of the extraction process was solublization of oven dried *Spirulina* powder in dilute alkali (pH 10). The solution was then stored at 4°C over night and then centrifuged at 3000 rpm at 4°C for 30min. The blue supernatant was collected and the pellet left was dissolved again in dilute alkali subjected to a second extraction procedure. Both supernatants were pooled, titrated to pH 2 with 0.1 M HCl to precipitate the phycobiliprotein-rich component. Precipitation at pH 2 helped to concentrate phycobiliproteins by excluding other pigments such as chlorophyll a. The precipitate (phycobiliprotein- rich crude extract or PBE) was then separated from the solution by centrifugation, neutralized by using 0.01M NaOH, freeze dried and stored at -20°C until analysis.

6.2.3 Chemical composition of phycobiliprotein–rich crude extract

Proimate composition was analyzed using AOAC procedures. Moisture content was determined by drying a representative sample for 4hrs at 105°C (AOAC, 2000; 925.09). Crude protein content (N x6.25) was determined by employing the Kjeldahl method (AOAC, 2000; 979.09) from a 0.5g sample. Crude fat was quantified gravimetrically by exhaustively extracting 2g sample in diethyl ether (boiling point, 55°C) in a Soxhlet apparatus (AOAC, 2000; 4.5.01). Ash was determined by incineration of 2.5g sample in a muffle furnace at 550°C until a gray ash was obtained (AOAC, 2000; 923.03). Carbohydrate content was determined as the weight difference using protein, lipid, moisture and ash content data (James, 1996). Phycobiliproteins were determined following the procedure developed by Yoshikawa and Belay (2008).

6.2.4 Amino acid composition

Amino acid analysis was performed according to AOAC (2005; 994.12). Briefly, samples were oxidized with a hydrogen peroxide/formic acid/ phenol mixture. Excess oxidation reagent was decomposed with sodium metabisulfite. The oxidized samples were hydrolyzed with 6 M hydrochloric acid for 24 h. The hydrolysate was adjusted to pH 2.2, centrifuged, and filtered. Amino acids were separated by ion exchange chromatography (Biochrom 20+ Amino Acid Analyser, Biochrom Ltd, Cambridge, UK) and determined after reacting with ninhydrin by using

photometric detection at 570 nm. The amino acids were identified and quantified by comparing peak profiles of the proteins with amino acid profiles from external amino acid standards.

6.2.5 Determination of *in vitro* protein digestibility

In vitro protein digestibility of PBE and crude *Spirulina* powder was carried out according to the method described by Elkhalil *et al.* (2001) with some modifications. Twenty mg of sample was digested in triplicate in 10 ml of trypsin (0.2 mg/mL in 100 mM phosphate buffer, pH 8.0). The suspension was incubated at 37 °C for 2 hours. Hydrolysis was stopped by adding 5 ml 50% trichloroacetic acid (TCA). The mixture was allowed to stand for 30 min at 4 °C and centrifuged at 4000rpm for 30 min using 80-2 centrifuge (China). The protein content of the supernatant was then determined by employing the Kjeldahl method and digestibility was calculated as percentage.

Protein digestibility (%) = A/B x 100

Where: A: total protein content (mg) in the supernatant.
B: total protein content (mg) in the sample.

6.2.6 Functional property of phycobiliprotein–rich crude extract

6.2.6.1 Protein solubility

Protein solubility was determined according to Bera and Mukherjee (1989), with some modifications. One hundred mg of PBE was dispersed in 10 mL of distilled deionized water. The suspensions were adjusted to pH 2.0 up to pH 12.0 using either 0.1 M HCl or 0.1 M NaOH. Suspensions were shaken in an orbital shaker for 30 min at room temperature (approximately 22°C) and centrifuged at 3000rpm for 30 min. The protein content of the supernatant was determined by the Kjeldahl method and percent nitrogen solubility was calculated as follows:
Protein solubility (%) = A/B x 100

Where: A: total protein content (mg) in the supernatant.
B: total protein content (mg) in the sample.

6.2.6.2 Water and oil absorption capacity (WAC and OAC)

Water and oil absorption capacity were determined according to Gandhi and Srivastava (2007). One gram of sample was mixed with 10 ml distilled water or sunflower oil in centrifuge tubes and then allowed to stand for 30 min. Samples were centrifuged at 3000rpm for 30 min. The supernatant was discarded and the tube was weighed. WAC (grams of water per gram of sample) was calculated using the equation;

$WAC = (W_2-W_1)/W_0$

where W_0 was the weight of the dry sample (g), W_1 was the weight of the tube plus dry sample (g) and W_2 was the weight of the tube plus sediment (g).

OAC (grams of oil per gram of extract) was calculated using the equation;

$OAC = (F_2-F_1)/ F_0$

where F_0 was the weight of the dry sample (g), F_1 was the weight of the tube plus dry sample (g) and F_2 was the weight of the tube plus sediment (g).

6.2.6.3 Bulk density

A known weight of sample was added to a graduated measuring cylinder. The cylinder was gently tapped and volume occupied by the sample was determined. Bulk density was calculated as weight per unit volume (g/mL)(Mohamed *et al.*, 2009).

6.2.6.4 Emulsifying activity (EA) and emulsion stability (ES)

6.2.6.4.1 Effect of protein concentration

The effect of protein concentration on the emulsifying activity (EA) and emulsion stability (ES) of the PBE and bovine serum albumin (BSA) (reference protein) was determined according to

Pearce and Kinsella (1978). Samples (15mL) at 10 to 50mg/ml in 0.01 M sodium phosphate buffer (pH 8.0) were stirred at room temperature for 30min and sun flour oil (5mL) was added to each. The mixtures were homogenized in a homogenizer (China) for 1min at a velocity of 12000rpm. Immediately after homogenization and emulsion formation, 50μL aliquots of the emulsion were taken at 0 and 10 min from the bottom of the tube and added to 5mL of 0.1% sodium dodecyl sulphate (SDS) solution (1:100 dilution). The diluted emulsion was shaken very briefly in a vortex mixer and the absorbance at 500 nm was read in UV/VIS/NIR spectrophotometer (Perkin Elmer, USA). The EA and ES were calculated as follows:

$$EA\ (m^2/g) = \{(2 \times 2.303) / [C \times (1-\theta) \times 10^4]\} \times A_{500} \times DF$$

Where C is the initial concentration of protein; θ is the fraction of sunflower oil used to form the emulsion; A is the absorbance and DF is the diluting factor (100).

$$ES\ (\%) = A_{10}/A_0 \times 100$$

Where A_0 and A_{10} are the absorbance of the diluted emulsions at 0 and 10 min

6.2.6.4.2 Effect of pH

To determine the effect of pH on the emulsifying activity and stability of PBE, samples (15ml) of 0.1% w/v of solutions were prepared and the pH of the suspensions was adjusted to pH 2.0 up to pH 12.0 using either 0.1 M HCl or 0.1 M NaOH. Emulsifying activity and stability were then determined as described above.

