TAMPERE UNIVERSITY
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THE GROWTH POTENTIAL OF ARTHROSPIRA PLATENSIS

Literature review and case study

Mikko Vaittinen

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ABSTRACT

Tampereen ammattikorkeakoulu
Tampere University of Applied Sciences
Energy and Environmental Engineering

Mikko Vaittinen:
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Literature review and case study

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The aim of this study is to evaluate and research the growth potential of Arthrospira Platensis. By analyzing the best practices found from previous studies, information regarding optimal growing conditions could be compiled. To support the literature research an experiment was conducted in the wastewater laboratory of TAMK to cultivate Spirulina for 22 day period using unmodified Zarrouk's nutrient medium.

It was concluded, that Spirulina culture is relatively simple to grow successfully. It has high protein content and is rich in important nutraseutical compounds, but requires only modest conditions and consumes less energy, water and nutrients, than many other alternatives.

Despite the growing interest towards Spirulina and micro-algae in general, they haven't been recognized as widely as their potential suggests. Development and research concerning nutrient media optimization and production methods are vital for more affordable commercial products.

Key words: arthrospira platensis, spirulina, zarrouk's medium, indoor cultivation, nutrition, therapeutical application, micro-algae

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ABBREVIATIONS AND TERMS

TAMK – Tampereen ammattikorkeakoulu (Tampere university of applied sciences)

ZN – Zarrouk's nutrient medium

PVC – Polyvinyl chloride

HCl – Hydrochloric acid

HIV - Human immunodeficiency virus

K – Potassium

P – Phosphorus

Na – Sodium

Ca – Calcium

Mn – Manganese

Fe – Iron

CO_2 – carbon dioxide

H_2O – water

H_2CO_3 – carbonic acid

HCO_3^- - bicarbonate

H – hydrogen

CH_2O – formaldehyde

O_2 - oxygen

°C - Celcius

PBR - Photobioreactor

Kg – kilogram

g – gram

ml – milliliter

l - liter

UV – Ultraviolet

1 INTRODUCTION

The use of animals to produce food is extremely inefficient in terms of land use and puts more stress on to the environment, than any other food production. It pollutes water and is the biggest source of anthropogenic greenhouse gas (methane, nitrous oxide) emissions among other problems. As the world population and income growth increases, there will be more demand for meat related products, which likely will magnify the problems. (FAO/OECD 2016, 113-115)
Micro-algae Spirulina has turned out to be of the most promising alternative sources for protein in the future. Microalgae or microphytes are microscopic, eukaryotic, photosynthetic organisms, found in freshwater, marine and damp terrestrial environments. They are considered generally autotrophs as they require only inorganic nutrients and light source to grow. Microalgae are a significant producers of oxygen and organic compounds, which heterotrophic organisms use. (Barsanti & Gualtieri 2006, 16)

According to Barsanti & Gualteri (2006, 3): "Oceans covering about 71% of earths surface contain more than 5000 species of planktonic microscopic algae, the phytoplankton, which forms the base of the marine food chain and produces roughly 50% of the oxygen we inhale."
Algae are of universal occurrence and found in all types of challenging habitats submitted to extreme conditions (changes of salinity, temperature, nutrients, UV-Vis irradiation), so they must adapt rapidly to the new environmental conditions, producing a great variety of metabolites, which cannot be found in other organisms. (Sarabhai & Arora 1995, 4; Sze 1996, 17)

The scientific field investigating the algae is called Phycology. The history of phycology is as old as human kind's interest in botany (Sarabhai & Arora 1995, 4). The earliest records found regarding Arthrospira Platensis, date back to 16th century South America where the Aztecs harvested Spirulina from lake Texaco for nutritional purposes. However it wasn't until 16th century, with the invention of microscope when the scientific progress regarding microalgae started to emerge in western cultures, discovering it's vast potential for different appliances. (Barsanti & Gualtieri 2006, 251)
Today the global interest towards algae is on increase. In terms of biomass harvested yearly algae are among the most cultivated marine organisms. Most of the commercial producers are focusing to Dunaliella, Haematococcus, Arthrospira, and Chlorella –

species cultivated mainly in open ponds for inexpensive, viable sources of carotenoids, pigments, proteins, and vitamins that are used for the production of nutraceuticals , pharmaceuticals, animal feed additives and cosmetics. (Barsanti & Gualtieri 2006, 251-252)

2 SCOPE

This thesis explores the growth potential of Arthrospira Platensis for food supplementary or therapeutical purposes. Spirulina has many health benefits and high protein content, but requires only relatively modest conditions to grow. Hence it can be seen as a potential part-solution to the increasing protein need in the future, but also to replace the current production means. Therefore affordable production platforms and more optimized nutrient ratios should be researched extensively.

The experiment was carried out as a case study to support the literature review. The primary aim of this experiment was to evaluate the growth potential of Spirulina (Arthorspira Platensis) in unmodified Zarrouk's nutrient medium during 22 days period by sampling the drymass content at regular time intervals.

Spirulina cultivation should be attainable in residential, or similar environment, using simple and cheap cultivation methods. The experiment was be carried out with this in mind, while still managing to keep the growth at optimum phase and meeting hygiene and toxicological aspects for high quality biomass yield for nutraceutical or therapeutical purposes. In the beginning following questions were topics of interest:

1. How much biomass can be produced with the selected experimental setup?
2. How does Zarrouk's nutrient medium perform as the growing medium in regards of pH stability and growth rate?
3. Is it economically worthwhile to maintain stable yield of Spirulina biomass in small-scale residential growing conditions?
4. Does the slightly decreased temperature from optimum conditions effect the growth rate?
5. Can the growing conditions be influenced to boost production of specific compounds?

3 THEORETICAL BACKGROUND

3.1 Characteristics of Spirulina

Arthrospira Platensis, commonly known as Spirulina, belonging to cyanobacteria phylum of Eubacteria kingdom of Bacteria domain is symbiotic, multicellular green-blue algae. The size of gas-vacuolate filaments vary from 3 to 12 micrometers (Ali & Saleh 2012). It is currently the most cultivated micro-alga in the world with production volumes ranging between 3000 and 20 000 tons yearly. (Delrue et al. 2017)

Cyanobacteria were the first algae to evolve over 3,5 billion years ago. Although not the first organism capable of photosynthetis, Cyanobacteria were the first ones to have two photosystems and to produce oxygen as a byproduct. This new ability was instrumental for the development of atmosphere, shaping Earth's biochemistry and subsequently contemporary human economy. This role is becoming increasinly important as human impact on ecosystems result in massive alteration of biochemical cycling of chemical elements. (Sze 1996, 21-24; Barsanti & Gualtieri 2006, 159)

3.2 Cell structure

The cell structure of Spirulina is typical to cyanobacteria, a prokaryotic organization described with soft cell walls and cell membrane enclosing the protoplasm containing chromosomes, various granules and the photosynthetic systems called thylakoids. The main photosynthetic pigment, phycocyanin is blue in color. Environmental conditions (temperature, physical, chemical) may subject alteration to the morphological characteristics, such as sheat consistency and cell size. (Sze 1996, 21-24; Barsanti & Gualtieri 2006, 159)

