Master in Bioengineering – Specialization in Biological Engineering

Arthrospira sp. cultivation from laboratory scale to pilot-scale

Dissertation for the Degree of Master in Bioengineering

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ABSTRACT

Arthrospira sp. is a cyanobacteria known worldwide for its several biotechnological applications, from human food and animal feed to pharmaceutical and cosmetic products. It is mostly known for its protein-rich biomass, which allows it not only to combat malnutrition in developing countries but also to act as a novel source of protein. In this project, this species was cultivated from laboratory to pilot-scale (raceways) and the influence of different growth media, bicarbonate concentrations, sodium sources and nitrogen concentrations in its growth was assessed. In the first two trials at laboratory scale, three growth media (Allmicroalgae standard medium (MNS), Zarrouk and BG11) and the concentration of sodium bicarbonate in the media were tested. They revealed that BG11 was not suitable for *Arthrospira* sp. growth and that MNS supplemented with sodium bicarbonate was the best medium. Another trial showed that a minimum sodium concentration of 50 mM in the culture medium is essential for the strain understudy, because, otherwise, its structure and morphology would be affected and it would eventually die. The last trial studied the effect of nitrogen concentration on *Arthrospira* sp. growth and, the medium with the highest concentration of nitrogen reached the highest dry weight. The results of the optimization trials proved that MNS with 30 mM of N supplemented with 16.8 g L^{-1} of sodium bicarbonate led to the highest growth performance and, thus, it was the medium chosen to proceed to the scale-up up to the pilot-scale raceways. During the scale-up process, several flat panel photobioreactors (80 L, 150 L, 500 L and 750 L) were inoculated, and the last one was used to inoculate a raceway pond with a standard configuration. The same raceway was inoculated in different periods, in order to develop two independent assays. The highest biomass content obtained in each assay was 1.494 g L^{-1} and 1.428 g L^{-1}. Later, the culture from the first assay was harvested by filtration using filters with several nets with different porosities and *Arthrospira* sp. got retained in the weave cloths with a pore diameter equal or lower than 122.900 µm. The protein content of the culture growing in the raceways was 41.8% of biomass dry weight, which is a little lower than the concentration this species typically exhibits (50 to 70% of biomass dry weight).

Keywords: Cyanobacteria, *Arthrospira* sp., raceway ponds, culture media, high protein content.

RESUMO

Arthrospira sp. é uma cianobactéria conhecida mundialmente pelas suas aplicações biotecnológicas em produtos para alimentação humana e animal, farmacêuticos e cosméticos. É conhecida principalmente pela sua biomassa rica em proteínas, o que lhe permite não só combater a subnutrição em países em desenvolvimento, mas também age como uma nova fonte de proteína. Neste projeto, a espécie foi cultivada desde a escala laboratório até à escala piloto (*raceways*), de forma a testar se o seu crescimento é afetado por diferentes meios de cultivo, concentrações de bicarbonato fontes de sódio e concentrações de azoto. Inicialmente, à escala laboratorial, foram testados três meios de cultivo (Zarrouk, o meio da Allmicroalgae (MNS) e BG11) e a concentração de bicarbonato de sódio nos meios. Os ensaios revelaram que o BG11 não foi indicado para o crescimento e que o MNS suplementado com bicarbonato de sódio foi o melhor meio. Posteriormente, outro ensaio provou que esta cianobactéria deverá ter uma concentração mínima de sódio no meio de 50 mM, pois caso não tenha, a sua estrutura e morfologia será afetadas e eventualmente irá provocar a morte. No último ensaio avaliou-se o efeito da concentração de azoto no crescimento da *Arthrospira* sp. e, foi no meio com a concentração de azoto mais elevada que se obteve o maior valor de peso seco. Os resultados dos ensaios provaram que o meio MNS com 30 mM de N suplementado com 16,8 g L^{-1} de bicarbonato de sódio foi o que obteve os resultados mais promissores, e por isso, foi este o meio escolhido para realizar o *scale-up* até aos *raceways*. Ao longo do scale-up, vários fotobiorreatores em placa (80 L, 150 L, 500 L e 750 L) foram utilizados e o último foi utilizado para inocular um *raceway* de configuração padrão. O mesmo *raceway* foi inoculado em períodos diferentes, de forma a realizar dois ensaios. O teor de biomassa mais elevado obtido por cada um dos reatores foi 1,494 g L^{-1} e 1,428 g L^{-1}, nessa ordem. Posteriormente, a cultura do primeiro ensaio foi colhida por várias redes com diferentes porosidades, tendo *Arthrospira* sp. ficado retida nas redes com um diâmetro igual ou inferior a 122.900 µm. O conteúdo proteico da cultura que cresceu nos *raceways* foi de 41,8% em peso seco de biomassa, sendo uma concentração inferior à que normalmente apresentada (entre 50 e 70% em peso seco da biomassa).

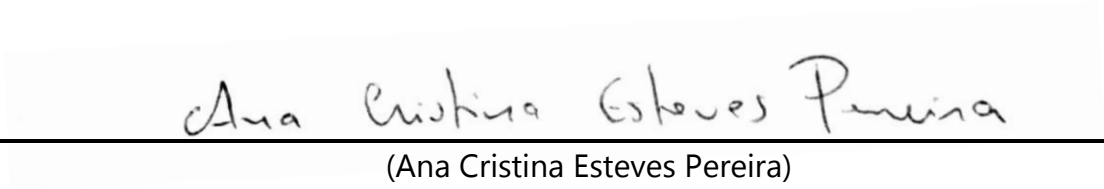
Palavras-chave: Cianobactéria, *Arthrospira* sp., *raceway ponds*, meios de cultivo, alto teor de proteína.

DECLARATION

I declare, under oath, that this work is original and that all non-original contributions were properly referenced with the source identification.

Eu declaro, sob compromisso de honra, que este trabalho é original e que todas as contribuições não originais foram devidamente referenciadas com identificação da fonte.

Porto, September 28th 2020 | Porto, 28 de Setembro de 2020



(Ana Cristina Esteves Pereira)

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NOMENCLATURE

APC	Allophycocyanin
FPP	Flat panel photobioreactor
GLA	γ-linolenic acid
LDPE	Low-density polyethylene
PBR	Photobioreactor
PC	Phycocyanin
PUFA	Polyunsaturated fatty acids

MEASURING UNITS

A	Area	m^2
DW	Dry weight	$g\ L^{-1}$
OD	Optical density	
P	Volumetric productivity	$g\ L^{-1}\ d^{-1}$
Pa	Areal productivity	$g\ m^{-2}\ d^{-1}$
r_x	Growth rate	$g\ L^{-1}\ d^{-1}$
V	Volume	m^3
X	Biomass concentration	$g\ L^{-1}$
μ	Specific growth rate	d^{-1}
t	Time	d

1. INTRODUCTION

1.1. The company (Allmicroalgae – Microalgae Production Unit)

Allmicroalgae (Figure 1) is a biotechnology company located in Pataias, Leiria, working with microalgae since 2013.

Figure 1. Industrial facilities plant of Allmicroalgae.

This company is shareholded by Secil group and its first goal was to reduce the emission of greenhouse gases by the adjacent Cibra-Pataias cement factory. Allmicroalgae is considered one of the largest microalgae production units in a close system in Europe, with a production capacity higher than 1300 m^3 (Allmicroalgae, 2020). Nowadays, the cultivation of microalgae has the intent to produce high nutritional value microalgae in order to create natural and healthy products for food and feed applications (da Fonseca *et al.*, 2016).

The food products (e.g. *Chlorella vulgaris*) are commercialized by Allma brand and the entire process and supply chain is certified under several ISO and Occupational Health and Safety Standards (OHSAS) (Allmicroalgae, 2020). With the goal of satisfying consumer needs, the company has been adopting many measures in order to guarantee continuous improvement, which is fundamental to establish a position in the increasingly demanding world market. This effort is proven by the acquisition of certifications, such as ISO 22000, ISO 9001, ISO 14001 and OHSAS 18000 certificates, which guarantee the execution of various requirements of hygiene and food safety, quality, environmental sustainability and safety of employees, respectively (Allmicroalgae, 2020).

1.2. Problem

The increasing consumption of animal-based products has a considerable impact on the environment, because animal farming and aquaculture are common sources of pollution (carbon dioxide, methane and nitrous oxide emissions), freshwater usage, deforestation and biodiversity loss (Macleod *et al.*, 2013; Huis, 2017). In order to produce one kilogram of beef in intensive farms, about 15 500 liters of fresh water is consumed to raise the animals and irrigate the fields where they are growing (Food, 2015). Genetic modifications have been encouraged in the industry in order to reduce the costs of animal production due to faster growth and adaptation to living in stalls. However, this approach leads to the loss of biodiversity on our planet (Food, 2015). Meat consumption in Portugal surpassed the production capacity of the country since 2003, increasing from 58 to 117 kg per capita since 1981 (Portal do INE, 2018). In order to mitigate the problems caused by the increasing population and have a sustainable food system, the research for alternative protein sources that are more environmentally responsible, healthy and nutritive is an essential task, while always taking into account the planet limitations to regenerate resources and provide ecological services (EUFIC, 2020). Some organisms, such as microalgae have various biotechnological applications due to their biomass being rich in added-value compounds (Aishvarya *et al.*, 2015). For instance, *Arthrospira platensis* biomass is rich in protein content, being useful as a meat substitute (Nutrition, 2020).

1.3. Goals

This master thesis project had three main goals: the optimization of growth medium at the laboratory scale for *Arthrospira* sp. cultivation, the scale-up up to the pilot-scale raceway ponds with the best culture medium resulting from the laboratory trials, and, lastly, the biomass harvesting. To accomplish these goals, several specific objectives were followed. The optimization process started in the laboratory, where the media Zarrouk, BG11 and Allmicroalgae medium were tested. Among those media, different conditions, such as, sodium bicarbonate concentrations, sodium sources and nitrogen concentrations were studied. Subsequently, in order to evaluate the optimization process developed at the laboratory scale, the best culture medium from this scale was used to inoculate several flat panel photobioreactors and, lastly, a pilot-scale reactor, namely raceway pond, where the

biochemical composition (pigments, more precisely, phycocyanin, and proteins) of its biomass was analysed. The biomass from the last optimization trial was also analysed. Lastly, the harvesting process was studied, where nets with different pore diameters were used.

1.4. Outline of the work

This work is structured in six chapters. The first chapter, *Introduction*, introduces the theme of the dissertation and problem, as well as the objectives defined to help face this problem. The second chapter, *State of the Art*, presents an overview of the current knowledge existent in the scientific community about microalgae, with a focus on their biochemical properties, applications and their cultivation in photobioreactors. Chapter 3, *Material and Methods,* gives detailed information about the methodologies used to perform the present work, including growth parameters calculations and methodology for biochemical composition analysis. Then, the fourth chapter, *Results and Discussion,* is divided according to the three main project goals, comprising the optimization of growth medium at the laboratory scale, the scale-up with the best culture medium resulted from the laboratory scale and, lastly, the biomass harvesting. The fifth chapter, *Conclusions*, presents the concluding remarks by assessing where the project goals are accomplished, and the final chapter, *Future Recommendations*, suggests some future assignments to complement this dissertation. Additionally, the references of this work can be consulted, as well as supplementary information in *Appendices*.

2. STATE OF THE ART

2.1. Algae – General overview

Algae are aquatic organisms that can also be found in terrestrial environments with humidity that, in general, have the ability to carry photosynthesis. Some may also obtain food and energy heterotrophically (Levine and Fleurence, 2018). These organisms are commonly found in a wide variety of habitats like rivers, oceans, ponds and lakes (Jacob-Lopes *et al.*, 2020). They can be classified into several groups: microalgae (usually unicellular, with some exceptions), macroalgae (multicellular organisms) and cyanobacteria (also called blue-green algae) (Soni *et al.*, 2017). In the past, some authors used to treat cyanobacteria as microalgae, but in fact, they belong to the kingdom Monera (prokaryote) (Soni *et al.*, 2017). Cyanobacteria are Gram-negative bacteria and appeared on earth billion years ago, being the primary oxygen producers, which lead to the later appearance of eukaryotic organisms like other microalgae, macroalgae and animal cells (Sharma *et al.*, 2014). For example, the microalgae *Archaeplastida* (or kingdom *Plantae*) lineage is related to cyanobacteria, since it inherited its chloroplasts from cyanobacteria by primary endosymbiosis billion years ago (Sharma *et al.*, 2014).

Cyanobacteria are ancient microorganisms with a cosmopolitan distribution, which means they have thrived in different environments with distinct conditions (Sharma *et al.*, 2014). Some cyanobacteria have the ability to fix atmospheric nitrogen due to the presence of heterocystous cells (Berman-Frank *et al.*, 2003; Jacob-Lopes *et al.*, 2020). Others can thrive in hostile environments because of either akinetes, which function is to store nutrients and metabolites for future consumption, or the extracellular polysaccharide (EPS) they produce that act as a buffer and contribute to desiccation tolerance (Sharma *et al.*, 2014). The cyanobacteria *Arthrospira platensis* appears naturally in high alkaline lakes and has a long history of being consumed as a food, for instance, by the people living near lake Chad (Africa), who harvested it and then left it to dry in a cake form under the sunlight, which is locally called "dihe" (Piccolo, 2012).

2.2. Morphological structure and biochemical composition of *Arthrospira platensis*

Throughout the years, cyanobacteria have endured different habitats with distinct conditions (temperature, salinity, UV radiation, etc) which led to several structural and functional modifications. They have a variety of morphologies, including unicellular, multicellular, filamentous and branched filamentous forms. This diversity made cyanobacteria a very versatile microorganism, which contributed to their competitive success in many environments (Levine and Fleurence, 2018).

A. platensis is a symbiotic, multicellular and filamentous non-N_2-fixing cyanobacterium (Figure 2) that belongs to the *Cyanophyceae* group and its taxonomic classification is given in Table 1 (Furmaniak *et al.*, 2017).