6.2.6.5 Foaming capacity and stability

6.2.6.5.1 Effect of protein concentration

The effect of protein concentration on foaming capacity (FC) and foam stability (FS) of PBE and egg albumin (reference protein) was determined according to Agyare *et al.* (2009) and Arogundade (2006). Protein dispersions (10 to 50 mg/ml, 40 ml) were stirred at room

temperature for 60 min and then homogenized in homogenizer for 1 min at a velocity of 12000rpm. The resulting foam was poured into a 100 ml cylinder. Total foam volume was recorded and foam capacity was expressed as the percent increase in volume. To determine foam stability (FS), foam volume was recorded 60 min after whipping and calculated as follows:

FS = foam volume after 60 min/initial foam volume x 100

Foam capacity (FC) was calculated using the following formula:

$$FC = \text{Volume } \frac{\text{after whipping} - \text{volume before whipping}}{\text{Volume before whipping}} \times 100$$

6.2.6.5.2 Effect of pH

To determine the effect of pH on the foaming capacity and stability of PBE and egg albumin, 0.5 g of sample was dissolved in 50 ml of buffer solutions, with different pH values (2, 4, 6, 8 10, 12). Then the suspensions were whipped at 12000rpm in a homogenizer (China) for 1 min at room temperature (22 °C). Foaming capacity and foaming stability were then determined as described above.

All data are expressed in terms of mean ± standard deviation. Variation in the chemical composition of *Spirulina* powder and PBE was analyzed using student's t-test. Significant level for the analysis was set to $p < 0.05$.

6.3 Results and discussion

6.3.1 Chemical composition

A protein product with higher concentrations of phycobiliproteins components (compared to the original *Spirulina* powder) was prepared by modifying the storage temperature, storage time and precipitation pH reported by Chronakis *et al.* (2000) for the preparation of *Spirulina* protein isolate. About 8.3% of phycobiliprotein-rich crude extract (PBE) was recovered with higher contents of proteins compared to the crude *Spirulina* powder (60.1 vs 45.2%) (Table 6.1). As is

shown in Table 6.1, the extract had 188.3mg g^{-1}, 89mg g^{-1} and 2.18 mg g^{-1} of crude phycocyanin, C-phycocyanin and allophycocyanin content respectively which were more than double of their concentration in the original *Spirulina* powder.

Table 6.1. Chemical composition of *Spirulina* powder and phycobiliprotein-rich crude extract (PBE)

Chemical composition	*Spirulina* powder	PBE
Moisture (%)	8.10±0.1[a]	8.8 ±0.03[b]
Protein (%)	45.2±0.55[a]	60.1±1.04[b]
Crude fat (%)	2.5±0.05[a]	0.4±0.09[b]
Ash (%)	13.8±0.49[a]	7.6±0.27[b]
Total carbohydrates (%)	30.4±0.73[a]	23.1±1.31[b]
Crude phycocyanin(mg g^{-1})	73.7±0.00[a]	188.3±0.32[b]
C- phycocyanin(mg g^{-1})	25.7±0.01[a]	89.0±0.06[b]
Allophycyanin(mg g^{-1})	16.9±0.01[a]	28.1±0.01[b]

In each raw different letters indicate significant differences ($p < 0.05$)

6.3.2 Amino acid composition

Since the nutritional quality of a food protein is related to the content, proportion and availability of its amino acids (Becker, 2007), the amino acid composition of PBE was determined and compared with the crude *Spirulina* powder (Table 6.2). All essential and non-essential amino acids were represented in both *Spirulina* powder and PBE but differences were observed in their total amino acid contents (Table 2). Higher quantities of amino acids were recorded for PBE (45.61%) compared to the crude *Spirulina* powder (39.0%). Furthermore, except lysine (Lys) and histidine (His), all other essential amino acids (Isoleucine (Ile), leucine (Leu), Methionine (Met), phenylalanine (Phe), Threonine (Thr) and Valine (Val)) were represented in higher quantities in

the extract compared to the crude *Spirulina* powder. These results show the good nutritional quality of the extract.

Table 6.2. Amino acid composition of *Spirulina* powder and phycobiliprotein-rich crude extract (PBE)

Amino acids	*Spirulina* powder	PBE
EAs	g/100g sample	g/100g sample
Ile	1.44	2.27
Leu	2.78	3.67
Lys	2.38	1.88
Met	1.28	1.28
Phe	1.65	1.78
Thr	1.92	1.95
Val	1.89	2.65
His	1.12	0.60
NEAs		
Cys	2.78	0.90
Tyr	1.22	1.45
Asp	3.12	4.94
Glu	6.88	9.01
Ser	2.00	2.01
Gly	1.90	1.80
Ala	1.84	3.00
Arg	2.29	3.36
Amm	2.49	3.07
Total AAs	39.00	45.61

6.3.3 *In vitro* protein digestibility

Although amino acid profile is important in evaluating the nutritional value of proteins, the digestibility of those proteins is the primary factor of the availability of their amino acids. The *in vitro* protein digestibility of PBE was therefore determined and compared to the crude *Spirulina* powder. The phycobiliprotein-rich crude extract was found to be more digestible than the crude *Spirulina* powder with a digestibility value as high as 86% (Table 6.3).

