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Cultivation of *Arthrospira* Strains in Tropical Conditions, with Particular Reference to Ethiopia

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Abstract Tropical soda lakes generally harbor abundance of *Arthrospira* sp. (Spirulina) and related phytoplankton communities due to their optimal water chemistry and ecological conditions. *Arthrospira fusiformis* was described as the dominant species in Lake Chitu. The main aim of this study was to cultivate *Arthrospira* sp. isolates from Lake Chitu, Lake Abijata and Lake Shalla. Morphological features were determined using light Microscope. We found three different morphotypes (H-type, S-type and C-type) of *Arthrospira* sp. of Lake Chitu on the basis of selected morphological parameters. In this study it has been also confirmed that a concentration of ammonium nitrate of 0.03 M is optimal for cultivation of Spirulina. Revision of taxonomic positions of *Arthrospira* of Lake Chitu by combining morphological, ultrastructural and molecular methods is required using a larger number of strains isolated from different sampling points in the lake and increasing sampling from other lakes from the Rift Valley. In conclusion, Lake Chitu is a potentially interesting area for isolating *Arthrospira* sp. in the wild and hence to provide genetic variation of possible interest for future small to large scale commercial production or research activities.

Keywords *Arthrospira*, Spirulina, Lake Chitu, Morphotypes, Soda Lakes

1. Introduction

Cyanobacteria species such as *Arthrospira fusiforms* (*A. platensis*), *Oocystis* and *Anabaenopsis* were reported as dominant species in the considered sampling sites of Lake Chitu, Abijata and Shalla during 1960 to 1988 periods (Fetahi, 2016). Since then, a change in dominance of species had occurred due to environmental changes (Fetahi, 2016; Otago *et al.* 2016). However, Lake Chitu is known for its almost monoalgal composition of *A. fusiformis* (Otago and Kifle, 2014).

1.1. History and Taxonomy of Spirulina (and related names)

1.1.1. The Name "Spirulina" Used in this Article Refers to a Commercial Name

Currently the commercially used Spirulina can be assigned taxonomically to two different names, not belonging to genus *Spirulina* Turpin ex Gomont, but instead to genus *Arthrospira* Sitzenberger ex Gomont, 1892 (Gomont 1892), that is *Arthrospira platensis* Gomont and *Arthrospira maxima* Setchell and Gardner. These two names are assigned to biological entities easily distinguishable on the basis of dimension, since *A. maxima* spires are much larger than those of *A. platensis*. *Arthrospira fusiformis* (Voronikhin) Komárek and Lund is a specific name assigned to samples of *Arthrospira* with compressed spires (it has a shape of a coiled spring), typically from some African soda lakes, such as Chitu lake.

What is then to be assigned to the name "Spirulina"? We must consider that species delimitation in cyanobacteria is a complex task, also on a logical basis, in relation to the difficult application of biological species concept in prokaryotes (Dvorak *et al.* 2015). Nevertheless, it was possible to distinguish at least the main groups of biological entities in coiled cyanobacteria. Genus *Spirulina* Turpin ex Gomont was based on a typical species (*Spirulina major* Kützing ex Gomont) that revealed not to belong to the same genus as the *Arthrospira* species used in cultivation, but even to a different order (Komarek *et al.* 2014). As a result of this taxonomic treatment, Spirulina in the sense of the cultivated one is a commercial/vernacular name, not a taxonomically correct genus name and comprises rather strains belonging to genus *Arthrospira* (such as *A. platensis*). For this reason in our text spirulina will not be written in italics, since we refer to the commercial Spirulina, that is biological entities belonging to genus *Arthrospira*.

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Cyanobacteria are considered relatively primitive organism and are believed to have evolved about 3.5 billion years ago (FAO, 2008; Ali and Saleh, 2012). Spirulina has been harvested as a food source by Aztecs from the great soda lakes of Mexico from historical records of the 16th century (Ciferri, 1893). The Aztecs (Mexicans) call it "tecuitlatl", meaning stone's excrement and they make it into small cakes and sold it at the local markets to be used as an addition to sauces and mixed with grains (Genene Tefera *et al.* 2016). Back in the early 9th century, Spirulina use had a long history in Chad during Kanem Empire (Ali and Saleh, 2012). The Kanembu population living around Lake Chad were also reported to consume a traditionally collected Spirulina cake called "dihé" which is mixed with different sauces and consumed by the majority of the Kanembu population still today (FAO, 2008). The Spirulina has been harvested traditionally from the lake by skimming the surface of the water with finely woven nets. The slurry water substance containing Spirulina is allowed to drain via a pre-prepared round hole in sandy areas leaving a round cake-like substance called "dihé", which can be sold in local markets to be used as a source of protein by the consumers. Spirulina was established as "wonderful future food source" in 1967 for its exceptionally high quality protein (70% dry weight) content (Mogale, 2016; Bleakley and Hayes, 2017). With the start of commercialization, the first large scale production plant was established during early 1970s by Sosa Texcoco company (Belay, 2013).

Despite being a bacterium (Cyanobacteria) Spirulina is usually considered as microalgae, due to its ability to photosynthesize and similar appearance. Cyanobacteria (blue-green algae) have, at first glance, a similar morphology and property with respect to microalgae. However, microalgae such as Spirulina and cyanobacteria in general have unique features, such as their principal photosynthetic pigment (chlorophyll a), oxygen as a photosynthetic product and their size (5 to 10 times higher) that distinguish them from most bacteria (Moreira, 2009; Ali and Saleh, 2012).

1.2. Morphology

Spirulina are oxygenic, multicellular, gram negative, photolithoautotroph, filamentous and non-heterocystous cyanobacterium characterized by coiled cylindrical filaments or trichomes (Fig. 1), with a length of 200-500 µm, width between 3 and 12 µm and diameter varies from 30 to 70 µm (Ciferri, 1983). Spirulina species are reported to have three different forms as spiral, straight and wavy (Moreira, 2009). Moreover, they also have other numerous relevant cell components and inclusions such as stratified cell wall, DNA region, thylakoids, ribosomes, gas vacuoles, carboxysomes, phosphate granules and cylindrical bodies (Rangsayatorn *et al.* 2002; Belay, 2013; Noyma *et al.* 2015; Deschoenmaeker *et al.* 2016). However, morphology of Spirulina species is influenced by certain environmental variables such as temperature, pH, salt, light and nutrient availability (Kebede, 1997; Wu *et al.* 2005; Rosario and Josephine, 2015).