3.3 Natural habitat

The characteristic helical shape of Spirulina culture is only maintained in aquatic conditions. In nature they prefers tropical or subtropical waterbodies with high carbonate/bicarbonate content, salinity and high pH, which may not support other plants. They grow in interwoven mats of filaments, that are capable of gliding movement to adjust the filament density in response to changes in environmental conditions.
The limited supply of nutrients is usually the limiting factor in the growth cycles. New nutrients are obtained from either an upwelling from inside the waterbody, or trough rivers or pollution. The population grows until it reaches a maximum possible density, and then starts to die off as nutrients are exhausted. A new seasonal cycle begins when decomposed algae release their nutrients or when more nutrients flow into the lake. (Ahsan et al. 2008; Sze 1996, 25, 30)

3.4 Reproduction mechanism

Spirulina reproduces through binary fission, the simplest type of reproduction. The parent organism divides into two similar organisms containing the hereditary information, including cytological, genetical, physiological and morphological structures of the parent. (Barsanti & Gualteri 2006, 8; Sarabhai & Arora 1995, 15)

3.5 Biochemical composition

Closer studies regarding the biochemical composition started in 1970. It was discovered that Spirulina is an excellent source for proteins, vitamins and minerals. (Ali & Saleh 2012)

3.5.1 Health benefits

The protein content of the drymass is about 55-70 %, but compared to other commonly used sources of protein such as milk, meat, eggs; the proteins contain less lysine and sulfured aminoacids (methionine, cysteine), but still much more than any other vegetable. Phycobiliproteins constitute major portion of proteins, the most important being phycocyanin with 7-13 % of the drymass. The carbohydrate content is 10-20 %, with lipids acconting for 9-14 % of that. Spirulina is rich in minerals including K, P, Na, Ca, Mn and Fe representing 6-9 % of the drymass. Vitamin A, B and C are also present, with an average B-carotene content of 1,4 mg /g^{-1} of drymass, translating to 0,25 mg of vitamin A. (Ali & Saleh 2012)

Growing conditions have been observed to influence multiple characteristics such as the amount of thylakoid membranes per cell or the rate of lipid production. For example, as the culture gets stressed more lipids are produced, while chlorophyll-a content and the overall biomass production decreases. (Barsanti & Gualtieri 2006, 282; Ali & Saleh 2012)

3.5.2 Therapeutical applications

The multiple extensive toxicological studies conducted are highlighting the vast potential of Spirulina for therapeutical applications in field of immunomodulation, anticancer, antiviral and cholestrol reduction. It is also found out to be beneficial against certain effects of HIV infection. (Barsanti & Gualtieri 2006, 280)

B-Carotene has been studied to have antioxidatory properties. As stated above, the growing conditions can be specified to stimulate the production on certain components such as B-Carotene. Ali et al. (2015) reported increased B-carotene production when the culture was stressed with excess NaCl (0,02 M control, 0,04 M, 0,08 M). Consequently the dry weight and chlorophyll content was decreased. (Miranda et al. 1998; Ali & Saleh 2012; Barsanti & Guartieri 2006, 282).

Summary of biochemical composition of Spirulina:

TABLE1: Vitamins in Spirulina powder (Ali & Saleh 2015)

Vitamins:	mg 100 g^{-1}:
Provitamin A	2,330,000 IU kg^{-1}
β- Carotene	140
Vitamin E	100 a-tocopherol eq:
Thiamin B_1	3,5
Riboflavin B_2	4
Niacin B_3	14
Vitamin B_6	0,8
Vitamin B_{12}	0,32
Folic Acid	0,01
Biotin	0,01
Phantothenic Acid	0,1
Vitamin K	2,2

TABLE 2: Fatty acid composition of Spirulina powder (Ali & Saleh 2015)

Fatty acid:	Fatty acids %:
(C_{14}) Myristic acid	0,23
(C_{16}) Palmitic acid	46,07
($C_{16:1}$) D^9 Palmitoleic acid	1,26
($C_{18:1}$) D^9 Oleic acid	5,26
($C_{18:2}$) $D^{9,12}$ Linoleic acid	17,43
($C_{18:3}$) $D^{9,12,15}$ g-Linolenic acid	8,87
other	20,88

TABLE 3: Minerals in Spirulina powder (Ali & Saleh 2015)

Mineral:	mg 100 g^{-1}:
Calcium	700
Chromium	0,28
Copper	1,2
Iron	100
Mangnesium	400
Manganese	5
Phosphorus	800
Potassium	1400
Sodium	900
Zinc	3

TABLE 4: Pigments in Spirulina powder (Ali & Saleh 2015)

Pigment:	mg 100 g^{-1}:
Carotenoids	370
Chlorophyll-a	1000
Phycocyanin	14000

3.6 Temperature

In artificial cultures the temperature should be maintained as close as possible to the temperature of its natural habitat. According to previous studies optimal temperature for Spirulina cultures range between 30 - 35 °C. (Barsanti & Gualtieri 2006, 213; Delrue et al. 2017; Ashan et al. 2008)

3.7 pH

The optimal pH range for Spirulina is between 8,5 - 10,5. Unacceptable pH, due unsufficient aeration may start to disrupt the cellular processes, which may lead to complete collapse of the culture. Also, in cultures with high densities the addition of CO_2 may correct the increased pH. This can be done either by pumping or by increasing the surface area exposed to atmosphere relative to the volume of culture. Higher concentrations of CO_2 in the medium due to the small size of culture and their incapability to take it up enough may result in significant decrease in pH. (Barsanti & Gualtieri 2006, 213, 228; Delrue et al. 2017; Ashan et al. 2008, 4)

3.7.1 Bicarbonate buffering system

The correct pH value is relatively easy to maintain, since the media which Spirulina prefers has large buffering capacity due to it's bicarbonate buffering system (Barsanti & Gualtieri 2006, 213, 228). Bicarbonate buffering system refers to an acid-base homeostatic mechanism. In which water and carbon dioxide react forming carbonic acid, which in turn dissociates into bicarbonic ions and hydrogen ions as shown below:

$$CO_2 + H_2O \leftrightharpoons H_2CO_3 \leftrightharpoons HCO_3^- + H^+$$

3.8 Light

3.8.1 Photosynthesis

Like all plants, Spirulina uses light as its energy source to drive photosynthetic reactions as well as in it's sensing processes. Spirulina captures light with it's phycobiliprotein compartment. These proteic pigments are located inside the thylakoid membranes, whose task is to absorb and channel the light energy to drive the photochemical and enzymatic reactions. Orange and red light (400-700 nm) is absorbed most efficiently, due to the light blue color of these pigments. (Barsanti & Guantieri 2006, 111, 136)
The generalized form of photosynthesis is written below:

$CO_2 + H_2O + (\text{light energy}) \Rightarrow CH_2O + H_2O + O_2$

3.8.2 Intensity & photoperiod

Light is the most important physical factor regulating the growth, so for this reason photoperiod and intensity need to be considered, along with the spectral quality under artificial growing conditions. Also the design of the culturing vessel plays important role in how the light is distributed among the culture. As the density of the culture increases self-shading starts to occur, with light absorbed rapidly in the water, the competition becomes even more severe. (Barsanti & Guantieri 2006, 104-105, 213)
In artificial conditions sunlight may not be found, so lamps need to be used as substitutes. Overheating by lamps or other source should be avoided at all times. For small-scale cultivations, where the volume of the pool is less than 10.000 liters, depending on the volume and density of the culture typical light instensities used range between 2500 to 5000 luxes, corresponding around 5 to 10 % of the intensity of daylight. Increase in irradiance level causes more electron and energy rich molecules to be produced, which accelerates the rate of photosynthesis. If other factors arent limiting, the photosynthetic rate become increasinly non-linear until saturation level is reached. Further increase in irradiance beyond the saturation level actually decreases the photosynthetic rate from the maximum saturation level and may be damaging to the cells due to the added energies of photons corresponding energy of UV photon. Irradiance level influences the light absorbtion properties of the algae cells by changing its pigmentation content. Cells

acclimated to high light intensities have higher carotenoid values relative to their chlorophyll-a content, whereas in cells exposed to low irradiance the chlorophylls per cell can increase five- to ten-fold. The molecules are also less effective in their light absorbtion capacity. (Barsanti & Guantieri 2006, 204-206)

