EVALUATION OF THE POTENTIAL OF SHALA HOT SPRING WATER FOR THE BIOMASS PRODUCTION OF *ARTHROSPIRA FUSIFORMIS (VORONIKHIN) KOMEWK & J.W.G. LUND*

Master of Science Thesis

by

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ACRONYMS

B	Biomass
CCA	Canonical Correspondence Analysis
chl-a	chlorophyll-a
dt	doubling time
GF/C	Glass microfiber filter/course
GF/F	Glass microfiber filter/fine
HSD	Honest Significant Difference
P	Corresponding probability value
RDA	Redundancy analysis
SM	Standard medium
SM25	25% standard medium- supplemented spring water-based medium
SM50	50% standard medium- supplemented spring water-based medium
SM75	75% standard medium- supplemented spring water-based medium
SSM	Shala hot spring water medium
USAID	U.S. Agency for International Development
μ	Specific growth rate (d^{-1})

ABSTRACT

Arthrospira is a super food, which has attracted the attention of many governmental and non-governmental organizations as a means of combating malnutrition. However, the cost of production especially the media cost has become prohibitive to efforts being made in this direction. The high media cost has necessitated the search for ways of reducing it. In this study the potential of hot spring water-based media for the production of Arthrospira biomass was evaluated by using media supplemented with 0% (SSM), 25% (SM25), 50% (SM50), 75 % (SM75) and 100 % (SM) standard Arthrospira medium. The specific growth rate (μ, day^{-1}), biomass (B, as Chl-a and dry weight) of Arthrospira in relation to some chemical parameters of the culture media were evaluated. μ and B values comparable to those in SM were achieved using SM25%, while increase in the level of supplementation to 50% supported higher B. pH of culture media had significant ($P<0.01$) effect on the growth of Arthrospira. Unlike pH, salinity didn't have significant effect on the growth of Arthrospira ($P>0.05$) despite its significant variations ($P<0.05$) among the culture media. PO_4^-P and NO_3^-N did not significantly ($P>0.05$) vary among media except SSM, which was relatively nutrient-deplete. The addition of the standard medium is therefore, necessary to add bicarbonate–carbonates and nutrients and maintain optimal pH level, to promote biomass production of Arthrospira over longer period of cultivation. The present results show that use of Lake Shala hot spring water-based media can reduce the media cost by 50-75%. Further study on outdoor cultures using this hot spring water with optimized growth condition is recommended.

Key words: Arthrospira, Lake Shala-hot spring, media cost, biomass production, Chemical factors

1. INTRODUCTION

1.1. Background

Conventional agriculture is no longer capable of producing adequate food for the ever-increasing human population. Current farming practices have depleted our soils of minerals, while overuse of chemical fertilizers destroys microorganisms, which normally enhance the cycling of minerals required by plants for normal growth (Henrikson, 2009; David and Michael, 2013). Moreover, the current agribusinesses choose hybrid strains based on appearance, and the ease with the crops can be harvested and stored, rather than their nutrient content. The long shipping and storage time between harvest and sale also reduces nutrient content (Henrikson, 2009). As a consequence, a significant portion of the world's human population is suffering from hunger and/or malnutrition. Even though in 19 developing countries, the number of hungry people dropped by 80 million over ten years, hunger is on the rise (Heierli, 2007). Some 795 million people in the world do not have enough food to lead a healthy active life. That's about one in nine people on earth (FAO, 2015) with more than one in four people remaining undernourished in the Sub-Saharan region, which is the highest prevalence of any region in the world (FAO *et al.,* 2014).

Although Ethiopia has been experiencing strong and broad-based growth over the past decade, malnutrition is still a problem. It affects some 2.7 million people who are acutely food insecure. According to USAID (2014), malnutrition is under-nutrition: stunting, under-weight, wasting and micronutrient deficiencies or over-nutrition: overweight and obesity. Owing to the limited household access to safe, nutritious, and diverse foods, and inappropriate feeding and caring practices within large families and in families with short birth intervals, child malnutrition alone costs the Ethiopian government about 5.5 million dollars every year, which is 16.5 percent of Ethiopia's GDP (Orner, 2014). However, Ethiopia is a country, which is endowed with natural resources of land and water that can be the foundation for any economic development endeavour geared towards ensuring food security and fulfilling other basic necessities of its people (CSI, 1998).

Malnutrition has necessitated the search for alternative sources of human food. One of the potential areas of good quality food production for humans is aquaculture including algal culture- the cultivation of algae with high nutritional value such as *Arthrospira*. Even though *Arthrospira* has been used as food for centuries by the native peoples around Lake Texcoco

in Mexico and Lake Chad in Chad, Africa (Vonshak, 1997), it's needed for our world more than ever considering the nutritional depletion of modern foods through time. Diseases like cancer are directly related to environmental factors and an ever-growing portion of world population needs to boost its immune system to protect itself against pollution-related ailments, retard the aging process and develop resistance to diseases (Henrikson, 2009). *Arthrospira* can be the answer to all of the above problems. *Spirulina* (*Arthrospira fusiformis Voronikhin) Komarek & Lund (1990)* is a super food, which contains a nutrient profile higher than any other food, plant, herb or grain and, which can be easily absorbed by the human body. It has the highest protein content of natural food (65 – 70 %), with greater concentrations of valuable vitamins, minerals, phytonutrients, essential fatty acids like gamma-linolenic acid and other nutrients (Boussiba and Richmond, 1980; Cohen *et al.,* 1987; Henrikson, 2009). Additionally, it has many therapeutic applications (Richmond, 1988; Amha Belay and Ota, 1993; Samuels *et al*., 2002).

Arthrospira requires an abundant supply of light and nutrients, relatively high temperature, and very high carbonate+bicarbonate alkalinity and pH (Habib *et al.,* 2008). Ethiopia harbours lakes characterized by high temperature and irradiance, nearly constant photoperiod, high carbonate–bicarbonate alkalinity and pH, which are ideal conditions favouring the high productivity of *Arthrospira* (Richmond and Grobbelaar, 1986; Vonshak, 1997; Talling and Lemoalle, 1998). Naturally, *Arthrospira* is found in Lake Chitu, which is a small closed and highly productive tropical soda lake with ideal chemical conditions that promoted the formation of it's nearly monoalgal population (Elizabeth Kebede *et al.*, 1994; Elizabeth Kebede, 1996). *Arthrospira*, which used to form surface blooms in such other soda lakes of Ethiopia as Arenguade, Abijata, Beseka and Hora-Kilole, is now disappearing from these lakes at an alarming rate (Elizabeth Kebede *et al.,* 1994; Brook Lemma, 1994, cited in Misgina Belachew *et al.,* 2012). Harvesting *Arthrospira* from natural lakes is not always a workable strategy as it can pose safety problems because natural lakes often consist of several species some of which may be toxic. Furthermore, harvesting *Arthrospira* biomass from a lake cannot be sustained due to the generally small size of *Arthrospira* lakes and the significant temporal variation in its standing crop. Cultivation is the preferred and workable strategy for *Arthrospira* biomass production.

1.2. Statement of the Problem

Even if *Arthrospira* is an easily cultured species, most of the advanced farms, which were designed to produce high quality *Arthrospira* products, have encountered high production costs of some 5-7 euros per kg of *Arthrospira* (Akvopedia, 2015). The main costs incurred by local production of *Arthrospira* are costs of labour, nutrients, packaging, capital and administration (Henrikson, 2009; Akvopedia, 2015). Next to labour, the second major cost item is nutrients, which accounts for 15-25% of the total production cost of *Arthrospira* biomass (Amha Belay, 1997; Vonshak, 1997; Habib *et al*., 2008). However, in developing countries like Ethiopia where labour and land costs are comparatively lower, high cost and readily unavailability of inorganic nutrients constitute the main barriers to *Arthrospira* cultivation. To overcome this problem, other nutrient sources, which can substitute the standard *Arthrospira* medium (Zarrouk's medium) especially its carbonate-bicarbonate content that is incurring high cost, should be sought for. Although a great deal of effort has been made to use enriched seawater, saline lake waters and domestic wastewaters and other potential sources of nutrients for the cultivation of this micro alga, diminutive consideration has been given to the potential of such saline spring waters as those found in the shores of Lake Shala for the production of *Arthrospira* biomass. The saline alkaline water of these hot springs has the potential to reduce the cost of production by minimizing the extent of supplementation with the standard medium. This research work, therefore, aimed at evaluating the potential of Lake Shala hot spring supplemented with Zarrouk's medium for the production of *Spirulina* (*Arthrospira fusiformis*) biomass at optimal temperature and light conditions. The information generated through this project will be useful in efforts geared towards reducing the cost of large-scale commercial production of *Arthrospira.*

1.3. Significance of the study

This study has great significance as it provide scientific information on the potential of Lake Shala hot spring water for the production of *Arthrospira*. The findings obtained from this study can encourage large scale production of *Arthrospira* by private investors, which are prohibited by the seemingly incredibly high cost of production by reducing nutrient cost of *Arthrospira* biomass by over 25%. In addition, by encouraging the production of *Arthrospira*, it will contribute to efforts geared towards combating malnutrition.

2. OBJECTIVES

2.1. General objective

- To evaluate the potential of hot spring water-based media for biomass production of *Arthrospira.*

2.2. Specific objectives

- To determine some chemical parameters of Lake Shala hot spring that has relevance to the growth of *Arthrospira.*
- To determine the specific growth rate (μ, day^{-1}) and biomass (as Chl-a and dry weight) of *Arthrospira* cultivated in lake Shala hot spring water-based media with different supplementation.
- To document some chemical features of the various growth media with a view to identify factors of overriding importance to *Arthrospira*'s growth parameters.
- To assess the potential of Lake Shala hot spring for the biomass production of *Arthrospira.*

3. LITERATURE REVIEW

3.1. Biology of *Arthrospira*

Arthrospira fusiformis (Voronikhin) Komarek & Lund (1990), which is commercially known as *Spirulina* (Elizabeth Kebede and Ahlgren, 1996; Tadesse Ogato *et al.*, 2014) is one of the most primitive cyanobacteria (blue green algae) that originated on Earth. It's a primarily photoautotrophic alga belonging to Division Cyanobacteria, Class *Cyanophyceae* and Order *Oscillatoriales.* This microscopic, spiral-shaped and filamentous alga has trichomes with a length of 50 to 500 μm and a width of 3 to 4 μm. The trichomes form floating mats owing to their gas-filled vacuoles and helical shape (Habib *et al.,* 2008). *Arthrospira* has the typical prokaryotic cellular organization, and hence lacks membrane-bound organelles (Tomaselli, 1997). Its cell wall is made up of peptidoglucan similar to that of gram-negative bacteria (Usharani *et al.*, 2012). The shape and osmotic protection of the cell wall depend on this lysozyme-sensitive heteropolymer- peptidoglycan (Habib *et al.,* 2008). *Arthrospira* forms solitary trichomes, which reproduce by binary fission (Tomaselli, 1997). Although *Arthrospira* as a species forms helical trichomes, it shows morphological variations in response to environmental stress associated with the salinity, nutrients, temperature and level of photosynthetically active radiation of its natural habitats (Tadesse Ogato and Demeke Kifle, 2014). Similar responses are induced in laboratory and large scale cultures of the species by the interaction of light with temperature (Li and Gao, 2008), nutrients (Bai and Seshadri, 1980) and salinity (Elizabeth Kebede, 1997) with the trichomes varying in helix dimensions, degree of coiling and shape of the trichome end and relative abundance. The morphotypes, which are seen in natural and culture systems, also vary from tightly coiled to straight forms (Tadesse Ogato and Demeke Kifle, 2014).

Arthrospira has three major pigments: phycocyanin (blue), chlorophyll (green) and carotenoids (red, orange or yellow). Phycocyanin is the most important pigment, which accounts for 14% of the protein complex and is believed to be the precursor of chlorophylls since the former evolved billion years before the latter. *Arthrospira* has 1% chlorophyll, which is one of the highest natural levels. It is known as a cleansing and detoxifying phytonutrient. It has also carotenoids, which are natural antioxidants (Henrikson, 2009).

The species, which is used for most commercial purposes, belongs to the genus *Arthrospira.* However, it has been known as *Spirulina* for a long time although it is taxonomically wrong.

The genus *Spirulina* was first established by P. J. Turpin in 1827 from a fresh water sample although Wittrock and Nordstedt reported about a blue-green microalga named *Spirulina jenneri* f. *Platensis,* which has a helical, septate character, in 1844. Years later, considering the presence of septa, helical and multicellular structure, Stizenberger introduced the genus *Arthrospira* in 1852 (cited in Ciferri, 1983).

The taxonomic confusion started in 1932 when Geitler reunified the genera *Spirulina* and *Arthrospira* because of the common helical morphology (cited in Ciferri, 1983). However, considering the difference in mean DNA base composition between them, Herdman *et al.* (1979) and Rippka *et al.* (1979) suggested taxonomic revision on them. Guglielmi and Cohen-bazireg's (1982) study revealed that the two genera have ultrastructural difference with *Spirulina* having several rows of pores at the concave site of the coil at each site of the cross-walls, while *Arthrospira* has only a single row of pores around the trichome at each site of the cross-walls. Cohen *et al.* (1987) also reported that all *Arthrospira* strains have gamma-linolenic acid unlike *Spirulina* strains. A detailed analysis of the molecular marker 16S rRNA (Nelissen *et al.,* 1994) showed that *Arthrospira* strains shows 99.7% similarity among them, while with *Spirulina,* the similarity was only 91.2%. The available molecular information has enabled algal taxonomists to clear the confusion and assign the two species to separate genera, *Spirulina* and *Arthrospira* (Tomaselli *et al*., 1996; Sanchez-Luna *et al*., 2004). *Arthrospira* has four species: *A. jenneri, A. platensis, A. fusiformis*, and *A. maxima. A. jenneri* and *A. platensis* were isolated from America, Europe and Africa. They have benthic character because of their non-gas vacuolate structure, while *A. fusiformis* and *A. maxima* are planktonic with gas vacuolate trichomes and occurring in Africa and tropical and central Asia (Komarék and Lund, 1990). The entire commercially produced algal product, which has been sold as *Spirulina,* taxonomically belonged to *Arthrospira*. Since a lot of money has been expended to commercialize *Spirulina* and the name *Spirulina* has been used for a long time, the commercial name for the genius *Arthrospira* is still *Spirulina* (Amha Belay, 2008). Among the *Arthrospira* species, *Arthrospira fusiformis* used to form blooms in such soda lakes of Ethiopia as Abijata, Chitu, Arenguade and Hora-kilole (Misgina Belachew *et al.,* 2012) and now with an almost natural monoalgal culture of *Arthrospira fusiformis* is found only in Lake Chitu (Elizabeth Kebede, 1997).