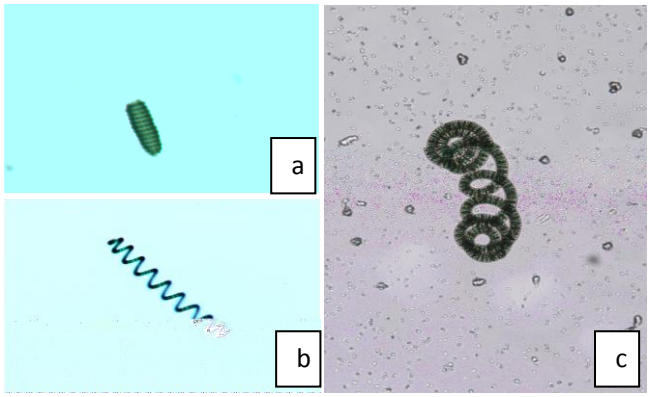

Figure 1. Morphotypes of *Arthrospira fusiformis* isolated from Lake Chitu: a = H-type, b = S-type, c = C-type. Microscopic observation from the present study

1.3. Ecology

Spirulina species are mainly found in tropical and subtropical regions of the world water bodies having high pH (8-11), carbonate and bicarbonate (Small, 2012; Belay, 2013; Kaggwa *et al.* 2013). They are alkalophilic, halophilic, thermophilic and extremophilic (Mandal and Rath, 2015). The extreme conditions of water bodies enable Spirulina growth with less possibility of contamination by other microorganisms and contribute for maintenance of monoalgal cultures in large scale production using outdoor ponds (Mühling, 2000). Both natural and human induced environmental changes are reported to influence the distribution of Spirulina and may favor other species of microorganisms (Belachew *et al.* 2012). In addition, nutrients in natural lakes, that either come from external sources as influxes, or from inside the water bodies via upwelling, are usually limited and influence the density of Spirulina population (FAO, 2008).

1.4. Spirulina Cultivation

1.4.1. Requirements of Spirulina Growth

Table 1. Composition of Zarrouk's medium (standard medium, SM)

Constituents	Composition (g/L)
$NaHCO_3$	18.0
$NaNO_3$	2.5
K_2HPO_4	0.5
K_2SO_4	1.0
NaCl	1.0
$CaCl_2.2H_2O$	0.04
Na_2EDTA	0.08
$MgSO_4.7H_2O$	0.2
$FeSO_4.7H_2O$	0.01
A 5 micronutrient sol.	1ml/L

A5 micronutrient solution consists of H_3BO_3, 2.86; $MnCl_2.4H_2O$, 1.81; $ZnSO_4.7H_2O$, 0.222; $CuSO_4.5H_2O$, 0.079; (NH_4) $6Mo7O_{24}$ (g/L) [Source: Nyabuto *et al.*, 2015].

Generally, microalgae require carbon, nitrogen and phosphorus as their major nutrients in addition to water and light for optimum growth (Harwati, 2013). Micronutrients such as potassium, magnesium, sulfur, calcium and iron are also commonly required by microalgae (Moreira, 2009; Markou *et al*. 2014). Zarrouk's medium is currently in use as a standard medium for the cultivation of Spirulina and contain various constituents (Tab. 1).

1.4.1.1. Carbon Source

Carbonates and bicarbonates are reported as best source of carbon for most microalgae particularly for *A. platensis* to carry out photosynthesis and biomass production (Jain and Singh, 2012; Sujatha and Nagarajan, 2013). Organic manures, Rice Husk Ash (RHA) and $NaHCO_3$, Na_2CO_3, organic carbon (such as sodium acetate, glucose, glycerol, etc.) are also reported as a source of carbon for Spirulina cultivation (Usharani *et al*., 2012). Carbon dioxide from the atmosphere and industrial source can also be used as a source of carbon (Singh and Singh, 2014). This fixation of CO_2 as a carbon source is important both from the economic and environmental point of view, since it tends to reduce the effects of global warming. Thus carbon may be taken up by Spirulina from both organic and inorganic forms.

1.4.1.2. Nitrogen and Phosphorous

Nitrogen is the most important nutrient playing an important role in the production of Spirulina biomass. It can be utilized for the synthesis of amino acids, nucleotides, chlorophylls and phycobilins (Harwati, 2013). Differently from other cyanobacteria that are nitrogen-fixing, Spirulina needs external nitrogen sources. Nitrate, ammonia and urea are reported as the commonly used sources of nitrogen (Avila-Leon *et al*., 2012; Madkour *et al*., 2012; Nor *et al*., 2015). Wastewater having high concentrations of nitrogen can also be used as a source of nitrogen (Nor *et al*., 2015). The nitrogen content of microalgal biomass is reported in the range of 1% to more than 10% and this may vary depending on its supply and availability, types of groups and species of microalga (Grobbelaar, 2004). Phosphorus is another essential nutrient playing an important role in the metabolism process of ATP, DNA, RNA and phospholipids (Nyabuto *et al*., 2015). It is commonly found in the form of $H_2PO_4^-$ or HPO_4^{2-} and wastewater can also be used as the cheapest source of phosphorus (Markou *et al*., 2015). Phosphorous can also contribute for the reaching the best production of highest biomass, chlorophyll a and protein content of Spirulina (Nyabuto *et al*., 2015).

1.4.1.3. Other Growth Nutrients

Graham *et al*. (2009) reported that magnesium, iron, silicon, sulfur and other trace elements are important growth nutrients for microalgal growth for the biosynthesis of different compounds. Iron is a cofactor for many enzymes such as, ferredoxins, catalases, glutamate synthetases, nitrogenases, nitrates and nitrite reductases (Kean *et al*., 2015). Sulfur, being a cofactor for the enzymes nitrogenase and CoA, it can also be required for the biosynthesis of some amino acids like cysteine and methionine. Magnesium is required mainly for the biosynthesis of chlorophyll (Usharani *et al*., 2012).

1.4.1.4. Environmental Factors

Major environmental parameters such as light, temperature, salinity, CO_2 addition, nutrient addition, inoculation size, stirring, pH, etc. have been studied and influence the growth, chemical composition and productivity of microalgae including Spirulina (Goksan *et al*., 2007; FAO, 2008; Ravelonandro *et al*., 2011; Almahrouqi *et al*., 2015). Therefore, these mentioned and other environmental factors should be maintained at the optimum range to increase production and biomass of Spirulina.