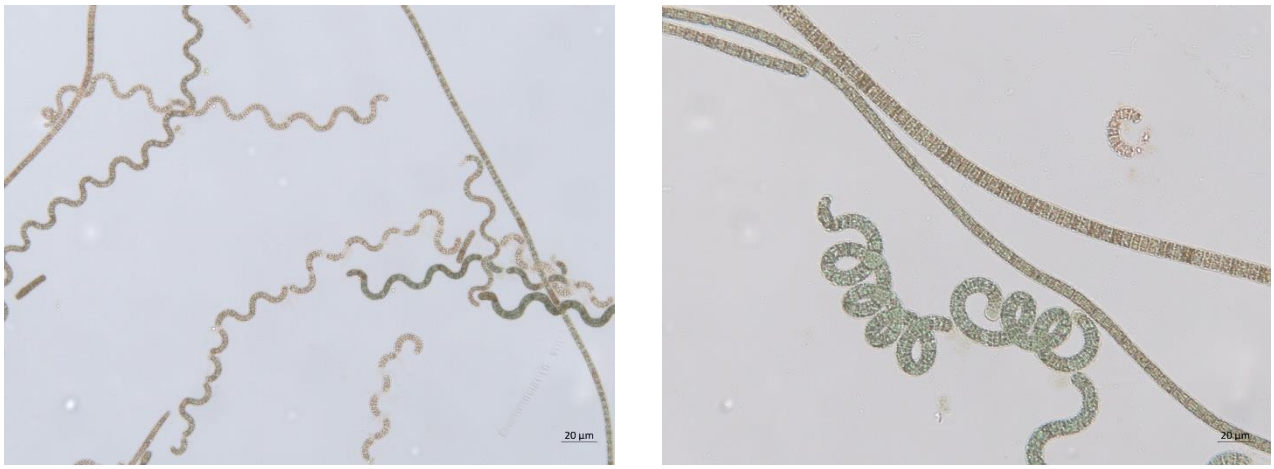

Figure 2. Microscopic observation of *Arthrospira* sp. cells (magnification 20× and 40×, from left to right).

Its filament (or trichomes) usually has a helical shape with a length of 50 to 500 µm and a width of 3 to 4 µm, which is regarded as a peculiar property of the genus *Arthrospira* (Habib *et al.*, 2008). However, its helical filament shape might change to a linear one, when exposed to different environmental conditions, like light or nutrients, and the need to better adjust to them in order to survive (Habib *et al.*, 2008). These cyanobacteria reproduce by binary fission (Koru, 2012).

Table 1. Taxonomic classification of *Arthrospira platensis* (WoRMS – World Register of Marine Species, 2020).

Kingdom	Bacteria
Phylum	Cyanobacteria
Class	*Cyanophyceae*
Order	*Oscillatoriales*
Family	*Microcoleaceae*
Genus	*Arthrospira*
Species	*Arthrospira platensis*

A. platensis is one of the most promising cyanobacterium species because of its biochemical composition, high protein content, essential amino acids (e.g. leucine, valine), B complex vitamins, minerals (e.g. potassium, calcium, magnesium, selenium), antioxidants (e.g. β-carotene, phycocyanin (PC)), polyunsaturated fatty acids (PUFA) (especially γ-linolenic acid) and phenolic compounds (Serban *et al.*, 2015). Therefore, *A. platensis* was declared by the World Health Organization as "one of the greatest superfoods on earth" and NASA considered it an excellent compact food for space travel because a small amount provides a wide range of nutrients (Soni *et al.,* 2017). The growing interest in these cyanobacteria is especially driven by its high-quality protein content (55-70% of its dry weight), making it a better protein source than other commonly used plants such as soybeans (35% of its dry weight), peanuts (25% of its dry weight) and grains (8-10% of its dry weight) (Jung *et al.*, 2019; Großhagauer *et al.*, 2020). *A. platensis* is also rich in PC, a pigment with a blue color. Some *A. platensis* strains have the pigment phycoerythrin, giving it a red or pink color (Koru, 2012).

The average percentage of lipids found in *A. platensis* is about 5 to 6% and, of those, 1.5 to 2.0% are PUFAs (Jung *et al.*, 2019). This cyanobacterium is the richest algae source of γ-linolenic acid (GLA), but also has the lipids α-linolenic acid (ALA), linoleic acid (LA), stearidonic acid (SDA) and arachidonic acid (AA) (Jung *et al.*, 2019). Table 2 summarizes the percentage of fatty acids commonly found in several *Arthrospira* species.

Table 2. Fatty acids composition (%) of different *Arthrospira* species (Cohen *et al.,*1987; Ötleş and Pire, 2001; Mühling *et al.*, 2005).

Fatty acids / Cyanobacteria	Palmitic acid **C16:0**	Palmitoleic acid **C16:1**	Stearic acid **C18:0**	Oleic acid **C18:1**	Linoleic acid **C18:2**	γ-linolenic acid **C18:3**
A. maxima	35.82	0.85	1.49	5.03	16.34	18.16
A. platensis	42.30	1.00	0.95	1.97	16.18	20.06
Arthrospira sp.[1]	44.60	4.40	0.50	6.40	17.10	27.00
Arthrospira sp.[2]	47.00	2.80	1.40	7.50	15.20	26.20

[1] Values of the strain SB. [2] Values of the strain D867.

2.3. Biotechnology applications of *Arthrospira platensis*

Arthrospira sp. biomass is rich in added-value compounds, like polysaccharides, enzymes, vitamins, pigments which are the reason for the interest of many industries, like cosmetic, nutritional and pharmaceutical (Chew *et al.*, 2017; Tang *et al.*, 2020). Nowadays, the commercial production of *Arthrospira* sp. has gained worldwide attention and approximately 200 species are being used worldwide in different biotechnological areas (Enzing *et al.*, 2014).

Some of the best worldwide known *A. platensis* producing companies are Earthrise Farms (USA), Cyanotech (USA), Hainan DIC Microalgae Co., Ltd (China), Murugappa Chettiar Research Center (India), Genix (Cuba) and Solarium Biotechnology (Chile). 70% of the current *A. platensis* market is targeted to human consumption (Koru, 2012). To monitor *A. platensis* based products, quality standards were established both nationally and internationally (Koru, 2012). Large-scale production, especially in open reactors, must have strict quality control in order to guarantee the absence of any other cyanobacteria capable of producing cyanotoxins, such as microcystins, anatoxin-a, saxitoxins, and nodularin (Großhagauer *et al.*, 2020). The risk posed by cyanotoxins should not be underestimated because their presence is detrimental to human health (Großhagauer *et al.*, 2020).

These cyanobacteria have gained more interest in the sector of food supplements and can be consumed in powder, capsules, or tablets. It can be used as a natural dye in many food products, like candy, chewing gum, ice cream and yogurt (Großhagauer *et al.*, 2020).

Although other species may be used for the same purpose, this one has the advantage of having a mucoprotein cell wall instead of a cellulose cell wall, which facilitates the digesting process (Koru, 2012). The consumption of products fortified with *A. platensis* is benefic for vegans and vegetarians because it is a relevant vitamin B_{12} (also called cobalamin, Cbl) source, which can only be obtained in substantial quantities by animal food (Levine and Fleurence, 2018). Figure 3 shows some of the *Arthrospira* sp.-based products available in the market.

A. platensis is claimed to be a non-toxic, nutritious food, with some pharmaceutical properties. The human intake of 1-8 g day^{-1} for 12 weeks led to several health benefits, ranging from weight loss to reduced blood pressure (Großhagauer *et al.*, 2020). The long-chain PUFAs, especially omega-6 fatty acids (e.g. GLA), are of pharmacological interest because they induce benefits on cardio-circulatory diseases, atherosclerosis, coronary heart diseases, hyperlipidemia, hypertonia and cancer (Ötleş and Pire, 2001). Serban *et al.* (2015) realized a trial with humans, where 1-10 g day^{-1} of *A. platensis* over treatment periods between 2-12 months were administered. A significant reduction of total cholesterol, low-density lipoprotein levels (LDL-C) and triglyceride levels with a simultaneous increase of high-density lipoprotein levels (HDL-C) was found (Serban *et al.*, 2015).

These cyanobacteria are also suitable for bioremediation because they can accumulate metals, which might be benefic to remove lead from wastewater (Großhagauer *et al.*, 2020). However, the specific cyanobacteria used in this process are not desired for dietary purposes (Großhagauer *et al.*, 2020). The European Commission has established a maximum limit of 4 mg kg^{-1} lead in their composition (Großhagauer *et al.*, 2020).

Finally, these cyanobacteria can be used in aquaculture as a feed additive to improve growth, feed efficiency, carcass quality and physiological response to some diseases in several species of fish (Madkour *et al.*, 2012). GLA is a precursor for the biologically active compound prostaglandin E1 (PGE1), which is necessary for the enhancement of the immune system in shrimp larvae (Madkour *et al.*, 2012).

Figure 3. Some commercialized products containing *Arthrospira* sp. (a) chocolate; (b) pills; (c) energy bars; (d) Wrap; (e) pasta fusilli; (f) hamburger. (Chocolate Salty pirulina, Iswari ©,2020; *Spirulina* Portugal, 2020; Buddha Protein Bar Chia Limão *Spirulina*, Iswari ©, 2020; Mini *Spirulina* Wraps, Wrawp Foods, 2020; Fusilli with organic *Spirulina*, Buon'Italia – Imported Italian Food, 2020; Nutrition, 2020)

2.4. Cultivation systems

Microalgae cultivation has existed for many years. Between early 1970s and late 1970s, commercial production of microalgae was initiated in East Europe, Israel and Japan (Habib *et al.*, 2008). During these periods, microalgae were grown commercially in open ponds as healthy food (Koru, 2012).

The cultivation of microalgae occurs in specialized bioreactors, with the purpose to maximize the exposure to sunlight, increasing the growth efficiency as much as possible (Hallmann *et al.*, 2020). Microalgae can grow in saline or freshwater environments and can be cultivated in open or closed systems, such as raceway ponds and tubular photobioreactors (PBR), respectively (Figure 4) (Ruane *et al.*, 2010). Choosing the right culture system can be challenging due to several factors, such as the algae used, toxic effects of the PBR material, leaching, fluid dynamics (flow velocity) and cleaning and sanitizing costs in each type of PBR (Pires *et al.*, 2012). Large scale production of microalgae occurs mostly

in open systems due to its effectiveness, simplicity and low production costs (Pires *et al.*, 2012). Table 3 compares these two systems (open, e.g. raceway ponds, and closed, e.g. tubular PBRs).

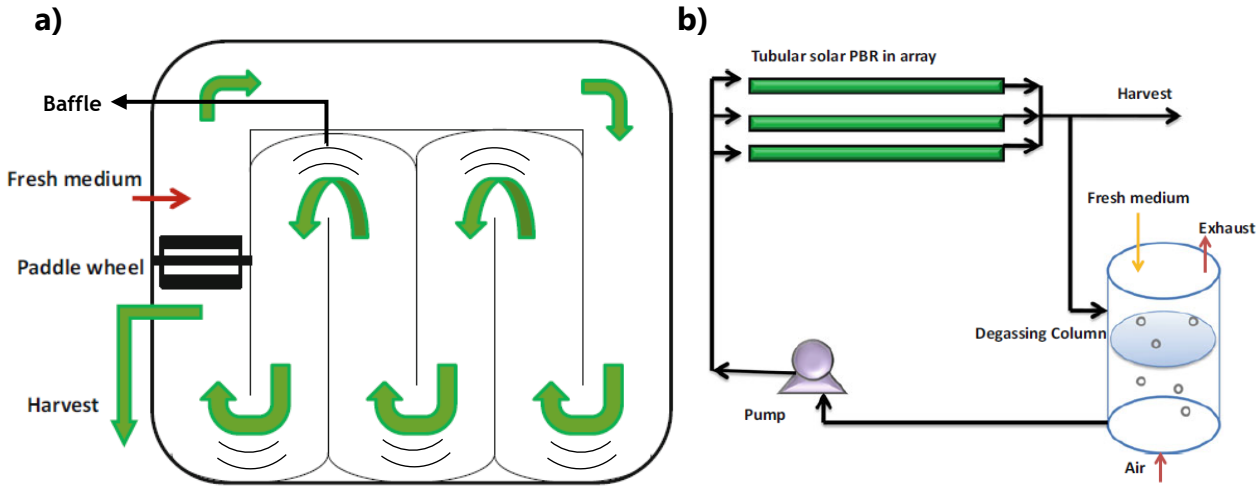

Figure 4. Cultivation systems used. a) Open raceway pond; b) closed system (tubular photobioreactor) (adapted from Aishvarya *et al.*, 2015).

A. platensis has been produced commercially for more than 40 years (Habib *et al.*, 2008). It can be grown in both systems (open and closed), where the major difference is that, in the first reactor, CO_2 mass transfer into the culture occurs directly from the atmosphere.

Raceway ponds are either dug in the ground or, more commonly, erected on the surface and usually have a depth of 0.15 m so that the sunlight gets to the maximum volume of culture, which subsequently will improve microalgae growth (Sharma *et al.*, 2014). The culture is mixed only by a paddlewheel and is recirculated around a racetrack loop and its course is enhanced by baffles, which function is guiding the flow at bends, reducing the dead zones (Suparmaniam *et al.*, 2019). In open ponds, the light exposure and temperature of the culture are not even and cannot be controlled, which affects the culture growth and cell concentration, because sunlight can only reach a certain depth (Suparmaniam *et al.*, 2019). Also, this reactor is more prone to contaminations, leading to a less controlled microalgae production, and its growth is influenced by local climate (indraft of rainwater) (Suparmaniam *et al.*, 2019). Contamination is a major drawback of this kind of cultivation systems, so the use of this cultivation system is restrict to more "robust" microalgae (e.g. *Dunaliella*, *Arthrospira* and *Chlorella*), which present a fast-growth or thrive in selective conditions (Pires

et al., 2012). The optimal growth of *Arthrospira* sp. is in high alkalinity environments (Pires *et al.*, 2012). In order to have temperature under control, raceway ponds must be placed inside of a greenhouse structure with air conditioning and vents that are often controlled automatically and can be open or closed according to the temperature inside (Hallmann *et al.*, 2020). Also, this reactor has the advantage of not needing a cooling system, which greatly reduces water costs, because temperature is reduced by active evaporation, which is affected by the ambient temperature (Hallmann *et al.*, 2020). However, in very hot and dry environments, water loss by evaporation may be excessive and, therefore, carbon dioxide is not being used effectively, which reduces productivity (Chisti, 2007). The assimilation of carbon dioxide by microalgae varies with several environmental factors like, for instance, in high temperatures, the CO_2 solubility in the culture is reduced (Morales *et al.*, 2018).