6.3.4 Functional property of PBE

6.3.4.1 Protein solubility

Protein solubility is an important prerequisite for the functional properties of a given food protein (Kinsella, 1976a; Schwenzfeier *et al.*, 2011). Evaluation of protein solubility at different pH values is being common as it is a useful indicator of protein functionality since it directly relates to many important properties of proteins such as emulsifying, foaming and gel forming abilities (Kinsella, 1976; Molina and Wagner, 2002). The phycobiliprotein-rich crude extract exhibited pH dependent protein solubility as shown in Fig 6.1; minimum solubility was observed at pH 2(8.3%) and an increase in solubility was observed above this point and at pH 7, its solubility reached 50.5 %. The solubility then decreased from pH 7 to pH 10 but at pH 12, it reached 62.2 %. It has been reported that the solubility of a single protein is typically minimal at its isoelectric point (pI), since at this point the overall net charge is zero and electrostatic repulsion is low which leads to the formation of protein aggregates and then precipitation of the protein (Singh *et al.*, 2005). However, the low solubility of PBE at the basic pH region was not clear as solubility is expected to increase at these pH values since the electrostatic repulsion between the individual protein molecules is expected to be high (Yin *et al.*, 1996). Similar results were reported by Nirmala *et al.* (1992) for a spray dried defatted *Spirulina* powder and attributed this as alkali induced protein association as well as disulphide oxidation. Although maximum solubility of PBE was observed at pH 12, at this pH value, discoloration of the pigment was noted. Similar findings were reported by Sapin (1988) and this was attributed to isomerization in the phycocyanobilin chromophore.

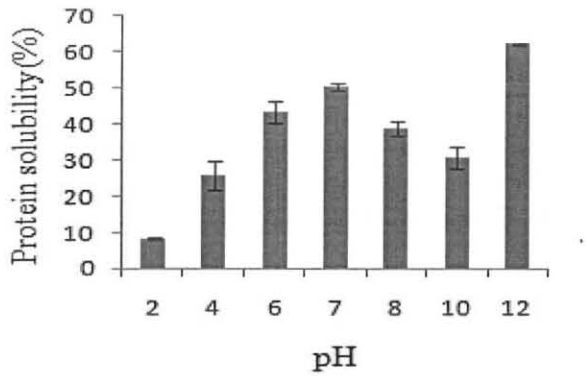


Fig 6.1. Effect of pH on the protein solubility of phycobiliprotein-rich crude extract. Error bars represent the standard deviation of means.

6.3.4.2 Water and oil absorption capacity

The water absorption capacity of proteins plays a major role in the functional property of proteins (Guil-Guerrero *et al.*, 2004) since interaction with water is the first step in imparting the desired functional property to any food system. The water absorption capacity of PBE was 1.76g water/g of extract and was less than the water absorption capacity of *Spirulina* powder, 2.05g water/g of powder (Table 6.3). However, the water absorption capacity of PBE was higher than that reported by Anusuya and Venkataraman (1984) for *Spirulina* protein concentrate (1.09 g water/g sample).

Oil absorption capacity is a critical determinant of flavor retention and enhancing the mouth feel of foods (Kinsella, 1976b). High oil absorption or oil holding capacity is an essential property for the formulation of foods such as sausages, cake batters, mayonnaise and salad dressings (Chandi and Sogi, 2007). The oil absorption capacity of PBE was 1.43 g oil/g of extract and thus higher than the crude *Spirulina* powder which had a capacity of 0.98g oil/g of powder (Table 6.3). The higher oil absorption capacity of PBE might be due to the higher protein content of the extract which led to a greater extent of hydrophobic interaction between protein and oil. However, the oil absorption capacity of PBE was lower than a *Spirulina* protein concentrate (2.80 g oil/g of sample) reported by Anusuya and Venkataraman (1984).

6.3.4.3 Bulk density

Bulk density indicates the behavior of a product in food formulations. For instance, high bulk density is disadvantageous for the formulation of weaning foods as low density is required in such type of formulations. Moreover, bulk density is an important parameter that determines the packaging requirement of a product (Chandi and Sogi, 2007). Phycobiliprotein-rich crude extract was found to have lower bulk density, 0.64g/ml, compared to the crude *Spirulina* powder it was extracted from which had a bulk density of 0.75g/ml (Table 6.3).

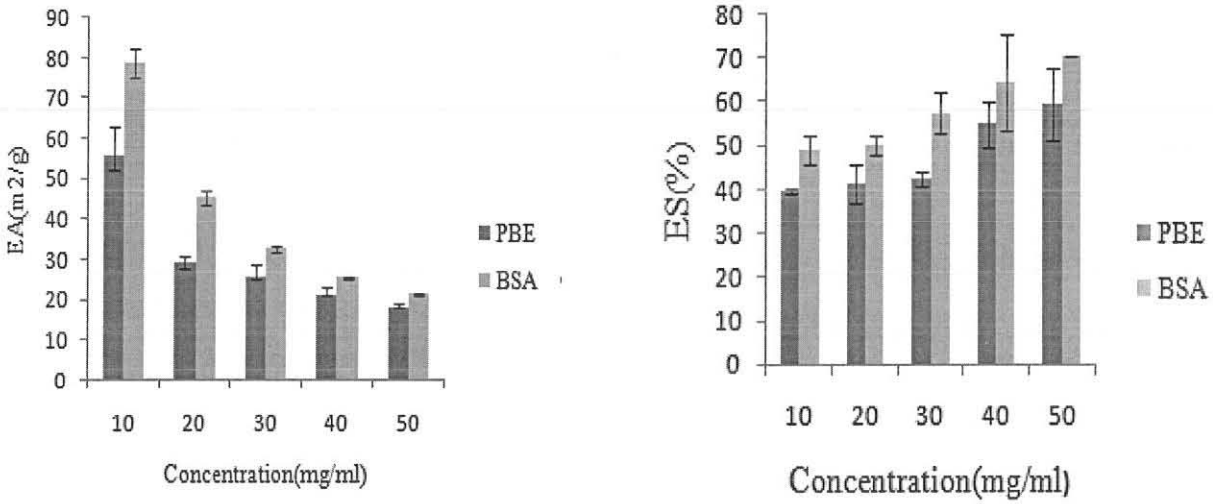
Table 6.3. *In vitro* protein digestibility, water absorption capacity, oil absorption capacity and bulk density of *Spirulina* powder and phycobiliprotein-rich crude extract (PBE)

Functional property	*Spirulina* powder	PBE
In vitro protein digestibility (%)	59.3±1.71	86.0±2.15
Water absorption capacity (g water/g sample)	2.15 ±0.04	1.76 ±0.1
Oil absorption capacity (g oil/g sample)	0.98 ±0.1	1.43 ±0.03
Bulk density(g/ml)	0.75 ±0.00	0.64 ±0.01