A) Light

Light is one of the important environmental factors required by Spirulina during different phases of its growth (Kebede and Ahlgren, 1996). Ogawa and Teuri (1970) pointed out that the optimum light intensity for Spirulina growth is between 20 and 30 K lux. This report has also showed that changing the color of the light intensity via covering the fluorescent bulbs can influence the yield of chemical compositions and products of Spirulina. The photosynthetic efficiency of most microalgae is in the range of 4.5 - 7% in the presence of low to moderate light levels in pond and closed system production technologies (Scragg *et al*., 2002).

B) Temperature

Temperature is also fundamental climatic factor influencing the growth rate of Spirulina (Rafiqul islam *et al*., 2003; Hizarci Uslu *et al*., 2009). Although optimal growth temperature varies by species, strain and constituents of the culture medium, most of Spirulina species optimally grow in the range of 30-35℃ (Jourdan, 2001). A temperature above 35℃ is dangerous, leading to possible bleaching of cultures (Usharani *et al*., 2012). Moreover, temperature can also affect the biochemical compositions of Spirulina (Rafiqul islam *et al*., 2003). Thus temperature is a sensitive parameter of algae regarding growth and metabolic activities.

C) pH

Spirulina is reported mainly to be found in natural alkaline lakes having pH value between 8-10 (Goksan *et al*., 2007; Rania *et al*., 2011; Ravelonandro *et al*., 2011; Joshi *et al*., 2014; Nyabuto *et al*., 2015). Conversely, extreme pH values potentially have a direct effect on the physiology of the algae and availability of nutrients (Usharani *et al*., 2012). However, optimal pH values vary with species types and strains, composition of the growth medium and cultivation conditions (Ismaiel, 2016). Some authors reported that pH determine the solubility of carbon source and minerals in the culture medium (Pandey *et al*., 2010). It also influence protein and chlorophyll content of *A. platensis* (Sharma *et al*., 2014).

D) CO_2

Increased CO_2 concentration can affect the pigment content of algae and thus decreases the maximum biomass yield (Gordillo *et al*., 1999). CO_2 can also be utilized as inorganic gaseous carbon source for photosynthesis process. However, excess amount of CO_2 can cause inhibition of biomass growth by excess carbon (Singh and Singh, 2014). The optimum range of CO_2 concentration required by *Spirulina platensis* is reported to be 10-15% (Kumar *et al*., 2010; Sydney *et al*., 2010). However, most microalgae are reported to grow well in the range of 1-5% (v/v) CO_2 concentrations (Harwati, 2013). This last report also showed that CO_2 also affect the biosynthesis of lipid by several species of microalgae. CO_2 can also affect the contents of pigments such as phycocyanin, chlorophyll a and carotenoides in *A. platensis* (Gordillo *et al*., 1999).

1.5. Applications of Spirulina

Nowadays the cultivation of Spirulina is recognized worldwide as important and profitable business in biotechnological industries and is considered a so-called super food, due to the high diversity of nutritional compositions. In addition to its use as a nutrient source for both human food and animal feed, it can also be applicable to food colorant industry, cosmetics, medicine, energy production, wastewater remediation and CO_2 mitigation as explained as follows.

1.5.1. Human Food and Animal Feed

Spirulina is becoming a known food for human worldwide being a rich source of many nutritional components such as proteins, vitamins, amino acids, fatty acids, minerals, pigments etc. (Ali and Saleh, 2012; Chu, 2012). In addition, nutritional components of Spirulina have been proven to play great role in promoting healthy body functions of consumers and thus reduce the risk of disease by enhancing the immune system (Hoseini *et al*., 2013; Rosario and Josephine, 2015). Its high organoleptic properties and non-toxicity make it a safe food or food supplement for human consumption. Studies have also shown that in many African countries Spirulina is directly collected from natural water bodies, dried and eaten as a cake, particularly by those who live around Lakes (FAO, 2008). Spirulina has also been proved to be a potential feed resource to many agriculturally important animal species such as chickens, pigs, cattles, ruminants, sheep and rabbits (Holman and Malau-Aduli, 2013). This last report also showed that it improves the animals' health, growth, productivity and quality. About 50% of the current world production of Spirulina is used as feed supplement to poultry and different animals (Zahroojian *et al*., 2013).

1.5.2. Aquaculture

Spirulina is also widely used in aquaculture activities as a supplement of diet mainly for fish larvae and juveniles of both zooplankton and fish (FAO, 2008; Sirakov *et al*., 2015). It has been also used for stabilization of the culture medium, stimulation of immune system and for their probiotic effects (Irianto and Austin, 2002). Spirulina can also be used as a source of natural pigments for the culture of prawns, salmonid fish and ornamental fish (Priyadarshani and Rath, 2012; Teimouri *et al*., 2013). It can also be consumed as live feeds by bivalve molluscs (e.g. oysters, scallops, clams and mussels) during all stages of their growth, whereas abalone, crustaceans and some fish species consume it during their juvenile stages (Sirakov *et al*., 2015).

1.5.3. Spirulina in Cosmetics Industry

Microalgal products can also play a great role in cosmetics industry as thickening agents, water-binding agents and antioxidants (Priyadarshani and Rath, 2012; Wang *et al*., 2015). The extracts can also be found in face, skin and hair care products and sun protection creams (Stolz and Obermayer, 2005). Representative species commonly used in the cosmetics industry are: Spirulina, *Chlorella*, *Chondrus crispus*, *Mastocarpus stellatus*, *Ascophyllum nodosum*, *Alaria esculenta*, *Spirulina platensis*, *Nannochloropsis oculata*, *Chlorella vulgaris* and *Dunaliella salina* (Priyadarshani and Rath, 2012). In addition, Spirulina extracts can also be used to produce cream for the treatment of wounds in animals.

1.5.4. Microalgae and Food Colorant

Microalgae produce various substances containing pigments such as beta carotene, astaxanthin, lutein, canthaxanthin, zeaxanthin, lycopene, bixin and chlorophyll used for coloring of food (Prasanna *et al*., 2007). Spirulina is a known source of natural phycocyanin which has commercial values in food industries as it is used as a natural food colorant and additive (Gouveia *et al*., 2008b). It can also be used as cosmetic coloring (blue color extract). However, the potential use of microalgal products as natural food coloring has limitations as it is not photo stable (Priyadarshani and Rath, 2012). In addition to being used as coloring agent in food industries, it can also give a pink color to the feather of flamingo birds which feed on Spirulina (Small, 2012).