Closed PBRs can be found in different configurations, such as vertical column reactors (airlift), tubular reactors and flat-plate reactors and each PBR has their own advantages (Pires *et al.*, 2012; Suparmaniam *et al.*, 2019). The design of a tubular PBR usually consists of horizontal translucent acrylic tubes, that can also be arrayed in vertical, fence-like, inclined, or helix configurations. Those configurations are meant to achieve the maximum amount of sunlight, since it is the main factor affecting biomass productivity (Jacob-Lopes *et al.*, 2018). Choosing the right design of tubular PBR is an important task because the area exposed to the sunlight varies in each tube (Pires *et al.*, 2012). For example, the tubes at the top of the configuration have more sunlight surface area available than the tubes beneath them. This system operates by continuously recycling the microalgae culture, which is obtained by a mechanical pump, maintaining a constant turbulent regime and hence a perfect mixture (Suparmaniam *et al.*, 2019). Tubular PBRs are preferred for high-value applications because there is less contamination, the cultivation growth parameters can be controlled, and they occupy less space. However, not all species are suitable to be cultivated in them (Pires *et al.*, 2012). Some microalgae (e.g. *Nostoc*) secrete polysaccharides and proteins into the growth medium, promoting the creation of a biofilm that will eventually decrease photosynthetic efficiency (Hallmann *et al.*, 2020). Moreover, the connection of the tubes in the tubular PBR leads to small gaps, in which microalgae cells can be deposited and, over time, form a biofilm (Hallmann *et al.*, 2020). In industrial environments, biofilm formation must be avoided, since

removing it can prove to be a costly endeavor (Sharma *et al.,* 2014). Sometimes, maintaining the flow velocity above 0.5 m s^{-1} can be sufficient to decrease or even remove the biofilm (Hallmann *et al.*, 2020). In general, cleaning and sanitizing a closed PBR is more difficult and expensive than doing it in an open system, due to the obligatory use of chemicals, since the inside of the tubes cannot be reached, and the need for large amounts of water, since this process must be performed more than once (Sharma *et al.*, 2014). On the other hand, in raceways, as the biofilm can be formed on the water surface, on the bottom or in the sidewalls and these reactors are open, industrial equipment with brushes attached can be used to clean it (Sharma *et al.*, 2014; Pires, 2017). This process is simpler and does not require the amount of water that closed systems demand (Hallmann *et al.*, 2020).

Table 3. Comparison of raceway ponds and photobioreactors.

Factor	**Raceway ponds**	**Photobioreactors**
Applicability to different species	Low	High
Biomass quality	Reproducible	Variable
Cell density in culture	Low	High
Contamination	High	Low
Cost per kg of dry biomass	Low	High
Ease of cleaning	Easy	Difficult
Ease of scale-up	Easy	Variable
Ease of maintenance	Easy	Difficult
Energy input for mixing	Low	High
Evaporation loss	High	Low
Gas exchange efficiency	Low	High
Harvesting efficiency	Low	High
Harvesting cost	High	Lower
Overheating probability	Low	High
Process control	Difficult	Less difficult
Shear stress	Low	High
Space required	High	Low

Modified from Grobbelaar (2009); Brennan and Owende (2010); Pires *et al.* (2012); Sharma *et al.* (2014) and Narala *et al.* (2016).

2.5. Growth conditions

Microalgae growth is affected by: abiotic factors like temperature, pH, light exposure, available nutrients, the type of reactor used and related processes like the intensity of mixing, frequency of algae harvesting and salinity; biotic factors such as bacteria, fungi, history of the microalgae and strain used in the process and competition with another species (Okoro *et al.*, 2019). Each type of microalgae has it needs and, therefore, a universal growth medium cannot be achieved. Besides water, microalgae must be supplied with all the essential nutrients required for growth. These can be divided into macro- or micronutrients according to the amounts needed by the cultures. Macronutrients include carbon (C), nitrogen (N) and phosphorous (P). Micronutrients like copper (Cu), cobalt (Co), manganese (Mn), boron (B), molybdenum (Mo), and zinc (Zn) are required in much smaller quantities (Jacob-Lopes *et al.*, 2018). The water used in the process is important because some of these micronutrients can be found in sufficient quantities in the water supply and their quality affects the biomass quality (da Fonseca *et al.*, 2016). Some producers, like Earthrise Farms and Cyanotech Co., use the Colorado river and deep seawater combined with freshwater as water sources, respectively, in order to obtain a wide range of minerals (Kim *et al.*, 2007).

A. platensis can grow in different media, such as, seawater, fresh water, sewage water, medium based on commercial grade chemicals and fertilizers, which indicates high adaptability to different environments (Madkour *et al.*, 2012). For many years, Zarrouk medium has successfully served as the standard medium. It is known that this species requires an alkaline and high carbonate content culture medium, with pH values ranging between 9.5 and 9.8 (Madkour *et al.*, 2012). The fact that the optimal pH is around 10 is an advantage for this species because only a few species thrive in those harsh conditions, which effectively inhibits contamination (Madkour *et al.*, 2012).

2.6. Biomass harvesting process

Microalgae biomass harvesting is an expensive process. This process is usually difficult and expensive because microalgae are small size microorganisms and their density is close to the medium they are growing, taking several hours to settle (Mohd-Sahib *et al.*, 2017). However, *A. platensis*' morphology softens this operation cost, because it creates clusters which facilitate the harvesting process (Amaro *et al.*, 2017). Biomass can be collected by

chemical, physical, electrical and biological methods, such as, filtration, centrifugation, sedimentation ultrasound, floatation, gravity sedimentation and flocculation (Tang *et al.*, 2020). Flocculation technique is attractive due to its lower cost and high efficiency and can be realized with chemicals or biological flocculants (Amaro *et al.*, 2017). The flocculants used can be classified into three categories: inorganic flocculants (metallic salts of iron and aluminum); inorganic polymers (polyelectrolyte, polyaluminium chloride and polyacrylamide); organic polymers (cationic starch, chitosan and glutamic acid) (Okoro *et al.*, 2019). When chemical flocculation is used, the slurry can be contaminated, reducing biomass market value, transesterification processes and the use of the biomass in food industry and animal feeds (Okoro *et al.*, 2019). The toxicity and detrimental health risks of these chemicals are the major reason for being the utmost important research topic, with the intent to find substitutes (Okoro *et al.*, 2019; Suparmaniam *et al.*, 2019).

A. platensis biomass is usually harvested from raceway ponds by filtration where weave clothes (screens) with a specific mesh are placed at the entrance of the raceway pond (Vonshak, 1997). There are several types of filtration: an inclined gravity screen, horizontal vibratory screen and vacuum-table or vacuum-belt filter (Sharma *et al.*, 2014). Later, the obtained biomass passes through an extruder, which transforms the biomass into a "spaghetti" form of 2 mm diameter, and lastly it is left to dry at low temperatures (Stunda-Zujeva and Rugele, 2017). In the past, some industries used a spray drier to dry *A. platensis* biomass. However, this method is not ideal because of the high temperatures it achieves, which deteriorates biomass content (Stunda-Zujeva and Rugele, 2017). The traditional and ancient method to dry this cyanobacteria consists in using sandy hollows, which was used on the shores of lake Chad (Großhagauer *et al.*, 2020). *A. platensis* biomass should be harvested in the morning due to its bigger stored protein content at that time of the day, since cool temperatures facilitate the operation and a long exposition to the sun would dry the final product (Soni *et al.*, 2017).

3. MATERIALS AND METHODS

All trials and biomass biochemical analysis were performed at Allmicroalgae facilities between February 10th and August 21st, 2020.

3.1. Cyanobacteria and culture medium

The species used in this study was the cyanobacteria *Arthrospira* sp., a blue-green algae, which is deposited in the Allmicroalgae culture collection under the internal code 0071AN. The media used were Zarrouk (Table A_1 to A_2, Appendix) (Richmond and Grobbelaar, 1986), with pH=10, adjusted before sterilization; MNS, Allmicroalgae industrial medium, which is based on Guillard F/2 (Guillard, 1975) and BG11 (Table A_3 to A_4, Appendix) (Rippka *et al.*, 1979) and were made up with the local water source of the adjacent cement factory. The first two media were also used with different sodium bicarbonate ($NaHCO_3$) concentrations, sodium sources and nitrogen concentrations.

3.2. Laboratory culture media optimization

The assay was conducted in triplicate in 1 L flask reactor set with constant aeration of 1.4 vvm. The cultures pH was kept at 10 by CO_2 injection (if necessary). Cultures were grown at 24 ºC under continuous radiation exposure of 700 µmol photons m^{-2} s^{-1}. The initial biomass concentration was set to 0.3 g L^{-1}. Sampling was taken every day at 9 am during the trial in order to monitor growth performance (OD at 540, 600 and 750 nm), pH, temperature and nitrates concentration. Besides that, every day, right before sampling, water was added to compensate evaporation losses. All laboratory assays were performed considering this setup.

3.2.1. Trial A: Culture media screening

The media Zarrouk, BG11 and MNS were tested with nitrogen concentration adjusted to 30 mM.

3.2.2. Trial B: Sodium bicarbonate concentration

The media Zarrouk and MNS were tested. The latter was also tested with different $NaHCO_3$ concentrations: 100%, which corresponds to Zarrouk's concentration, 200 mM (16.8 g L^{-1}), 50% and 25%. Zarrouk and MNS without $NaHCO_3$ were the positive and negative control, respectively.

3.2.3. Trial C: Sodium bicarbonate *vs.* potassium bicarbonate

This trial was realized in duplicate. The media Zarrouk, MNS with $NaHCO_3$, MNS with potassium bicarbonate ($KHCO_3$) and MNS with $KHCO_3$ plus vitamins were tested. The concentration of $KHCO_3$ was calculated in order to maintain the same molarity as in $NaHCO_3$, which corresponds to 200 mM.

3.2.4. Trial D: Sodium source

The media Zarrouk, MNS with $NaHCO_3$, MNS with $KHCO_3$, MNS with $KHCO_3$ plus Sodium thiosulphate ($Na_2S_2O_3$), MNS with $KHCO_3$+NaEDTA and MNS with $KHCO_3$+vitamins were tested. The compounds added to the main media (Zarrouk and MNS) maintained the culture media with a sodium concentration of 50 mM. Zarrouk and MNS with $KHCO_3$ were the positive and negative control, respectively.

3.2.5. Trial E: Nitrogen concentration optimization

The media Zarrouk, MNS with a nitrogen concentration of 30 mM, 20 mM, 10 mM and 5 mM were tested. All four MNS media were supplemented with 16.8 g L^{-1} of $NaHCO_3$.

3.3. Culture scale-up up to pilot-scale

The first step of the scale-up occurred at the laboratory, under controlled temperature and light exposure (24 ºC and 50 W (6332 Lm) (PROTEK SECOM, Industrial Flood Light, Spain)). Microalgae cultures were grown in Zarrouk in 2 L and 5 L round bottom flask, connected to an air feed, which was sterilized by a 0.2 µm air filter (Midisart® 2000 PTFE, Sartorius, Germany). An identical filter was placed at the air outlet (Figure 5).

Figure 5. *Arthrospira* sp. growth in Zarrouk medium in 5 L reactors under laboratory-controlled conditions.

The next phase of the scale-up occurred outdoors and four 5 L flask reactors, with the necessary DW, were used to inoculate a FPP of 80 L (outdoors). There were several scales of FPPs (80 L, 150 L, 500 L and 750 L), which were sequentially inoculated one with the other. Several culture media were used in the different FPPs (Table 4).

Table 4. Culture media used in the several flat panel photobioreactors during the second phase of the scale-up process.

Flat panel photobioreactor	Culture medium used
80 L	Zarrouk
150 L	MNS + $KHCO_3$ (200 mM)
500 L	MNS + $NaHCO_3$ (200 mM)
750 L	MNS + $NaHCO_3$ (200 mM)

These reactors (Figure 6) consisted of a bag made with low-density polyethylene (LDPE) contained between two rigid frames confining a relatively thin vertical panel. In this outdoor PBR, natural light was the source of photons and the temperature was regulated by spraying water at the surface of the reactor, if necessary. Microalgae growth kinetics was analyzed until the stationary phase was reached.

Figure 6. Cultivation of *Arthrospira* sp. in a flat panel photobioreactor with roughly 750 L in the medium MNS supplemented with $NaHCO_3$ at 16.8 g L^{-1} under outdoor conditions.

The last step of the *Arthrospira* sp. scale-up process consisted in the pilot-scale raceway pond inoculation, in the medium MNS supplemented with $NaHCO_3$ (200 mM). The pilot-scale raceway pond is represented in Figure 7.

Figure 7. Cultivation of *Arthrospira* sp. in a raceway pond with a surface area of 27 m^2 and a working volume of 3.5 m^3. Growth occurred in MNS with $NaHCO_3$ under outdoor conditions.

This reactor has a total length and width of 15 m by 2 m and a superficial area of 27 m^2. A culture depth of 13 cm, and a circulation velocity of 0.3 m s^{-1} was set, since is the most commonly used flow velocity for biomass production (Hallmann *et al.*, 2020). A central baffle divides the reactor into two channels, each with 1 m of width. Two sets of three baffles were placed at the end of the reactor in order to guide the flow, reduce dead zones, maintain the culture velocity constant and reduce pressure losses at the bends. A paddlewheel with the same width as the channel was placed at the beginning of the channel right after the bend with the intent to blend the culture, homogenize the temperature and enhance its aeration. The culture usually self-maintains the optimum pH because of the $NaHCO_3$ buffering capacity, but in the necessity of decreasing pH, this reactor has a CO_2 diffuser placed right after the paddlewheel at the bottom of the raceway.