3.9 Nutritional media

Succesfully maintained culture requires correct specie-dependent nutritional media. Spirulina needs inorganic nutrients and light energy to produce organic compounds. The growth rate of the algae population is regulated by the nutrient available in the smallest quantity in the growing medium. For example, if there is an abundance of all other required nutrients, but nitrogen is the limiting one; photosynthetic and respiratory rates will be reduced. (Barsanti & Gualtieri 2006, 160-161, 215)
Trace metal buffer (chelators), preferably EDTA; is required to maintain free ionic metal in the medium, but also to prevent precipitation and accumulation of toxic metal concentrations. (Barsanti & Gualtieri 2006, 226-227)

3.10 Culturing methods

Artificial cultures consist of three important components: the culturing container with the medium, the biomass growing in it and air to allow CO_2 to be exchanged between the medium and atmosphere. In addition to CO_2 and water, only nutrients, trace elements and light is needed. Like all algae, Spirulina is able to produce all necessary biochemical comounds it need by means of photosynthesis. (Barsanti & Gualtieri 2006, 211, 213)

3.10.1 Cultivation and production of Spirulina in artificial environment

In theory the artificial conditions in which Spirulina grow should be as close as possible to its natural habitat. In nature algae are subjected to fluctuating environmental conditions and seasonal cycles, which however can be eliminated in artificial conditions. Isolating the culture from possible predators and invasive species should be considered, despite the fact that Spirulina is quite pest resistant, since it requires high pH and bicarbonate content, where most predators can't live. (Barsanti & Gualtieri 2006, 211, 213)
Spirulina can be cultivated in closed-indoor or outdoor conditions. The system can be batch based, continuous or semi-continuous. There are several things to consider when choosing the cultivation method and materials. The scope of production and the type of final product, but also the cost of land, energy, water, nutrients and climate naturally influence the choices. (Barsanti & Gualtieri 2006, 241)
Common method to cultivate Spirulina indoors is to grow it in photobioreactor.
They are closed systems, where the algae are isolated from atmosphere preventing direct gas exchange and contaminations, but also evaporation thus reducing water consumption. Photobioreactors allow full control over all important culture parameters, such as pH, oxygen and CO_2 concentration, and temperature to maximize the cell growth relative to operating cost. Still they are many fold more expensive to maintain than outdoor cultures, so their use should be limited only to produce high value compounds that cannot be cultivated elsewhere. (Barsanti & Gualtieri 2006, 241, 243)
Outdoor are cheaper to maintain and can operate only using sun light as their light source, but also more difficult to control due predators, contamination and varying environmental conditions, hence it is harder to sustain production for longer period of time. The achievable cell density is lower compared to controlled indoor culture. Artificial outdoor cultures are usually shallow, uncovered ponds, pools or tanks, but eutrophic lakes can be used also. (Barsanti & Gualtieri 2006, 241, 243)
According to sosio-economic study conducted by Delrue et al. (2017); production in photobioreactors was found out to be 2-20 times more expensive than in open ponds (18,71-74,29 euros/ kg in PBR to 3,86-9,58 euros/ kg outdoor). In this study Spirulina was cultivated in 1000 L photobioreactor for 40 day period, with maximum daily productivity of 58,4 g/m^2 /day.

3.10.2 Batch, continuous and semi-continuous cultures

Batch based cultures are closed systems, with limited volume and there is no input or ourput of matter. Batch cultures are easy to maintain due to their simplicity and low cost. After the lag phase, the density of algae increases exponentially until some factor becomes limiting and the density starts to decrease. Also the concentration of produced metabolites increase over time. Subculturing can be done by transfering a small volume of existing algae at regular intervals into a fresh medium where it continues to grow. (Barsanti & Gualtieri 2006, 237)

Continuous cultures aim to maintain the exponential growing phase at all times. The resources are potentially unlimited, since fresh culture medium is added regularly proportional to the growth rate of the algae, while same volume of medium is removed. Continuous cultures benefit from automation, which makes the maintenance of all culture parameters much more precise. Controlling the flowrate of nutrients also controls the limiting nutrient available, which directly influences the growth rate. In practice the dilution rate should be set to be lower than the maximum growth rate or total washout of the entire culture eventually happens. The upkeep of continuous systems is high, complex and is exclusively restricted to indoor areas, which also limits its scale. Semi-continous system refers to a technique, where fresh medium is added all at once, while the ready biomass is harvested. (Barsanti & Gualtieri 2006, 239, 241)

3.10.3 Mixing

For preventing sedimentation the culture should kept mixed at all times. Mixing ensures that all indivduals of the population gets exposed equally to light and nutrients, but also to important for carbon dioxide to be distributed evenly for photosynthetic processes. As stated above dense cultures benefit from adding about 1 % additional carbon dioxide to the air being bubbled into the medium through suitable filter (to avoid contamination). Mixing should be gentle, since the the soft cells may experience damage from extreme turbulence. Suitable mixing can be achieved only with sufficient aeration in small cultures, but also variously designed automated paddle wheels and stirrers can be utilized depending on the size of the culture (Barsanti & Gualtieri 2006, 214).

3.10.4 Culture vessel properties

Important properties to consider when choosing the culturing vessel are transparency, to ensure proper light penetration, it should be easily cleaned, non-toxic and most importantly able to provide good surface area to volume ratio. However, the more exposed the container is to air, the more evaporation occurs and more water is wasted. According to Barsanti & Gualtieri (2006, 214) recommended materials for culturing vessels include Teflon, polycarbonate, polystyrene and borosilicate glass, while all types of rubber and PVC should be avoided to be in contact with Spirulina. For experimental purposes borosilicate glass conical Erlenmeyer flasks of various volumes are often used. The flasks are capped with cotton-wool to prevent evaporation and contamination, still allowing gas exchange. Before any equipment is used, it has to be sterilized to get rid of all microbial life and chemical traces with phosphate-free detergent, rinsed with tap water, soaked in HCl for preferably many days, then rinsed extensively with distilled water. (Barsanti & Gualtieri 2006, 214, 235)

3.11 Growth phases

The growth is described in five phases. In continuous cultures, the goal is to maintain the exponential growing phase to maximise the production. In addition to reduced biomass production, the nutritional value of algae cultiated under growth-limiting conditions is inferior to algae cultivated in optimal conditions.