1.5.5. Environment and Agriculture

Microalgae have been used in bioremediation techniques to protect the environment from contaminants, hazardous substances and organic pollutants. Spirulina has been confirmed to have great role in wastewater treatment process as heavy metal remover for treating water contaminated with metals such as copper and cadmium (Small, 2012). Spirulina can also survive in highly alkaline and other extreme environmental conditions where contaminants do not grow, indicating its importance from environmental application point of view. Moreover, Spirulina can also be used as a source of biofertilizer, as it has high nitrogen and phosphorous content and thus it is important for the growth and development of agricultural plants and for soil

conditioning (FAO, 2008). Therefore, Spirulina could be a cost effective alternative to chemical fertilizer in agricultural sectors (Wuang *et al*., 2016).

1.5.6. Biofuels

Microalgae are considered good sources of biofuel production in comparison to other crops. Their high protein content and the rapid biomass production make them to be preferred and become an interest of researchers, entrepreneurs and the general public (Priyadarshani and Rath, 2012). The extraction of oil from microalgae (different from Spirulina) is estimated to be greater than 80% (on dry weight basis) and average annual biodiesel yield of 98.4 m^3 per hectare (Rajvanshi and Sharma, 2012). Biofuel production from microalgae genera including Spirulina has been reported to reduce CO, hydrocarbons and other particulate matter emissions (Sarpal *et al*., 2016). Biofuel production from cyanobacteria is also profitable from the economic point of view as it can grow easily using wastes or seawater (Small, 2012).

1.5.7. Microalgae in Pharmaceutical Industries

Products of microalgae play great role in the pharmaceutical industry. These products are mostly bioactive compounds containing carbohydrates, organic acids, amino acids and peptides, vitamins, growth substances, antibiotics, enzymes and toxic compounds of pharmaceutical importance (Rania and Hala, 2008; Chu, 2012). Spirulina produces various bioactive metabolites (primary and secondary metabolites) having antifungal, antibacterial, antiviral, antibiotic, anticancerous, antidiabetic, antianaemic and antileucopenic effects (Ali and Saleh, 2012; Vijayakumar and Menakha, 2015; Mazard *et al*., 2016; Sowjanya and Manjula, 2016). The co-occurrence of different algal species in the natural aquatic habitat contributes the existence of these bioactive compounds because of antagonistic interactions among them (Priyadarshani and Rath, 2012).

Various studies have been done on ecology, morphology, diversity, cultivation, biological composition and applications of Spirulina by various researchers (Chu, 2012; Priyadarshani and Rath, 2012; Harwati, 2013; Marrez *et al*., 2014; Otago and Kifle, 2014; Hafidh *et al*., 2015; Nyabuto *et al*., 2015). But investigation on strains of potential interest for growth specifically in tropical regions is not well documented and needs to be explored. In the present study, laboratory cultivation, characterization, identification and maintenance of those strains sampled from tropical alkaline lake of Ethiopia was carried out. Moreover, malnutrition is a major problem in developing countries having high population growth resulting in high food demand. Thus, there is inadequate supply of nutrient rich foods and high children mortality rate has been reported frequently (FAO, 2008; Moreira, 2009; Albert *et al*., 2012). Therefore, using Spirulina as a source of those nutrients could be the best alternative to combat malnutrition in different parts of the world. In addition, Spirulina isolated from tropical climatic conditions have been reported as potential candidates as a source dietary supplements and several bioactive compounds (Ciferri, 1983; Pal *et al*., 2011; Harwati, 2013; Otago *et al*., 2014). Thus, isolation, cultivation and maintaining those potential strains is required for their potential applications such as for human and animal nutrition, energy, environment, medical and cosmetics. Ethiopian alkaline soda lakes could be a potential source of Spirulina (Fetahi, 2016; Genene Tefera *et al*., 2016; Otago *et al*., 2016). Nowadays, Spirulina has got worldwide attention for being rich source of different nutrients and their benefits. Therefore, this study would give valuable baseline information about tropical microalgae, particularly Spirulina, for further study and use.

2. Materials and Methods

2.1. Descrition of the Study Area

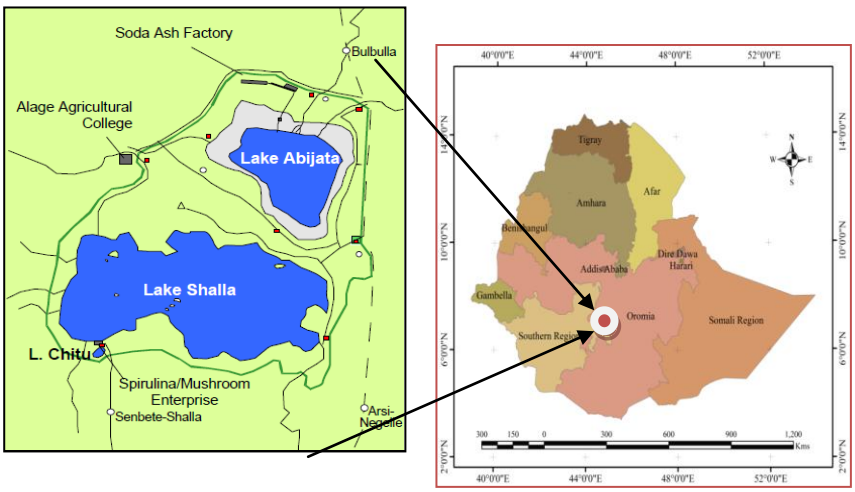

Figure 2. Location of study sites in Ethiopia (*Source: Adapted from Reaugh-Flower, 2011*)