6.3.4.4 Emulsifying activity (EA) and Emulsion stability (ES)

Emulsifying activity (EA) is the ability of a protein to help formation and stabilization of the emulsion created. Emulsion stability (ES) is a measure of the ability of a given protein to impart strength to the emulsion to resist changes to its structure such as coalescence, creaming, flocculation or sedimentation over a defined time period (Boye *et al.*, 2010). The effect of protein concentration on the EA and ES of PBE and BSA are presented in Fig 6.2a and b. The EA of PBE at a concentration of 10mg/ml was 55.5 m^2/g and this value was reduced to 18.1m^2/g at 50mg/ml concentration. In general, the emulsifying activity of both PBE and BSA decreased as the concentration of protein increased. According to Sze-Tao and Sathe (2000), at low protein concentration, protein adsorption at the oil–water interface is diffusion controlled, since it will

spread over the surface before it can be adsorbed. On the other hand, at high protein concentration, the activation energy barrier does not allow protein migration to take place in a diffusion-dependent manner as such emulsifying activity decreases with increased protein concentration. Compared to BSA, PBE had lower emulsifying activity especially at low protein concentration but as the concentration increased it showed comparable emulsifying activity. On the other hand increasing protein concentration increased the emulsion stability of both PBE and BSA. For instance the ES of PBE at a concentration of 10mg/ml was 39.7 % and this number has increased to 59.6% at a concentration of 50mg/ml.

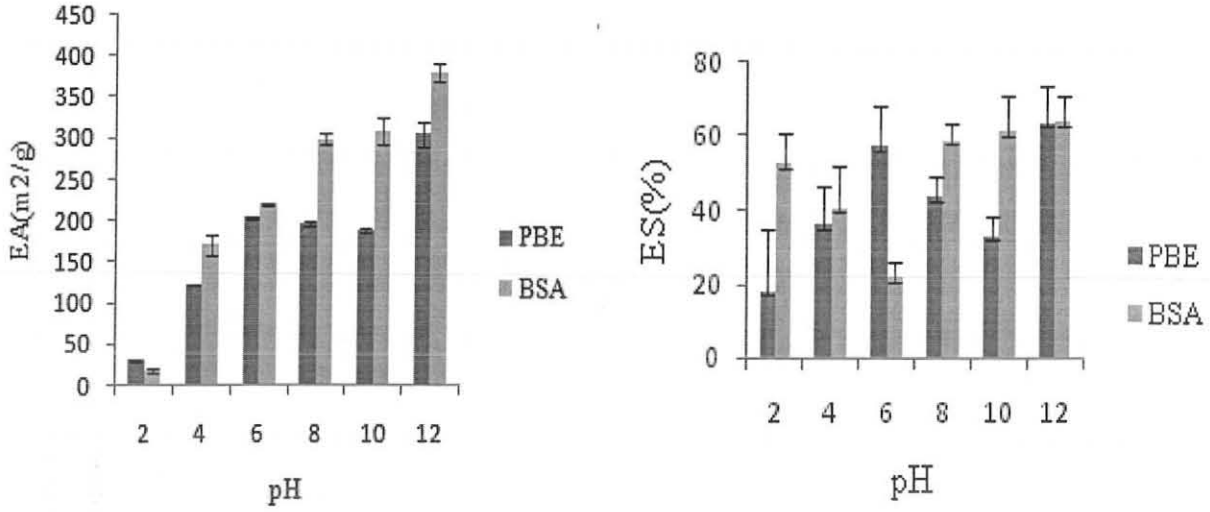


(a) (b)

Fig 6.2. Effect of protein concentration on the emulsifying activity (a) and emulsion stability (b) of phycobiliprotein-rich crude extract (PBE) and bovine serum albumin (BSA). Error bars represent the standard deviation of means.

The effect of pH on the EA and ES of PBE and the reference emulsifier, BSA is shown in Fig 6.3a and b. The EA of PBE was low at pH 2 (29.1m^2/g) and increased as the pH increased, 305.8m^2/g at pH 12. At pH 2, protein solubility was low, protein adsorption at the oil–water interface would be diffusion controlled. However, at a pH range of 4.0–12.0 with protein solubility increased, the activation energy barrier did not allow protein migration to take place in a diffusion dependent manner. Increase in protein solubility facilitated enhanced interaction between the oil phase and the aqueous phase (Mao and Hua, 2012). The results indicated that

alkaline pH was found to improve the EA of PBE more than acidic pH. The EA of PBE had some resemblance with its protein solubility profile. Similar findings were reported by Guil-Guerrero *et al.* (2004) in a study on the functional properties of three microalgal species. In general, the emulsifying activity of PBE was low compared to BSA at all pH values considered in this study.



(a) (b)

Fig 6.3. Effect of pH on the emulsifying activity (a) and emulsion stability (b) of phycobiliprotein-rich crude extract (PBE) and bovine serum albumin (BSA). Error bars represent the standard deviation of means.

The ES of PBE had a similar pattern with its protein solubility since it showed minimum stability at pH 2 (18.1%) and maximum stability at pH 12(63.7%). At pH 6, PBE showed extremely higher ES than BSA (57.2% vs 21.8%) and the ES of PBE at pH 12 was also comparable with BSA. In other pH values the ES of PBE was lower than BSA.

6.3.4.5 Foaming capacity (FC) and stability (FS)

The basic requirements for a protein to be a good foaming agent is its ability to rapidly adsorb at the air–water interface during bubbling and its ability to undergo rapid conformational changes

at the interface (Schwenzfeier *et al.*, 2013a). The effect of protein concentration on the foaming capacity (FC) and stability (FS) of PBE and egg albumin is presented in Fig 6.4 a and b. At a concentration of 10mg/ml, the FC of PBE was 47.5% and at a concentration of 50mg/ml, its foaming capacity reached as high as 75%. Similarly, the FS of PBE was 89.8% at the concentration of 10mg/ml and it increased to 98.5% stability when its concentration increased to 50mg/ml. In general, the FC and FS of PBE increased with increasing protein concentration. It was also observed that increasing the protein concentration not only increased the foam volume but also increased the thickness of the foam. This is because high protein concentration increases the viscosity and facilitates the formation of a multilayer, cohesive protein film at the interface (Damodaran, 1997) which is also beneficial to FS (Akintayo *et al.*, 1999). However, relatively lower FS was recorded for PBE than egg albumin especially at lower protein concentrations. Unlike egg albumin which is composed of ~90% of protein, PBE contains 60% protein. The low FS of PBE might be therefore associated with the difference in protein concentration since usually proteins are surface active components (Anusuya and Venkataraman, 1984).