3.4. Cyanobacteria growth analysis

With the intent of monitoring cyanobacteria growth, both in the laboratory trials and in the scale-up process, daily samples were taken, and biological and operational parameters were measured.

OD was measured at 540, 600 and 750 nm using a spectrophotometer (Genesys 10S UV-VIS, Thermo scientific). DW was determined by filtering a known volume of culture through a 0.7 µm glass fiber filter (VWR, Portugal), using a vacuum pump (N86 LABOPORT, KNF, USA). After washing with water, the filter was dried in a moisture analyzer (DBS 60-3, KERN & SOHN GmbH) and later measured in an analytical balance (PA114, ohaus

corporation, USA). The obtained results were plotted together in a calibration curve (Figure A_1, Appendix), as represented by the equation $y = 0.5608x + 0.0601$, where "x" is OD at 540 nm and "y" DW in g L^{-1}).

The volumetric biomass productivity (Equation 1), P (g L^{-1} d^{-1}), quantified the evolution of biomass concentration over time, where X_1 and X_2 are the DW in g L^{-1} in different times (days), t_1 and t_2, respectively.

$$\mathrm{P}\ (\mathrm{g\ L^{-1} d^{-1}}) = \frac{\mathrm{X_2 - X_1}}{\mathrm{t_2 - t_1}} \quad \textbf{(1)}$$

Areal productivity of biomass (Equation 2), P_a (g m^{-2} d^{-1}) was calculated as the product of the volumetric biomass productivity (P, g L^{-1} d^{-1}), given by Equation 1, and the working volume of the reactor (V), divided by the ground area occupied by the reactor (A, m^2). In raceway ponds the ground area corresponds to the surface area of the reactor.

$$P_a\ (g\ m^{-2} d^{-1}) = \frac{P \times V}{A} \quad \textbf{(2)}$$

Specific growth rate (μ) was given by Equation 3 and represented in d^{-1}. It was expressed as the difference between the final and initial Napierian logarithm of biomass concentrations (X_2 and X_1, respectively) and its respective time (t_2 and t_1, respectively).

$$\mu\ (d^{-1}) = \frac{1}{X}\frac{dX}{dt} = \frac{ln\ (X_2) - ln\ (X_1)}{t_2 - t_1} \quad \textbf{(3)}$$

3.5. Nitrates determination

To study the culture growth, nitrates consumption was evaluated overtime. First, a small sample was taken and centrifuged for 10 min at 2000 g (MiniStar Silverline, VWR, Portugal), in order to obtain the supernatant. After, a dilution of 1:80 was realized, where 125 μL of the previous supernatant was added to a tube with 300 μL of hydrochloric acid (1 M), totaling with water until 10 mL. Lastly, the nitrates were determined at 220 and 275 nm using a spectrophotometer (Genesys 10S UV-VIS, Thermo scientific). In order to remove the interference caused by organic matter, two times the absorbance reading at 275 nm was subtracted from the absorbance reading at 220 nm. The nitrates concentration was then given by a calibration curve ($y = 3.73x + 0.000356$, where "y" is (OD_{220}-(2×OD_{275})) and "x"

is the concentration of NO_3^- (mM)). The temperature and pH were registered by a pH meter (Combo pH/Conductivity/TDS Tester, Hanna instruments, Portugal). The integrity of the cultures was assured by microscopic observations (Primo Star, ZEISS, USA).

3.6. Biochemical composition

3.6.1. Pigments content

The phycobiliproteins were extracted using the protocol described by Bennett and Bogorad (1973). This protocol was tested with different amounts of biomass (2 mg, 5 mg and 10 mg). The necessary volume was centrifuged in glass tubes at 9 ºC, 3500 *g* for 10 minutes (Z 400 K, Hermle Labortechnik GmbH, Germany) and biomass was washed three times. To the pellet, 1.5 mL of zirconia beads and 6 mL of 0.1 M sodium phosphate buffer (pH 7) were added (Table A_5, Appendix). Subsequently, the tubes were homogenized in the vortex (classic vortex mixer, Velp Scientifica) in the dark for 5-10 minutes. The tubes were again centrifuged under the same conditions and at the end, the supernatant was collected to a new clean vial. This procedure (add phosphate buffer, vortex and centrifuge) was repeated until the pellet was white (which means that there is nothing else to extract) or until the supernatant was clear (which means that the solvent could not extract more pigments). Before reading the pigments in the spectrophotometer (Genesys 10S UV-VIS), 1 mL was centrifuged (MicroStar12, VWR) for 2 minutes. Lastly, the supernatants (Figure 8) were measured at the following wavelengths: 280 nm, 615 nm, 652 nm, 675 nm and 750 nm, using 1 cm light path quartz cuvettes. The spectrophotometer was calibrated using the phosphate buffer as blank. All analysis were performed in duplicated. The PC and allophycocyanin (APC) content was calculated with Equation 4 and 5.

$$[PC] = \frac{(A_{615} - (0.474 \times A_{652}))}{5.34} \quad \textbf{(4)}$$

$$[APC] = \frac{(A_{652} - (0.208 \times A_{615}))}{5.09} \quad \textbf{(5)}$$

Figure 8. Phycocyanin extraction. a) The three samples (2 mg, 5 mg and 10 mg, from left to right) of *Arthrospira* sp. b) Color obtained after centrifugation of 1 mL of extracted volume.

3.6.2. Protein content

The total protein content was determined by the method described by Lowry *et al.* (1951). This method was realized with a final biomass concentration of 1 g L^{-1} (DW_f) and a final volume of 6 mL (V_f). Equation 6 gives the necessary volume to have the required DW.

$$DW_i \times V_i = DW_f \times V_f \leftrightarrow V_i = \frac{1\ (g\ L^{-1}) \times 6\ mL}{DW_i} \quad \textbf{(6)}$$

The volumes given by the previous equation were transferred to 50 mL falcon tubes (Normax) and then biomass was washed three times. The pellet was resuspended in 6 mL of water and was homogenized in the vortex (classic vortex mixer, Velp Scientifica). 2 mL of this suspension was transferred to a glass tube, where 2 mL of NaOH (2 mM) was added. The glass tube that will serve as the blank solution had 2 mL of water instead of culture. Subsequently, the tubes were placed in a water bath at 99 ºC for 10 minutes (1003, 14 L, GFL) and cooled at room temperature. The tubes were again centrifuged and to 1 mL of the supernatant, 5 mL of the reagent 1 (sodium carbonate (5% m v^{-1}), potassium sodium tartrate (2% m v^{-1}) and the copper sulfate solution (1% m v^{-1}) reagents, Table A_6 (Appendix)) were added. The tubes were left in the dark for 10 minutes and 1 mL of reagent 2 (Folin-Ciocalteau reagent and Water, Table A_7 (Appendix)) was added. The tubes were left in the dark for more 30 minutes and were measured in the spectrophotometer at the wavelength of 750 nm. The same procedure was realized with a stored sample of *Chlorella vulgaris,* which was the control and has approximately 55% of protein content. The analysed samples are represented in Figure 9.

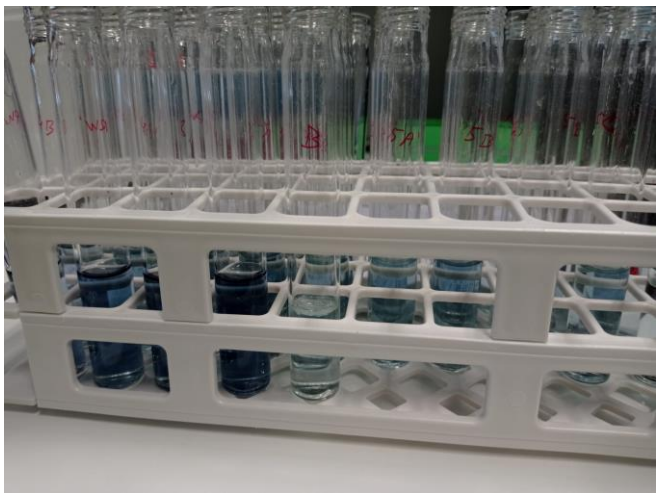

Figure 9. Glass tubes after realizing the extraction of protein from *Arthrospira* sp. by the Lowry *et al.* (1951) method.

3.7. Harvesting

The culture was harvested from the first assay that occurred in the raceway pond by a physical method (filtration), where gravity was used as the driving force. Six synthetic weave cloths (nylon) with different porosities (233.813 µm; 205.479 µm; 122.900 µm; 45.099 µm; 18.037 µm; 6.374 µm) were tested.

3.8. Statistical treatment

Results are presented as the mean and the standard deviation (SD) of triplicate determinations. The differences between samples were statistically evaluated using IBM SPSS Statistics 24 software. The values were evaluated at a 95% significance level using ANOVA with a post-hoc Tukey Test.

4. RESULTS AND DISCUSSION

4.1. Laboratory trials

4.1.1. Trial A: Screening of three different culture media

Arthrospira sp. was cultivated in three culture media (Zarrouk, BG11 and MNS) in 1 L flask reactors for 14 days (Figure 10).

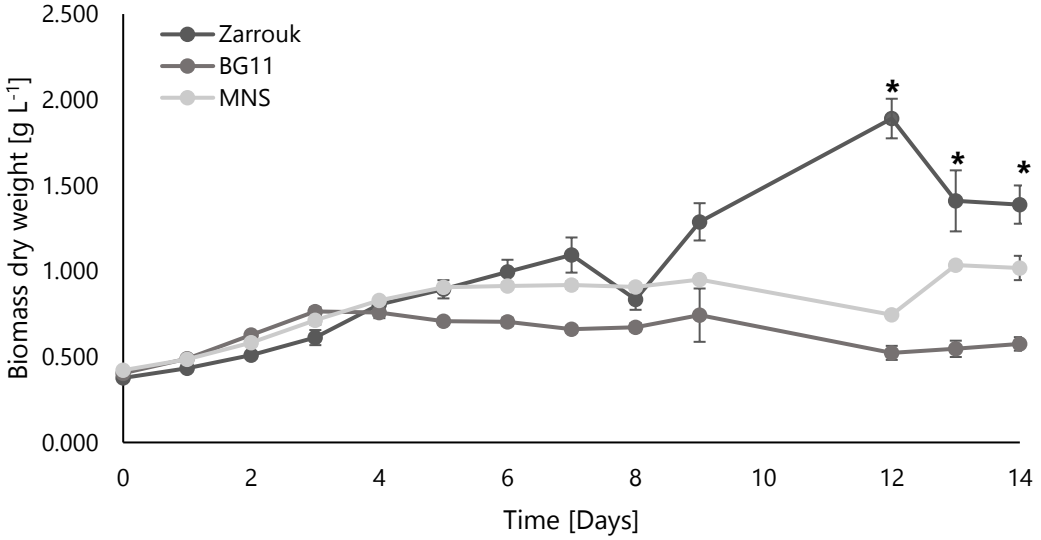

Figure 10. *Arthrospira* sp. growth curves in three different media (Zarrouk, BG11 and MNS) for 14 days. The assay was performed in 1 L flasks, under laboratory-controlled conditions. All conditions were run in triplicates and the values presented are the average of triplicates and the error bars are the respective standard deviations. "*" indicates a statistical difference (p<0.05).

The culture in all media presented a lag phase of only 1 day, which revealed great adaptability to the media. The kinetic parameters are described in Table 5.

Table 5. Productivities and specific growth rates of three different culture media in 1 L flasks (Zarrouk, BG11 and MNS).

Culture medium	Productivity (g L^{-1} d^{-1})		Specific growth rate (d^{-1})	
	Global	Maximum	Global	Maximum
Zarrouk	0.072±0.008^{a}	0.452±0.060^{a}	0.093±0.006^{a}	0.432±0.036^{a}
BG11	0.012±0.003^{b}	0.138±0.033^{a}	0.025±0.005^{b}	0.248±0.061^{b}
MNS	0.043±0.005^{c}	0.289±0.031^{a}	0.063±0.005^{c}	0.328±0.034^{c}

Values are mean ± standard deviation. Columns with different letters are statistically different (p<0.05).

Figure 10 shows that *Arthrospira* sp., when growing in Zarrouk, has a longer exponential phase, which lasts 9 to 11 days. After that, the cells enter the stationary phase until day 14. The obtained results revealed that, despite the similar growth patterns in the first few days, Zarrouk ended up achieving the highest DW, 1.890±0.116 g L^{-1}, global productivity, 0.072±0.008 g L^{-1} d^{-1} and specific growth rate, 0.432±0.036 d^{-1} (p<0.05). Pumas and Pumas (2016) realized a study with *A. platensis* and registered similar specific growth rates (1.02 d^{-1}), but presented a lower global productivity (0.044 g L^{-1} d^{-1}). Delrue *et al.* (2017) registered in modified Zarrouk a higher global productivity (0.092±0.004 g L^{-1} d^{-1}) than the observed in this trial (0.072±0.008 g L^{-1} d^{-1}), which could be justified by the intensity of light, which was 700 µmol photons m^{-2} s^{-1} in this trial and 11 µmol photon m^{-2} s^{-1} in the study performed by that author, as light has great impact in *Arthrospira* sp. growth since this species is photosensitive (Vonshak, 1997).

Shi *et al.* (2016) studied the growth of *Arthrospira* sp. in Zarrouk medium under different wavelengths (red, green, blue and a fluorescent light as control) and proved that red light (620-630 nm) induces *Arthrospira* sp. growth, since the biomass DW increased from 0.859 g L^{-1} (control) to 1.346 g L^{-1} (Shi *et al.*, 2016). In this trial the cultivation was induced by white light, which means that the species could grow even further if a red light were used, despite the biomass obtained being higher that was obtained by that author.

Additionally, the cyanobacterial initial biomass concentration could have an impact on its growth. One study realized by Delrue *et al.* (2017), which cultivated *Arthrospira* sp. for 27 days in a modified Zarrouk medium, the authors proved that the initial biomass concentration influenced its growth, achieving the maximum DW of 3.40 g L^{-1} with an initial DW of 1 g L^{-1}, and 2.76 g L^{-1} when the trial started with a lower DW (0.35 g L^{-1}). In this study the initial biomass concentration in Zarrouk was 0.376±0.000 g L^{-1}, which indicates that higher values of DW could be reached in future assays if the initial concentration of biomass is increased.