Lake Abijata, Shalla and Chitu are among the tropical creater soda lakes of Ethiopia. They are located in the Ethiopian rift valley at a distance of about 285 km south of Addis Ababa at a geographical position of 7° 37′ 0″ N, 38° 36′ 0″ E, 7° 29′ 0″ N, 38° 32′ 0″ E and 7°24'13"N 38°25'16"E respectively (Legesse *et al.* 2002; Otago and Kifle, 2014). Lake Abijata is relatively the shallowest (<7m deep) with area of 180 km^2 (Kumssa and Bekele, 2014). It is small alkaline closed lake in central Ethiopia, along the Ziway Shalla basin (Fig. 2). It lies in a saucer-shaped hollow within a deep faulted trough at an altitude of 1580 m above mean sea level (Tenalem, 2002). The water in Lake Abijata is saline and soda-type having pH of about 10 and salinity of 16.2 g/l (Tenalem, 2002; Lemma, 2016). Lake Chitu is the smallest soda lake having an area of 0.8 km^2 and maximum depth of 21 m and is known for almost its monospecific population of *Arthrospira fusiformis* (Otago and Kifle, 2014). These authors also reported that the lake is characterized by environmental conditions such as high pH, salinity, alkalinity, Na^+ and Cl^- ions and limiting levels of inorganic nitrogen compounds which are proved to be ideal for the optimum growth of Spirulina species. In addition, the location of the lake is characterized by semi-arid to sub-humid type of climate with mean annual precipitation and temperature of 600 mm and 25℃, respectively (Legesse *et al.* 2002). Lake Shalla is relatively the deepest (266 m deep) and covering an area of 370 km^2 having high saline-alkaline water but less saline-alkaline than Lake Chitu (Tenalem, 2002; Otago *et al.* 2014). They also reported that Lake Shalla lies at an altitude of 1550 m a.m.s.l, having a water surface temperature in the range between 22 and 26℃ and less productive, although its saline-alkaline conditions are suitable for the growth of Spirulina.

2.2. Sample Collection

Spirulina dominated water samples were collected 10 cm below the surface from Lakes: Abijata, Shalla and Chitu following standard methods described by (Rout *et al*., 2013) using plankton net and sample bottles. Salinity, pH and temperature were measured in situ using a portable water quality checker (U-10, USA). Photosynthetically Active Radiation (PAR) on the surface of the lake was also measured using photometer (HS1010, USA). After that all samples were transported to the laboratory of general botany, University of Florence, Italy for Spirulina isolation, cultivation and characterization.

2.3. Isolation and Culturing

A few drops of samples were put on to glass slides and observed with the optical microscope to verify the presence of Spirulina, after thoroughly mixing them. Slides having Spirulina were washed with Zarrouk medium and the mixture was collected in small sterilized beaker. Afterwards, the aliquot was transferred into sterilized flasks for growth. In addition, 1-2 ml from the original sample was also taken using pipette and inoculated in to 250 ml of Erlenmeyer flasks containing sterilized zarrouk medium. All inoculated tubes were incubated in a growth chamber at 27 ± 3 ℃ under continuous illumination with fluorescent white lamp with light intensity of 215 μ mol photon m^{-2} s^{-2} for 30 days to allow them to grow with greater variability of morphotypes. To improve CO_2 availability, agitation of the flasks and tubes containing the growth were carried out 3 times a day as proposed by Nyabuto *et al*. (2015). Continuous follow up of the growth conditions of cultures were done by observing their thallus under the microscope. During the process of their growth fresh sterilized Zarrouk media was also added to the cultures to improve nutrient availability for their growth. Several successive transfers have been also carried out using Zarrouk media to purify the cultures and refresh them to increase their concentration for further analysis and treatment.

2.4. Cultivation under Different Concentrations of Nitrogen Source

The experimental organisms, Spirulina species isolated from water samples of Lake Chitu were cultivated under different concentrations of NH_4NO_3 using Zarrouk media. These different concentrations of nitrogen source were considered to evaluate their effect on the growth and biomass of Spirulina species according to methods described by Costa *et al*. (2004); Madkour *et al*. (2012); Sharma *et al*. (2014); Castro *et al*. (2015). The nitrogen source in the Zarrouk media sodium nitrate ($NaNO_3$) was replaced by ammonium nitrate (NH_4NO_3) with concentrations: 0.01 M, 0.03 M and 0.05 M. Spirulina (10%) was added to 250 ml sterilized Erlenmeyer flasks containing sterilized Zarrouk media with (0.01 M, 0.03 M and 0.05 M) concentrations of NH_4NO_3. The experiment was carried out in triplicates allowing the cultures to grow under growth chamber at 27 ± 3℃ for 33 days. Cultures were shaked manually three times a day with 12h: 12h photoperiod cycle and illuminance of 215 μ mol photon m^{-2} s^{-2}.

2.5. Characterization and Identification of Isolates

Characterization of the isolates based on their morphological parameters such as degree of coiling, number of coils per trichome, color of trichome, trichome end attenuation, trichome length, trichome diameter, helix diameter (at the middle and at the end) and helix pitch were carried out using optical microscope (Leica Microscopie & Systeme GmbH. Wetzlar, Germany). On the basis of their level of coiling, isolates were grouped into three different morphotypes as H-type (tightly coiled), S-type (loosely coiled) and C-type (intermediate). For each morphotype both qualitative and quantitative description of the above morphological parameters were made using optical microscope (Leica Mikroskopie & Systeme GmbH Wetzlar, Germany) at 45X and 100X magnifications. Molecular characterization using 16S rRNA sequences of the cultivated isolates was also carried out for further checking the variability among the species as described below based on

standard protocols.

2.5.1. Morphological Identification Using Optical Microscope

Drops from each triplicate samples were taken and put to glass slides. The different forms of morphotypes were identified and images were captured with a Nikon camera inbuilt in the microscope (DIGITAL SIGHTDS-LT, JAPAN). The above mentioned morphological parameters were carefully observed, measured and counted under 45X and 100X magnification power of optical microscope (Leica Microscopie & Systeme GmbH. Wetzlar, Germany). All examined morphological parameters were compared with previously described standard keys.

2.6. Growth Measurements

Biomass concentrations of the growing cultures during cultivation were determined by measuring optical density (OD) using direct reading spectrophotometer (HACH DR/2000, U.S.A). Absorbance of Spirulina samples were read at 680 nm in the interval of three days. Each measurement of optical density was recorded to make a curve indicating Spirulina biomass concentration versus the growth period and concentration of nutrients. Optical density measurement was continued until decline phase of the cultures was attained (33 days). Protocols described by Costa *et al*. (2004); Harwati, (2013) and Joshi *et al*. (2014) were used.

2.7. Maintenance of Selected Strains

The pure isolates of Spirulina strains were maintained using Zarrouk medium as modified (composition: 10 g $NaHCO_3$, 0.5 g K_2HPO_4, 2.5 g $NaNO_3$, 1.0g K_2SO_4, 1.0 g NaCl, 0.20 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.08 g EDTA and 0.04 g $CaCl_2$) by (Ravelonandro *et al*., 2008) in Erlenmeyer flasks (250 ml capacity). During the process of growth they have been provided with a continuous illumination of 215 µ mol photon m^{-2} s^{-2} at 27 ± 3℃ with manual shaking three times a day under 12:12 hour light-dark cycles (Joshi *et al*., 2014). The pH of the media was maintained to the optimum (9.5-10) range and checked continuously for being within the specified range. They can be maintained (preserved) for short, medium and long term (Rout *et al*., 2013).