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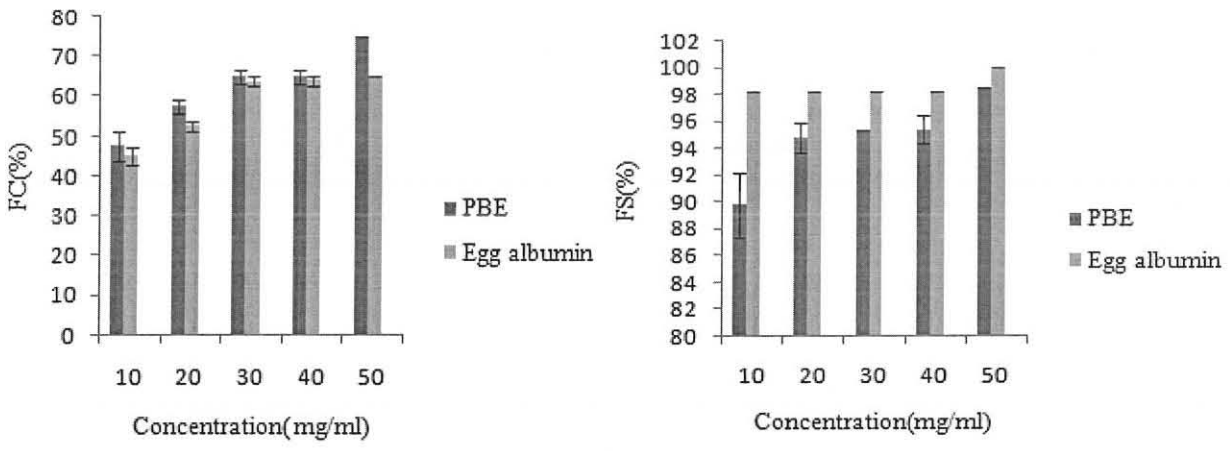
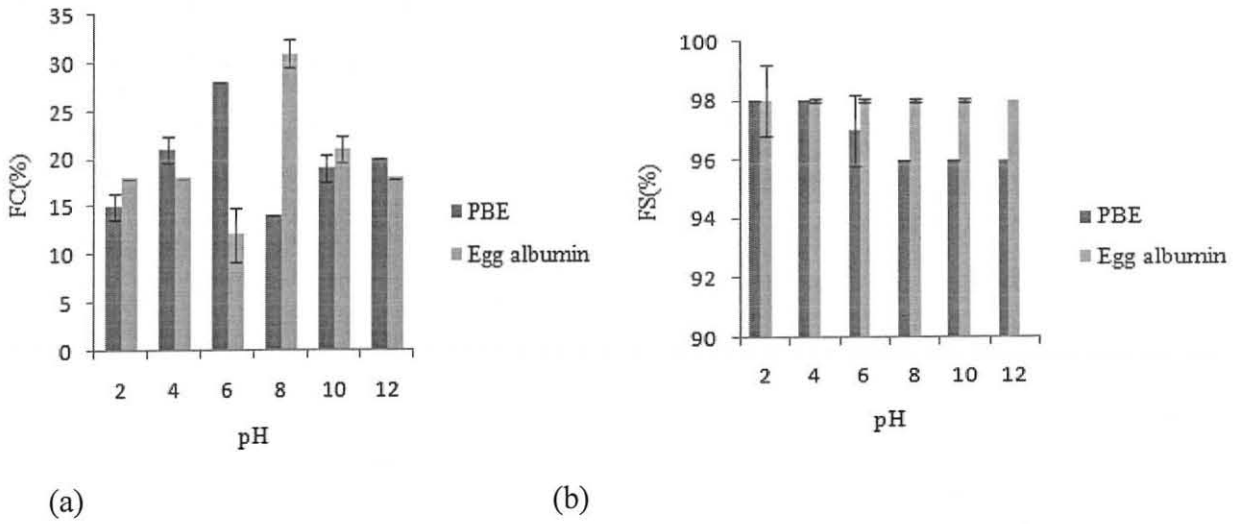


(a) (b)

Fig 6.4. Effect of protein concentration on the foaming capacity (a) and foaming stability (b) of phycobiliprotein-rich crude extract (PBE) and egg albumin. Error bars represent the standard deviation of means.

The effect of pH on the FC and FS of PBE is illustrated in Fig 6.5 a and b. The FC of PBE was low at pH 2(15%) and increased sharply to pH 6(28%) which was where maximum foaming capacity was recorded and decreased from pH 8 onwards. The trend had close resemblance with its protein solubility and emulsifying activity. This might be due to an increase in the net charge of the protein which weakens hydrophobic interaction and increases protein solubility and flexibility, allowing the protein to spread to the air–water interface more quickly, encapsulating air particles and thus increasing foam formation(Lawal *et al.*, 2005). While PBE showed maximum FC at pH 6, the maximum FC of egg albumin (31%) was recorded at pH 8. In other pH values, PBE and egg albumin had comparable foaming capacity. In general, in most pH values considered in this study, the FC of PBE was comparable and in some cases higher than egg protein. Nirmala *et al.* (1992) also reported a higher FC than egg protein for a spray dried defatted *Spirulina* powder.



(a) (b)

Fig 6.5. Effect of pH on the foaming capacity (a) and foaming stability (b) of phycobiliprotein-rich crude extract (PBE) and egg albumin. Error bars represent the standard deviation of means

The FS of PBE was as high as 98% in the acidic pH region and it didn't show significant reduction as its stability was ≥ 96% in the neutral and basic pH regions. The FS of PBE was also comparable with egg albumin especially in the acidic pH region.

6.4 Conclusions

The results revealed the possibility of preparing an extract rich in phycobiliproteins from *Spirulina* with good nutritional profile, *in vitro* protein digestibility and techno-functional property. Here it is also worth mentioning about the multiple advantage of such type of protein preparation would have if it is used as an ingredient in processed foods as the health benefits of phycobiliproteins especially phycocyanin is well documented. Therefore, further study on the application of this extract in food formulations is recommended.