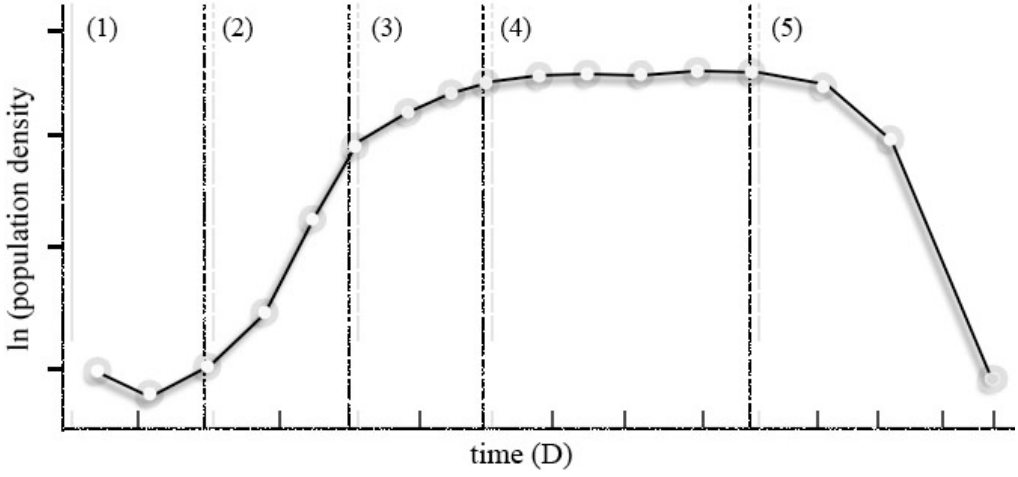


FIGURE 1: Five growth phases of micro-algal culture. (1) Lag phase, (2) Exponential phase, (3) Declining phase, (4) stationary phase, (5) crash phase.

The figure above shows the five different phases of growth. As the culture is transferred into the new growing medium, physiological adaptation occurs. This phase is called induction or lag phase (1). During this relatively long phase only little increase in cell density is excepted. (Lavens & Sorgeloos 1996)
After the lag phase the cells start to divide exponentially. Exponential phase (2) is relatively short, because the cells start to shade each other as the density increases.
In phase of declining growth (3) some physical or chemical factor, such as availability of nutrient(s), light or pH start to limit the growth the cell division will slow down from the exponential phase. (Lavens & Sorgeloos 1996)
In theory the increase in cell density can be estimated with following equation:

$C_t = C_0.e^{mt}$

C_t, C_0 cell concentrations at time t and 0

m specific growth rate

Exponential phase is followed by stationary phase (4), where the culture is in a state of balanced growth and relatively constant cell density.
The crash phase or death phase (5) may be triggered by many reasons. For example the deterioration of water quality, depletion of nutrients, overheating, unacceptable pH or contamination may drive the culture into a phase characterized by rapid decline of cell density. (Lavens & Sorgeloos 1996)

4 MATERIALS & METHODS

4.1 Materials

3 x 5 ml vials of Arthrospira Platensis was obtained from UCL (London's global university) via airmail to initiate the cultivation process.
Even though the cultivation was done to observe the cost-efficiency , laboratory grade equipment and materials were used in all stages of the experiment. Zarrouk's nutrient medium was choosen as the media of choice based on the positive results obtained from the other studies (Amara & Steinbüchel 2013; Delrue et al. 2017; Madkouret al. 2012). Compromises were made with keeping the cultures at indoor temperature of the laboratory at about 25 to 27 °C in absence of external heating, excluding the heat emitted from the lamps, considering the lower than optimal temperature might influence the growth rate. Furthermore; traditional fluorescent lamps were used instead of lamps with optimized spectral quality, although it was assured, that the cultures received the needed illuminance (3000-4000 lux).
The aeration was provided by custom "airstones" made from 5 ml polyethylene-pipette by piercing a hole into the thicker end of the pipette and attaching it to teflon hose connected to airpump. Traditional airstones may contain metals, that will leach into the culture medium in toxic concentrations.

4.1.1 Zarrouk's media

Spirulina prefers alkaline marine media. Different nutritional compositions for growing Arthrospira Platensis can be found in the literature. As stated above, according to recent studies, Zarrouks –nutrient medium has been found to be the most successful in terms of harvested dry biomass.

The media was prepared beforehand by weighing the macro- and micro-nutrient elements obtained from TAMK laboratories; respectively to the reported concentrations published by Amara & Steinbüchel (2013). The containers were covered with parafilm, sealed with masking tape and covered with aluminium foil, to prevent evaporation.

TABLE 5: Micro- and macro-nutrient components of Zarrouk's nutrient media (Amara & Steinbüchel 2013)

nutrient solution:		
macronutrients:		g/L
$NaHCO_3$	sodium bicarbonate	16.80
$NaNO_3$	sodium nitrate	2,5
K_2HPO_4	dipotassium phosphate	0,5
K_2SO_4	potassium sulfate	1
NaCl	sodium chloride	1
$CaCl_2\ 2H_2O$	calcium chloride dihydrate	0,04
Na_2EDTA	disodium salt dihydrate	0,08
$MgSO_4\ 7H_2O$	magnesium sulfate heptahydrate	0,2
$FeSO_4\ 7H_2O$	iron(ii) sulfate heptahydrate	0,01
micronutrient solution		1 mL/L
micronutrients:		
H_3BO_3	boric acid	2,8
$MnCl_2\ 4H_2O$	manganese chloride tetrahydrate	1,8
$ZnSO_4\ 7H_2O$	zinc sulfate heptahydrate	0,2
$CuSO_4\ 5H_2O$	copper sulfate pentahydrate	0,08

4.2 Methods

The actual experiment was set to last for 22 days straight, excluding the precultivation period 12th of june, 2017 onwards and finishing 11th of july, 2017.
The photo-period was chosen to be 12/12 light/dark ratio and controlled with automated timer.

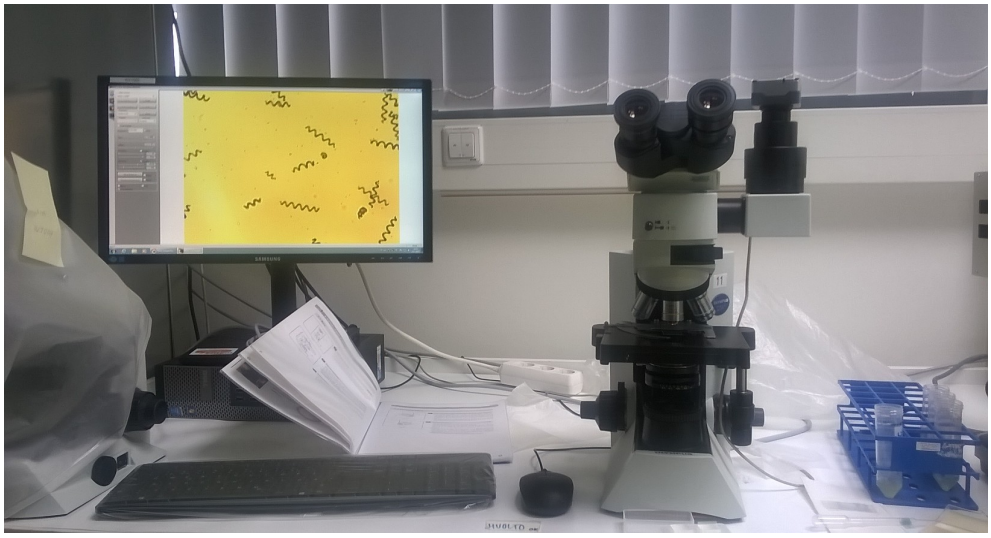
According to literature (Madkour et al. 2012) the doubling time of Spirulina using Zarrouk's medium is found out to be around 2 days. In this experiment the cultivation volumes are relatively small in the beginning comapred to the reference study, so the upscaling of the volume should be reasonably frequent. Due to the schedule it was decided that the doubling would happen every Tuesday and Thursday.