When comparing the three media, BG11 was indeed not suitable to grow *Arthrospira* sp. and, therefore, this medium will not be considered for further trials. The major difference between these three media consists in the carbon concentration, with Zarrouk having its carbon source, $NaHCO_3$, at the highest concentration of 0.2 M. High

concentrations of bicarbonate favor the growth of *Arthrospira* sp. (Richmond *et al.*, 1982; Sujatha and Nagarajan, 2013). Despite the results showing that Zarrouk induced the higher productivity and growth rate, MNS showed promising results and represented a non-specific medium for the growth of *Arthrospira* sp., being selected for further optimization.

4.1.2. Trial B: Sodium bicarbonate concentration optimization

After the optimization, several concentrations of $NaHCO_3$ with the aim to find the concentration needed to supplement the medium MNS, were tested. As shown in Figure 11, *Arthrospira* sp. grew in Zarrouk, MNS without supplementation and MNS supplemented with 25% $NaHCO_3$; 50% $NaHCO_3$ and 100% $NaHCO_3$, which corresponds to 4.2 g L^{-1}, 8.4 g L^{-1} and 16.8 g L^{-1} (0.2 M), respectively in 1 L flask reactor during 8 days. The kinetic parameters are described in Table 6.

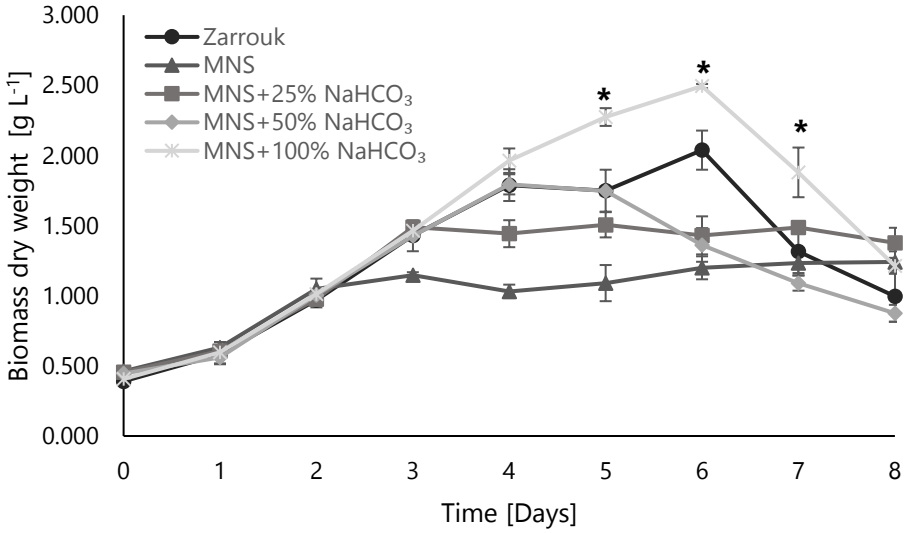

Figure 11. *Arthrospira* sp. growth curves in (Zarrouk, MNS, MNS with 25%, 50% and 100% of $NaHCO_3$) during 8 days. Trial was performed in 1 L flask reactor, under laboratory-controlled conditions. All conditions were run in triplicates and the values presented are the average of triplicates and the error bars are the respective standard deviations. statistical differences are marked with "*" (p<0.05).

Table 6. Productivities and specific growth rates of five growth media in 1 L flasks (Zarrouk, MNS, MNS with 25%, 50% and 100% $NaHCO_3$).

Culture medium	Productivity (g L^{-1} d^{-1})		Specific growth rate (d^{-1})	
	Global	Maximum	Global	Maximum
Zarrouk	0.076±0.023[a b]	0.462±0.078[a]	0.115±0.022[a b]	0.512±0.124[a]
MNS	0.097±0.009[a b]	0.417±0.110[a]	0.123±0.007[a b]	0.507±0.128[a]
MNS+25% $NaHCO_3$	0.115±0.013[a]	0.513±0.028[a]	0.138±0.009[a]	0.475±0.007[a]
MNS+50% $NaHCO_3$	0.054±0.008[b]	0.445±0.065[a]	0.084±0.008[b]	0.588±0.102[a]
MNS+100% $NaHCO_3$	0.101±0.007[a]	0.500±0.077[a]	0.137±0.006[a]	0.528±0.062[a]

Values are mean ± standard deviation. Columns having with different letters are statistically different (p<0.05).

The cultures in all media remained in the lag phase for one day and had a similar growth rate until the 3rd day. After that, the cultures had different behaviors and the one growing with MNS supplemented with 100% $NaHCO_3$ end up achieving the highest DW, 2.495±0.015 g L^{-1} (p<0.05). However, it did not achieve neither the highest productivity nor specific growth rate. A study conducted by de Castro *et al.* (2015) proved that biomass production tended to increase with an increase in the $NaHCO_3$ concentrations, since it improved from 1.070 mg L^{-1} (with 10.82 g L^{-1} of $NaHCO_3$) to 1.540 mg L^{-1} (with 16.18 g L^{-1} of $NaHCO_3$). In this same study the productivity increased from 48.571 mg L^{-1} d^{-1} to 70.952 mg L^{-1} d^{-1} (de Castro *et al.*, 2015). These results indicate that the supplementation of $NaHCO_3$ to the MNS medium is crucial for *Arthrospira* sp. growth.

4.1.3. Trial C: Sodium bicarbonate *vs.* potassium bicarbonate

HCO_3^- is the most common carbon source used in the growth of *Arthrospira* sp. and is crucial to maintain pH around 10.5. To assess whether the source of bicarbonate is relevant, *Arthrospira* sp. was grown in 4 conditions (Zarrouk, MNS+$NaHCO_3$, MNS+$KHCO_3$ and MNS+$KHCO_3$+vitamins) for 7 days in 1 L flask reactors. This trial was performed in duplicate, so no statistic or standard deviations were included. Growth curves are plotted in Figure 12 and the kinetic parameters described in Table 7.

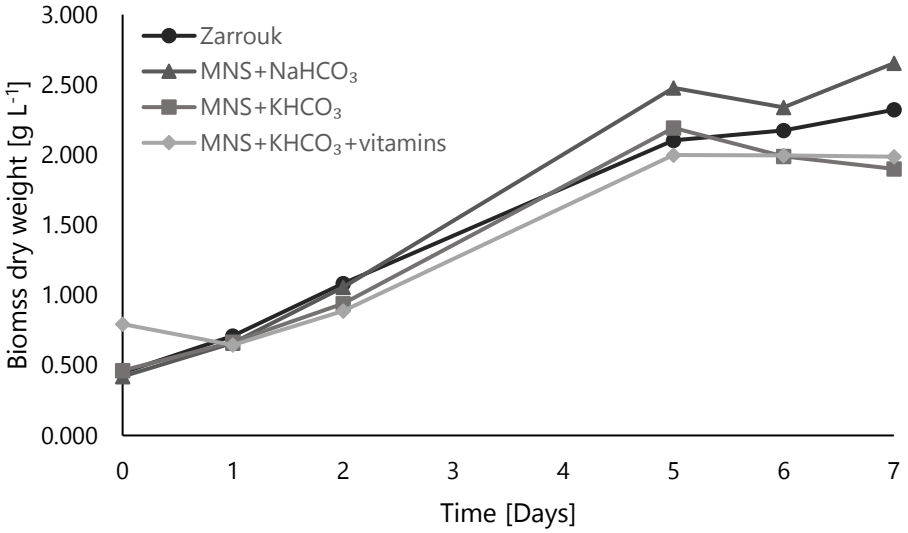

Figure 12. *Arthrospira* sp. growth curves in 4 media (Zarrouk, MNS supplemented with $NaHCO_3$ at 16.8 g L^{-1}, MNS with $NaHCO_3$ at 20.0 g L^{-1} and MNS with $NaHCO_3$ and vitamins) for 7 days. Trial was performed in 1 L flasks, under laboratory-controlled conditions. All conditions were run in duplicates and the values presented are their average.

The growth rate of *Arthrospira* sp. was similar when supplemented with all media and all (except for the culture growing with Zarrouk) entered the stationary phase on the fifth day.

Table 7. Productivities and specific growth rates of four growth media in 1 L flasks (Zarrouk, MNS with $NaHCO_3$ (16.8 g L^{-1}), MNS with $KHCO_3$ (20.0 g L^{-1}) and MNS with $KHCO_3$ and vitamins.

Culture medium	Productivity (g L^{-1} d^{-1})		Specific growth rate (d^{-1})	
	Global	Maximum	Global	Maximum
Zarrouk	0.268	0.375	0.234	0.457
MNS+$NaHCO_3$	0.319	0.474	0.263	0.471
MNS+$KHCO_3$	0.205	0.417	0.202	0.364
MNS+$KHCO_3$+vitamins	0.171	0.370	0.131	0.324

The culture achieved the highest DW (2.654 g L^{-1}), productivity (0.319 g L^{-1} d^{-1}) and specific growth rate (0.263 d^{-1}) in the medium MNS supplemented with $NaHCO_3$. *Arthrospira* sp. growing in MNS supplemented with $KHCO_3$ and vitamins had the lowest global productivity of 0.171 g L^{-1} d^{-1}, showing that vitamins are not a key factor in

Arthrospira sp. cultivation. This is economically advantageous because vitamins are very expensive and usually, industrial cultivations (outdoors) do not contain vitamins.

Figure 13 are microscopic pictures of *Arthrospira* sp. growing in MNS supplemented with $NaHCO_3$ (a) and with $KHCO_3$ (b) and reveal the importance of the source of HCO_3^-, since the absence of a sodium source lead to a linear morphology and fragmentation. Cyanobacteria in general need a minimum sodium concentration of 50 mM to grow, because otherwise their physiology and morphology would be affected and eventually would lead to cell death (Schlesinger *et al.,* 1996). Zarrouk medium has four sodium sources in its composition, having a total sodium concentration of 69.6 mM, with 54.7 mM being derived from $NaHCO_3$. Meanwhile, the total sodium concentration in MNS medium is only 0.164 mM. This medium, when supplemented with $NaHCO_3$, allows it to achieve a sodium concentration of 54.9 mM. However, when supplemented with $KHCO_3$, it only has 0.164 mM of sodium.

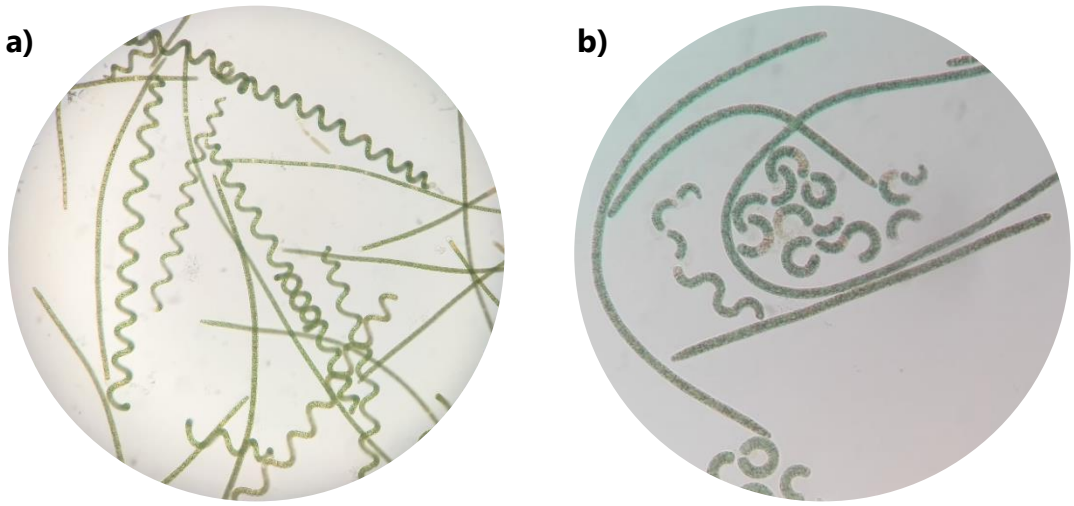

Figure 13. Microscope observations (magnification of 40×) of cells in the medium MNS supplemented with $NaHCO_3$ (Figure 13.a) and MNS supplemented with $KHCO_3$ (Figure 13.b).

These results indicate that, despite MNS with $NaHCO_3$ performing slightly better than Zarrouk, the difference is not significant, and the latter continued to be used as the positive control in the following trials. The results also showed that a sodium concentration of 50 mM must be maintained in the culture media, in order to preserve *Arthrospira* sp.'s physiology and morphology.

4.1.4. Trial D: Sodium source

Arthrospira sp. was cultivated using several sodium sources: Zarrouk; MNS with $NaHCO_3$; MNS with $KHCO_3$; MNS with $KHCO_3$ and $Na_2S_2O_3$; MNS with $KHCO_3$ and NaEDTA; and, lastly, MNS with $NaHCO_3$+vitamins, for 8 days using 1 L flask reactors. Growth curves are plotted in Figure 14 and the kinetic parameters described in Table 8.