Chapter 7: General discussion

As described in the Introduction part of this dissertation, the use of unconventional protein sources such as *Spirulina* contributes considerably as an alternative protein source. In spite of the indicated importance of *Spirulina* in alleviating malnutrition and its general health related benefits, no studies on the possibility of using *Spirulina* as a dietary supplement were available in Ethiopia. Therefore, the aim of the present study was to investigate the possibilities of using the high standing *Spirulina* biomass of Lake Chitu as a human food supplement or as a source of high value products for possible applications (techno-functional) in foods. As a first step to achieve the aim, a preliminary assessment about the quality and safety of the biomass was undertaken. The result obtained from this investigation has led to further evaluation of the biomass's nutrient profile on seasonal basis. The variation in the nutrient profile of the biomass on seasonal basis was then determined. This was followed by evaluation of the safety of the biomass based on criteria set for safety evaluation of food-grade *Spirulina*. Finally, a phycobiliprotein-rich crude extract was prepared from the biomass and evaluated for its composition, *in vitro* protein digestibility and techno-functional properties for possible applications in foods.

7.1 Growth of *Spirulina* as a uni-algal population in Lake Chitu

Lake Chitu is characterized by high pH (>10), high alkalinity (>500 meq L^{-1}) and high salinity (>50 g L^{-1}). It has been reported that in lakes with salinity greater than 30g L^{-1}, the algal population is almost exclusively composed of *Arthrospira* and *Spirulina* species (Richmond, 1990). High pH and alkalinity also favors the presence of *Arthrospira* as a dominant species (Melack, 1979 cited in Belay, 2013). Therefore, the dominance or unchallenged growth of *Arthrospira* in Lake Chitu could be associated with the physicochemical characteristics of the lake. In fact, microscopic observation of the lake water failed to show other algal contaminants in the lake.

An interesting feature of the lake region is the absence of great variability in the maximum and minimum temperature of wet and dry seasons which might be one factor for the growth of *Arthrospira* (*Spirulina*) in the lake throughout the year. However, high algal biomass was

observed in wet months compared to dry ones during this study. Although there is a long time gap between their sampling, the results of Wood and Talling (1988) and Kebede *et al.* (1994) also support this observation. Wood and Talling (1988) measured a biomass concentration of 2600μg L^{-1}(in terms of Chl *a* concentration) for the lake in a wet month (August 1966) and Kebede *et al.* (1994) reported relatively lower biomass (Chl *a*) concentration (224 μg L^{-1}) for a sample taken immediately after the dry season (March 1991). Similar observations were made by Abdulqader *et al.* (2000) for another *Spirulina* dominated lake, Lake Kossorom in Chad. The release of nutrients into the euphotic zone during mixing of water bodies that results from cooling and winds associated with rains may contribute to the high biomass concentration during this season (Zinabu and Taylor, 1989).

During the study period, harvest of *Spirulina* biomass from Lake Chitu was possible during morning times and during late afternoon. This is because flocculated mats of *Spirulina* were at their most dense during these times: morning (7:00am-11:00am) and afternoon (4:00pm – 6:00pm). When the level of solar radiation became high during noon, all the flocculated mats submerged and harvesting was impossible. Similar observations were made by Ma and Gao (2009) in which the buoyancy of *Spirulina* peaked near sunrise, decreased with increasing solar radiation towards noon and increased with declining sunlight towards sunset. While Sinha *et al.*, (2001) indicated the down movement of cyanobacteria during high levels of solar radiation as a strategy to avoid harmful radiation, Ma and Gao (2009) indicated such a migration pattern as a mechanism that could enable cyanobacteria to receive more light under light-limiting conditions and to lessen photoinhibition during high levels of solar radiation (noon period).

7.2 Possibility of using Lake Chitu's *Spirulina* as a human food supplement

Natural blooms of *Spirulina* have been noted in Lake Chitu many decades ago (Talling *et al.*, 1973; Wood and Talling, 1988). The bloom appears throughout the year supporting high flocks of Lesser Flamingos. The occurrence of abundant *Spirulina* biomass in Lake Chitu and the presence of protein malnutrition in the country (EHNRI, 2010) have led to the evaluation of the biomass for its suitability to be used as a human food (protein) supplement. Given the relatively high production costs of artificial production as one of the main limitations in *Spirulina*

production (Vonshak and Richmond, 1998), the proliferation of *Spirulina* in lakes and its direct harvest and processing could present an economically feasible production system, as pond and media costs are reduced. The only costs involved in this case are those of harvesting and drying. In this case, a preliminary study was made to get background information about the chemical composition and safety of the high standing biomass in the lake. The result was encouraging to pursue the study about the quality and safety of the biomass on seasonal basis as the biomass had good chemical composition (a protein content of 43% which is higher than conventional high protein plant sources such as legumes) and safe in terms of its microbial profile and absence of acute toxicity on mice fed high doses (30g/kg body weight, with 85% moisture content) of the fresh biomass which is equivalent to an acute dose of 1800g of fresh biomass for a 60kg adult human.

7.3 Seasonal variation in the nutrient profile of Lake Chitu's *Spirulina*

Spirulina growing in open ponds or especially in natural lakes are affected by environmental factors such as temperature, salinity, light and nutrients. Most of these environmental factors vary according to season and the changes in these conditions can stimulate or inhibit the synthesis of several nutrients (Lobban *et al.*, 1985). Seasonal variations were observed in the nutrient profile of Lake Chitu's *Spirulina*. For instance, high protein contents were recorded for wet season samples compared to dry ones which can be associated with nutrient availablity. The positive correlation between nitrogen or nutrient availability and protein content has been established from several studies. For instance, in the study of seasonal variation on the chemical composition of seaweeds, the protein levels of the seaweeds were positively correlated with the nitrogen content of the sea water (Marinho-Soriano *et al.*, 2006). Similarly, light limitation and nutrient availability that prevail in the lake during wet season also induced biosynthesis of pigments (phytonutrients) to maximize light capture (Zinabu, 2002). Dry season samples had relatively higher carbohydrate concentrations compared to wet season samples which can be attributed to combined effect of nutrient deficiency and high salinity that prevail in the lake during this season (Zinabu, 2002). Nutrient (nitrogen) deficiency has been also found to stimulate the synthesis of carbohydrates in other cyanobactrial groups such as *Phormidium laminosum* (Fresnedo and Serra, 1992) and *Cyanothece* sp. (De Philippis *et al.*, 1993).