Arthrospira is known to have a high degree of adaptation, which enables it to grow in a wide range of habitats, ranging from fresh-alkaline to saline waters, marine and even

thermal springs (Kaggwa, 2013). It is adapted to survive in aquatic environments with salinities from 5 to 60 g L^{-1}. Among the ions K^+, Na^+, CO_3^{2-}, HCO_3^- and SO_4^{2-} are needed at high concentrations, while Cl^- and Ca^{2+} and Mg^{2+} are required at moderately high and very low concentrations, respectively (Iltis, 1971, cited in Sehadril *et al*., 1980). *Arthrospira* prefers relatively high temperature (32 - 40 ^{0}C), pH (9 - 11), and abundant supply of light, and nutrients with very high carbonate–bicarbonate alkalinity, which makes this alga an alkaliphile. Its alkaliphilic nature helps to prevent the growth of other algae thereby enabling the alga to form an almost monoalgal natural culture in tropical and subtropical water bodies (Rafiqual *et al.*, 2005; Vareschi, 1978, cited in Kaggwa, 2013). *Arthrospira* grows with such other algae as *Anabaenopsis, Oscillatoria*, certain diatoms and sometimes dinoflagellates when the physicochemical conditions particularly alkalinity and pH become less favourable to *Arthrospira* (Iitis, 1870 cited in Sehadril *et al*., 1980). Rotifers including *Brachionu*s spp., *Cephalodella* and *Pedialia*, and such ciliates as *Euplotes, Holophrya* and *Urotrichia* are usually seen with this species (Pourriot *et al*., 1967, cited in Sehadril *et al*., 1980). Naturally, *Arthrospira* is found mainly in Africa (Leonard and Compere, 1967, cited in Schager *et al*., 2015) especially in the rift valley lakes of Ethiopia (Talling *et al.*, 1973; Elizabeth Kebede, 1997), Kenya (Krienitz and Kotut, 2010), Sudan (Fott and Karim, 1973), Chad and Zambia (Leonard and Compere, 1967; Ciferri, 1983, cited in Schager *et al*., 2015) It is also found in South American and Asian regions and is produced worldwide for commercial purposes (The National Centre for Biotechnology Information, 2011).

3.2. What Makes *Arthrospira* World Most Powerful Food?

Arthrospira is an amazing food, which contains almost every nutrient at unusually high concentration (Figure 1). Its 55 to 70% protein content by dry weight (Phang *et al*., 2000) is the highest of any natural foods (Henrikson, 2009). It comprises almost all amino acids even if it has lower amounts of methionine, lysine and cysteine than animal product proteins (Habib *et al*, 2008).

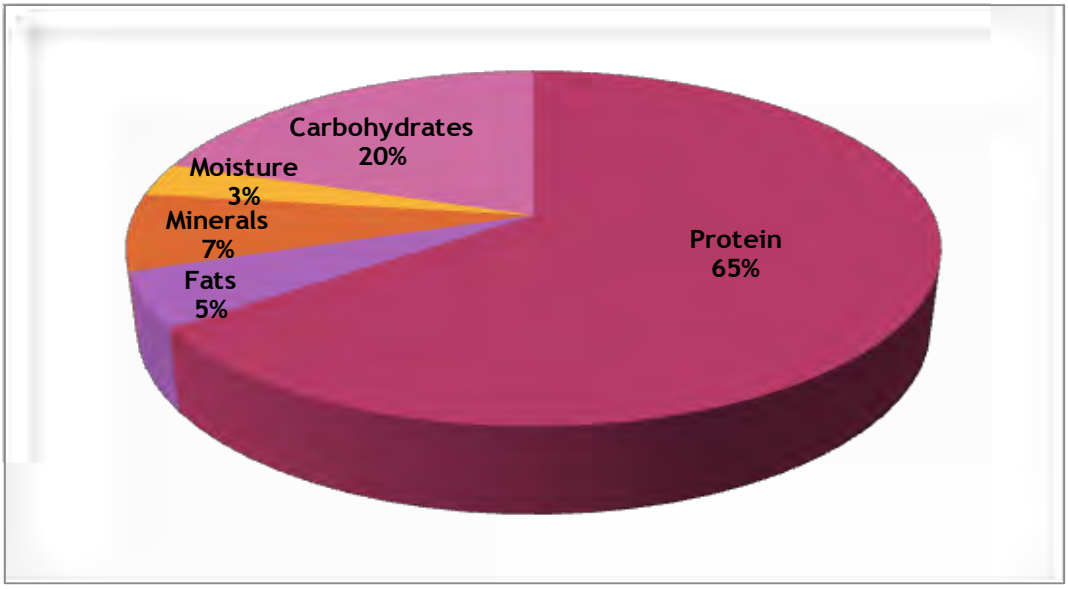

Figure 1. *Arthrospira* composition (Source: Henrikson, 2009).

Another advantage of using *Arthrospira* protein is that it helps to avoid the problem of consuming high fat, cholesterol and calorie food (Henrikson, 2009). There is utmost 4 - 7 % of lipid in *Arthrospira* constituted by such essential fatty acids as omega 3, 6, and 9, which are very important to control the hormone system. Of these, gamma linolenic acid (GLA, omega 6), which accounts for 36 % of the total polyunsaturated fatty acids, has immense nutritional importance as it is the precursor of the hormones prostaglandins that control many functions (Nichols and Wood, 1968; Henrickson, 2009). The remaining percentage of lipids goes to other important omega-3 fatty acids like linoleic acid (LA, 36 percent of total), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), arachidonic acid (AA) and stearidonic acid (SDA) (Habib *et al.,* 2008).

Arthrospira contains many vitamins including thiamine (B1), riboflavin (B2), nicotinamide (B3), pyridoxine(B6), folic acid(B9), cyanocobalamin (B12), vitamin C, vitamin D and smaller amount of vitamin E. It also contains many minerals including calcium, copper, chromium, germanium, iron, magnesium, manganese, phosphorus, potassium, selenium, sodium and zinc (Habib *et al.,* 2008; Henrickson, 2009), which are used to fight free radicals that come from poor diet, pollution, stress or other sources (herbwisdom.com, 2015). Such phytonutrients of *Arthrospira* as phycocyanin, chlorophyll and carotenoids have the advantage of stimulating the immune system, cleansing, detoxifying and providing protection against oxidants (Anitha *et al.,* 2006). The cell wall of *Arthrospira* is made up of soft mucopolysaccharides, which have the advantage of being easily digested and assimilated by

the digestive system. These make *Arthrospira* even more suitable for malnourished people (Mani *et al*., 2008; Henrickson, 2009).

Besides being composed of a lot of nutritionally beneficial constituents, *Arthrospira* also has many therapeutic applications including stimulating and strengthening the immune system by promoting regeneration of cells including new blood cells, enhancing function of macrophages, natural killer cells, T-cells, bone marrow stem cells, spleen and Thymus glands. Phycocyanin of *Arthrospira* stimulates the immune system (Boussiba and Richmond, 1980) and reduces risk of cancer, while intake of β-carotene (provitamin A), which is abundantly found in *Arthrospira* (Richmond, 1988), is known for its deactivation of free radicals that damage cells and lead to cancer risk (Henrickson, 2009). Hernandez (2001) also reported that phycocyanin can lower the toxic side effects of chemotherapeutic drugs, which are used in cancer management. Considering the ever increasing problem of cancer resulting from many environmental factors, antioxidants are beneficial to reduce risk of cancer. Beta-carotene is one of the antioxidants abundantly found in *Arthrospira*. Additionally, *Arthrospira* has immune modulation effect, which is needed in cancer prevention.

A research conducted between Harvard medical school and Earthrise farms has shown that *Arthrospira* extracts have the potential to reduce viral replication and even stop it at higher concentration without being toxic to human beings (Hernandez-Corona *et al.,* 2002). Japan scientists also reported that *Arthrospira* inhibits the replication of Influenza A virus, Herpes Simplex, Mumps virus, Human Cytomegalovirus and Measles virus *in vitro* without affecting human cells (Hayashi *et al.,* 1993; Hayashi *et al.,* 1996). Especially Sulfolipids from Arthrospira protect cells from viral attachment or penetration, which is important to treat cancer and AIDS virus (Henrikson, 2009). Besides increasing anti-viral activity of human immune system, *Arthrospira* products have cholesterol-reduction effects in humans (Amha Belay, 2002) without loss of weight because of their effect on the metabolism of lipoproteins (Nayaka *et al.,* 1988).

The GLA in *Arthrospira* is a precursor of prostaglandins, which help fight against obesity by preventing the accumulation of cholesterol in the body (Samuels *et al*., 2002), arthritis, heart disease, zinc deficiency, Parkinson's disease, multiple sclerosis, hives atopic eczema, while reducing premenstrual syndrome symptoms (Kernoff *et al.,* 1977; Vadaddi and Horrobin, 1979; Huang *et al.,* 1982; Kunkel *et al.,* 1982; Amha Belay, 2002). Moreover, *Arthrospira* can be used to treat herpes infection, iron anaemia, mineral deficiency, hay fever and reduce

radiation sickness, and toxicity that results from different chemical pollutants in kidney and liver (Takemoto, 1982; Yamane, 1988; Radhika, 2008). It also has wound healing capacity owing to its antibiotic effect **(**Yoshida, 1977) and helps build healthy lactobacillus populations in the intestines, which enhances the digestive process **(**Archer and Glinsmann, 1985), and reduce sources of allergies and inflammation (oilgae.com, 2016). A finding by Parikh *et al.* (2001)*,* showed its potential to control blood glucose level and also improve the lipid profile, which is advantageous in the treatment of diabetics (Takai, 1991). The pro-vitamin A from *Arthrospira* is also used to treat eye problem caused by the deficiency of it. Most importantly, the high nutritional profile of *Arthrospira* can be used to eliminate malnutrition (Henrikson, 2009).

Furthermore, excellent results were obtained when *Arthrospira* was used as a fertilizer (FAO, 1981; Zeenat *et al*., 1990; Habib *et al.,* 2008), protein supplement with high vitamin and minerals in poultry and livestock feeds (Venkataraman *et al.,* 1994; Elsayed, 1994; Britz, 1996), in aquaculture feeds as protein replacement and supplement in cultures of Mozambique tilapia (*Oreochromis mossambicus,* Vonshak, 1997), Nile tilapia (*Oreochromis niloticus,* Lu and Takeuchi, 2004), Abalone (*Haliotis midae*, Stott *et al.,* 2004), giant freshwater prawn (*Macrobrachium rosenbergii,* Nakagawa and Gomez-Diaz, 1975), silver seabream (*Rhabdosargus sarba,* El-Sayed, 1994) and as colorant of food products using the pigments phycocyanin (blue), chlorophylls (green) and carotenoids (red, orange and yellow) (Toyomizu *et al*., 2001).

3.3. Culture Conditions of *Arthrospira*

Arthrospira usually forms almost unialgal blooms in tropical and subtropical lakes with high carbonate and bicarbonate alkalinity, very high pH, high irradiance and temperature (Rafiqual *et al.*, 2005). Optimal culture conditions for *Arthrospira* were derived from these observations (Amha belay, 2008). Many factors affect *Arthrospira* growth and composition although light, temperature, nutrients (Cornet *et al.,* 1992), pH and salinity (Pandey *et al*., 2011) are of overriding importance.

3.3.1. Light

The presence of light is a necessary condition in algal culture since intensity, duration and quality of light are the vital factors influencing photosynthetic organisms (Robin, 1981). High

growth of *Arthrospira* is achieved in the presence of high intensity light. The optimum light level, according to Ogawa and Teuri (1970, cited in Usharani *et al*., 2012) ranges from 270.3 to 405.41 μmol photons m^{-2} s^{-1}. However, Zarrouk (1966, cited in Habib *et al.,* 2008) argued that *Arthrospira*'s growth would saturate at 150 - 200 μmol photons m^{-2} s^{-1}. Apart from influencing growth rate and biomass production, the light condition affects the nutritional content of *Arthrospira* as high light intensity decreases protein content, while increasing carbohydrate and β-carotene content (Chaiklahan *et al.,* 2007; Habib *et al.,* 2008). Moreover, light affects *Arthrospira* growth due to self-shading and photoinhibition. Self-shading precludes the distribution of optimum light level to every single alga through the depth. This problem should be tackled by controlling the culture density. Especially in outdoor cultures, when the culturing depth is between 12 and 15cm, self-shading presides over the availability of light for the single cell. The main limiting factor of outdoor culture of *Arthrospira* in the summer is light because higher temperature in summer exacerbates the effect of self-shading more than the lower temperature of spring and winter (Vonshak, 1997). The other problem associated with light called photoinhibition ensues when the light intensity is too high especially if the temperature is low since *Arthrospira* culture found at a lower temperature than the optimal condition is more sensitive to excess photons, which result in lowered photon utilization efficiency of PSII, which is commonly observed in outdoor cultures of *Arthrospira* (Vonshak and Guy, 1992; Torzillo *et al.,* 1996; Vonshak *et al*., 1996; Torzillo *et al.,* 1998, cited in Lu and Vonshak, 1999). This lower photosynthetic efficiency, which makes the cell needy of more light to compete with non-photoinhibited cells, makes the cells light-limited at the same time (Vonshak, 1997). The light: dark photoperiod to which algal cultures are exposed needs to be as close to natural as 10:14-12:12 and illumination of 24 hours a day in artificial cultures is not recommended since this condition would inhibit dark reactions including synthesis of proteins and respiration (Jourdan, 2001; oilgae.com, 2016).

3.3.2. Temperature

Temperature is another important physical factor for all living things since it affects the metabolic activity of cells. It also controls the physical properties of water, nutrient availability and uptake of the same by cells (Vonshak, 1997). The usual optimal temperature for *Arthrospira* is between 35°C and 38°C since it is a mesophilic alga (Chanawongse, 1992; Vonshak, 1997). The net productivity of *Arthrospira* is dependent on temperature since photosynthesis and respiration are very much influenced by temperature. It is often the main

limiting factor affecting the biomass production of *Arthrospira* as outdoor cultures and natural systems don't have the optimum temperature for most of the day time. Diurnally, temperature varies from 15°C to 38°C out of tropics. Although in tropics the culture temperature is below the optimal ranges for most of the day. Growth of *Arthrospira* is almost nil when the temperature is below 18°C. At night, high temperature increases respiration rate, which leads to loss of biomass. The loss may reach 30 % of the previous day production (Vonshak, 1997). Temperature beyond 38°C is harmful to *Arthrospira* (oilgae.com, 2016).

3.3.3. Nutrients

Even though Zarrouk's medium has served as a standard medium successfully since 1966, use of it for large scale production is unaffordable. Zarrouk's medium, which is the first medium formulated for *Arthrospira* (Zarrouk, 1966, cited in Amala and Ramanathan, 2013), is rich in nutrients and has high alkalinity of carbonate and bicarbonate and salinity (Ogawa and Teuri, 1970, cited in Usharani *et al*., 2012). *Arthrospira* medium is composed mainly of carbonate-bicarbonate as a carbon source, which have the highest concentration and cost. Other than carbon, *Arthrospira* requires the macronutrients N, P, S, K, Ca, Mg and such trace metals as Zn, Mn, B, Co, Na, Mo, Fe and Cu (Jourdan, 2001). Phosphorus and nitrogen are the major macronutrients, which affect algal cell metabolism and growth. Nitrogen is the basic element required for the synthesis of nucleic acids, proteins and ATP, while phosphorus is a constituent of ATP, DNA, RNA and phospholipids. N and P play an essential role in determining the extent of biomass production of microalgae (Mostert and Grobbelaar, 1987). However, in natural environments, these nutrients can be limiting to alga growth (Harris, 1986, cited in Juneja *et al.,* 2013). Other than Zarrouk's medium, different media have been developed for *Arthrospira* through modification/ substitution of some components of the standard medium or formulation of such new media as CFTIR medium (Venkataraman *et al.*, 1995), OFERR medium (Singh, 2006), Revised medium (Raoof *et al*., 2006), Rao's medium (Singh, 2006), Bangladesh medium (Khatum *et al.,* 1994), Mysore medium, MCRS medium, Madras medium, CNRS medium, and others (Bai, 2006). Considering the suitability of the gross chemistry of brackish and marine environments, other cost effective media have also been prepared using their water (Vonshak, 1997).