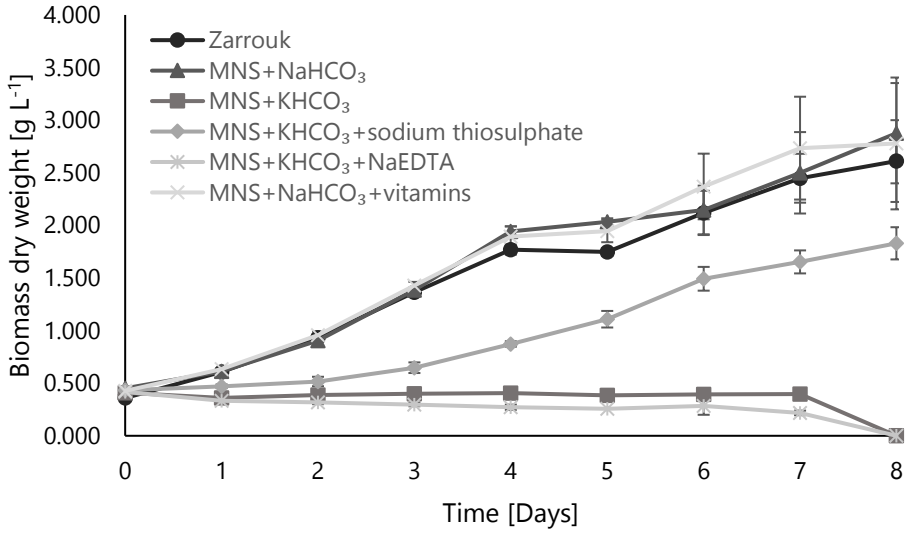

Figure 14. *Arthrospira* sp. growth curves in six media (Zarrouk, MNS supplemented with $NaHCO_3$ at 16.8 g L^{-1}, MNS with $NaHCO_3$ at 20.0 g L^{-1}, MNS with $KHCO_3$ and $Na_2S_2O_3$, MNS with $KHCO_3$ and NaEDTA and MNS with $NaHCO_3$ and vitamins) for 8 days. Trial was performed in 1 L flasks, under laboratory-controlled conditions. Presented results are the mean and standard deviation of three individual biological replicates.

Table 8. Productivities and specific growth rates of six culture media in 1 L flasks (Zarrouk; MNS with $NaHCO_3$; MNS with $KHCO_3$; MNS with $KHCO_3$ and $Na_2S_2O_3$; MNS with $KHCO_3$ and NaEDTA; and MNS with $NaHCO_3$ and vitamins.

Culture medium	Productivity (g L^{-1} d^{-1})		Specific growth rate (d^{-1})	
	Global	Maximum	Global	Maximum
Zarrouk	0.281±0.049[a]	0.433±0.043[a]	0.246±0.019[a]	0.521±0.016[a]
MNS+$NaHCO_3$	0.303±0.060[a]	0.557±0.046[a]	0.230±0.020[a]	0.426±0.014[a]
MNS+$KHCO_3$	0.000±0.001[b]	0.025±0.002[b]	0.021±0.030[b]	0.066±0.007[b]
MNS+$KHCO_3$+$Na_2S_2O_3$	0.175±0.019[a]	0.384±0.069[a]	0.180±0.011[a]	0.302±0.052[a]
MNS+$KHCO_3$+NaEDTA	0.000±0.000[b]	0.027±0.082[b]	0.000±0.000[b]	0.058±0.267[b]
MNS+$NaHCO_3$+vitamins	0.294±0.078[a]	0.472±0.001[a]	0.231±0.030[a]	0.408±0.046[a]

Values are mean ± standard deviation. Columns with different letters are statistically different (p<0.05).

Arthrospira sp. had a similar growth in every media, except the one growing in the media MNS with $KHCO_3$ and MNS with $KHCO_3$ plus NaEDTA. There was no statistical difference between the media MNS supplemented with $NaHCO_3$ plus vitamins, Zarrouk and MNS with $NaHCO_3$. However, it was the latter that achieved the highest biomass DW (2.876±0.476 g L^{-1}) and productivity (0.303±0.060 g L^{-1} d^{-1}). *Arthrospira* sp. growing in the media MNS+$KHCO_3$ and MNS supplemented with $KHCO_3$ and NaEDTA had a null productivity and specific growth rate, which indicates that these media are not suitable to grow this species. These results showed that the source of sodium concentration is significant and cannot be any compound. It also proved a second time that the presence of vitamins is not necessary, as the culture in MNS+$NaHCO_3$ without vitamins achieved the highest DW and productivities, making it more economically viable from the company perspective.

4.1.5. Trial E: Nitrogen concentration optimization

Arthrospira sp. growth under different nitrogen concentration (5, 10, 20 and 30 mM) was studied during 15 days, with the intent to determine if this concentration can be reduced in order to cut production costs. In previous trials, the MNS medium used had a 30 mM nitrogen concentration, which was equivalent to Zarrouk's concentration. Growth curves are plotted in Figure 15 and the kinetic parameters described in Table 9.

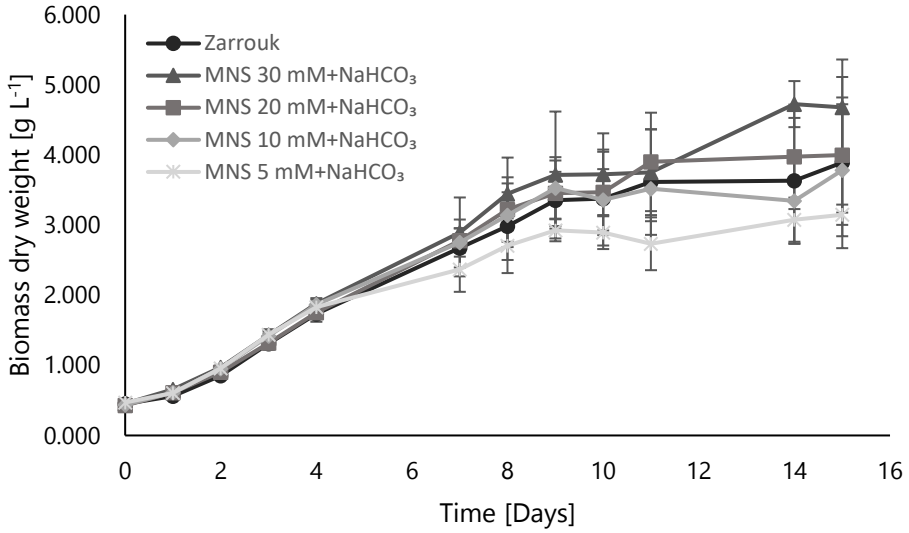

Figure 15. *Arthrospira* sp. growth curves in five media (Zarrouk; MNS with a nitrogen concentration of 30, 20, 10 and 5 mM. All media were supplemented with $NaHCO_3$ at 16.8 g L^{-1}. Trial was performed in 1 L flasks, under laboratory-controlled conditions and the presented results are the mean and standard deviation of three individual biological replicates.

Arthrospira sp. had a similar growth in all media tested and presented an exponential phase of 8-9 days. After that, it entered the stationary phase until the end of the trial.

Table 9. Productivities and specific growth rates of five culture media in 1 L flasks (Zarrouk; MNS with a nitrogen concentration of 30, 20, 10 and 5 mM). All media were supplemented with $NaHCO_3$.

Culture medium	Productivity (g L^{-1} d^{-1})		Specific growth rate (d^{-1})	
	Global	Maximum	Global	Maximum
Zarrouk	0.229±0.081[a]	0.459±0.056[a]	0.140±0.022[a]	0.430±0.044[a]
MNS 30 mM+$NaHCO_3$	0.282±0.045[a]	0.549±0.111[a]	0.155±0.009[a]	0.395±0.036[a]
MNS 20 mM+$NaHCO_3$	0.238±0.055[a]	0.440±0.302[a]	0.148±0.014[a]	0.390±0.040[a]
MNS 10 mM+$NaHCO_3$	0.223±0.063[a]	0.466±0.058[a]	0.142±0.017[a]	0.442±0.049[a]
MNS 5 mM+$NaHCO_3$	0.178±0.010[a]	0.479±0.041[a]	0.127±0.003[a]	0.457±0.037[a]

Values are mean ± standard deviation. Columns with different letters are statistically different (p<0.05).

The culture in all media had no statistical differences in the biomass DW, productivities and growth rate. de Castro *et al.* (2015) performed a study with different nitrogen concentrations and proved that its concentration could be reduced, since the highest biomass production occurred with 1.5 g L^{-1}, while the medium Zarrouk has a nitrogen concentration of 2.5 g L^{-1}. Colla *et al.* (2007) had similar results since it proved that varying

the nitrogen concentration from 0.625 g L^{-1} to 2.5 g L^{-1} did not affect *A. platensis* growth. El-Baky (2003) performed a trial using growth media with different nitrogen concentrations and found that low nitrogen concentrations led to a decrease in PC content. These reports are consistent with the results obtained in this study, since *Arthrospira* sp. DW and productivity was not affected by variations of the nitrogen concentration within the range of 5 mM to 30 mM. However, a study conducted by Piorreck *et al.* (1984) revealed that increasing nitrogen concentration, also increased, in blue-green algae, biomass DW (from 8 to 450 mg L^{-1}), protein content (from 8% to 54% of DW) and chlorophyll.

In the media with lower nitrogen concentrations, after nutrients ran out, the culture became more yellow because cells used pigments as a source of energy (process known as chlorosis or bleaching), represented in Figure 16, which indicates that this species needs considerable amounts of nitrogen. Therefore, in order to maintain the blue-green color and the PC content of the culture, the nitrogen concentration of 30 mM is preferred.

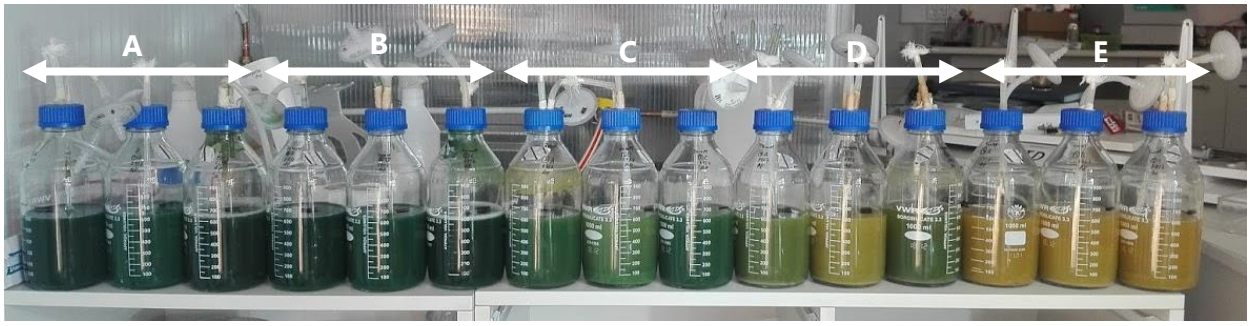

Figure 16. *Arthrospira* sp. in 1 L flask reactors at the end of the trial. Being, A-Zarrouk; B-MNS with a nitrogen concentration of 30 mM; C-MNS with N-20 mM; D- MNS with N-10 mM; E-MNS with N-5 mM.

Phycobiliproteins act as a reserve of nitrogen in stressful situations, where the protein presented in pigments are detached in order to release the necessary and essential metabolites (Jacob-Lopes *et al.*, 2020). However, if nutrients were re-added to the culture, these molecules would be resynthesized and the culture would eventually recover its color (Jacob-Lopes *et al.*, 2020).

Literature also describes several studies testing different sources of nitrogen (ammonium, nitrate, urea, etc) in order to reduce production costs. Some studies mentioned that urea is a better nitrogen source when compared to nitrate or ammonium, which are toxic at concentrations higher than 2 mM (Choi *et al.*, 2003; Madkour *et al.*, 2012;

Deschoenmaeker *et al.*, 2017). However, these results indicate that *Arthrospira* sp. requires high nitrogen concentrations and using nitrate instead of other sources (urea and ammonium) is benefic because it is not toxic for cyanobacteria growth (Khazi *et al.*, 2018).

With all studies conducted, the results obtained show that MNS with a nitrogen concentration of 30 mM, when supplemented with $NaHCO_3$ at 16.8 g L^{-1} is comparable to the Zarrouk medium, as the results between the two media were not statistically different. However, *Arthrospira* sp. growing in MNS+$NaHCO_3$ performed better in numerous trials in comparison to Zarrouk and, therefore, this was the chosen medium to realize the scale-up. This choice of medium for the scale-up process was also advantageous to the company, since it meant a lower investment, due to it being the company own medium.

4.2. Scale-up

The second phase of the scale-up occurred outdoors under the local meteorological conditions, with the FPPs and raceway pond being south and east oriented, respectively. This process was realized in 92 days (60 days until the last FPP (750 L) plus the 2 assays in the raceway pond) and the main goal was producing the highest biomass content and, in parallel, testing a harvesting technique with nets when the culture reached the raceway stage. Several reactors (4 FPPs and 1 raceway pond) with different volumes (80 L, 150 L, 500 L, 750 L and 3500 L) were sequentially inoculated one with the other (Figure 17).

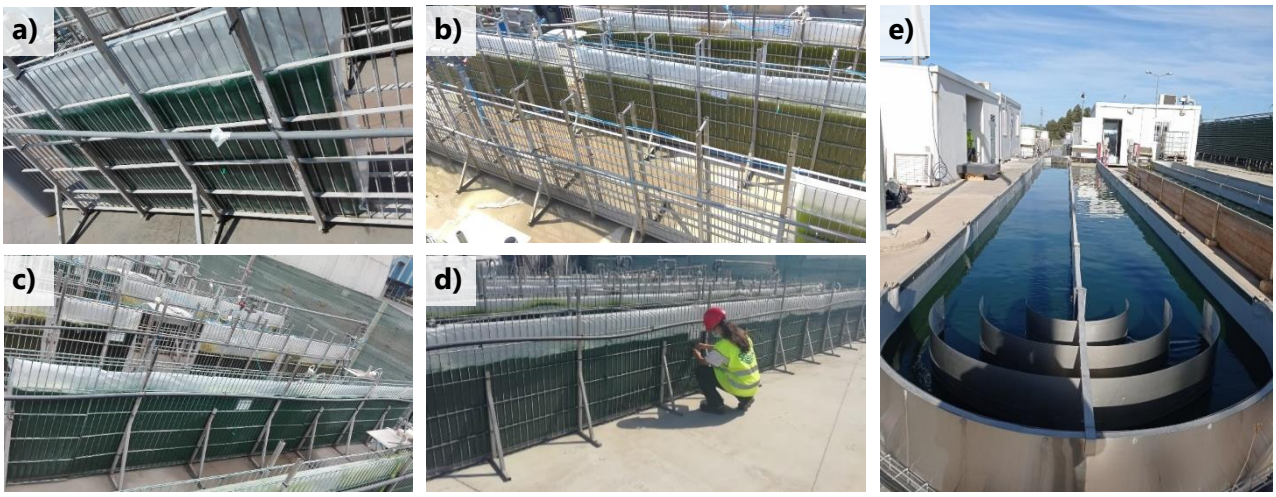

Figure 17. Reactors used in the scale-up of *Arthrospira* sp. (outdoors). (a) FPP 80 L, (Zarrouk); (b) FPP 150 L, (MNS+$KHCO_3$); (c) FPP 500 L, (MNS+$NaHCO_3$); (d) FPP 750 L, (MNS+$NaHCO_3$) and (e) raceway pond 3500 L, (MNS+$NaHCO_3$), where 2 assays were performed.