4.2.1 Sampling the dryweight

The first samples to determine the dryweight were taken on the first day of the experiment. The subsequent sampling was done according to schedule, between 10 am and mid-day. Each time 5 ml sample was taken from each replica. The samples were taken from well mixed, homogenous culture using 5ml glass pipette and peleus ball. Then were taken onto a glass fiber filter (Whatman GFC, 25 nm diameter) using a glass-rod for more accurate transfering coupled with vacuum filtering system. Using pincers, the sample was then taken into aluminium drying plate (labeled accordingly and weighed beforehand) and placed into 105 °C drying oven for one hour. Which should be enough to get rid of the excess water. Finally the samples were weighed again and the mass of the glassfilter was substracted from the final mass.

4.2.2 Experimental procedure

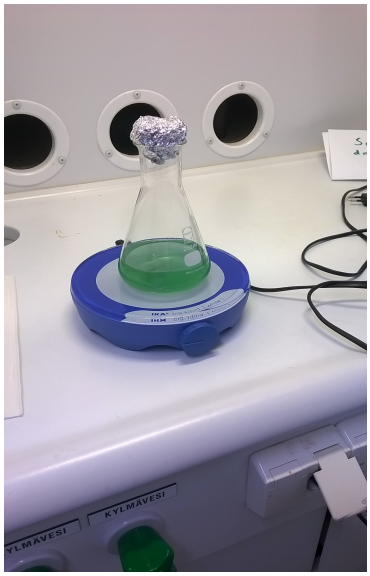
As the initial flaks ordered were obtained, they were immediately taken into the laboratory, and inspected under the microscope. The experimental setup was located under the fume hood, where the vials were transferred into 250 ml Erlenmeyer flask; filled with 50 ml Zarrouk's nutrient medium. The flask was then placed on top of magnetic stirrer, with moderately calm stirring speed and introduced to fluoresecent light (of around 3200 lux). The flask was capped with cotton-wool, then sealed with aluminium-foil with added holes to allow gas exchange.



PICTURE 1: Inspecting a sample with a microscope.

The motherculture was sampled to find out when the concentration was at 0,25 g/l at 600 ml volume. Then it was divided into three replica cultures. 250 ml Erlenmeyer flasks were used as the culturing vessels for the replica cultures as well, filled with 150 ml of motherculture as initial volume.

At 600 ml volume the replicas were transfered carefully under the fume hood, into 2000 ml Erlenmeyer flasks, as more growing volume was needed. On 15th day of the experiment the volume of each culture was increased to 2000 ml from 1200ml. They were kept at that volume for one week exactly, after which the experiment finished.



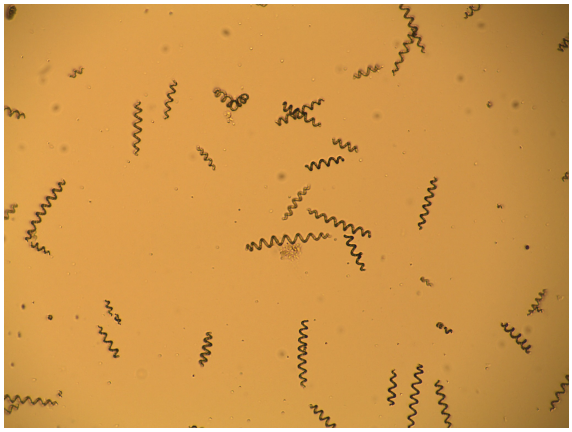
PICTURE 2: The initial mother culture on the first day of the experiment.

TABLE 6: Summary of selected culturing parameters.

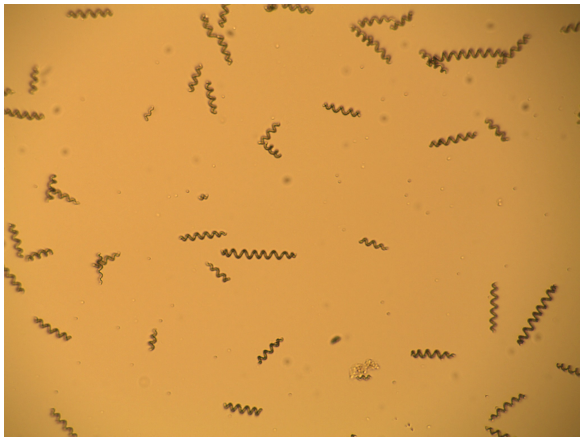
Parameter:	Value:
Duration of experiment	22 days
Photoperiod	12/12 light/dark ratio
pH range	9-10
Illumination	3000-4000 lux
Temperature	25-30 °C

TABLE 7: The volumes of culturing flasks according to the schedule. The volumes indicate the amount of Zarrouk's nutrient medium contained inside the vessel.

Day of experiment:	Volume:	Date:
1	150 ml	20.6.2017
4	300 ml	23.6.2017
8	600 ml	27.6.2017
11	1200 ml	30.6.2017
15	2000 ml	4.7.2017
18	2000 ml	7.7.2017
22	2000 ml	11.7.2017



PICTURE 3: Laboratory picture of the Spirulina sample taken from "Culture 2 [Z1]"

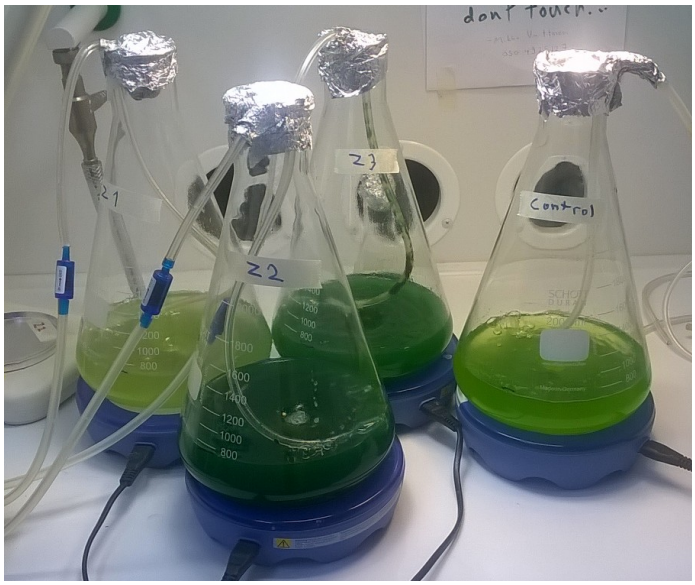


PICTURE 4: Photo of Culture 4 [Z3] taken through microscope.

These pictures wer taken with microscope during the experiment to inspect the condition of the culture. It also shows the characteristic filamentous shape of Spirulina. No visible signs of contamination could be observed from any sample during the cultivation period.

5 RESULTS

Observations made and results obtained during the laboratory experiment conducted between 20.6.2017 and 11.7.2017 are presented in this chapter.



PICTURE 5: The culturing vessels pictured. The volume of each flask is set to 600ml corresponding day 8 of the experiment.

PICTURE 6: The cultures pictured at the final day of the experiment. Volume of each flask is set to 2000 ml.

The colour difference among the cultures can be observed at quite early stage of the experiment from picture 3 and 4 above. At later stages of the experiment, the differences between Culture 1 [C] on the right and culture 2 [Z1] on the left compared to cultures 3 [Z2] and 4 [Z3] can be seen even more clearly. Some picture-to-data similarity can be observed when comparing the drymass values of each culture in figure 8 to the color of corrseponding flask.