3.3.4. Salinity

Even though *Arthrospira* is naturally found in a wide range of habitats of different salinity (Vonshak and Tomaselli, 2000), it is an important factor that influences the biochemical composition of algal cells (Juneja *et al.,* 2013). The exposure of *Arthrospira* to high salinity, which usually refers to high concentration of NaCl, causes cessation of growth for a while and subsequent onset of a lag phase to adapt to the new salt concentration. The growth of the alga will be much less than that of the same strain grown in a medium with normal salt concentration. The reduced growth is attributable to the higher energy demand (Vonshak, 1997), which cannot be met by decreased photosynthetic capability of stressed cells (Sreevani *et al.,* 2011).

3.3.5. pH

pH is the major factor affecting the availability and solubility of CO_2, essential nutrients and minerals (Goldman, 1973; Chen and Durbin, 1994) which have direct effect on algal physiology and metabolism (Vincent and Silvester, 1979). pH also affects the protein and chlorophyll-a content and growth of *Arthrospira* (Pandey *et al.,* 2010). pH of the media is determined by the relative concentrations of inorganic carbon species (Azov, 1982, cited in Juneja *et al.,* 2013) as the rise in pH occurs when the alga removes inorganic carbon from the media (Hansen, 2002). *Arthrospira* grows well at pH values between 9 and 11(Vincent and Silvester 1979; Richmond *et al*., 1993) although the optimal pH level is in the range 9.5 – 10.5, which has the advantage of minimizing the risk of contamination by such other organisms as *Chlorella*, diatoms and cyanobacteria. However, when the pH is above 10.5, the growth rate declines (Fox, 1996; Amha Belay, 1997; Vonshak, 1997; Carlos *et al.,* 2003)

3.4. Worldwide *Arthrospira* Production and Its Drawbacks

Due to its several benefits, *Arthrospira* is produced commercially throughout the world. The production has increased through time since it started in 1970s. It is estimated that the global production reached 12000 tonnes in 2013 (algaeworld.org, 2015). The alga is produced by harvesting from natural lakes or using such commercial farming systems as open raceway pond system, enclosed photobioreactors, tanks, integrated systems, etc (Henrickson, 2011) and its products sold by more than 40 countries as tablets, capsules and powder. *Arthrospira* products emanate from cultivation systems ranging from very small to very large scale

production systems. However, producing *Arthrospira* with the right quality incurs high cost, which often inhibits efforts geared to its production. This makes the product expensive and less distributed through the world especially in developing countries. As Akvopedia (2013) pointed out, the main cost items of *Arthrospira* production are labour, nutrients, packaging, capital and administration. The total cost of *Arthrospira* production differs from country to country owing to the differences in biological, chemical, physical and environmental conditions (Amha belay, 2008).

Since *Arthrospira* is adapted to tropical and sub tropical water bodies, which are characterized by high irradiance and temperature, places located outside these regions are likely to have additional costs for *Arthrospira* production. Even though the tropics have seasonal rains, there can be production during most of the year since there is virtually no place in the world where production of *Arthrospira* takes place throughout the year. Desert areas seem to be better sites for *Arthrospira* production owing to their consistent climatic conditions, which tend to give higher quality and yield (Amha Belay, 2008) but unavailability of adequate water supply may be a problem.

Although labour is regarded as the main cost item in *Arthrospira* production, this may not be the case with many countries particularly the developing countries where labour is very cheap (Akvopedia, 2015). However, nutrients remain the second major cost item behind labour accounting for 15 to 25% of the total production cost. Thus, using standard media for *Arthrospira* production is too expensive at least for developing countries (Amha Belay, 1997; Vonshak, 1997; Habib *et al*., 2008). As a result, there is a need to look for something that can substitute the costly reagent grade chemicals especially the carbonates and bicarbonates, which are indispensable nutrients serving as carbon source for photosynthesis (Cornet *et al.,* 1992), and as chemical factors that can be manipulated to optimize pH condition thereby minimizing the risk of contamination by other algae.

3.5. Efforts Made So Far To Minimize Cost of Nutrients for the Production of *Arthrospira*

Many investigators have made efforts to minimize cost of *Arthrospira* production through the use of various growth media, which can potentially substitute the standard *Arthrospira* medium. Cursory treatments of the efforts made by different researchers to substitute the standard medium by low cost media are given in the following sections.

3.5.1. Using sea water as a low cost medium

Considering the prerequisites for mass production of *Arthrospira,* lessening cost of media using sea water is inevitable especially in tropical arid areas where the climatic condition is favourable, but freshwater resource is scarce (Marerassi *et al*., 1984) The potential of sea water has been assessed by different researchers including Devanathan and Ramanathan (2012) who used seawater with the addition of sodium bicarbonate ($NaHCO_3$) and animal waste (poultry dry manure) as a substitute of nitrate since animal waste is low cost nitrogen source (Olguin *et al.,* 2001, cited Devanathan and Ramanathan, 2012). The results show that natural sea water has the potential to support *Arthrospira* along with dry manure as cost effective medium. Another investigation by Bharat *et al.* (2011) in khambhat also involved the use of sea water enriched with bicarbonate ($NaHCO_3$) and nitrate ($NaNO_3$) salts at different concentrations. The media didn't have significant adverse effect on the growth of *Arthrospira*, which suggests that using sea water for commercial cultivation is worth-considering. Sandeep *et al*. (2015) also developed a low cost medium by using sea water and prawn hatchery waste water and argued that sea water, when properly mixed with hatchery waste water, have the potential to produce good quality biomass of *Arthrospira.* Leema *et al*. (2010) also used sea water supplemented with various levels of the critical nutrients and reported that the treatment, which had better supplementation of phosphate and nitrate produced almost the same results as the standard Zarrouk's medium with significantly higher lutein content of the algal product. Tredici *et al.* (1986) reported that sea water containing urea as a nitrogen source and lower concentrations of phosphate and carbonate prevent their precipitation of them as insoluble salts. Another investigation by Wu *et al.* (1993) has also demonstrated the potential of sea water enriched with commercial fertilizer containing N, P, K (in the ratio12: 12:12), $FeSO_4$ and $NaHCO_3$ and in which precipitation of the fertilizer is prevented by adding phosphate-containing compound with a little amount of $NaHCO_3$ through tubular dialysis. Thus, it can be seen that high yield of high quality products can be obtained using less expensive media and without involving precious farm lands. However, production of *Arthrospira* using sea water should consider physiological aspects such as stress tolerance, acclimatization, pigment production and growth (Materassi *et al.,* 1984).

3.5.2. Using lake and lagoon water as low cost media

Alkaline saline lake waters contain the basic nutrients and their salinity has the potential to minimize the cost of the media produced from them. Since these environments are often the natural systems colonized by *Arthrospira* (Belov and Giles, 1997; Richmond, 1990, cited in Costa *et al.,* 2003), their potential for *Arthrospira* production is immense. An investigation by Tadesse Ogato *et al.* (2014) using the Soda lakes of Ethiopia, Chitu and Shala, has shown that soda lake waters which supplemented by standard medium have the potential to support good growth of *Arthrospira* and considerably reduce cost of production. Costa *et al.* (2003), who also assessed the potential of fresh water from a lagoon at Mangueira, Brazil supplemented with different levels of nutrients for the biomass production of *Arthrospira,* demonstrated the feasibility of better biomass production with the addition of enough sodium bicarbonate and other nutrients, which minimize the cost.

3.5.3. Using domestic wastewaters and other potential sources of nutrients

Using waste waters and other nutrient resources is advantageous with regard to cost minimization and environmental quality. Amala and Ramanathan, (2013) reported that growth rate as changes in chlorophyll concentration, dry biomass, protein and lipid content comparable to that can be obtained with Zarrouk's medium was achieved using a new rice mill effluent medium (RME) although trace amount of pesticide residue was detected in the product. Muhiye Endrie's (2009) study showed that a local product, Trona ($Na_2CO_3.NaHCO_3.2H_2O$), can be used to substitute the major parts of the standard Zarrouk's medium without having significant adverse effect on the growth or composition of *Arthrospira,* while reducing the nutrient cost by 40%. The growth of *Arthrospira* in wastewater, desalinated wastewater and salinated synthetic medium prepared by adding 1 g L^{-1} NaCl to Paoletti medium (Babadzhanov *et al.,* 1999) was also studied by Volkmann *et al.* (2008). The results of cultivation in desalinated wastewater approached those obtained by Rafiqul *et al.* (2005) using Zarrouk's medium, and resulted in higher protein content than salinated water, which was attributed to the absence of salinity stress in the desalinated wastewater as salinity stressed cells have lower protein synthesizing capacity (Zeng and Vonshak, 1998). Michele *et al*. (2007) also suggested the use of molasses, which contains 50% sugar, as low-cost substrate for the mixotrophic cultivation of *Arthrospira platensis* and opened up the possibility of using low-cost agricultural by products for the growth of this microorganism. A study by Seshadri and Thomas (1979) showed the suitability of bone-meal

and biogas for the growth of *Arthrospira* as low-cost substitutes for Ca, P and N. Waste water from a fertilizer company in Nigeria (Anaga and Abu, 1996) and rice waste water from a fermented noodle factory in Thai (Vetayasuporn, 2004) were also used as cost-effective media for the cultivation of this micro alga and biomass, crude protein and phycocyanin levels comparable to those that can be achieved using the standard medium, were obtained with different nutrient supplementations.

3.5.4. Using thermal spring waters and underground water resources

The use of underground water for the cultivation of *Arthrospira* is advantageous owing to its high natural mineral content and minimized risk of contamination. A study by Kim *et al*. (2007) also showed that improved biomass productivity and quality are obtainable using underground water rich in minerals. Albeit the high concentration of Ca in the ground water, the risk of precipitate formation is avoidable by precluding addition of $CaCl_2$ and other minerals in the standard media, which obviously reduces media cost. Nevertheless, little research work has been done with regard to the potential of hot spring water for the biomass production of *Arthrospira*. As a result, assessing the potential of Shala hot spring, exhibiting high carbonate-bicarbonate alkalinity, pH and containing many minerals, for the biomass production of *Arthrospira* is relevant and will narrow the existing research gap.

4. MATERIALS AND METHODS

4.1. Description of Lake Shala Hot Spring Used for *Arthrospira* Cultivation

Lake Shala hot springs are some of the saline springs in Ethiopia, which are concentrated in the eastern and south western shores of Lake Shala (RATSON, 2004). Lake Shala is surrounded and fed by many saline hot springs, which have different size, temperature and discharge rate. The hot spring, whose water was used for *Arthrospira* cultivation, is the largest and hottest spring water with a temperature of 97^0C and daily discharge rate of 50,000 liters (Humber and Elizabeth Kebede, 1998). It is located in the lake's north eastern corner at an altitude of 1556 m.s.l. and global position of $07^0$28.677'N $038^0$38.085'E, some 286 km south of Addis Ababa (Figure 2). The hot springs are used by the local people and visitors for the treatment of dermatological, respiratory and neurological diseases (RATSON, 2004), recreation, sanitation, and livestock watering (Tadesse Ogato, 2015). The selected hot spring has high pH and fairly high carbonate-bicarbonate alkalinity and salinity, (Table 1) which is probably related to the geological processes of dying Pleistocene to recent volcanism (Baumann *et al.,* 1975). This indicates its suitability for *Arthrospira* cultivation and considered to have the potential to be used for large scale production of *Arthrospira.* The region where this hot spring and Lake Chitu are located has semi-arid to sub-humid type of climate with air temperature ranging from 25 to 28 °C and annual mean precipitation of 955 mm (Tadesse Ogato, 2015). They located in a region of high temperature and irradiance and nearly constant photoperiod, which are ideal environmental conditions for the production of *Arthrospira* (Richmond and Grobbelaar, 1986; Vonshak, 1997; Talling and Lemoalle, 1998). In addition, the region has higher rate of evaporation than precipitation (Tadesse Ogato *et al*., 2014). Large concentration of trachytic and rhyolitic rocks with abundant alkali elements and residuals of rock decay containing pseudo-lateritic soils characterize the region of Lakes Chitu and Shala hot spring (Klemper and Cash, 2007)(EWNHS, 2009, cited in Tadesse Ogato *et al*., 2014). The area is covered by open acacia woodland with sparse acacia trees, which are overgrazed by livestock (Tadesse Ogato *et al*., 2014).

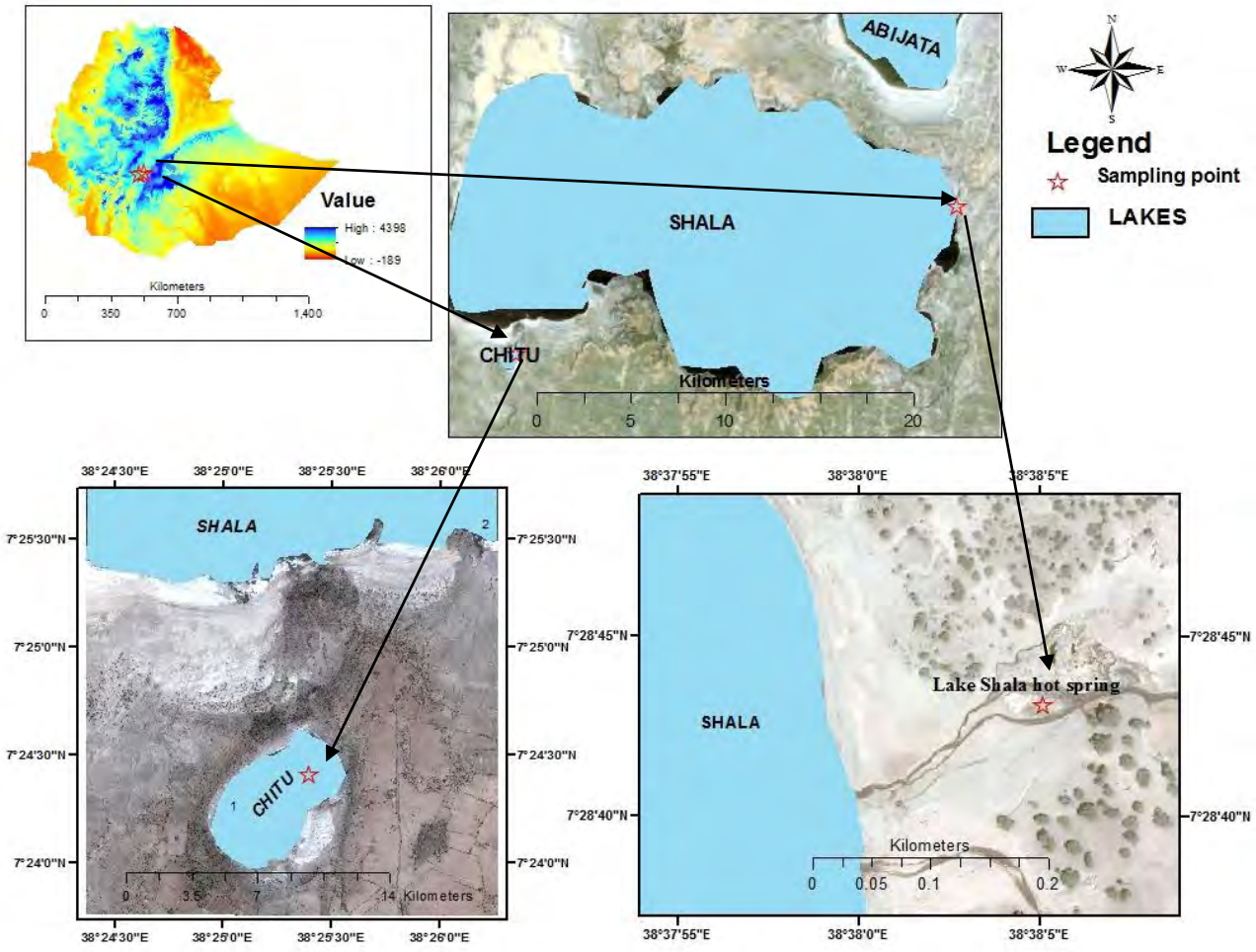

Figure 2. Location of Shala hot spring and Lake Chitu with sampling sites, Southern Ethiopia.