The respective growth curves of the FPP and raceway pond used to grow *Arthrospira* sp. are presented in Figure 18 and Figure 19, while productivities and specific growth rates are presented in Table 10. In this step only individual assays were realized, so no statistical data was included.

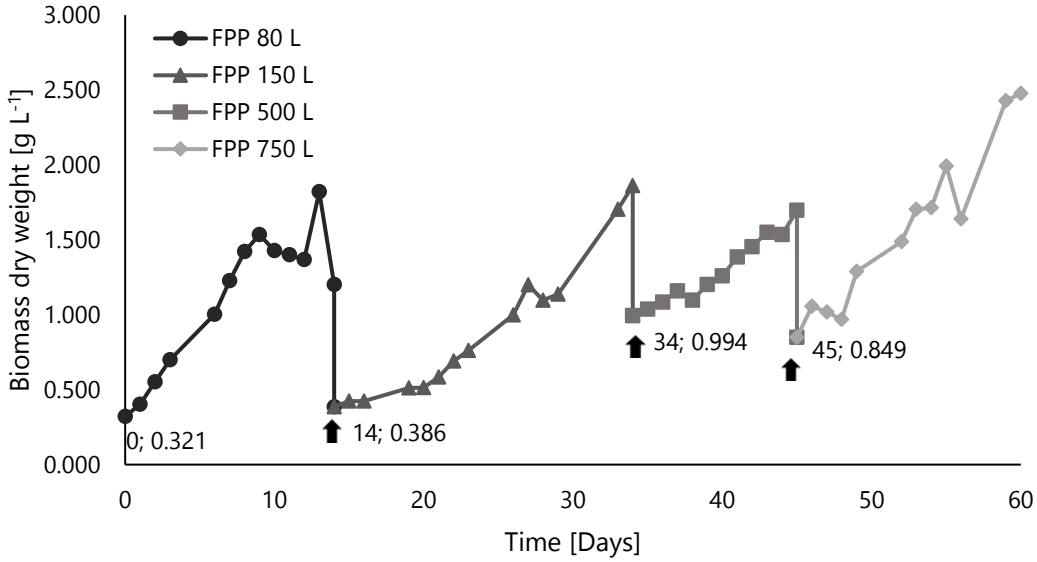

Figure 18. *Arthrospira* sp. growth curves in several FPP used in the scale-up process (80 L, growing in Zarrouk; 150 L, growing in MNS+$KHCO_3$; 500 L, growing in MNS+$NaHCO_3$; 750 L, growing in MNS+$NaHCO_3$). (⬆) indicates the day that the particular reactor was inoculated. The presented results are individual assays performed in different periods.

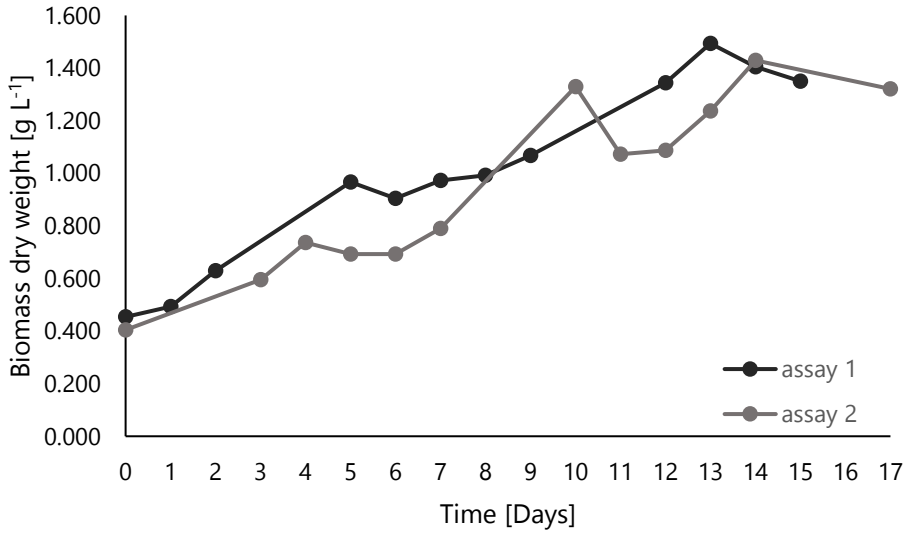

Figure 19. *Arthrospira* sp. growth in 2 assays performed in a raceway pond with 3500 L in the medium MNS with sodium bicarbonate. Growth occurred under outdoor conditions for 15 and 17 days for assay 1 and 2, respectively.

Table 10. Global and maximum productivities (g L^{-1} d^{-1}) and specific growth rate (d^{-1}) of the 4 FPP and the 2 raceway ponds used in *Arthrospira* sp. scale-up process.

Reactor/scale	Productivity (g L^{-1} d^{-1})		Specific growth rate (d^{-1})	
	global	maximum	global	maximum
FPP (80 L)	0.063	0.453	0.094	0.314
FPP (150 L)	0.074	0.200	0.079	0.182
FPP (500 L)	0.064	0.162	0.049	0.100
FPP (750 L)	0.109	0.321	0.071	0.286
Raceway (assay 1)	0.060	0.149	0.073	0.245
Raceway (assay 2)	0.054	0.192	0.070	0.211

Since both the growth medium optimization phase and the scale-up process are slow procedures, the outdoor reactors' assays had to be started before the laboratory trials were concluded. So, Zarrouk was the chosen medium to inoculate the FPP of **80 L**, since it is the most cited medium in the literature, as mentioned before. This assay was performed for 14 days. When comparing the results obtained in the medium Zarrouk from the last laboratory trial with this FPP, the transition to the pilot-scale reactors led to a decrease in the global productivities from 0.229±0.081 g L^{-1} d^{-1} to 0.063 g L^{-1} d^{-1} and global specific growth rate from 0.140±0.022 d^{-1} to 0.094 d^{-1}, which could be explained by the outdoor natural light, changes in irradiation and temperature (Borowitzka and Vonshak, 2017). In the laboratory there is not a light/dark cycle because the temperature and radiation are kept constant, but in the outdoor assays, this is a very significant factor since the cultures only had available light for 16 hours.

Fourteen days later, when the FPP of **150 L** was inoculated, although the results of the medium optimization were already known, and MNS supplemented with $NaHCO_3$ was chosen to continue the scale-up, $NaHCO_3$ was not available at the time. So, $KHCO_3$ was chosen instead to supplement MNS. At the time of this inoculation, the culture was microscopically in bad conditions (low blue coloration), which could influence the performance of this species. However, during the assay, *Arthrospira* sp. gained its characteristic green-blue color and achieved a higher DW (1.861 g L^{-1}) and productivity

(0.074 g L^{-1} d^{-1}) in this reactor than in the previous one, despite the global specific growth rate being lower, which indicates that the culture needed more days to achieve the same DW.

After 20 days, the FPP of **500 L** and all further reactors, were inoculated with the medium MNS supplemented with $NaHCO_3$, which was the best performing medium from the laboratory optimization phase. In comparison with the reactor of 150 L, this reactor started with a very high biomass concentration, but it had lower global productivities (0.064 g L^{-1} d^{-1}) and growth rates (0.049 d^{-1}). This may be caused due to insufficient radiation during the culture growth, since the absorbed light is directly proportional to the biomass concentration, which can be explained by the Beer-Lambert law. Additionally, the shading provided by a nearby larger FPP could also be the cause. The spiral morphology and length of *Arthrospira* sp. is also an aggravating factor due to self-shading (Kroumov *et al.*, 2017).

Later, when the necessary DW was reached, the FPP of **750 L** was inoculated. *Arthrospira* sp. had a similar growth in all reactors and media, and even with a lag phase disparity of 1 to 5 days, they ended up achieving a similar DW. However, the FPP of 750 L, reached the highest DW (2.478 g L^{-1}) and global productivity (0.109 g L^{-1} d^{-1}). A few days after inoculation, the culture started to develop a greenish color, but, this time, the productivity and photosynthesis were not influenced by the sun exposure and neighbor FPPs. These parameters are important because productivity and photosynthesis can be influenced by the location where the reactors are placed and respective latitude, which in turn influences the sun exposure and the neighboring FPPs (Slegers *et al.*, 2011). This is concerning since some cyanobacteria, when exposed to too many hours to full sunlight, may be photoinhibited (Vonshak, 1997). Subsequently, this FPP is completely exposed to the sunlight and a study proved that low irradiance favors pigment synthesis in cyanobacteria because low light intensities stimulates phycobiliproteins (Begum *et al.*, 2016).

The last reactor of the scale-up process could be either the tubular PBR or the raceway ponds. Since tubular PBRs usually comprise a centrifugal pump, and due to *Arthrospira* sp.'s morphology, they were not suitable to cultivate this species, because of the mechanical stress that damages the trichomes. However, if an airlift tubular PBR was used, this problem would no longer exist. Naqqiuddin *et al.* (2014) grew *A. platensis* in a cylindrical PBR (20 L) with bottom aeration (with a standardized aeration rate of 0.7 L L^{-1} min^{-1}) under outdoor

conditions and achieved a productivity of 0.071±0.001 g L^{-1} d^{-1}. Therefore, since no airlift PBRs were available at the facilities, the raceway pond was chosen for the last reactor of the scale-up process.

The same raceway pond was inoculated twice (assay 1, 15 days, and assay 2, 17 days) and the meteorological conditions (air temperature and solar radiation) are represented in Appendix (Figure A_{12} to Figure A_{17}). This reactor had the lowest global productivity (0.054 g L^{-1} d^{-1} in assay 2, which corresponds to an areal productivity of 7.005 g m^{-2} d^{-1}), which is usually obtained in these rectors. One study conducted by Cunha *et al.* (2020), despite being realized with another species, *Nannochloropsis oceanica,* was performed in the same reactor under the same conditions (culture depth and velocity) and had a areal productivity of 7.089 g m^{-2} d^{-1}, which is similar to the obtained results. A study conducted by Pushparaj *et al.* (1997) with *A. platensis* in raceway ponds with a surface area of 3.8 m^2 registered the highest productivity of 14.5 g m^{-2} d^{-1} at the optimum population density (0.75 g L^{-1}). In comparison, in the first assay performed in the raceway the highest areal productivity (17.103 g m^{-2} d^{-1}) was achieved with a DW of 0.992 g L^{-1}, while in the second assay the highest areal productivity of 27.862 g m^{-2} d^{-1} was achieved with a DW of 0.736 g L^{-1}. This indicates that *Arthrospira* sp. performance in the raceway pond was better than what is described in the literature. The specific growth rate in the two assays performed in the raceway pond started to decrease after the highest DW was achieved, which indicates that if the company ever wants to scale-up this species, in order to maximize the growth, the culture should be scaled-up to the next reactor after reaching these DW and the reactor should then be renewed.

At the end of the scale-up process, from the laboratory phase up to pilot-scale, the obtained results showed that *Arthrospira* sp. growth is influenced by the culture conditions, the scale (volume) of the reactors, weather changes (temperature, light/dark cycle, etc) and the initial biomass DW. Raceway pond had the lowest productivities, but this reactor requires a low capital cost, making it a profitable investment.

4.2.1. Flat panel photobioreactor assays

In parallel with the scale-up process, and due the duration of the medium optimization phase conducted in the laboratory, some assays were performed outdoors with the aim to study some conditions (Figure 20).

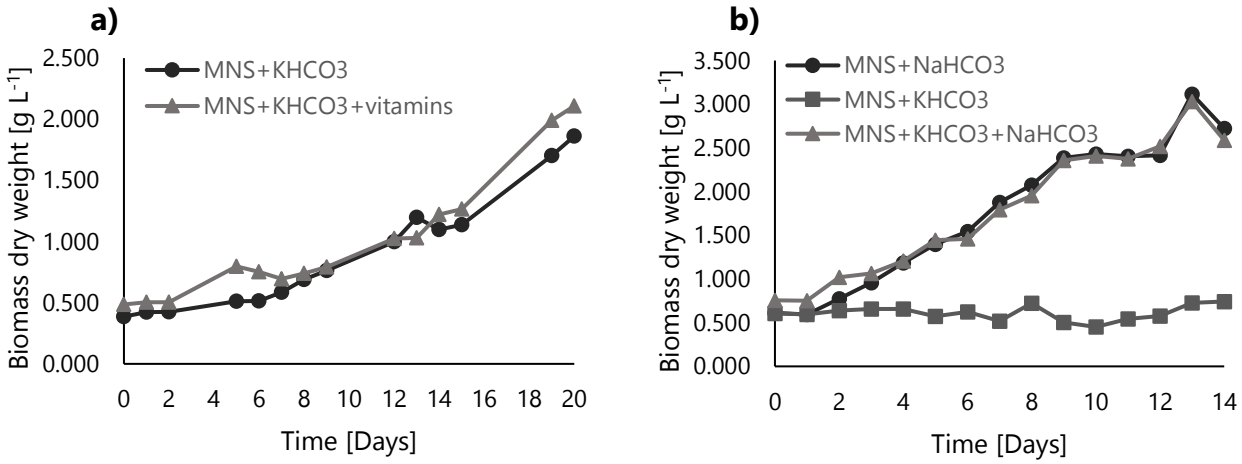

Figure 20. *Arthrospira* sp. growth outdoors in (a) 2 FPP of 150 L (MNS+$KHCO_3$; MNS+$KHCO_3$+vitamins); (b) 3 FPP of 80 L (MNS+$NaHCO_3$; MNS+$KHCO_3$; MNS+$KHCO_3$+$NaHCO_3$).