5.1 Growth

Hereby are presented the results obtained from drymass weighing. The values are converted from the mass observed in 5 ml sample and presented here in grams per liter. Figure 2 is a graphical illustration of drymass measurements, whereas table 8 represents the numberical data obtained from the biomass filtering and drying process, figure 3 the biomass growth in percentages.

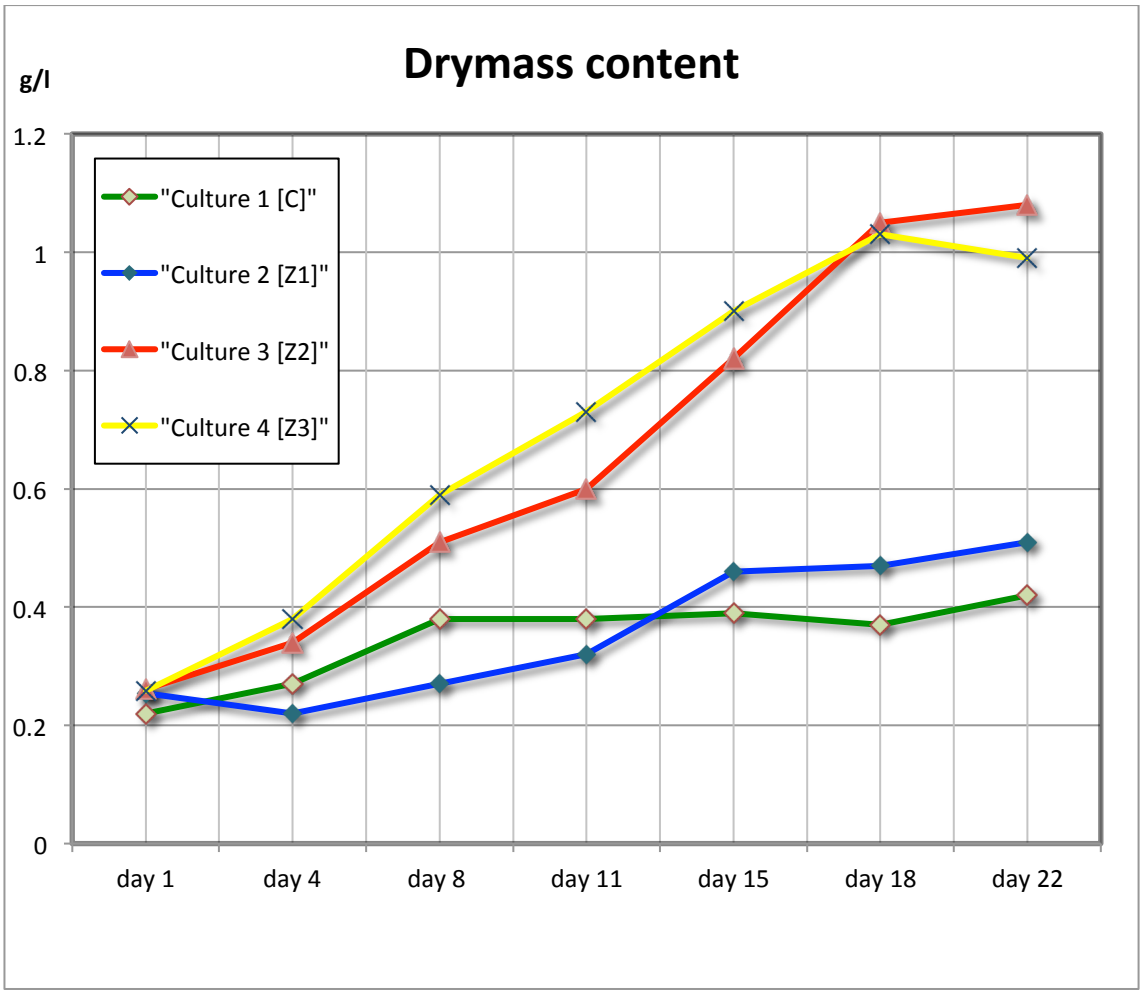


FIGURE 2: The measured dryweight according to time

Figure 2 show the filtered drymass mass measured in different days. All replicas show signs of slow cell division (lag phase) during the first three days of the experiment. Cultures 1 and 2 show considerably slower increase in algae density compared to culture 3 and 4. However all cultures show, that the increase in cell density was more than expected, considering to the doubing frequency of the volume. The cell division in cultures 3 and 4 started to show signs of declining growth rate on day 18, 4 days after the volume was settled to 2000 ml.

TABLE 8: The drymass content presented in grams per liter in different days.

Drymass (g/L):	Day 1	Day 4	Day 8	Day 11	Day 15	Day 18	Day 22
culture 1 [C]	0,22	0,27	0,38	0,38	0,39	0,37	0,42
culture 2 [Z1]	0,254	0,22	0,27	0,32	0,46	0,47	0,51
culture 3 [Z2]	0,261	0,34	0,51	0,6	0,82	1,05	1,08
culture 4 [Z3]	0,258	0,38	0,59	0,73	0,9	1,03	0,99

The table above presents the filtered drymass in numerical values. The initial concentrations of all cultures are between 0,22-0,261 g/l as they were sampled right after dividing the motherculture, which was at around 0,25 g/l. As stated above, as the experiment progressed the cultures show considerable variation in their biomass accumulation.

TABLE 9: Final drymass yield in 2000ml of medium, at the end of the experiment.

	Final drymass concentration (g) in 2l:
Culture 1 [C]	0,84
Culture 2 [Z1]	1,02
Culture 3 [Z2]	2,16
Culture 4 [Z3]	1,98

The table above represents the final concentration of each culture, measured according to the last drymass sampling on 11.7.2017. The final drymass concentration in two liters of medium was calculated by multiplying the final drymass sampled in g/l by two. Cu-

ture 1 had the lowest yield at 0,84 g, while culture 3 had the highest biomass production at 2,16 g (157,1% higher than culture 1). Average yield between all cultures being 1,5 g.