4.2. Description of Lake Chitu – The Ultimate Source of *Arthrospira* Inocula

Lake Chitu is a tropical soda lake located some 287 km south of Addis Ababa at a geographical position of 7°24.236' N 38°25.480' E and altitude of 1600 m (Figure 2). This lake, which is found 1 km southwest of Lake Shala, is a small (0.8 km^2), closed cup-shaped crater lake with relatively shallow depth (≈ 13 m). It is a highly productive soda lake with ideal environmental conditions for the formation of a nearly monoalgal population of *Arthrospira fusiformis* (Elizabeth Kebede *et al.*, 1994; Elizabeth Kebede, 1996). The lake is characterized by high pH, HCO_3^- + CO_3^{2-} alkalinity and salinity (Table 1) and with surface temperatures ranging from 21 to 24 °C (Tadesse Ogato *et al.*, 2014). Even though different

morphotypes with variability in abundance have been observed due to environmental stress, the same species- *A. fusiformis*- is found in Lake Chitu (Tadesse Ogato and Demeke Kifle, 2014). The concentrations of the major cations are in the order Na>K>Ca>Mg unlike those of most temperate lakes in which ionic dominance is in the order of Ca>Mg>Na>K (Tudorancea *et al.*, 1999). As the lake is closed, evaporative concentration is the prime factor for its saline–alkaline nature (Wood and Talling, 1988 cited in Tadesse Ogato *et al.,* 2014; Zinabu Gebre–Mariam, 2002). Lake Chitu supports a zooplankton community dominated by rotifers including *Brachionus* and *Hexarthra* and harpacticoid and cylopoid copepods (Tadesse Ogato, 2015).

Table 1. Some chemical parameters recorded for the Shala hot-spring and Lake Chitu (data source: Elizabeth Kebede *et al.,* 1994; Tadesse Ogato, Pers. Comm.)

Features	**Lake Chitu**	**Lake Shala hot spring**
Geographic position	7°24' N 38°25' E	7 ° 28'N 38 °38' E
Altitude (m)	1600	1556
pH	10.1	8.77
Conductivity (ms/cm)	56.33	8.16
Salinity (g L^{-1})	37.50	4.523
TA(meq L^{-1})	-	45.79
$N0_3^-N$ (µg L^{-1})	ND	0.055
NH_3-N(µg L^{-1})	ND	213.5
$P0_4^-P$ (µg L^{-1})	1985	63.51
$Si0_2$(mg L^{-1})	222	64.89
Na^+(mg L^{-1})	20,500	-
Ca^{2+}(mg L^{-1})	0.39	-
K^+(mg L^{-1})	1,200	-
Mg^{2+}(mg L^{-1})	0.06	-
Cl^-(mg L^{-1})	12,336.6	-
$SO4^{2-}$(mg L^{-1})	187.5	-
$HCO3^- + CO3^{2-}$ (alkalinity mg L^{-1})	36,848.5	-
Chlorophyll-a (chl-a, µg L^{-1})	224	-

4.3. Measurement of Some Physicochemical Parameters of Lake Shala Hot-spring

Shortly before the collection of the hot spring for the cultivation of *Arthrospira*, some physico-chemical parameters of the spring were measured *in situ* and in the laboratory. pH and conductivity were measured using digital HACH multi parameter meter (HQ40d model). Salinity (g L^{-1}) was estimated from conductivity measurements according to UNESCO (1983). Spring water samples were collected with two five-litre plastic containers, and used for the analysis of chemical parameters in the laboratory. Alkalinity of unaltered water sample was also determined by titration with 1 N HCl to pH 4.5 using a mixed indicator (Bromocresol green-methyl red) within a few hours after sample collection. Carbonate–bicarbonate alkalinity as $CaCO_3$ and its constituent ions were estimated from total alkalinity and pH according to APHA *et al.* (1999). Water samples filtered through Whatman GF/F were used for the analyses of inorganic nutrients except total phosphorus using the standard methods described in APHA *et al*. (1999). NO_2-N and NO_3−N, and $NH_3 + NH_4^+$ -N (hereafter ammonia) were determined by the Salicylate and Phenate methods, respectively. Soluble Reactive Phosphorus (SRP) and Total Phosphorus (TP) after persulfate digestion were analyzed by the Ascorbic Acid method. Sulphate (SO_4^{2-}) and Molybdate reactive silica ($SiO_{2)}$ were determined by the Turbidimetric and Molybdosilicate methods, respectively (APHA *et al.*, 1999). Samples acidified to a pH of 2 with HNO_3, were used for the analysis of major cations and some micronutrients according to the standard analytical methods outlined in APHA *et al.* (1999): Ca^{2+} by direct nitrous oxide-acetylene flame method, Na^+ and K^+ by flame photometric method; Fe, Mg^{2+}, Mn, Co, Cu, and Zn by Air-Acetylene Flame Atomic Absorption Spectrometry method according to APHA *et al.* (1999); Cl^- by Argentometric method according to APHA *et al.* (1999).

4.4. Isolation of *Arthrospira* and Scaling-Up of Its Seed Cultures

Algal samples were collected from Lake Chitu and transported in ice boxes to Addis Ababa University. *Arthrospira* was isolated by the serial filtration and dilution technique as described in Andersen and Kawachi (2005). The lake water was serially filtered to eliminate large and small organisms using different pore size meshes. The filtered lake water was diluted with liquid Standard medium (Zarrouk medium) as modified by Aiba and Ogawa

(1977). Concentrated trichomes of *Arthrospira* were checked under an inverted microscope and transferred to test tubes and a series of dilutions and subsequent checking were done under the microscope. The trichomes (Plate 1), which were picked by Pasteur micropipettes, were introduced into two 15 ml test tubes containing 5 ml standard medium. The trichomes in the test tubes were allowed to grow at a photon flux density of about 56-96 µmol photons m^{-2} s^{-1}, which was produced by 5-6 fluorescent tubes (58 W each) and at temperatures of 28 - 35 °C at different stages of the scaling up process. The cultures were mixed manually eight to ten times a day. Contamination by ciliates was eliminated using 60 mg L^{-1} Urea and 100 mg L^{-1} Ammonium bicarbonate (Carlos and Eduardo, 2012). Dense cultures were scaled up to a larger volume by diluting to 700 ml using the standard medium. This culture served as a source of inocula.

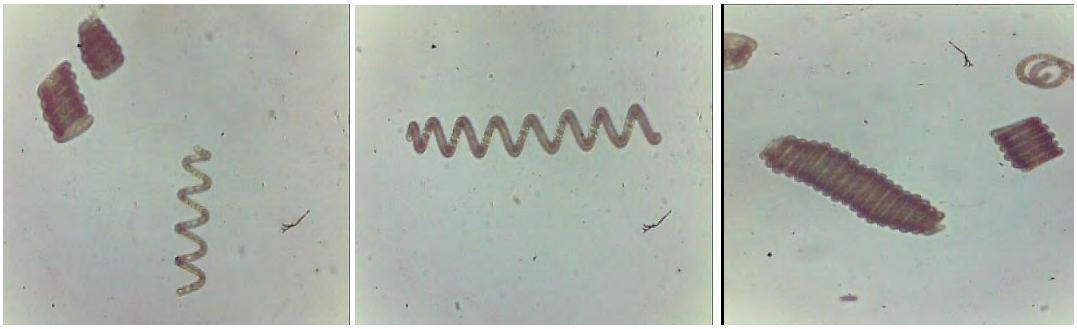

Plate 1. Isolates of *Spirulina* (*Arthrospira fusiformis*) collected from Lake Chitu.

4.5. Preparation of Growth Media for Experimental Cultures

Two 10-litre and one 25-liter plastic containers were used to collect water samples from the hot spring. The hot-spring water was filtered through a plankton net (70 µm pore size) to remove large particulate materials. The filtered water was sterilized by a chemical sterilization technique (bleaching) and neutralized with sodium thiosulfate solution following the procedure outlined in Kawachi and Noel (2005). In the laboratory, the spring water was filtered by 15 µm pore size net to remove impurities. The hot spring water was supplemented with the standard Zarrouk's medium as modified by Aiba and Ogawa (1977) in five different proportions as indicated in the following table (Table 2) to be used for assessing the growth responses of *Arthrospira* and evaluating the potential of the hot spring water for *Arthrospira* biomass production with a view to come up with a lower-cost culture medium.

Table 2. Types and composition of growth media used for experimental cultures of *Arthrospira.*

Types of media	**Designation**	**Composition (Volume of different components added to prepare the media)**		
		Inoculum	**Standard medium**	**Hot spring water**
Standard medium	SM	60ml	540ml	-
Shala hot spring medium	SSM	60ml	-	540ml
25 % standard medium- supplemented spring water medium	SM25	60ml	135ml	405ml
50% standard medium- supplemented spring water medium	SM50	60ml	270ml	270ml
75% standard medium- supplemented spring water medium	SM75	60ml	405ml	135ml

4.6. Experimental Design and Growth Conditions

The experiment was designed to investigate growth and biomass production of *Arthrospira* under laboratory conditions using media produced from previously hot spring water. The constant growth conditions were light, temperature and mixing with the SM-supplemented spring water-based growth media as independent variable. The response variables to be assessed were biomass production (B), doubling time (dt) and specific growth rate (μ). 1000 ml Erlenmeyer flasks filled with 600 ml culture medium were used to conduct the experiment. 60ml aliquots, constituting 10% of final culture volume, were inoculated into the replicate experimental culture flasks containing the five types of media prepared as shown in Table 1. (Amha Belay, 1997). The inocula were removed from exponentially growing cultures. Eight 58 W fluorescent tubes providing a total photon flux density of about 136 μmol photons m^{-2} s^{-1} at the surface of the culture were used as an artificial light source for the cultures. The cultures were grown in a temperature-regulated water bath (model DKZ series) at 35 °C in a light–dark cycle of 12:12 for 20 days (Plate 2). These light and temperature conditions are considered optimal for the growth of *Arthrospira* under laboratory conditions as reported by different researchers (e.g. Chanawongse 1992; Torzillo and Vonshak 1994; Vonshak 1997; Oliveira *et al*., 1999; Rafiqul *et al.,* 2003; Andersen and

Kawachi, 2005; Chaiklahan *et al.,* 2007). Mixing of the cultures was achieved manually by gentle shaking of the culture flasks six times a day at two-hour intervals.

Plate 2. Experimental setup in the limnology laboratory, Addis Ababa University.

4.7. Analytical Methods

Measurement of pH, conductivity and estimation of chlorophyll-a, of culture media were made every day except on the 19th day. Dry weight estimation of culture media was made every day during the first five days and then every other day until the 20th day. PO_4-P and NO_3-N analyses of culture media were made four times at 5-day intervals and on the final day to detect changes in growth conditions and performance of the alga over the growth period.

Chlorophyll-a (Chl-a) was determined spectrophotometrically from 5-mL samples filtered onto GF/F and extracted in 90% acetone. The absorbance of pigment extracts was measured at 665 and 750 nm with a UV–VIS Spectrophotometer (model 6405, Jenway) and chl-a concentration was estimated using the equation of the Monochromatic Method (Lorenzen, 1967).

$$\textbf{Chl-a } (\mu g\ L^{-1} \text{ or } mg\ m^{-3}) = \frac{26.73\,(E_{665b}-E_{665a})\,(V_E)}{(V_{SF})\,(Z)}$$

Where E_{665b} = Turbidity-corrected absorption at 665 nm before acidification= $A_{665b}-A_{750b}$, where A is absorption value.

E_{665a} = Turbidity-corrected absorption at 665 nm after acidification= $A_{665a}-A_{750a}$,

where A is absorption value.

$\mathbf{V_E}$ = Volume of extract, in ml

$\mathbf{V_{SF}}$ = Volume of sample filtered, in litres

Z = Length of the light path through the cuvette, in cm

Dry weight determination was made using 25 ml sample of algal suspension, which was filtered through an oven-dried and weighed Whatman GF/C filter paper of 47 mm diameter (Rafiqul *et al.,* 2003) and oven-dried at 103-105^{o}C for 2 hours. The dried filter paper containing *Arthrospira* biomass was cooled for 20 minutes and weighed. Dry weight was calculated by using the equation in APHA (1998) and Steinman and Lamberti (1996).

$$\text{Dry weight (Dwt, mg L}^{-1}) = \frac{(\text{A} - \text{B}) * 1000 \text{ ml}}{\text{Sample volume , in ml}}$$

Where A= weight of filter + dried residue, in mg; B= weight of filter, in mg

Specific growth rate (μ) and doubling time (dt) were calculated using the following equations used for batch culture of microalgae found at the exponential growth phase (Guillard, 1973; Vonshak, 1997). N_2 and N_1 are concentrations of the biomass indicator (chl-a, mg L^{-1}) at the end and beginning of the time intervals, t_2 and t_1, respectively.

$$\mu = \frac{ln\text{N}2 - lnN1}{t2 - t1}, \quad dt = \frac{ln2}{\mu} = \frac{0.6931}{\mu}$$

Biomass estimation was done using both chl-a (mg L^{-1}) and dry weight (g L^{-1}) (Table 5). Specific growth rate (μ) and doubling time (dt) were however calculated from chl-a, which is regarded as a better indicator of algal biomass owing to the fact that dry weight often includes dead and non-algal materials (APHA *et al.,* 1998).

NO_3-N and PO_4^-P were determined using samples filtered through Whatman GF/F, with the sodium salicylate (APHA, 1995) and ascorbic acid (APHA *et al.*, 1999) methods, respectively.