2.8. Statistical Analysis

Both SPSS (version 24) and PAST (version 3.15) statistical programs were used to analyze the morphological data obtained from this study. Morphological parameters used to characterize morphotyes of Spirulina were subjected to One-way ANOVA and Tukey's HSD (Honest Significant Difference) test using SPSS at 95% confidence interval with marginal error of 5% to verify the variability of these parameters among and within morphotypes of Spirulina. Histograms, graphs and tables were used to display some of the main outputs of SPSS. The data obtained from optical density measurements were also subjected to One-way ANOVA using SPSS at 95% confidence interval to see statistical significant differences among growth of Spirulina under different concentrations of NH_4NO_3. Statistical values of $P < 0.05$ were considered significant.

3. Results

3.1. General Overview of Morphology and Morphological Parameters

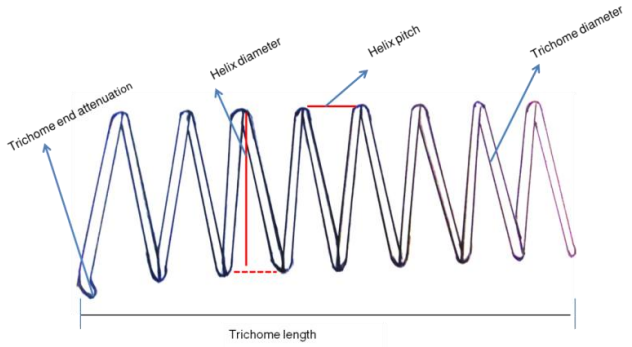

Figure 3. Parts of Spirulina filament (morphological parameters considered in this study)

Based on the observations with optical microscope Spirulina isolates in the present study were categorized in three morphotypes mainly on the basis of degree of coiling of trichomes and trichome end attenuation. Moreover, other parameters such as trichome length, trichome diameter, helix pitch, helix diameter and number of coils (Fig. 3) were measured and quantitatively determined. The three identified morphotypes were H-type, S-type and C-type (Fig. 4 A-C). These morphotypes may represent or share morphological characteristics between more *Arthrospira* species such as *Arthrospira fusiformis*, *Arthrospira maxima* and *Arthrospira platensis* even though they are not stable, since transformation of one morphotype to another may occur as a function of growth conditions during cultivation.

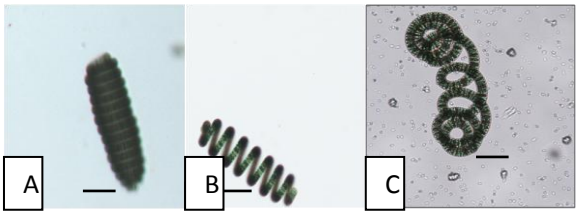

Figure 4. Microscopic view of Spirulina morphotypes of Lake Chitu: (A) H-type; (B) S-type; (C) C-type. Scale bars = 250 µm

As shown above the morphotypes were mainly explained by the difference in degree of coiling as highly coiled (H-type), loosely coiled (S-type) and intermediately coiled (C-type). Similarly differences in other parameters have been observed, measured and compared using Tukey's HSD test for Post Hoc multiple comparisons (Fig. 5 A-E; App. 1). Change or breakage of spiral could occur if there is intense light, even under the microscope during the observation.

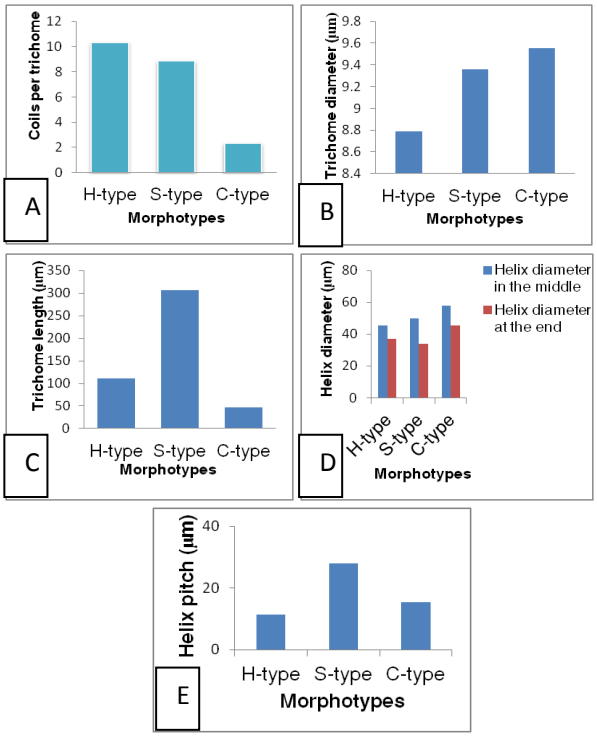

Figure 5. Mean value comparison of morphological parameters of the morphotypes of Spirulina of Lake Chitu: (A) coils per trichome; (B) trichome diameter; (C) trichome length; (D) helix diameter; (E) helix pitch

Results of Post Hoc tests for multiple comparisons indicated that both H-type and S-type are significantly different ($p < 0.05$) from C-type in the number of coils per trichome (MD = 6.50, SE = 0.68 and MD = 6.50, SE = 0.68 respectively) (Fig. 5 A, App. 1). Similarly, trichome length (MD = 195. 95, SE = 21.82) and helix pitch (MD = 16.57, SE = 2.94) are significantly different between H-type and S-type and S-type and C-type. In contrast, trichome diameter, helix diameter in the middle and helix diameter at the end did not show significant differences ($P > 0.05$) among the three morphotypes (App. 1). H-type and S-type did not also show significant difference in terms of number of coils per trichome (MD = 1.5, SE = 0.68, $p > 0.05$). Color varied from deep green to light green among the morphotypes.