By comparing the findings of this study with commercial *Spirulina*, it is possible to say that the amounts of Ca, Zn, and Cu in Lake Chitu's *Spirulina* were below the expected limits for this microalga. However, it should be noted that supplementation of the culture media with essential mineral salts is common in artificial production and thus result in enrichment of the final product with essential minerals.

In general, changes in environmental (growth) conditions not only affect the chemical composition of Lake Chitu's *Spirulina* but also its morphology. A study by Ogato and Kifle (2014) on Lake Chitu's *Spirulina* indicated that the dominance of H-type (tightly coiled morphotype) was strongly associated with NO_3^- and HCO_3^- deficiencies, high levels of radiation and temperature where as decrease in the salinity of the lake favored the dominance of S-type (spiral or loosely coiled morphotypes).

7.4 Safety of Lake Chitu's Spirulina

Spirulina biomass grown under controlled conditions usually has a specified chemical composition with little or no threat of contamination from external sources. However, growth in relatively variable conditions such as in lakes presents uncontrollable conditions, with quality and safety guaranteed only by the prevailing chemical composition of the lake. In order to give a preliminary go-ahead of Lake Chitu's *Spirulina* for human use, the main areas of concern should be addressed on seasonal bases. In relation to this, the biomass was evaluated for various safety parameters.

Since microbial contamination of algal biomass cannot be completely avoided during cultivation especially in open pond culture systems and natural lakes (Gors *et al.*, 2009), regulations with regard to the tolerable limits of microbial contaminations have been set by major producers. These regulations or limits vary according to the countries where the products are marketed (Jassby 1988; Becker 1994; Belay, 1997). The microbial profile of Lake Chitu's *Spirulina* was within standards set by major producers in the world (Japan, France, USA, Sweden, etc) which

can be associated with the physicochemical characteristics of the lake (high pH and high salinity) which are inhibiting for the growth of most pathogenic microorganisms.

Algal and cyanobacterial toxins have a broad range of potencies since ingestion of these toxins by human beings can cause a range of effects from mild illness to death. The presence of microcystins in commercial *Spirulina* and other algal supplements in concentrations that can potentially affect human health was reported by various authors (Gilroy *et al.* 2000; Bruno *et al.* 2006). Lake Chitu's *Spirulina,* both wet season and dry season harvested samples, were thus evaluated for this toxic substance and none of the samples were found to contain the toxin. Unlike other *Spirulina* lakes such as Lake Bogoria and Sonachi (Kenyan lakes) in which mass mortalities of filamingos occured due to ingestions of toxins (Ballot *et al.*, 2002), there are no reports about mass death of filamningos associated with feeding of Lake Chitu's *Spirulina.* This may thus demonstrate Lake Chitu's *Spirulina* as a non-toxin producer. Willen *et al.* (2011) also reported the absence of microcytin in both biomass and water sample taken from this lake. Furthermore, these results might also indicate the absence of other contaminant algae (especially toxin producers) in the lake.

Evaluation of Lake Chitu's *Spirulina* for its heavy metal contents indicated the presence of some metals in higher concentrations compared to the lake water. It has been reported that under high pH conditions, heavy metal concentrations found in association with sediments and particles are higher than their dissolved concentrations in surface and deep waters since the high pH favors stability of the metal fraction bound on to particles and sediments (Davies 1996: Zinabu and Pearce, 2003). Therefore, although the concentrations of some metals were very low in the Lake water, these metals might be found in the biomass forming complexes with adsorbed salts. As a post-harvest treatment, Becker and Venkataraman (1980) proposed the washing of the algal biomass with 0.01 M EDTA (pH 8), as this treatment reduced the concentration of accumulated Hg and Cd in the alga *Scenedesmus* by 90%. Similarly, Johnson and Shubert (1986b) reported that shaking of *Spirulina* (having a mercury level of 9.8ppm) with 0.01M Na_2EDTA for one hour reduced the algal mercury content by 60%. In their study of the ability of *S. platensis* to accumulate mercury, Cain *et al.*, (2008) found a maximum uptake value 428 mg Hg^{2+}/g dry biomass when *S. platensis* was placed in a solution containing an initial Hg^{2+} concentration of ca.

1000 ppm. Washing with 0.1M HCl once however desorbed 100% of mercury adsorbed by the biomass. Almela *et al.* (2006) recommended bioavailability studies (i.e., the fraction of absorbed contaminant that reaches the systemic circulation) in order to make a more realistic evaluation of safety of algal products in terms of heavy metals.

Phototoxic substances such as pheophorbides may occur in algal food supplements due to various reasons: natural degradation during senescence or death (Gors *et al.*, 2009), degradation due to microbial activity (Brown *et al.*, 1991) or degradation due to unfavourable and extreme light or temperature conditions, oxygen, acidic or basic conditions (Jeffrey *et al.*, 1997; Cubas *et al.*, 2008) during processing or storage. Lake Chitu's *Spirulina* contained low amounts of pheophorbides which thus indicate the presence of minimal degradation during growth and processing of the biomass.

The nucleic acid content of Lake Chitu's *Spirulina* was found to be low compared to literature data. This might be due to variations in growth conditions as well as the method used for the determination of these macromolecules. Diphenylamine and orcinol reaction are the common methods used for the quantification of DNA and RNA in microalgal products. Both methods are colorimetric methods where absorbance is read at a particular wavelength, 595nm for DNA and 665nm for RNA. The concentration of each nucleic acid is extrapolated from standard curves constructed from DNA and RNA standards. The method used in the present study was a bit different as it involved extraction and purification of these acids from the biomass using DNA and RNA extraction kits and determining the concentration of the purified acids in a Nanodrop Spectrophotometer. Determining the purified acids might have reduced the quantity and could explain the large difference observed between the results obtained in this study and what is reported in various literatures.

In addition to evaluating algal biomasses for the levels of biological and non-biological contaminants, they should be also subjected to animal studies or bioassays (Becker, 1995). Short term feeding of Lake Chitu's *Spirulina* to mice at levels higher than the anticipated human consumption didn't bring any undesirable effects to the experimental animals. The maximum daily recommended intake of *Spirulina* is 30g. The acute dose given to mice (10g/kg body weight) in the present study was equivalent to an acute dosage of 600g for a 60 kg individual.