4.8. Statistical Analyses

The differences in growth parameters and chemical factors among the various culture media were analyzed by One-way ANOVA and Tukey's HSD post-hoc test was used for multiple comparisons. Multiple regression analysis with stepwise procedure was also performed to identify the variables, which contribute to the observed variations in growth and biomass production of *Arthrospira*. The relationships between the response variable (B) and chemical factors were analyzed using Pearson' correlation. These analyses were done using IBM SPSS statistical program (Version 21). Multivariate analyses was performed using CANOCO for Windows 4.5 to assess the relationship between biomass indices (response variables), chemical factors (explanatory variables) and sampling dates, which were related to the levels of various chemical and biological parameters. Detrended correspondence analysis (DCA) was used to determine the appropriate response model (linear or unimodal) for biomass production data. Accordingly, the length of gradient was < 3 (Leps and Smilauer, 2003), which implies that response variables (Chl-a and dry weight) exhibit linear response to environmental gradients (Ter-Braak and Smilauer, 2002). Thus, the linear method of gradient analysis, redundancy analysis (RDA), was performed using log transformed data except for pH. The environmental variables that best explain the variance in response variables were selected and tested by RDA-associated automatic selection and Monte Carlo permutation tests (unrestricted permutations= 499, P = 0.05).

5. RESULTS

5.1. Physico- Chemical Features of Lake Shala Hot-spring

Table 3. Physico-chemical features of Lake Shala hot-spring and the standard *Arthrospira* medium, which were used for experimental cultures. Units are mg L^{-1}, unless otherwise indicated; ND=Not Detectable.

Parameter	Shala hot-spring	Standard medium
pH (*in situ*)	8.79	-
pH (In the Lab.)	9.05	9.35
Conductivity (*In situ*) (ms cm^{-1})	10.24	-
Conductivity (In the Lab.) (ms cm^{-1})	9.13	19.13
Temperature (*in situ*) (°C)	79.4	-
Salinity (g L^{-1})	5.103	11.353
Alkalinity (meq L^{-1})	48.4	-
CO_3^-	384	-
HCO_3^{2-}	1634.8	-
PO_4-p	1.189681	273,000
TP	49.7595	-
NO_3−N	0.25741	1,820,000
NO_2-N	0.42798	-
NH_3-N	0.03252	-
SiO_2	79.023	-
Ca^{2+}	1.6	11
Mg^{2+}	1.46	20
Na^+	2100	6,550
SO_4^{2-}	51.86	634
K^+	29.5	672
Soluble Iron (Fe)	0.71	-
Soluble Manganese (Mn)	0.03	-
Soluble Cobalt (Co)	ND	-
Soluble Zinc (Zn)	ND	-
Soluble Copper (Cu)	ND	-
Turbidity(NTU)	10.5	-
Taste	Salty	-

The spring water was characterized by physicochemical features (Table 3), which make it suitable for *Arthrospira* production. Although values of pH and conductivity measured *in-situ* and in the laboratory were different, the differences, which resulted from the large disparity in water temperature, were small. As is the case with the saline alkaline soda lakes of the Ethiopian Rift Valley, the hot-spring water was characterized by high pH and carbonate+bicarbonate concentrations, and predominance of Na and HCO_3^- among the cations and anions, respectively. The concentrations of the major divalent cations, Ca and Mg, were, however, noticeably low. Even though the nutrients critical for *Arthrospira* growth, phosphate and nitrogen, were found in amounts smaller than those in the standard medium, they seem to be at concentrations large enough to make considerable contribution to its biomass production.

5.2 Some Chemical Features of Experimental Culture Media

pH of all culture media (Figure 3) exhibited an increasing trend although the differences among the mean pH (Table 4) values of the various culture media were not statistically significant ($P > 0.05$, Table 4).

Table 4. Mean ± Standard Deviation (SD) of physico-chemical parameters of the various growth media.

Types of media	**Mean ± SD**			
	pH	Salinity	**PO_4-P**	**NO_3-N**
SSM	10.300 ± 0.47	6.016 ± 0.40431	36.204 ± 29.86	133.749 ± 251.30
SM25	10.740 ± 0.87	7.599 ± 0.453	77.653 ± 12.43	606.753 ± 10.463
SM50	10.576 ± 0.8104	8.983 ± 0.476312	79.751 ± 19.05	614.371 ± 1.4623
SM75	10.285 ± 0.56	10.272 ± 0.338	85.537 ± 28.41011	613.082 ± 1.045
SM	10.205 ± 0.58	11.639 ± 0.164	99.895 ± 30.73	613.152 ± 1.486

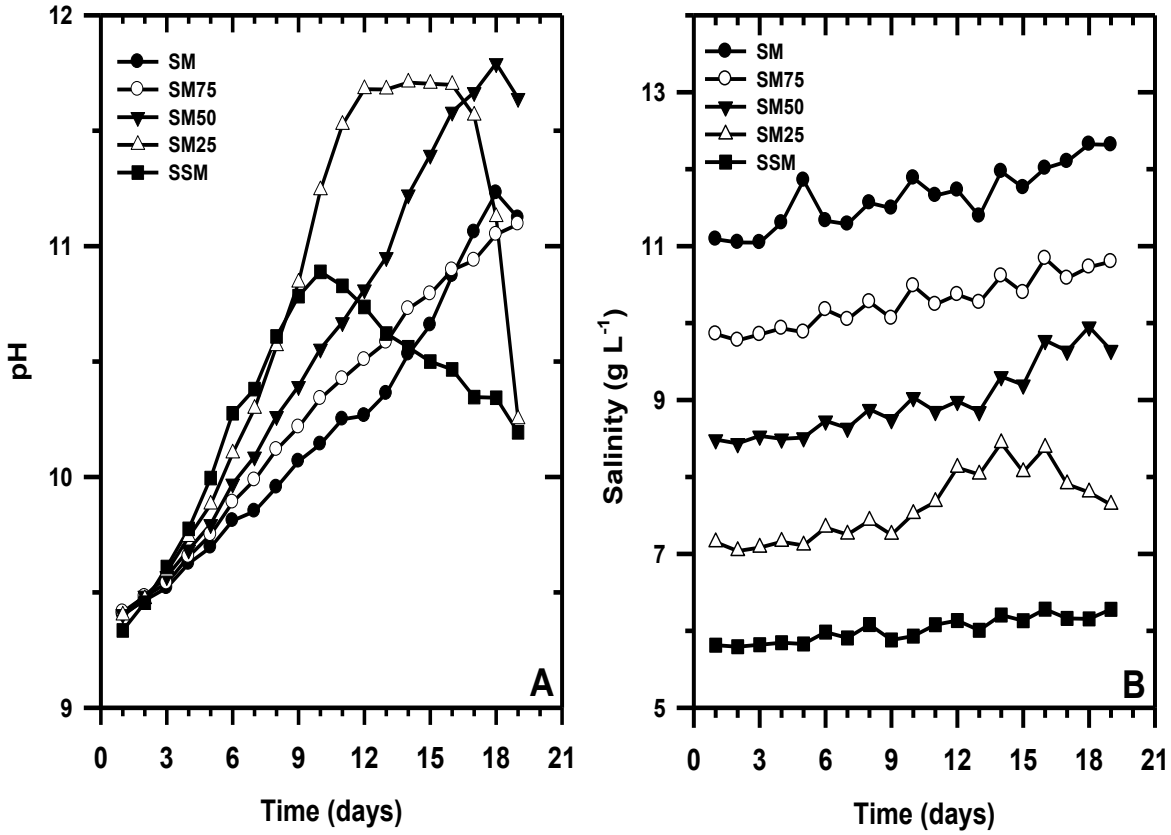

Figure 3. Changes in pH (A) and salinity (B) with time during the growth of *Arthrospira fusiformis* cultivated in the standard medium and various Lake Shala hot-spring water-based media at a temperature of 35 ˚C and photon flux density of 136 µmol photons m^{-2} s^{-1}.

The mean pH values corresponding to the various culture media were in the order of SM< SM75 < SSM < SM50 < SM25. The increase in the salinity of the growth media, which followed its intermittent drops, was slight although abrupt declines in salinity were observed in SM25 and SM50 towards the end of the growth period. SM had the highest mean salinity with the mean salinity levels of the various media declining with decreasing amounts of supplementation with the standard medium in the order of SM >SM75 >SM50 >SM25 >SSM. The mean salinity (Table 4) values of the various culture media were statistically significant ($P < 0.05$).

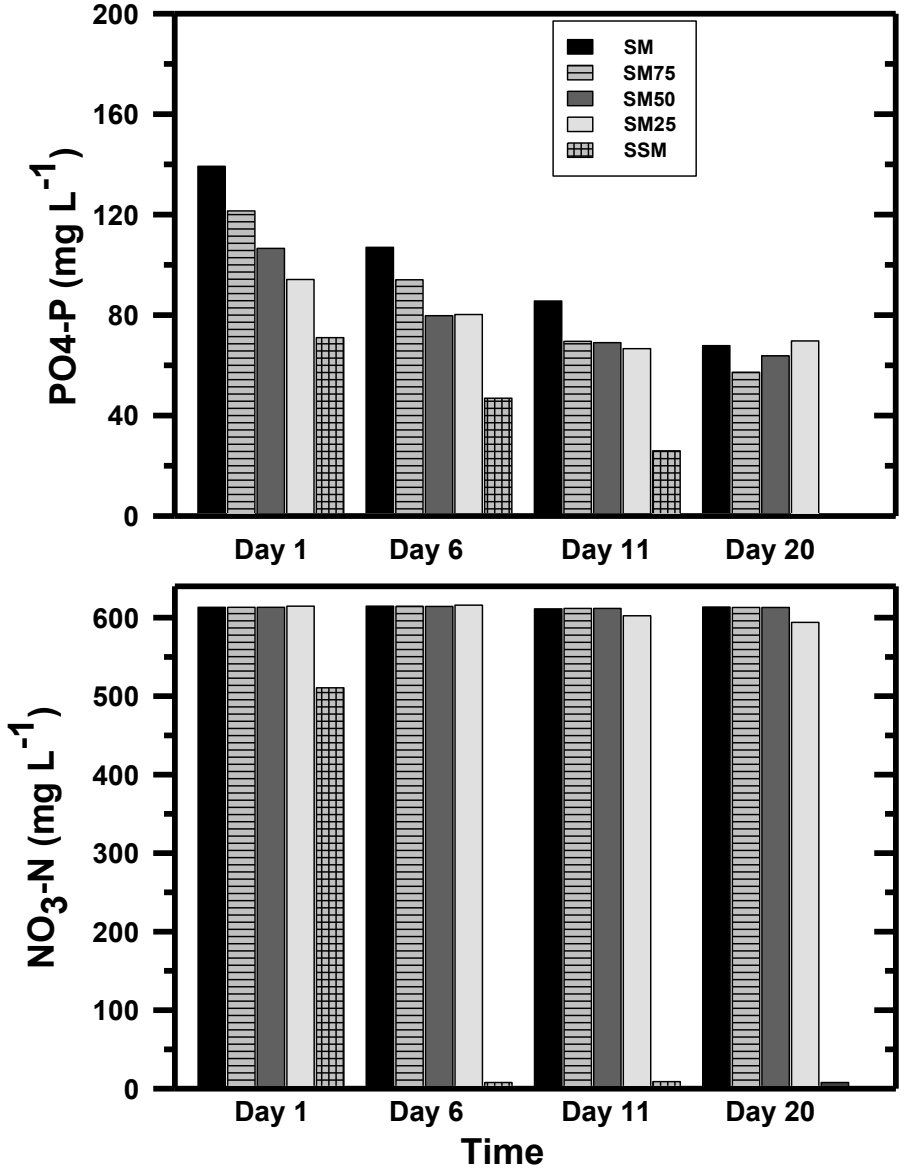

Figure. 4. Changes in the levels of nitrate (NO3-N) and Soluble Reactive Phosphate (PO4-P) during the growth period of *Spirulina (Arthrospira fusiformis*) cultivated in the standard medium and various Lake Shala hot-spring water-based media.

Phosphate concentrations in the different media decreased consistently throughout the growth period (Figure 4). The mean PO_4-P (Table 4) contents of all the media except SSM were not significantly different ($P > 0.05$). The increases in PO_4-P concentrations from SSM to SM corresponded to the increase in the amount of supplementation with the standard medium (i.e. PO_4-P of SM >SM75 >SM50 >SM25 >SSM). The decrease in the concentration of NO_3-N in the standard medium and all supplemented media was small, while that in SSM, which had

the lowest mean concentration, was large and significantly different from those of all other media ($P < 0.05$).

5.3. Growth and Biomass Production

The growth curves of *Arthrospira* cultivated in different media (Figure 5) did not exhibit a lag phase, with the higher biomass in SM75 and SM50 varying closely with that in SM. The biomass production of *Arthrospira* was relatively lower in both SM25 and SSM although culture collapse was much faster in the latter. The highest B, μ and shortest dt were observed for *Arthrospira* cultivated in SM, with slightly lower values of B and μ and longer dt for *Arthrospira* SM25 (Tables 5 and 6). Biomass estimated as dry weight (g L^{-1}) increased with an increase in the amount of supplementation with the standard medium although the differences among the values associated with the different media were not statistically significant ($P > 0.05$). When chl-a (mg L^{-1}) was used as biomass indicator, *Arthrospira* biomass in SM50 approached the highest level achieved in SM. The lowest level of biomass estimated as both chl-a and dry weight was observed for SSM, while intermediate biomass values were recorded for SM75 and SM25 although statistically significant differences were observed only between those of SM and SSM (ANOVA, $P < 0.01$).

Table 5. Maximum and mean values of *Arthrospira* biomass cultured in various media for 20 days.