3.2. Growth under Different Concentrations of Ammonium Nitrate

We prefer to use ammonium nitrate as an alternative nitrogen source due to its cost effectivness and ease of availability in Africa. It can be locally available being used as a normal agriculture fertilizer. Therfore, it can be used for the biomass production of Spirulina in small and large scale commercial production industries. Moreover, a comparable yield and biomass production using standard medium and cheaper medium containing alternative nitrogen sources have been reported (Costa *et al.* 2001; Nor *et al.* 2015; Mashor *et al.* 2016). The results about the relationship between the OD and growth period graphs (Fig. 6) revealed that, the optimum growth of Spirulina depends on the concentration of NH_4NO_3. At the beginning of the growth period there was lower growth but it gradually increased through time. The best growth of Spirulina has been observed at 0.03 M NH_4NO_3 concentration in comparison with 0.01 M and 0.05 M (Fig. 6). However, the growth recorded at 0.05 M NH_4NO_3 concentration was significantly lower (M = 0.28 nm; P = 0.033; SD = 0.18) than values observed at 0.01 M and 0.03 M concentrations. There was no significant difference between growth of Spirulina observed at 0.01 M (M = 0.36 nm; P = 0.056; SD = 0.23) and 0.03 M (M = 0.47nm; P = 0.062; SD = 0.24) concentrations of NH_4NO_3. The growth in the control with $NaNO_3$ increased continuously until day 18 unlike growth of different concentrations of NH_4NO_3 in which increments lasted longer (Fig. 6). Lower growth rates have been observed in the end of growth periods in 0.01 M and 0.03 M concentrations of NH_4NO_3 and in the control. On the contrary gradual increments towards the end have been observed at 0.05 M concentration.

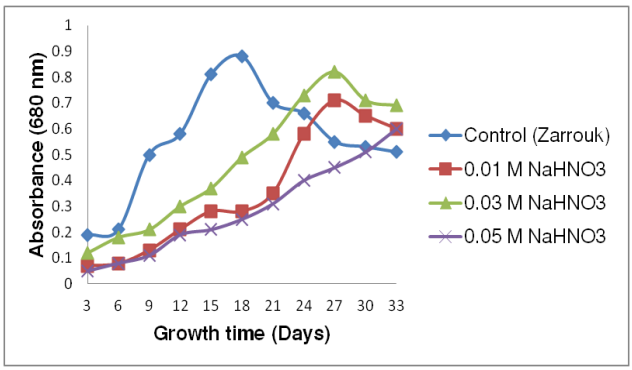

Figure 6. Optimization of Spirulina growth using different concentrations of ammonium nitrate as an alternative nitrogen source

4. Discussion

Morphotypes of *Arthrospira* based on morphological parameters and optimization of growth conditions under different concentrations of ammonium nitrate were investigated in this study. Furthermore, morphological parameters that mainly describe the morphotypes of *Arthrospira* were identified and suggested to use in further studies.

4.1. General Overview of Morphology and Morphological Parameters

Based on microscopic observation of *Arthrospira* isolates from the current study, three distinct morphotypes of *Arthrospira* as H- type (tightly coiled), S- type (loosely coiled) and C- type (intermediately coiled) have been recognized. Degree of coiling of the trichomes and trichome end attenuation were frequently used to group the morphotypes during microscopic observations. This is due to the phenotypic variation among morphotypes in terms of these two parameters. However, morphological parameters only were proved to be not reliable criteria for the taxonomic classification of *Arthrospira* (Jeeji Bai and Seshadri, 1980,

Li *et al.* 2001) and see the taxonomic note in this thesis. Furthermore, most of these morphological parameters can vary with the influence of environmental parameters and nutrient conditions (Mü-hling, 2000; Kim *et al.* 2007; Kaggwa *et al.* 2013; Otago and Kifle, 2014). As reported from Belay (1997), the degree of coiling has a direct relationship with temperature. Besides that, these authors also mentioned mechanical stress during harvesting is another factor causing morphological changes in *Arthrospira*.

These three morphotypes of *Arthrospira* were already identified by Otago and Kifle (2014) in the same study site including their variability as a response to changes in environmental variables. Similarly, they have been also recognized in other parts of the world including: India (Jeeji Bai and Seshadri, 1980) and Kenya (Kaggwa *et al.* 2013). However, these authors did not include ultrastructural and molecular aspects that are not well documented in previous studies of *Arthrospira*.

H-type and S-type have significantly larger number of coils than C-type. As the tendency of coiling increases, the number of coils per trichome also increases, which implies the length of trichome also increases. Hence all these measures are positively correlated. Helix pitch has also shown a significant difference among the morphotypes, as it is an indicator of the degree of coiling and therefore could vary between the filaments of H-type, S-type and C-type morphotypes. On the other hand, trichome diameter and helix diameter (in the middle and at the end) did not show any significant differences among the morphotypes. Anyway, these morphotypes are not stable as there could be transformation of one morphotype to another since they are highly dependent on nutrient conditions and environmental parameters (Ballot *et al.* 2004). They may be hence useful as bioindicators of a mutation of the environmental conditions of medium in cultivation of the natural medium in the lakes where *Arthrospira* sp. can be found.

In support to our finding, Otago and Kifle (2014) and Jeeji Bai and Seshadri (1980) reported that trichome end attenuation is a good parameter for recognizing the morphotypes in addition to helix diameters (in the middle and at the end). Although these and other morphological parameters are regarded as not sufficiently reliable to recognize the taxonomic position of *Arthrospira* species, they still are in use to distinguish them (Mü-hling , 2000; Thammathorn, 2001; Kaggwa *et al.* 2013; Rout *et al.* 2015).