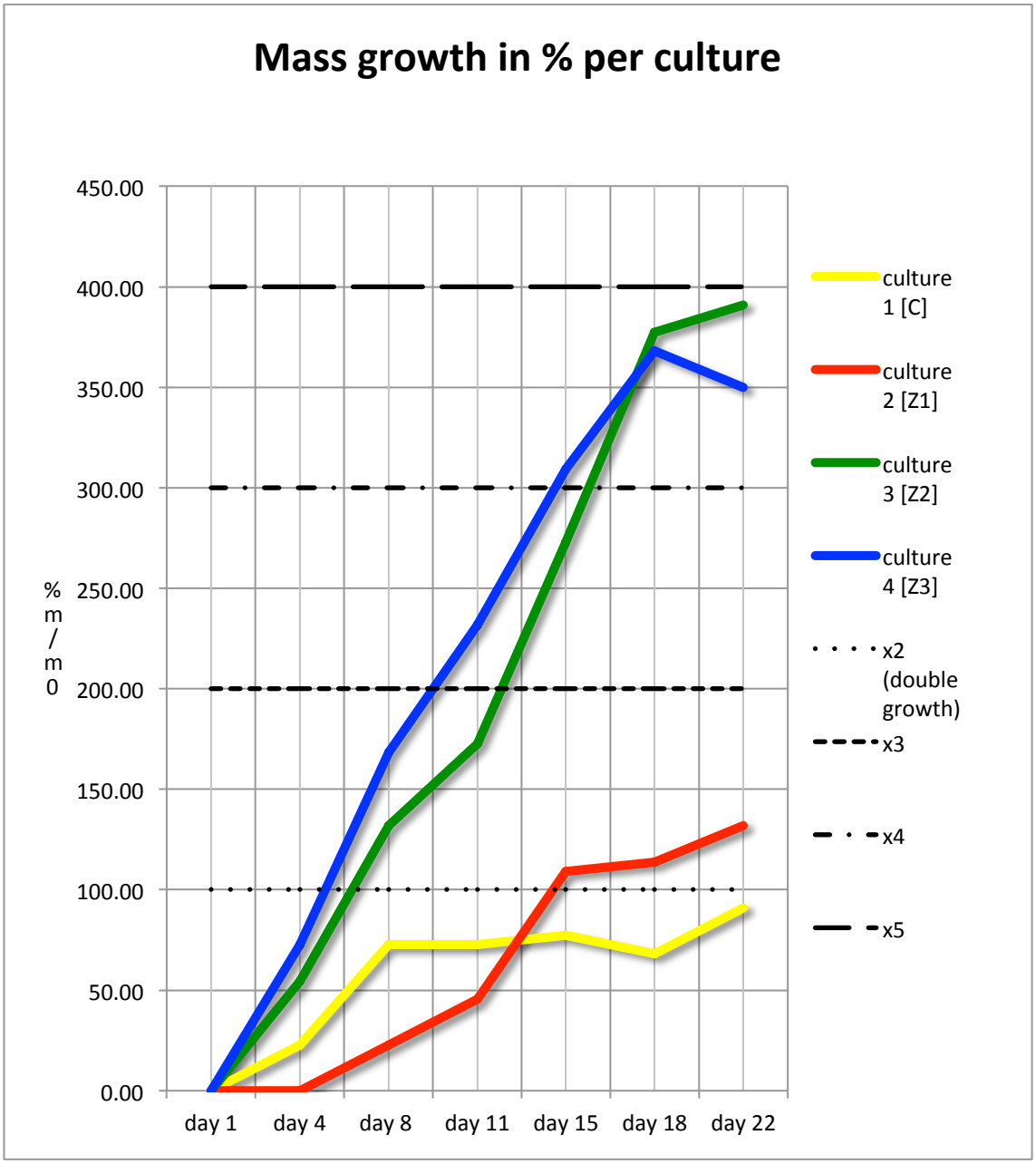


FIGURE 3: Mass growth in percentages per culture. The y-axis represents the total growth in % compared to the initial concentration.

The mass growth in percentage per culture figure shows, that culture 1 is the only culture, that didn't reach atleast 100% increase in it's biomass content relative to volume of the culture according in 22 day cultivation period. Culture 2 reached 100% increase between day 11 and day 15 of growth. However culture 3 reached 100% increase in biomass between days 4 and 8, and continued to do so for another 100%, every 5 days up to 400% increase. Culture 4 shows similar behaviour in growth, but it seems to have

reached 200 % increase few days earlier and has slight decrease towards the end of the experiment.

5.2 pH values

Hereby are presented the pH values measured during the experiment. The values were measured using Mettler Toledo FE20 desktop pH Meter.

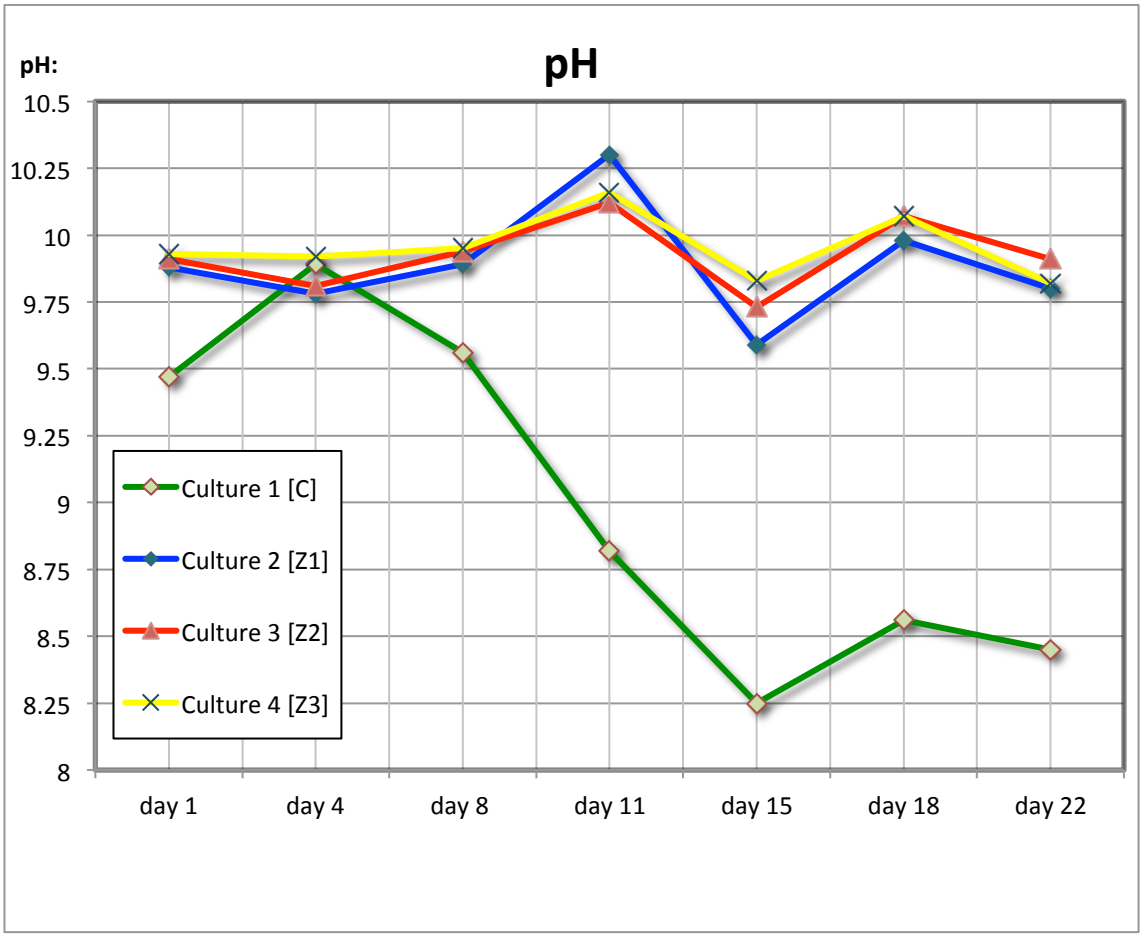


FIGURE 4: Graphical presentation of the measured pH values according to time.

The pH was measured together with the drymass sampling. Cultures 2,3 and 4 behave similarly in regards to changes in pH values. Culture 1 has lower value from day 1, but seems to get ajusted to same level than the rest, only to steadily decrease until day 15. The pH values in all cultures, including culture 1 were measured to be in the acceptable range of 8,5-10,5 for the duration of experiment, excluding culture 1 on day 15 and 22. Also cultures 2,3 and 4 show slightly increased values as the volumes were expanded from 300 ml to 600 ml followed by a decrease when the cultures where further increased to 1200 ml.