Types of culture media	**B_{max}***		**Mean B ± std. deviation***	
	chl-a	Dry weight	chl-a	Dry weight
SSM	3.6	1.029	2.4832 ± 0.99615	0.77 ± 0.2822
SM25	11.11	1.296	6.052 ± 3.689	0.911023 ± 0.33999
SM50	12.9632	1.69133	6.693 ± 4.2943	1.082 ± 0.510581
SM75	12.1	1.9267	6.693 ± 4.08	1.1867 ± 0.53105
SM	13.05	1.9333	6.97 ± 4.46	1.2356 ± 0.56846

*Maximum (B_{max}) and mean biomass (mean B) estimated as chl-a (mg L^{-1}, n=19) and dry weight (g L^{-1}, n=12)

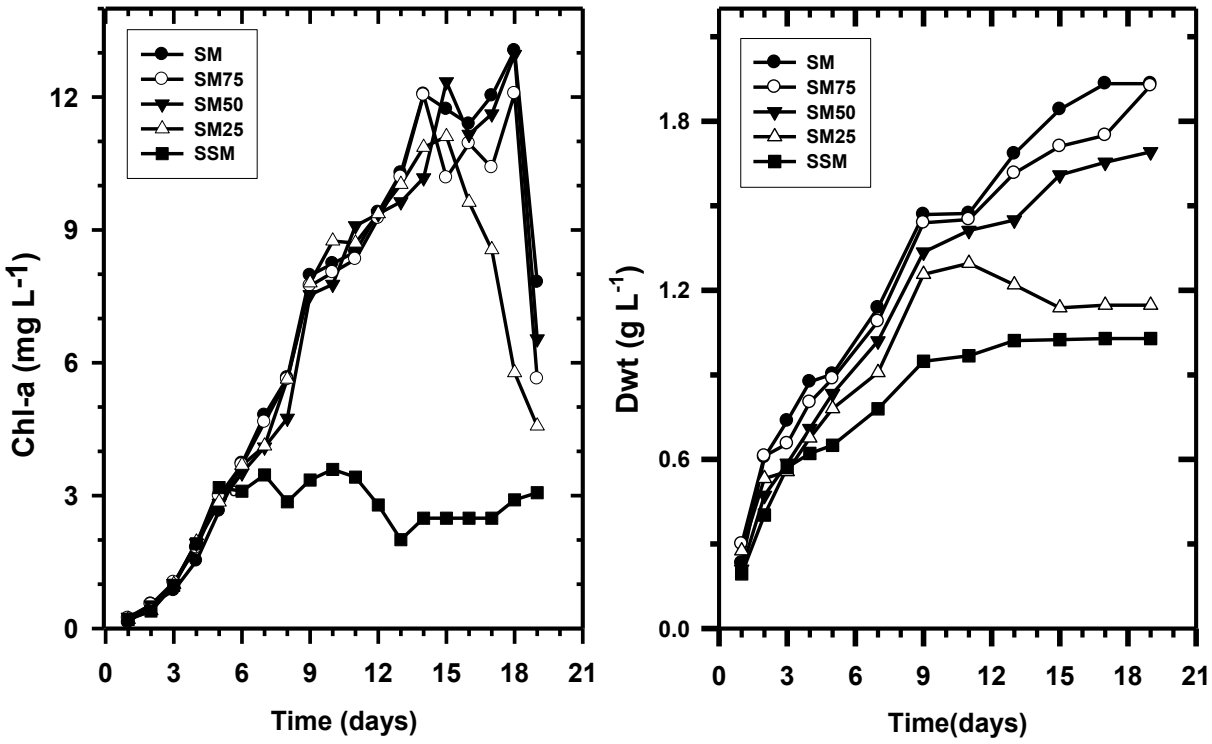

Figure 5. Growth curves determined using Chlorophyll-a (Chl-a) and dry weight (Dwt) as biomass indicators for *Spirulina(Arthrospira fusiformis)* cultivated in the standard medium and various Lake Shala hot-spring water-based media at a temperature of 35^{o}C and photon flux density of 136 μmol photons m^{-2} s^{-1}.

Table 6. Growth parameters of *Arthrospira* cultured for 20 days.

Types of culture media	**Specific growth rate (μ, day^{-1})***	**Doubling time (days)***
SSM	0.3505(0.69)	1.98
SM25	0.5	1.5
SM50	0.424	1.64
SM75	0.4403	1.6
SM	0.51	1.4

*μ, (day^{-1}) and dt (days) were calculated using chl-a by taking 9 days of exponential growth phase except for SSM, which had only 5 days of exponential growth phase.

5.4. The Relationship between Biological parameters and Chemical Factors

B and μ varied in relation to the extent of supplementation, and the associated differences in salinity and pH (Figure 6). The highest B and μ in SM coincided with the highest salinity and relatively lower pH compared to those of other Media. Broadly speaking, the increases in μ and B along the gradient of supplementation with SM seem to be associated with increasing salinity but decreasing pH although the differences in the latter among the various culture media were very small. *Arthrospira* in SSM, which exhibited the shortest growth period, had the lowest B and μ, which corresponded to the lowest salinity and pH of all media.

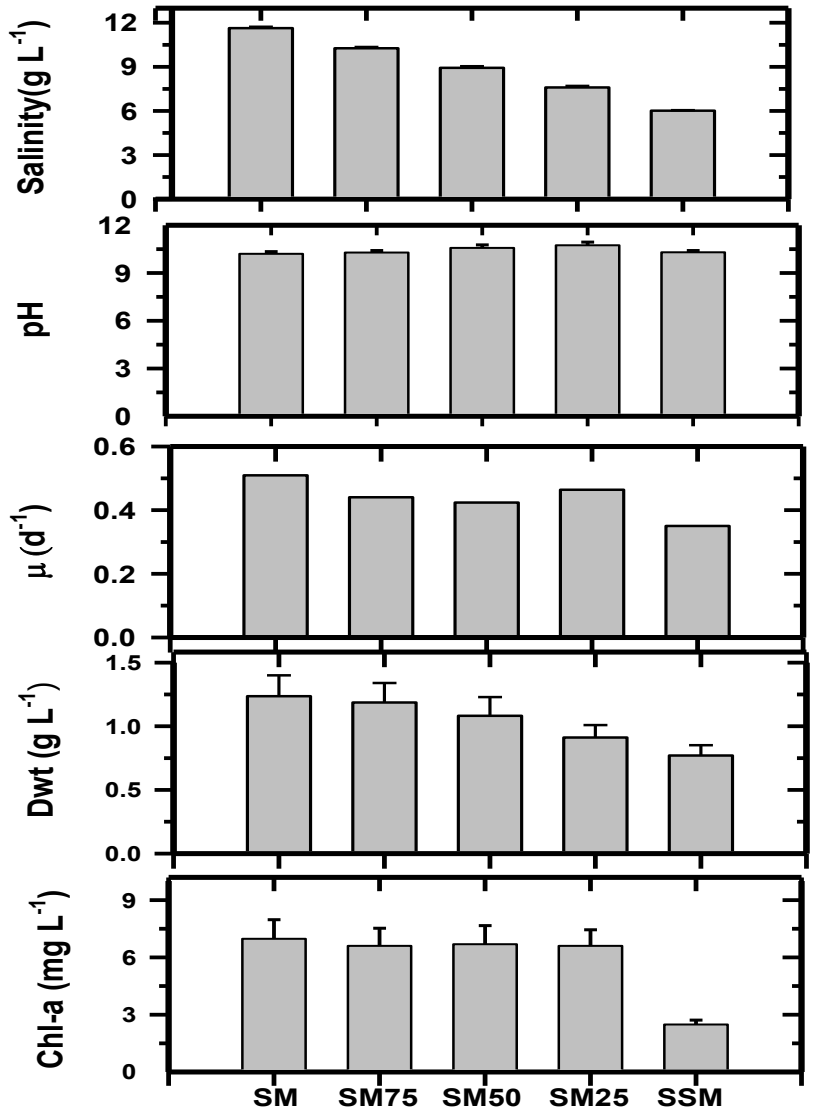

Figure 6. Variations in mean Chl-a and Dry weight (Dwt) biomass and specific growth rate (m) in relation to mean pH and salinity of the various culture media.

Table 7. Pearson correlation (ρ) of response variables (chl-a, mg L^{-1} and Dwt, g L^{-1}) and explanatory variables (PO_4^-P (mg L^{-1}), $N0_3$-N(mg L^{-1}), Salinity(g L^{-1}) and pH)

		Chl-a	Dwt	pH	Salinity	PO_4-P	NO_3-N
Chl-a	ρ	1					
	Sig. (2-tailed)						
Dwt	ρ	.887**	1				
	Sig. (2-tailed)	0.000					
pH	ρ	.791**	.774**	1			
	Sig. (2-tailed)	0.000	0.000				
Salinity	ρ	.476**	.484**	.036	1		
	Sig. (2-tailed)	0.000	0.000	0.730			
PO_4-P	ρ	-.342	-.494	-.546*	.518*	1	
	Sig. (2-tailed)	0.140	0.065	0.013	0.019		
NO_3-N	ρ	.168	.065	-.085	.619**	.718**	1
	Sig. (2-tailed)	.480	.818	.722	.004	.000	

**Correlation is significant (Sig.) at the 0.01 level (2-tailed).

*Correlation is significant (Sig.) at the 0.05 level (2-tailed).

Chl-a was significantly and positively ($P < 0.01$) correlated with dry weight, pH and salinity However, its correlation with PO_4-P and NO_3-N was not statistically significant ($P > 0.01$) (Table 7). The correlation of PO_4-P with chl-a was negative showing the expected decrease in the concentration of PO_4-P subsequent to an increase in algal biomass and the consequent

increase in the rate of PO_4-P removal from the medium. Similarly, dry weight exhibited significant correlation ($P < 0.01$) with pH and salinity though its correlation with PO_4-P and NO_3-N was not statistically significant. The correlations of dry weight with all the chemical factors except PO_4-P and salinity were lower than those of chl-a with the chemical factors. The results of multiple regression analysis (Appendix 1) of the relationship of biomass (B) indices, chl-a and dry weight, with such chemical factors as pH, salinity, PO_4-P and NO_3-N show that pH is a factor of overriding importance to the variations in chl-a and dry weight accounting for 62.8 % and 76.9 % of their variations, respectively. All chemical factors collectively account for 98% of the variation in dry weight biomass (Appendix 1B).

RDA was also used to explore the relationships among the biomass indices (response variables), chemical factors, treatments and sampling dates. The first two axes of RDA ordination (Figure 7) explained 99.9% of the variance in response variables. The first Axis (Horizontal) is the most important axis accounting for 82.3% of the variance in sampling dates (related to the levels of the various chemical and biological parameters), which was significantly but negatively correlated with PO_4^-P ($P < 0.05$) and PO_4^-P accounting for 28% of the variations in the sampling date. During the early sampling dates, PO_4-P was high, but decreased consistently through time, as was the case with NO_3-N whose correlation with sampling dates was not, however, significant. The other explanatory variable, which accounted for 72% of the variations in response variables is pH, whose effect was positive and significant ($P < 0.01$). pH is found at the late sampling dates indicating its increase through the sampling dates. Salinity is the other chemical factor, which influenced positively the variations in response variables although the changes in its level were not significant ($P > 0.01$).

The response variables increased through the growth period, which was observed in both chl-a and dry weight, and were influenced by such chemical factors as pH and PO_4^-P and sampling dates. The second axis (vertical) accounted for 17.6% of the variations clearly separating the concentration (or levels) of both response variables and chemical factors. The upper half of axis 2 (the positive part) is associated with lower concentrations (or levels) of measured parameters relative to the lower half of the same axis (negative part).

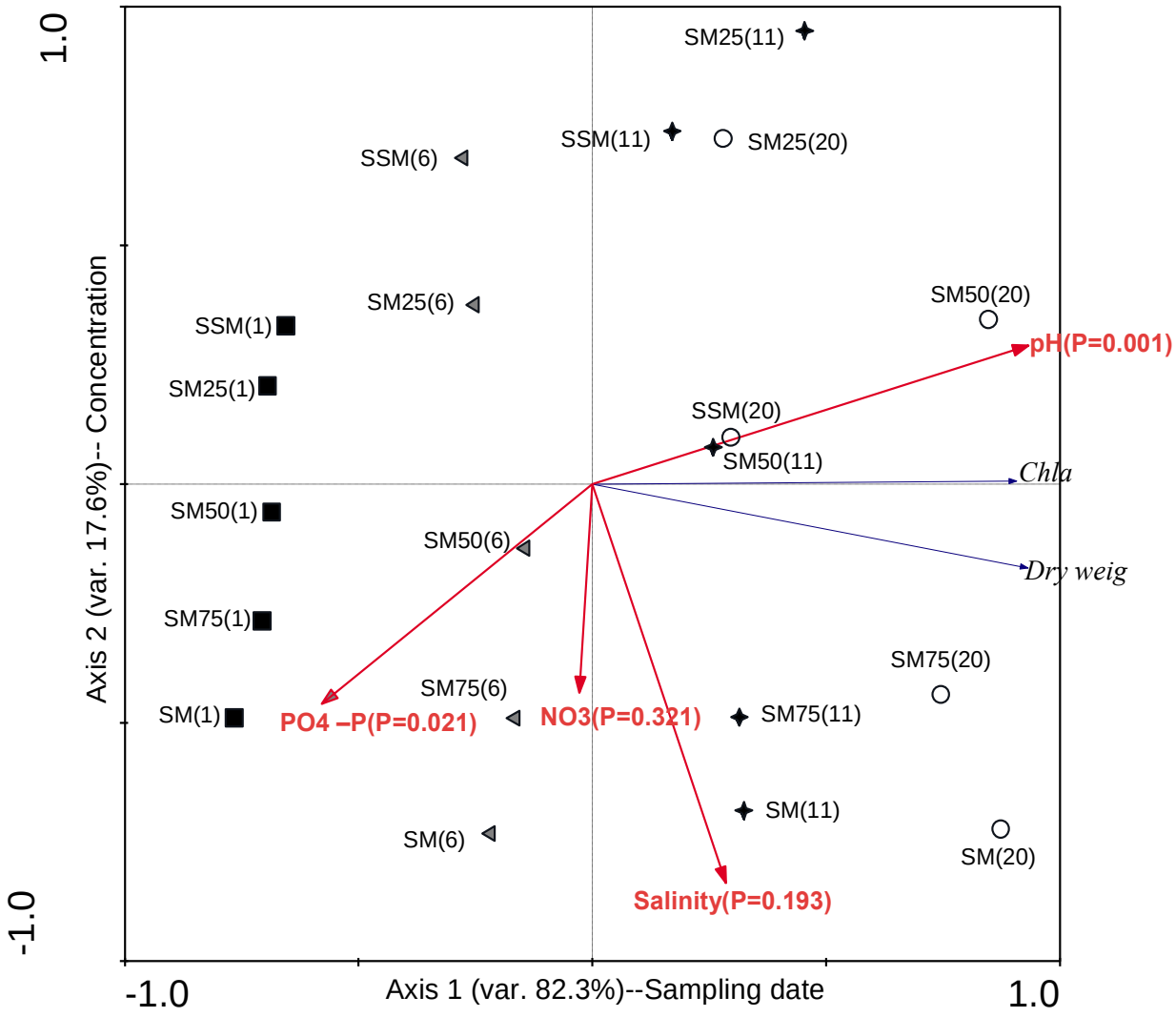

Figure 7. RDA ordination diagram of response variables and explanatory variables (chemical factors) for the first two axes. Axis 1 (horizontal) accounting for 82.3% and Axis 2 (vertical) accounting for 17.6% of the variance in culture media, sampling dates, chemical factors and response variables The response variables are chl-a and Dry weight. The chemical factors are pH, Salinity, PO_4-P and NO_3^-N. The points are Culture Media with sampling dates in brackets.

6. DISCUSSION

6.1 Physico-Chemical Features of Lake Shala Hot-spring

The observed values of alkalinity, salinity and conductivity of Shala hot spring are higher than those recorded by Tadesse Ogato (2015). The major ion composition of Shala hot spring water was characterized by the predominance of Na^+ and HCO_3^- and exceptionally low levels of Ca and Mg, a chemical condition regarded as a feature of Soda springs, which are known to be inclined towards the Na+K apex, with low levels of calcium and magnesium (Forstner, 1975). The gross chemistry of the spring water is suitable for the preparation of culture media for *Arthrospira* production. Sodium and potassium carbonates are more soluble than calcium and magnesium carbonates, which form precipitates when the pH is raised above 8.3 and carbonate is produced due to vigorous photosynthesis (Wurts and Durborow, 1992). The high bicarbonate level, which was also observed in another study (Tadesse Ogato *et al.,* 2014) is due to the weathering processes of the dying Pleistocene to recent volcanism(Forstner, 1975), while the high concentrations of Na and K and the relatively very low levels of calcium and magnesium are related mainly to reverse weathering processes (Wood and Talling, 1988). The present PO_4-P level of the spring water is higher than that reported previously (57.67 µg L^{-1}; Tadesse Ogato, un published data), while the inorganic nitrogen sources, NO_3−N, NO_2-N and NH_3-N exceeded the levels measured in previous studies conducted by Tenalem Ayenew (2003) and Tadesse Ogato (unpublished data). The SiO_2 content of the spring water was also found higher than reported by Tadesse Ogato (2015).