Therefore, tolerance of this dose by mice can be used as a good predicator about the potential safety of the biomass during long term feeding.

7.5 Composition and functionality of a phycobiliprotein-rich crude extract obtained from Lake Chitu's Spirulina

Extraction of high-value protein products for techno-functional applications in foods has been studied by various reseasrchers (Anusuya and Venkataraman, 1984; Nirmala *et al.*, 1992; Guil-Guerrero *et al.* 2004; Schwenzfeier *et al.*, 2011). Microalgae can be used as ingredients into many types of foods, adding not only nutritional value, but also new, unique and attractive tastes (Richmond, 2004). The phycobiliprotein- rich crude extract obtained from *Spirulina* had some important properties such as high protein content, high digestibility and foamability. The solubility of the extract was also good which could have been further improved if it was extracted using mild isolation techniques such as bead beating which do have little effect on denaturation of proteins (Schwenzfeier *et al.*, 2011).

High value proteins or products in general can be isolated from algae eventhough the algal biomass is not a type of 'food-grade'. For instance, Niu *et al.* (2007) reported the possibility of extracting phycocyanin from *Spirulina* they call it 'inferior *Spirulina*' due to its heavy metal contents. The other more important aspect of using algal biomass or extracts as food ingrdients is their multiple advantage as they may function for instance as food colorants, to increase the nutritional value of a food product where at the same time can act as a functional ingredient with potential health benefits.

7.6 Limitations of the study

Samples (fresh biomass) were transported for long distance (about 300 kms) which may result in degradation of some sensitive nutrients and thus affect their final concentaration in the dried biomass.

The sample preparation method was crude which resulted in variation in the ash content and might have an effect on the concentrations of other components of the biomass. Moreover, the presence of high ash in our samples made the freeze drying process of the biomass to take long time (more than a week) which may result in degradation of some useful components of the biomass.

Although the quality of the high standing biomass was evaluated, the actual concentration of the biomass at different sampling months was not determined which might have been useful to suggest months with good biomass concentrations for harvesting.

Chapter 8: Conclusions, recommendations and perpectives

8.1 Conclusions

In the present thesis, the possibility of using Lake Chitu's *Spirulina* in human nutrition was studied. In this regard, the biochemical composition and nutritional quality (in terms of its amino acid and fatty acid profile) of the biomass were evaluated on seasonal basis. Similarly, safety of the biomass was determined (on seasonal basis) using criteria set for safety evaluation of food-grade *Spirulina*. Finally, a phycobiliprotein rich crude extract was prepared from the biomass and evaluated for its functional property for possible food applications.

Seasonal variations were observed in the nutrient profile of the biomass and in general wet season samples had high protein and phytonutrients (pigments) (chlorophyll *a*, carotenoids, and phycobiliproteins) content compared to dry season samples. This variation could be attributed to the availability of nutrients in wet season due to the frequent mixing up of the lake by rain and wind. Furthermore, light limitation in wet season directly influences the phyonutrients (pigments) as they would be synthesized in higher quantities to optimize the light capturing process. The protein, amino acids and pigment composition of wet season samples were comparable with commercial *Spirulina*. Except the contents of Ca and Zn, which were found in lower quantities, the mineral composition of Lake Chitu's *Spirulina* was also comparable with that reported for commercial *Spirulina*.

Evaluation of the nutritional quality of the biomass showed the presence of seasonal variation in the amino acid profile of the biomass. In general, the amino acid profile of wet season *Spirulina* was better than dry season *Spirulina*. Although Lake Chitu's *Spirulina* had low fat content, the fatty acid profile of the biomass had some interesting characteristics. Both wet season and dry season samples had good fatty acid profiles with high concentrations of essential fatty acids (PUFAs).

Preliminary investigation on the safety of the biomass showed that Lake Chitu's *Spirulina* has no detectable toxicological constraints, in terms of its microbial profile, microcystins,

pheophorbides, nucleic acids and heavy metals(except arsenic) contents. The survival of mice when given high doses of the biomass also showed its potential safety for further long term feeding trials.

The functional property of the protein preparation (phycobiliprotein - rich crude extract) from Lake Chitu's *Spirulina* was comparable with other standard proteins which are commonly used in processed foods. For instance, its foamability was comparable with egg albumin and it had also comparable emulsifying activity with the commonly used emulsifying agent, bovine serum albumin. These results are encouraging for the use of such type of protein preparations in foods as besides their functional property they do have high nutritional and potential therapeutic values.

8.2 Recommendations

To improve the quality of the biomass, acid-washing is necessary to remove adsorbed carbonates and other salts. This procedure may increase the protein content/of the biomass, when considering the high inorganic salt and mineral content of Lake Chitu's *Spirulina*. Acid washing may also reduce the heavy metal contents of the biomass since these metals may also found on the cells as adsorbed forms.

8.3 Perspectives

In order to declare the safety of Lake Chitu's *Spirulina* for human use, long term animal feeding studies are needed.

Although not intensive, Lake Chitu is situated in an area where agricultural activity is practiced and thus pesticide accumulation can be a toxicological concern due to run-off from the surrounding agricultural land. This therefore necessitates the evaluation of the pesticide content of the biomass.

The use of *Spirulina* as a human food supplement has already been well documented (Dillion and Phan 1993; Fox, 1996). However, acceptability of this protein source with Ethiopian context should be addressed.

The specific aim of this study was to demonstrate if naturally grown *Spirulina* can be used as a human food supplement. From an economic viewpoint, however, the exploitation of naturally harvested *Spirulina* as a protein source can only be fully realized if the cost benefits associated with *Spirulina* production override those of the economics of the use of other protein sources. There is, therefore, a need for economical feasibility study.

There is also a need to evaluate the ecological feasibility of production and use of naturally grown *Spirulina* in Lake Chitu as the lake supports birds (Lesser filammingo) which depend on the biomass as their main food.

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ADDIS ABABA UNIVERSITY

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The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled ""**Quality and safety evaluation of Spirulina (Arthrospira) biomass harvested from an Ethiopian soda lake, Lake Chitu for possible applications in human nutrition"** by **Hirut Assaye** in partial fulfillment of the requirement for the degree of **Doctor of Philosophy**.

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