6 DISCUSSION

6.1 Discussion of results

Contamination could be a reasonable explanation for the slower growth rates of cultures 1 and 2 compared to cultures 3 and 4. Because wastewater laboratory was used as a cultivation environment sources of contamination due invasive bacteria can be considered. The cultures were theoretically exposed to contamination every time sample was taken from there, even though it was done under the fume hood and no accessory species were obserbed with microscope. Furthermore there is a possibility, that the aluminium used as a cap for the culture vessel could have ended up inside the flask. However no aluminium was observed inside the flasks during the culturing or while cleaning the flasks when the experiment was finished.

Another possible reason for slower growth rate in culture 1 is insufficient aeration. The decreased pH values shown in FIGURE 4 and TABLE 10 could be due abundance of CO_2 present, which decreased the pH.

Zarrouk's nutrient medium was found out to be excellent choice for the nutrition medium. Alongside good results obtained from previous studies (Delrue et al. 2017; Ali & Saleh 2012; Madkour et al. 2012) the exponential growth rates in cultures 3 and 4 suggest this. Secondly, the bicarbonate buffers provided relatively stable pH (TABLE 10) throughout the experiment, since there was no need to adjust the pH manually.

Since all flasks were cultivated using the same nutrient solution, temperature (average 26 °C), illuminance (average 3400 lux) and stirring speed, it is unlikely that any of these factors were the cause for the varied growing speed among the cultures. However to be sure of that, more measurements would have been needed. For more precise experiment in the future, photobioreactor should be used.

6.2 Literature review discussion

Recent studies have revealed, that ZM can be diluted without impacting the growth potential. According to Delrue et al. (2017, 12) ”To reduce Spirulina production cost, dilutions of the modified ZM were tested and it was shown that modified it could be diluted up to five times without impacting the biomass productivity up to 21 days after inoculation”. So there is still potential to reduce the cost of nutrients by optimising the medium even further.

Other modified versions of Zarrouk’s nutrient medium have been studied by Madkour et al. (2012). The study aimed at finding cost effective medium for large-scale production of Spirulina Platensis, by substituting the nutrients in ZM with cheaper and commercially available chemicals and fertilizers. Single super phosphate, commercial sodium bicarbonate, Muriate of potash and crude sea-salt along with ammonium nitrate and urea was used as substitutes. The study concluded, that the modified media could be used as a substitute for ZM for large-scale mass production of protein rich Spirulina.

Comparing to other foods Spirulina indeed has superior crude protein content. In addition to better food safety it is easier to cultivate than meat. According to Ahsan et al. (2008) Spirulina uses less water per kilogram produced, than any other plant (2100 liters/kg). Also, according to the same report; small-scale cultivation of Spirulina is considered as a potential income-generating activity for households or villages.

TABLE 10: Protein contents of different foods (Ali & Saleh 2012)

Food type:	Crude Protein %:
Spirulina powder	65
Whole dried egg	47
Beer Yeast	45
Skimmed powdered milk	37
Whole soybean flour	36
Parmesan cheese	36
Wheat germ	27
Peanuts	26
Chicken	24
Fish	22
Beef meat	22

Malnutritions means micro-nutrient deficiency. Report published by Birot, de Jouvencel, Raginel & Rouillé (2012) states that: ”More than hunger, the true challenge in global health is malnutrition”. International solidarity associations such as Antenna Foundation (N.d.) has studied and implemented Spirulina production in developing countries to combat malnutrition. In the near future, as the cultivation volumes become larger, the main goal is to mass-produce it for western markets, while still preventing malnutrition locally. According to Antenna Foundation (N.d.) 1 to 3 grams of Spirulina a day for four to six weeks will cure a malnourished child. Birot et al. (2012) state, that only few countries along with FAO, who have taken Spirulina to their national health nutrition programmes have recognized its potential. This would suggest, that perhaps more political will along with marketing and scientific research is needed in other countries who are struggling with malnutrition.

The genome of Spirulina is registered into GenBank by Antenna Technologies, two private Swiss companies; Biorigin AS and Fasteris and the Haute École Spécialisée Hepia of Geneva. This restricts it from being patented and is freely available for anyone who wants to study it further (Birot et al. 2012). Future research could include topics of interest, such as genetically modifying Spirulina to grow in dark or accelerated compounds specific production. According to Miranda et al. (1998): “There is a current worldwide interest in finding new and safe antioxidants from natural sources such as plant material to prevent oxidative deterioration of food and to minimize oxidative damage to living cells”. Engineering the production rate of compounds such as β-Carotene, α-tocophenol and phenolic acid would provide cheap, sustainable source for antioxidants. According to Priyadarshani & Rath (2012) micro-algae cultivation is already the most profitable business in biotechnological industry, due to lack of waste and minimal use of resources and energy.

The industry has received some critisism regarding its misleading marketing. For example, some growing companies have claimed Spirulina to have high B12-vitamin content, thought the vitamin contained is pseudovitamin B12, which in turn is inactive in humans as such, but is an precursor for the active compound (Nicoletti 2016). This kind of example suggests, that the industrial sector needs more control and responsibility, but also more careful research regarding the health benefits should be done.

7 CONCLUSION

In authors opinion the experiment can still be concluded as succesful, since it supported the literature review it was based on. The results show similar behaviour in biomass accumulation compared to reference studies conducted in similar kind of environments. The results obtained from this experiment also show, that there may be differences in growth potential among Spirulina cultures despite careful maintenance of identical conditions. Hence the growth should be precisely controlled. In my opinion, hardest part in Spirulina cultivation is avoiding contamination, since other growing factors are relatively easy to keep at stable state.

Many reasons contribute to the fact, that Spirulina can be seen as a notable component of nutraceutical and food industry in the future. As more research is being done the culturing methods are becoming cheaper by better optimization. It has superior nutritional value compared to virtually any other plant and is easy to grow. Also due environmental impact of contemporary food production methods, alternative solutions to feed the world's growing population is needed.

Further study regarding consumer interest of home-culturing systems can be suggested to encourage designs of affordable photobioreactors or similar growing systems and kits. Also, it is yet to be seen if biotechnology is able to "design" some characteristics of micro-algae to be more spesificly suited for certain purposes. Other topic of interest would be to implement algae growing at small community or housing cooperative level, along with other eco-tech solutions. Encouraging political- and market-actors to raise more awareness towards Spirulina production to combat malnutrition where needed can also be seen beneficial from many aspects.

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APPENDICES

Appendix 1. the pH values:

pH:

	day 1	day 4	day 8	day 11	day 15	day 18	day 22
culture 1 [C]	9,47	9,89	9,56	8,82	8,25	8,56	8
culture 2 [Z1]	9,88	9,78	9,89	10,3	9,59	9,98	
culture 3 [Z2]	9,91	9,81	9,94	10,12	9,73	10,07	9
culture 4 [Z3]	9,93	9,92	9,95	10,16	9,83	10,07	9

Appendix 2. total yield in grams

total yield (g):

	day 1	day 4	day 8	day 11	day 15	day 18	d
culture 1 [C]	0,033	0,081	0,228	0,456	0,78	0,74	
culture 2 [Z1]	0,0381	0,066	0,162	0,384	0,92	0,94	
culture 3 [Z2]	0,03915	0,102	0,306	0,72	1,64	2,1	
culture 4 [Z3]	0,0387	0,114	0,354	0,876	1,8	2,06	