The pH of the spring water was found between 9 to 11, which is known to allow good growth of *Arthrospira* (Vincent and Silvester, 1979; Richmond *et al*., 1993). Thus, the spring water is unquestionably suitable with regard to one of the most important factors, which influence the photosynthesis activity, growth rate, cellular metabolism and composition of *Arthrospira* (Chen *et al*., 1994).

Even though the amount of bicarbonate+carbonate in the spring water is considerably lower than that in the standard medium, the low CO_3^{2-} to HCO_3^- ratio of the saline spring water, which is used for buffering the system, is almost the same as that in the standard medium. The spring water has, however, higher concentration of HCO_3^-, which is preferentially used by *Arthrospira* (Binaghi *et al.,* 2003).

6.2. Some Chemical Features of Experimental Culture Media

The increasing trend of pH, which was observed in all media (Figure 5), has also been reported in other studies (Richmond, 1986; Tadesse Ogato *et al.,* 2014; Hafidh *et al.,* 2015; Sandeep *et al*., 2015). This is due to the ecophysiology of *Arthrospira*, which preferentially uses bicarbonate and results in carbonate accumulation and subsequent creation of carbonate/bicarbonate imbalance in the medium, which causes a rapid increase in pH (Richmond and Grobbelaar, 1986; Richmond, 2002). During periods of rapid photosynthesis by dense algal biomass, CO_2 depletion and the consequent utilization of HCO_3^- leads to the release of carbonate, which is associated with dramatic pH rise (Wurts and Durborow, 1992; Andrad and Costa, 2007).

The increment in pH beyond the optimal range (above 10.5) after day 8, 10, 13, and 15 in SSM and SM25, SM50, SM75 and SM, respectively, caused the decline in growth rate as indicated by Amha Belay(1997) and (Fox 1996, cited in Carlos *et al.,* 2003). The high initial increment and eventual abrupt decline in pH, which were evident in the media supplemented with smaller volumes of the standard medium (SSM and SM25) are attributable to the lower buffering capacity of the spring water due to its less carbonate + bicarbonate content relative to that of the standard medium (Tadesse Ogato *et al.,* 2014). This rapid increase in pH in SM25 to beyond the range optimal for *Arthrospira* production seems to be suggestive of the need to adjust the pH for large scale production using this particular medium.

The observed increase and fluctuation of salinity of the growth media also occur in natural *Arthrospira* population growth systems (Elizabeth Kebede, 1997). The pronounced decline in the salinity of SM25 and SM50 towards the end of the cultivation period may have resulted from the dilution of growth media through biological removal and chemical transformation of carbonate–bicarbonate species, which are the major salinity contributors (Tadesse Ogato *et al.*, 2014).Even low salinity levels as that found in SSM may be considered to be optimum for the growth of *Arthrospira* since the former Lake Arenguade with a salinity of only 5 g L^{-1} used to support an almost monoalgal dense natural culture of *Arthrospira* (Elizabeth Kebede, 1997). Hafidh *et al.'s* (2015) experiments have also demonstrated that media with salinities similar to that of the spring water may support much higher *Arthrospira* biomass productivity than those with higher salinities. The association of decreased salinity with higher growth rate was also reported by several other workers (e.g. Reed *et al.,* 1985; Elizabeth Kebede, 1997;

Moisander *et al.,* 2002). However, the constituent ions especially carbonate and bicarbonate should be at concentrations large enough to support good growth of *Arthrospira* for longer time period. Although Ravelonandro *et al.* (2011) contended that 13 g L^{-1}, which is close to that of the standard medium, is the optimum salinity for *Arthrospira* growth.

The observed decline in phosphate concentration through the growth period resulted from the utilization of phosphate by the growing *Arthrospira*, a trend which was also reported by Lodi *et al.* (2003) and Sandeep *et al.* (2015). However, the decrease in phosphate concentration with an increase in biomass of *Arthrospira* may have also resulted from chemical precipitation, which is known to be favored by the increased pH (Laliberté *et al*., 1997; Lodi, 2003), The recorded low growth of this alga in SSM, which was relatively P-deplete, is a clear indication of the fact that P is a nutrient, which plays an important role in the high productivity of this micro alga (Mostert and Grobbelaar, 1987). The moderate decline in NO_3-N concentration through the cultivation period of the alga was also observed by Sandeep *et al.* (2015). The rapid and abrupt decline in NO_3-N concentration in SSM was probably related to the fact that the removal of nitrate by *Arthrospira* depends on the level of it in the medium with the removal of it becoming higher when the concentration is low Lodi *et al.* (2003).

The spring water has lower salinity, pH, PO_4-P and NO_3-N than the standard medium. Thus, supplementation of the spring water is necessary to overcome the problem of nitrate and phosphate limitation (Grobbelaar, 2004; Soletto *et al.,* 2005). Deficiency of nitrogen sources causes limited growth, altered pigmentation and biochemical composition (Mostert and Grobbelaar, 1987) and accumulation of carbohydrate rather than protein (Tadros, 1988). Shortage of carbonate and bicarbonate, which regulate the salinity and pH of the media, is also solved by supplementation with the standard medium. The present results have shown that supplementation of the spring water with 25% standard medium can support the growth of *Arthrospira* to a level, which is comparable to that in the standard medium.

6.3 Growth and Biomass Production

The growth curves (Figure 5) of *Arthrospira* cultivated in different media showed the exponential growth of the alga without any lag time, and with the higher growth rates in SM, SM25, SM75 and SM50 respectively. The absence of lag time during the growth of the alga may be attributed to the source culture of the inoculum, in which the alga was growing

exponentially and the size of the inoculum, which constituted 10% of the final culture volume (Amha Belay, 1997). Even though similar exponential growth was seen in all media for earlier dates growth difference and collapse probably caused by increased pH, carbonate-bicarbonate and nutrient limitation. This condition progressively makes the culture yellow (Tadros, 1988) which is clearly seen in plate 3.

Even though biomass and specific growth rate of the alga in SM25 were comparable to those in SM-supplemented media, culture collapse occurred at an earlier date. The culture collapse may have been caused by high pH, which reduce the growth rate and productivity of the alga (Tadros, 1988). The spring water was conducive for *Arthrospira growth.* However, for biomass production comparable to that in the standard medium supplementation with the latter is obviously needed to prevent limitation by inorganic carbon source, pH and nutrients. The present results seem to suggest that supplementation by at least 50% is needed to maintain the production of *Arthrospira* biomass for a longer period of time. The specific growth rate and biomass observed in the present study are higher than those reported by Tadesse Ogato *et al.* (2014) for *Arthrospira* cultivated in soda Lakes Chitu and Shala water-based media.

Plate 3. *Arthrospira*'s culture at the beginning (Left) and end (20th day) of the cultivation period. (Left: SM, SSM, SM25, SM50, SM75 left to right) and Right: SM, SM75, SM50, SM25, SSM right to left).

6.4. The Relationship between Biological parameters and Chemical Factors

The results of statistical analyses suggested significant correlation ($P < 0.01$) and high interaction (99.9%) between biomass indices (chl-a and dry weight) and aggregate chemical parameters (pH and salinity). This was expectable since *Arthrospira's* growth and biomass production are known to be affected by pH (Vincent and Silvester 1979; Tadros, 1988), salt concentration (Zeng and Vonshak, 1998) and the nutrients phosphate and nitrate (Mostert and Grobbelaar, 1987; Celekli *et al.,* 2009a). PO_4-P, which accounted for 28% of the variation in sampling dates, plays an essential role in biomass production of *Arthrospira* (Mostert and Grobbelaar, 1987). The overriding importance of pH, which according to RDA, accounted for 72% of the variation in biotic indices, was corroborated by the results of multiple regression. pH was a stronger determinant of B (chl-a and dry weight) than salinity, PO_4-P and NO_3-N, a finding that is consistent with the results of Pelizer *et al.* (2002). Similarly, pH was reported to have accounted for 52% of the variation in *Arthrospira* biomass in SM-supplemented alkaline-saline lake water-based media (Tadesse Ogato *et al*., 2014). The study made by Çelekli *et al*. (2009b) has also shown that pH, phosphate concentrations and cultivation time regimes significantly affected ($P < 0.01$) the amount of chlorophyll measured. The observed negative correlation between pH and PO_4-P may be explained by the chemical precipitation of PO_4-P, which is favoured by increased pH (Laliberté *et al.,* 1997; Lodi, 2003).

The present results suggest the overriding limiting role of pH in *Arthrospira* biomass production. pH of SSM and SM25, which reached above 10.5 after 7 days curtailed the growth and biomass production of *Arthrospira*. Since the optimal pH is known to be between 9.5 –10.5, a rise in pH above 10.5 is expected to lead to a decline in the growth rate (Fox, 1996: Amha Belay, 1997; Vonshak, 1997). However, *Arthrospira* is known for its tolerance to a wide range of pH 8 to 11 (Tadros, 1988). As pH affects the solubility of minerals, carbon source and metabolism of algae directly and indirectly (Vincent and Silvester. 1979; Guterman *et al.,* 1989, cited in Tadesse Ogato *et al.*, 2014) regulation of pH is highly recommended to ensure longer period of biomass production. Supplementation by SM, which has high carbonate+bicarbonate, enables to maintain optimal pH by preventing carbon depletion, which can also lead to shorter growth period (Richmond 1990; Vonshak 1997, cited in Tadesse ogato *et al.,* 2014). High levels of carbonate-bicarbonate are associated with higher biomass production as shown by the studies made by Costa *et al.* (2003) using lagoon water, which was supplemented with up to 2.88 g L^{-1} sodium bicarbonate. Tadros (1988) also

stated that increased bicarbonate concentration led to increased productivity and even further increase above 16 g L^{-1} (190 mM) did not affect the growth rate of *Arthrospira*. Moreover, higher nutrient concentration, which precludes nutrient depletion, is necessary for longer period of biomass production as the low levels of PO_4^-P and NO_3-N in SSM, together with very high pH, presumably resulted in the shortest growth period (Rosa. *et al.,* 2015). These nutrients were also reported to be limiting to *Arthrospira* in natural inland water bodies (Tadesse Ogato *et al.,* 2014) and sea water (Materassi *et al.*, 1984; Tredici *et al.,* 1986). Thus, supplementation with the standard medium provides the necessary nutrients often leading to high biomass yield, which is slightly less than that in the standard medium.

Different physiological process including photosynthesis, chemical production and growth of *Arthrospira* are affected by the salinity of a growth medium (Vonshak *et al.,* 1996; Elizabeth Kebede, 1997, cited in Tadesse Ogato *et al*., 2014). Even though the optimal salinity for *Arthrospira* production is considered to be that of the standard medium, the alga seems to exhibit a wide range of salinity tolerance. Relatively lower salinities of culture media associated with higher growth rate and biomass production have also been reported by different researchers(e.g. Reed *et al.,* 1985; Elizabeth Kebede, 1997; Vonshak, 1997; Moisander *et al.,* 2002; Mussagy, 2006). To sum up, Shala spring water has the potential to produce *Arthrospira* with small supplementation (25-50%) with the standard medium. According to Habib *et al.* (2008), 79.5 USD is needed to prepare 1 L SM. In light of this, the cultivation of *Arthrospira* in SM25 whose cost is estimated at 19.879 USD would enable one to save 59.625 USD. If SM50 is used to cultivate the same micro alga, the cost of its growth medium will be reduced by 50% (i.e. 39.75USD). Currently, 1 L *Arthrospira* medium costs 43.74 USD (Amazonbusiness, 2016). Thus the cost of this medium can also be reduced by more than 50% using Shala hot-spring water-based media.

7. CONCLUSIONS AND RECOMMENDATIONS

7.1. Conclusions

The chemistry of Lake Shala hot spring makes it suitable for the production of *Arthrospira* biomass with small supplementation with the standard medium. Growth and biomass production of *Arthrospira* comparable to those in the standard medium were achieved using spring water with 25% supplementation. Increasing the supplementation to 50% enhanced growth and biomass production and prolonged the period of growth and biomass production since the supplementation added carbonate–bicarbonates, and nutrients and maintained the optimal pH for longer period. The results of the present study showed the overriding importance of pH to the growth and biomass production of *Arthrospira*. Since pH is mainly affected by the level of carbonate-bicarbonate, addition of it is necessary to maintain optimal pH. To conclude, using Lake Shala hot spring supplemented with the standard medium, 50 to 75% of media cost can be reduced based on the rough estimation of the spring water proportion.

7.2. Recommendations

Based on the findings of this study, the following recommendations are given:

- Even though hot-spring waters seem to have high potential to support *Arthrospira* production, outdoor *Arthrospira* production using these spring water-based media has to be investigated before considering large scale production.
- Considering the effect of such chemical factors as pH, further study is needed to optimize the chemical environment for the growth of *Arthrospira* in these media.
- The nutritional profile of *Arthrospira* product, which can be obtained by growing the alga in hot-spring water-based media, has to be evaluated before considering large-scale production.

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9. APPENDICES

Appendix 1.Results of multiple linear regression with step-wise calculation to relate chl-a with chemical factors (A) and to relate Dry weight with chemical factors (B).

(A)

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	-31.246	6.452		-4.843	.000
	pH	3.460	.628	.792	5.509	.000

a. Dependent Variable: chlamgL1

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.792[a]	.628	.607	1.954792495492405

a. Predictors: (Constant), pH

(B)

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.877[a]	.769	.751	.3206170599842
2	.921[b]	.848	.823	.2703661565837
3	.969[c]	.940	.923	.1776774381901
4	.990[d]	.980	.973	.1063044685411

a. Predictors: (Constant), pH

b. Predictors: (Constant), pH, SalinitygL1

c. Predictors: (Constant), pH, SalinitygL1, PO4PmgL1

d. Predictors: (Constant), pH, SalinitygL1, PO4PmgL1, NO3mgL1

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	T	Sig.
		B	Std. Error	Beta		
1	(Constant)	-6.153	1.097		-5.608	.000
	pH	.696	.106	.877	6.571	.000
2	(Constant)	-6.462	.933		-6.923	.000
	pH	.650	.091	.819	7.127	.000
	SalinitygL1	.088	.035	.288	2.506	.028
3	(Constant)	-3.954	.867		-4.563	.001
	pH	.397	.086	.500	4.620	.001
	SalinitygL1	.176	.031	.574	5.582	.000
	PO4PmgL1	-.009	.002	-.488	-4.097	.002
4	(Constant)	-2.740	.583		-4.700	.001
	pH	.277	.058	.349	4.785	.001
	SalinitygL1	.166	.019	.541	8.733	.000
	PO4PmgL1	-.015	.002	-.821	-8.039	.000
	NO3mgL1	.001	.000	.366	4.553	.001

a. Dependent Variable: DryweightgL

Appendix 2. ANOVAs and multiple comparison analysis output for Dry weight, chl-a, pH, PO_4^-P and NO_3^-N.