4.2. Growth under Different Concentrations of Ammonium Nitrate

Nutrient type and concentration are the major limiting factors influencing the growth of microalgae including *Arthrospira* (Dejsungkranonta *et al.* 2012; Harwati, 2013). At the beginning of the cultivation not much growth has been observed (Fig. 6). This indicates that *Arthrospira*, like other prokaryotes, follows different growth phases (Qasim *et al.* 2012). First a lag phase at the start, is a phase in which they adapt to a new culture medium. After the lag phase a log phase (exponential phase) follows. In this phase cells are growing at higher rate due to optimal growth conditions. The third phase is the stationary phase, in which nutrients start to decline together with the biomass of cells. Finally cells enter the decline (death) phase, in which nutrients are totally depleted and cells are dying at high rate with the exception, in our experiment, of the 0.05 M concentration in which still gradual increments towards the end of our experimental period have been observed. This result could possibly be explained by its inhibitory effect at the beginning of growth due to its concentration and a better adaptation at the end of the growth period. Generally, the best growth of *Arthrospira* in terms of growth speed, has been observed at 0.03 M NH_4NO_3 concentration, indicating that *Arthrospira* requires NH_4NO_3 at this concentration as an optimal growth medium. This is in agreement with results in similar studies (Nor *et al.* 2015; Ismaiel *et al.* 2016), in which a higher concentration of nitrogen sources could have inhibitory effect on the growth of *Arthrospira*. Nitrogen is also reported as one of the main requirements for *Arthrospira* anabolism, such as for the synthesis of amino acids (proteins), phycocianin and other cellular components (Uslu *et al.* 2011). Ammonium nitrate can be used as an alternative nitrogen source for small and large scale commercial cultivation of *Arthrospira* biomass. It is relatively inexpensive with respect to other nitrogen sources (as $NaNO_3$) and is locally more easily available than other nitrogen sources. It has been preferred not only for being cheaper and easily available but also for its high productivity. Moreover, the different concentrations of ammonium nitrate used could contribute for better understanding of preferred optimal concentrations for growing *Arthrospira*. Similarly, it has been proven by other studies (Colla *et al.* 2007; Belay, 2013; Nor *et al.* 2015; Mashor *et al.* 2016) that ammonium nitrate is the best alternative cheaper nitrogen source for biomass production of *Arthrospira*, thanks to its widely distributed use in traditional agriculture. This could also contribute to scale-up Spirulina production with reduced cost.

4.3. Overall Justification, Recommendations and Perspectives

We planned to address *Arthrospira* species in three soda lakes of Ethiopia: Chitu, Abijata and Shalla. Unfortunately, we did not find *Arthrospira* growth from samples of Lake Abijata and Shalla during the successive laboratory cultivation. However, the main aim of this study was to cultivate *Arthrospira* sp. in tropical conditions in order to evaluate it for possible industrial applications. Therefore, the absence of *Arthrospira* sp. isolates from the above mentioned sampling sites did not affect the desired goal of the research. Thus, *Arthrospira* sp. isolates reported in this study were isolated only from samples of Lake Chitu. Reports indicated that Lake Chitu is known for its monoalgal population of *Arthrospira fusiformis* (Kebede *et al.* 1994; Kebede 1996). Nonetheless, we should keep in mind the

complex taxonomic situation about many species belonging to genus *Arthrospira* (section 1.2) and in general the difficult delimitation of species in Cyanobacteria (Dvorak *et al.* 2015).

In any case, on the basis of our study, we cannot conclude that other *Arthrospira* species are absent from Lake Abijata and Lake Shalla for the following reasons: (1) We collected a smaller number of purposive samples targeted for cultivation, (2) part of the sampling points in the lake may not harbor *Arthrospira* as this cyanobacterum may be distributed in different parts of the lake, (3) they may not survive in the time gap between transportation and cultivation in the lab, and (4) current physicochemical and biological changes in the lake induced by natural (seasonal climatic variation) or anthropogenic activities which could affect the abundance of phytoplankton communities including *Arthrospira* species. Due to these changes particularly in the Lake Abijata, *Arthrospira* species are currently largely replaced by non *Arthrospira* species (Kumssa and Bekele, 2014; Fetahi, 2016). In addition we suggest that a larger number of samples with representative sampling points in each lake during different season needs to be addressed to reach a certain consensus and to avoid confusions about the presence or absence of *Arthrospira* species in these lakes. The abundance of *Arthrospira* may vary with seasons of a particular area. This could also help to compare the variability of *Arthrospira* species in each lake. *Arthrospira fusiformis* was one of the dominant species of algae in Lake Abijata during 1960 to 1988 (Wood and Thalling, 1988). However, there are no research reports showing the presence of *Arthrospira* sp. from Lake Shalla even though the water chemistry of the lake and climatic conditions of the location were reported to be suitable for *Arthrospira* sp. growth (Otago *et al.* 2014). This partly supports our current report. Generally, tropical soda alkaline lakes are assumed to be hotspots of *Arthrospira* species and thus further multidisciplinary investigation needs to be carried out to have better understanding of the biology of the lakes including their potential to support the growth of *Arthrospira*. To further clarify the taxonomic positions and the variability among *Arthrospira* species of the above mentioned lakes, we suggest assessing large number of strains using morphological, ultrastructural and molecular approaches considering the present work as a baseline data. As the ultrastructural study of *Arthrospira* sp. of Lake Chitu is not documented previously, further details of ultrastructural features may be required for better understanding the morphological variations within the genus and could be an input to the taxonomic study. Nowadays Spirulina obtain worldwide attentions due to its overall high nutritional quality and quantity of compositions and values. Therefore, small scale and large scale production of Spirulina is required to solve malnutrition problems in developing countries in which the problem is prominent till to date. Besides these, its safety and non toxicity are important characteristics contributing to its acceptability. From this point of view an exact knowledge about the contaminants as those identified in this investigation may be fundamental for assessing the quality of the Spirulina produced locally.

There is a need to establish a collaborative research projects between institutions, Non-Governmental Organization (NGO), entrepreneurs and researchers to explore this important natural resource of tropical alkaline lakes such as Lake Chitu for large scale commercial production. This will help to develop various biotechnological applications by Spirulina producing industries. Although a lot of research have been done previously in various aspects of Spirulina of Lake Chitu, from the practical and biotechnological point of view further investigations such as outdoor Spirulina farm and indoor laboratory cultivations are required.

4.4. Conclusions

Laboratory cultivation of Spirulina under controlled conditions is an important prerequisite which could help to set optimal growth parameters as well as to obtain pure cultures for outdoor or large scale biomass production. This may in turn contribute a lot for malnutrition problems in addition to its economical advantages. From the morphological data of our study it has been confirmed that there are three different morphotypes of *Arthrospira* belonging most probably to a single species. The morphological parameters described in this study have shown variation among the morphotypes. Since the occurrence of the different morphotypes appear to be related to the environmental parameters of the medium, they may be hence useful as bioindicators of a mutation of the environmental conditions in cultivation of of the natural medium in the lakes where *Arthrospira* sp. can be found. Further investigation will be necessary to exactly understand the related ecological conditions that lead to specific morphotypes.

Spirulina isolates were further grown under different concentrations of ammonium nitrate (alternative nitrogen source) and a relatively low concentration (0.03 M) could be optimum for their growth. In general, from the data of this study it can also be concluded that Lake Chitu is one of the most important natural area to investigate on several aspects of *Arthrospira* species since this lake appear to be a part of the original distribution area of the species.

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