ANOVA

Dry weight g L^{-1}

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.813	4	.453	2.135	.089
Within Groups	11.672	55	.212		
Total	13.484	59			

ANOVA

pH

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	3.910	4	.978	2.150	.081
Within Groups	40.918	90	.455		
Total	44.828	94			

ANOVA

PO_4-P, mg L^{-1}

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	9049.184	4	2262.296	3.579	.031
Within Groups	9480.608	15	632.041		
Total	18529.792	19			

Dependent Variable: PO_4-P, mg L^{-1}

Tukey HSD

(I) TR	(J) TR	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
SM	SM25	22.241975000	.723	-32.65193280	77.13588280
	SM50	20.143595000	.787	-34.75031280	75.03750280
	SM75	14.358122500	.924	-40.53578530	69.25203030
	SSW	63.691377500*	.020	8.79746970	118.58528530
SM25	SM	-22.241975000	.723	-77.13588280	32.65193280
	SM50	-2.098380000	1.000	-56.99228780	52.79552780
	SM75	-7.883852500	.991	-62.77776030	47.01005530
	SSW	41.449402500	.189	-13.44450530	96.34331030
SM50	SM	-20.143595000	.787	-75.03750280	34.75031280
	SM25	2.098380000	1.000	-52.79552780	56.99228780
	SM75	-5.785472500	.997	-60.67938030	49.10843530
	SSW	43.547782500	.155	-11.34612530	98.44169030
SM75	SM	-14.358122500	.924	-69.25203030	40.53578530
	SM25	7.883852500	.991	-47.01005530	62.77776030
	SM50	5.785472500	.997	-49.10843530	60.67938030
	SSW	49.333255000	.089	-5.56065280	104.22716280
SSW	SM	-63.691377500*	.020	-118.58528530	-8.79746970
	SM25	-41.449402500	.189	-96.34331030	13.44450530
	SM50	-43.547782500	.155	-98.44169030	11.34612530
	SM75	-49.333255000	.089	-104.22716280	5.56065280

*. The mean difference is significant at the 0.05 level.

ANOVA

Salinity, ppt

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	368.558	4	92.140	625.725	.000
Within Groups	13.253	90	.147		
Total	381.811	94			

Multiple Comparisons

Dependent Variable: salinity, ppt

Tukey HSD

(I) TR	(J) TR	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
SM	SM25	4.040368429579*	.000	3.69377860825	4.38695825091
	SM50	2.655517630000*	.000	2.30892780867	3.00210745133
	SM75	1.367377231053*	.000	1.02078740972	1.71396705238
	SSW	5.622369333158*	.000	5.27577951183	5.96895915449
SM25	SM	-4.040368429579*	.000	-4.38695825091	-3.69377860825
	SM50	-1.384850799579*	.000	-1.73144062091	-1.03826097825
	SM75	-2.672991198526*	.000	-3.01958101985	-2.32640137720
	SSW	1.582000903579*	.000	1.23541108225	1.92859072491
SM50	SM	-2.655517630000*	.000	-3.00210745133	-2.30892780867
	SM25	1.384850799579*	.000	1.03826097825	1.73144062091
	SM75	-1.288140398947*	.000	-1.63473022028	-.94155057762
	SSW	2.966851703158*	.000	2.62026188183	3.31344152449
SM75	SM	-1.367377231053*	.000	-1.71396705238	-1.02078740972
	SM25	2.672991198526*	.000	2.32640137720	3.01958101985
	SM50	1.288140398947*	.000	.94155057762	1.63473022028
	SSW	4.254992102105*	.000	3.90840228078	4.60158192343
SSW	SM	-5.622369333158*	.000	-5.96895915449	-5.27577951183
	SM25	-1.582000903579*	.000	-1.92859072491	-1.23541108225
	SM50	-2.966851703158*	.000	-3.31344152449	-2.62026188183
	SM75	-4.254992102105*	.000	-4.60158192343	-3.90840228078

*. The mean difference is significant at the 0.05 level.

ANOVA

chlamgL1

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	263.323	4	65.831	4.875	.001
Within Groups	1215.383	90	13.504		
Total	1478.706	94			

Multiple Comparisons

Dependent Variable: chl-a, mg L^{-1}

Tukey HSD

(I) TR	(J) TR	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
SM	SM25	.916936143684312	.939	-2.402160680847179	4.236032968215603
	SM50	.279603552631679	.999	-3.039493271899811	3.598700377162970
	SM75	.365556200000101	.998	-2.953540624531390	3.684653024531392
	SSM	4.485731374210627*	.003	1.166634549679336	7.804828198741919
SM25	SM	-.916936143684312	.939	-4.236032968215603	2.402160680847179
	SM50	-.637332591052732	.984	-3.956429415584023	2.681764233478758
	SM75	-.551379943684311	.990	-3.870476768215601	2.767716880847180
	SSM	3.568795230526415*	.029	.249698405995124	6.887892055057706
SM50	SM	-.279603552631679	.999	-3.598700377162970	3.039493271899811
	SM25	.637332591052732	.984	-2.681764233478758	3.956429415584023
	SM75	.085952647368522	1.000	-3.233144177162969	3.405049471899813
	SSM	4.206127821579048*	.006	.887030997047757	7.525224646110339
SM75	SM	-.365556200000101	.998	-3.684653024531392	2.953540624531390
	SM25	.551379943684311	.990	-2.767716880847180	3.870476768215601
	SM50	-.085952647368522	1.000	-3.405049471899813	3.233144177162969
	SSM	4.120175174210625*	.007	.801078349679334	7.439271998741917
SSM	SM	-4.485731374210627*	.003	-7.804828198741919	-1.166634549679336
	SM25	-3.568795230526415*	.029	-6.887892055057706	-.249698405995124
	SM50	-4.206127821579048*	.006	-7.525224646110339	-.887030997047757
	SM75	-4.120175174210625*	.007	-7.439271998741917	-.801078349679334

*. The mean difference is significant at the 0.05 level.

ANOVA

NO_3-N, mg L^{-1}

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	731568.665	4	182892.166	14.454	.000
Within Groups	189799.810	15	12653.321		
Total	921368.474	19			

Multiple Comparisons

Dependent Variable: NO_3-N, mg L^{-1}

Tukey HSD

(I) TR	(J) TR	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
SM	SM25	6.398763000	1.000	-239.21569659	252.01322259
	SM50	-1.219365000	1.000	-246.83382459	244.39509459
	SM75	.069678000	1.000	-245.54478159	245.68413759
	SSW	479.403027250*	.000	233.78856766	725.01748684
SM25	SM	-6.398763000	1.000	-252.01322259	239.21569659
	SM50	-7.618128000	1.000	-253.23258759	237.99633159
	SM75	-6.329085000	1.000	-251.94354459	239.28537459
	SSW	473.004264250*	.000	227.38980466	718.61872384
SM50	SM	1.219365000	1.000	-244.39509459	246.83382459
	SM25	7.618128000	1.000	-237.99633159	253.23258759
	SM75	1.289043000	1.000	-244.32541659	246.90350259
	SSW	480.622392250*	.000	235.00793266	726.23685184
SM75	SM	-.069678000	1.000	-245.68413759	245.54478159
	SM25	6.329085000	1.000	-239.28537459	251.94354459
	SM50	-1.289043000	1.000	-246.90350259	244.32541659
	SSW	479.333349250*	.000	233.71888966	724.94780884
SSW	SM	-479.403027250*	.000	-725.01748684	-233.78856766
	SM25	-473.004264250*	.000	-718.61872384	-227.38980466
	SM50	-480.622392250*	.000	-726.23685184	-235.00793266
	SM75	-479.333349250*	.000	-724.94780884	-233.71888966

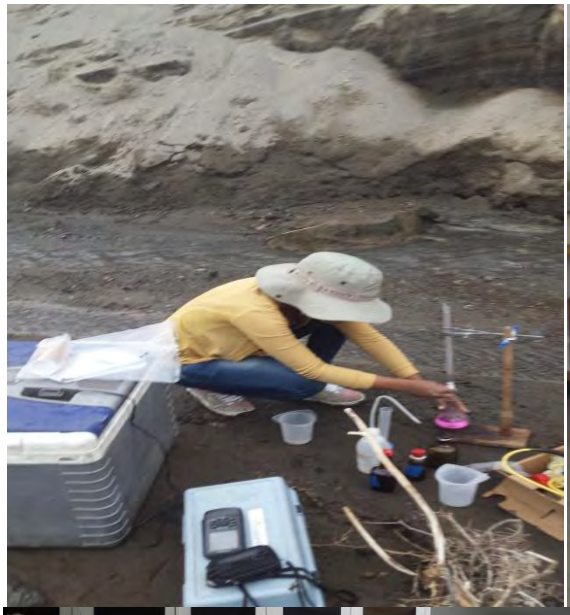
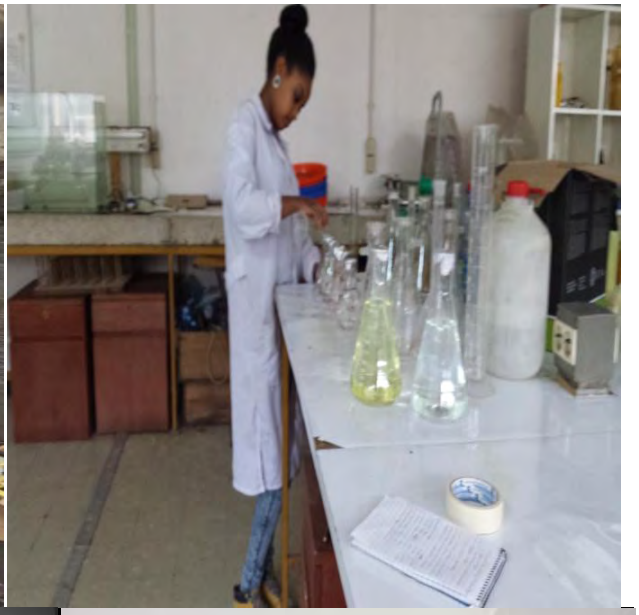
*. The mean difference is significant at the 0.05 level.

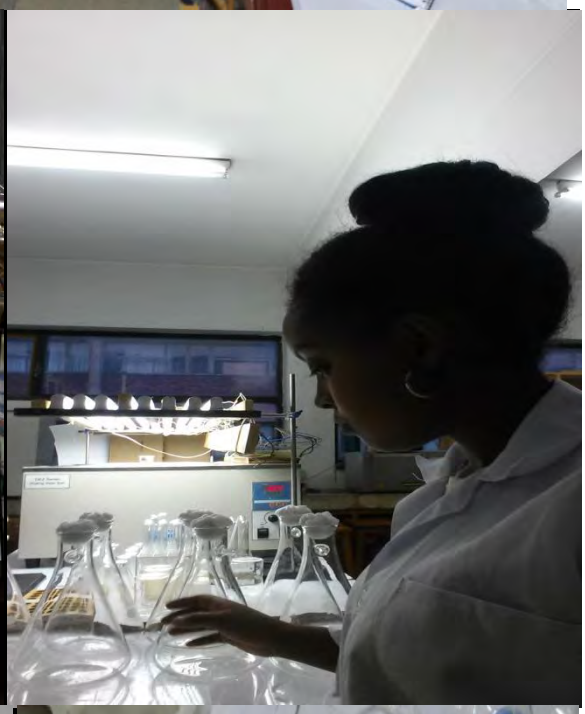
Appendix 3. Summary of RDA results of the relationship between biomass indices (response variables), chemical factors (explanatory variables) and sampling dates.

Axes	1	2	3	4
Eigen values	0.823	0.176	0.001	0
Species-environment correlations	0.908	0.408	0.000	0.000
Cumulative percentage variance of species-environment relation	82.3	99.9	100	100

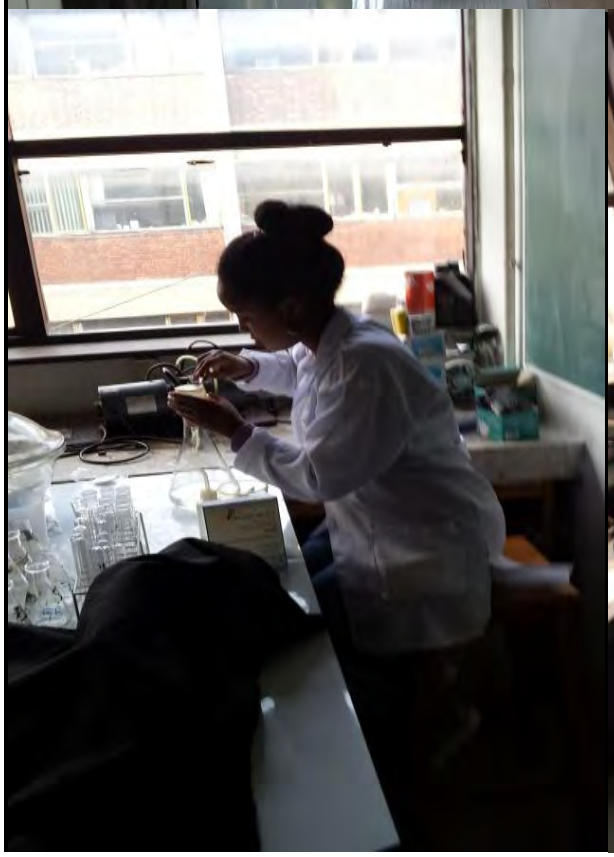
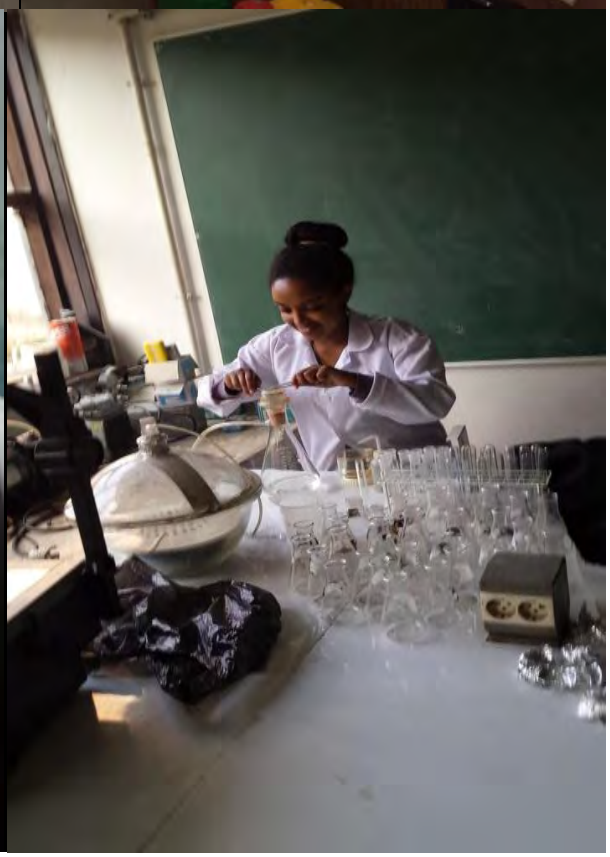
Appendix 4. Pictures of Lake Chitu and Shala hot-spring.

Appendix 5. Some pictures which taken from the *in situ* and laboratory analysis.





DECLARATION

I, the undersigned, declare that this thesis is my original work and that all sources of materials used for the thesis have been duly acknowledged.

Name: Elsabeth Asmare Signature: _____ Date: _____