The first assay (Figure 20, a) was developed in a FPP of 150 L with the aim to test *Arthrospira* sp. growth with vitamins in order to perceive if it is enhanced when vitamins are available in the culture medium. The culture growing with vitamins reached a higher DW (2.108 g L^{-1}) and global productivity (0.081 g L^{-1} d^{-1}), however, the ratio productivity/cost is not worth the investment in vitamins, since they are very expensive. Hence, the reactor without vitamins was used to continue the scale-up, while the reactor with vitamins was used to inoculate 3 FPP of 80 L, which corresponds to the second assay (Figure 20, b).

The second assay had *Arthrospira* sp. growing in MNS+$NaHCO_3$; MNS+$KHCO_3$ and MNS+$NaHCO_3$+$KHCO_3$, with the aim to study if there was a synergic effect of the combination of two bicarbonates. *Arthrospira* sp. growing with $KHCO_3$ stayed in the lag phase until the end of the assay, which indicates this medium is not suitable to grow this species, which is consistent with some of the previous laboratorial assays. *Arthrospira* sp. growing only with $NaHCO_3$ achieved the highest DW (3.117 g L^{-1}) and productivity (0.151 g L^{-1} d^{-1}). However, the culture growing with the two bicarbonates had similar values (0.131 g L^{-1} d^{-1}), which indicates that, in the case of shortage of sodium bicarbonate, combining the two bicarbonates is an alternate option. However, the culture growth in this

medium could only result because of the presence of $NaHCO_3$, since the culture did not grow with $KHCO_3$, although the same did not happen in one trial in the laboratory phase.

4.3. Biochemical composition

4.3.1. Pigments determination

In order to optimize the phycobiliproteins extraction method, a sample from the first assay realized in the raceway pond was collected and three masses (2, 5 and 10 mg of DW) were tested. The respective values are presented in Table 11.

Table 11. Pigment concentration of three amounts of biomass (2 mg, 5 mg and 10 mg). The presented values are the mean of two conditions.

Biomass dry content (mg)	Phycocyanin concentration (mg $g_{biomass}^{-1}$)	Allophycocyanin concentration (mg $g_{biomass}^{-1}$)
2	3.099	1.360
5	3.463	1.228
10	8.255	2.502

The highest content of PC and APC was extracted from the sample with 10 mg of biomass DW, being 8.255 mg $g_{biomass}^{-1}$ (which corresponds to 82.55 µg mL^{-1}) and 2.502 mg $g_{biomass}^{-1}$, which corresponds to 25.02 µg mL^{-1}, respectively. The obtained results indicate that the extraction of phycobiliproteins from future samples should be realized with 10 mg of biomass DW.

PC production is mainly influenced by abiotic factors (temperature, pH, salinity, light, etc). However, light (photoperiod, color of the light, intensity, etc) is the most important factor. For instance, a study conducted by Kumar *et al.* (2011) with *Spirulina platensis* proved that phycobiliproteins increased until a light intensity of 2000 lux, where the highest content of PC (7.73±0.52% of DW) and APC (3.46±0.15% of DW) was achieved. Further increase in light intensity promote the decreasing of phycobiliproteins. Some studies indicate that the red light is better for PC production (Jacob-Lopes *et al.*, 2020). Marrez *et al.* (2013) extracted phycobiliproteins from *A. platensis* with sonication and the highest amount of PC (55.37 µg mL^{-1}) and APC (51.73 µg mL^{-1}) was achieved in modified BG11 medium.

4.3.2. Protein determination

The total protein content of *Arthrospira* sp. from the last laboratory trial (nitrogen concentration optimization) and from the raceway ponds assay was determined and the results obtained are represented in Table 12.

Table 12. Total protein content (%) of the last laboratory trial (nitrogen concentration optimization) and the first assay in the raceway pond. The results are the mean and standard deviation of three individual biological replicates. The raceway pond was a single sample.

Sample	Total protein content (% DW)
Zarrouk	9.50±0.73[b]
MNS 30 mM with $NaHCO_3$	18.80±1.58[a]
MNS 20 mM with $NaHCO_3$	19.40±4.57[a]
MNS 10 mM with $NaHCO_3$	11.90±1.71[a b]
MNS 5 mM with $NaHCO_3$	6.80±2.79[b]
First raceway	**41.8**

The highest protein contents were achieved in the medium with a nitrogen concentration of 20 mM (19.40±4.57% of biomass DW) and 30 mM (18.80±1.58% of biomass DW). The results from these two media are statistically different from the media Zarrouk and MNS with N-5mM (p<0.05). *Arthrospira* sp. growing in the raceway pond produced more protein content (41.8% of biomass DW) than the cultures from the last laboratory trial.

The results obtained are not consistent with those commonly found in commercial products, which is between 50% and 70% of protein content (Dillon *et al.*, 1995). Madkour *et al.* (2012) extracted 52.95±0.53% of protein content from the species *A. platensis* growing in the medium Zarrouk. The culture in the media with low nitrogen concentration (N-5 mM and N-10 mM) had a low protein content due to nitrogen limitation and degradation of proteins to compensate that (Jacob-Lopes *et al.*, 2020). The culture of the raceway pond was the one that achieved more characteristic values for this species.

This method for protein determination is not very accurate because there is a lot of steps that can lead to biomass loss. For instance, one step of the protocol consists in pre-washing the biomass a few times (at least three) in order to reduce the interference of the

culture medium. Another problem lies in the fact that this species is very difficult to centrifuge, which can lead to losses along the process and the obtained results may not correspond to the reality. This problem was decreased by diluting the culture with distilled water and the difference in salinity, or the lack of salinity from the distilled water, facilitated the sedimentation. According to Archimedes' principle, a buoyancy force (from the bottom to the top) is applied over a body when it is immersed in a fluid. This force is higher in high density fluids (e.g. high salinity), so when this density is reduced, the body is kept closer to the bottom (Wang, 1988).

4.4. Harvesting

The harvesting process occurred by filtration and consisted in flowing 1 L of culture through several nets with different porosities. In this operation, we have the permeate flow, which is the part of the flow that permeates through the filter and the retentate, which, in this case, corresponds to the biomass that got trapped in the net (Jagschies *et al.*, 2018). The culture OD was measure before and after passing through the weave cloth and the results are represented in Table 13.

Table 13. Optical density at 540 nm of the culture before and after passing through weave cloths with different mesh sizes (233.813 µm; 205.479 µm; 122.900 µm; 45.099 µm; 18.037 µm; 6.374 µm).

	Pore diameter (µm)	**Optical density at 540 nm**
Before filtration (Raceway)	-	2.394
After filtration	233.813	1.060
	205.479	0.850
	122.900	0.090
	45.099	0.083
	18.037	0.069
	6.374	0.056

Arthrospira sp. was retained in all the weave cloths with a pore diameter of less than 122.900 µm, which was visible to the eye, since the permeate did not exhibit any green color. These results are sustained by data from Table 13, where the lower ODs (0.056 to 0.090) occurred in the nets with a pore diameter of less than 122.900 µm, which was a huge

difference from the OD of the culture growing in the raceway before filtration (2.394). According to the literature, a mesh with 380 to 500 with a filtration area of 2-4 m^2 $unit^{-1}$ is able to harvest 10-18 m^3 of *Arthrospira* sp. per hour (Habib *et al.*, 2008). Filtration is a process where separation occurs based on the particle size and, therefore, the mesh size of the filter is important in order to remove water (culture medium) from the biomass. Consequently, the pore diameter of the different fiber cloths, Figure A_{18} (Appendix), and two cells of *Arthrospira* sp. with different morphologies, Figure A_{19} and Figure A_{20} (Appendix), were measured using a specific tool of the microscope.

After filtration, for every net used, a thin layer of biomass was stored (along with the net) in a warehouse without humidity and nearby windows, in order to facilitate the drying process. Biomass dried in one week and in the end, it was possible to collect its powder (Figure 21). The biomass drying process was a rudimentary method (utilizing the sun), which means this operation was very inexpensive (Taylor *et al.*, 2015).

Figure 21. *Arthrospira* sp. powder after being dried for one week with a rudimentary method (using sunlight).

These results indicate that harvesting *Arthrospira* sp. by filtration was a successful technique, since the culture got retained in the filters with a pore diameter less than 122.900 μm and also that the rudimentary drying process that followed was able to dry the collected biomass. However, although the drying process had satisfactory results, it must be optimized, because this specific method reduces the product quality. Due to the lower temperatures it is exposed to during the process, it is necessary more time to completely dry the biomass, which favors bacterial growth.

5. CONCLUSIONS

Global consumption of animal protein has increased continuously over the years and novel protein sources must be studied. In addition, novel food habits demand the development of alternative sources of protein. *Arthrospira* sp. is a photosynthetic microorganism and a good candidate to substitute meat-based proteins because of its high protein content. In order to have the highest cost-effective ratio and obtain the richest biomass in added-value compounds, several approaches to produce the medium that stimulates the maximum efficiency of *Arthrospira* sp. growth were studied in the laboratory.

The cultivation of *Arthrospira* sp. from laboratory scale to raceway ponds, using a low-cost medium (Allmicroalgae medium, MNS) was viable when adding $NaHCO_3$ and adjusting the nitrogen concentration, reaching higher values of DW and productivity than when using Zarrouk. A minimum sodium concentration of 50 mM is crucial for *Arthrospira* sp. growth, to maintain its physiology and morphology. The culture medium must have a nitrogen concentration of 30 mM since *Arthrospira* sp. achieved the highest DW (4.724 ± 0.329 g L^{-1}) in these conditions and entered the stationary phase first in the culture medium with lower nitrogen concentration. Such results allow to conclude that this species needs high amounts of nitrogen.

During the scale-up process, several factors (temperature, light/dark cycle, etc) influenced *Arthrospira* sp. growth. The highest DW (2.478 g L^{-1}) and global productivity (0.109 g L^{-1} d^{-1}) was achieved in the FPP of 750 L. However, despite raceway ponds not being the most productive reactors, they are the most commonly used since it is a profitable investment, with a low capital cost. The culture growing in the raceway ponds was successfully harvested by filtration (using nets) and dried (using sunlight). The filtration technique was efficient in the nets with a pore diameter equal or lower than 122.900 µm. The biomass of *Arthrospira* sp. is usually rich in protein and, despite not achieving the 50 to 70% of protein content it usually exhibits, a value of 41.8% of biomass DW was obtained in the culture growing in the raceway. This value is greater than the protein content obtained during the laboratory assays, which indicates that the cultivation of this cyanobacteria in raceway ponds is potentially viable.

6. FUTURE RECOMMENDATIONS

Arthrospira sp. growth is enhanced by high temperatures (ideally 35 ºC), which is hard to achieve in some regions. So, in order to reduce this problem, the growth should occur in a greenhouse or with the addition of several halogen lamps to the bottom of the raceway ponds, since they can illuminate and heat the culture simultaneously.

After harvesting, biomass should be dried quickly in order to obtain a high-quality product. If that is not possible and a traditionally method (sunlight) is used, it should be left to dry in a "spaghetti" form instead of layers, which can be achieved by extrusion, in order to maximize evaporation due the surface area being higher.

The protocol to extract pigments and proteins could be optimized in future experiments, since this was a first approach to extract them from *Arthrospira* sp. in the company. For pigments extraction, sonication should be tested since it was reported it had better results and it is well known in literature. For proteins extraction, the equipment Skalar should be considered since it has proven to be the most reliable and economical choice. If this equipment is not available, biomass should be dried in a freeze dryer, in order to reduce losses.

It would also be interesting to realize a final assay comparing the media Zarrouk, MNS with $NaHCO_3$ at 16.8 g L^{-1} and the biological medium used in the company.

Since the strain of *Arthrospira* sp. used in this project was photo sensitive, it would be interesting to develop several trials in order to study the effects that light intensity, PBR orientation relating to the sun, different culture depths and different designs have on the growth of the culture.

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APPENDICES

This section contains supplementary information about preliminary screening experiments and the description of growth medium compositions. The two correlation curves which estimate biomass DW and measure nitrate concentration are also described.

Culture media formulations

Before moving forward to scale-up, an initial screening was realized in order to optimize the growth medium. The composition of the different growth media used to evaluate the growth of *Arthrospira* sp. are presented below.

Zarrouk medium consists of three different solutions: main solution, A_5 solution and B_6 solution. B_6 solution is composed of $Co(NO_3)_2 \cdot 6H_2O$, at a concentration of 4.40×10^{-2} mg L^{-1}, while the other two solutions are described in Tables A_1 and A_2.

Table A_1. Composition of Zarrouk medium main solution.

Reactants	Concentration (g L^{-1})
$NaHCO_3$	16.8
$CaCl_2 \cdot 2H_2O$	0.04
$NaNO_3$	2.50
$MgSO_4 \cdot 7H_2O$	0.20
NaCl	1.00
$FeSO_4 \cdot 7H_2O$	0.01
NaEDTA	0.08
K_2HPO_4	0.50

Table A_2. Composition of Zarrouk medium A_5 solution

Reactants	Concentrations (g L^{-1})
H_3BO_3	2.86×10^{-3}
$MnCl_2 \cdot 4H_2O$	1.81×10^{-3}
$ZnSO_4 \cdot 7H_2O$	2.22×10^{-4}
$CuSO_4 \cdot 5H_2O$	7.90×10^{-5}
MoO_3	1.50×10^{-5}

BG11 medium consists of two different solutions: main solution and a trace elements solution (Table A_{3-4}).

Table A_3. Composition of BG11 medium main solution.

Reactants	Concentration (g L^{-1})
$NaNO_3$	1.50
K_2HPO_4	0.040
$MgSO_4 \cdot 7H_2O$	0.075
$CaCl_2 \cdot 7H_2O$	0.036
$C_6H_8O_7 \cdot H_2O$	0.006
Na_2EDTA	0.001
Na_2CO_3	0.020

Table A_4. Composition of BG11 medium trace elements solution.

Reactants	Concentration (g L^{-1})
H_3BO_3	2.86
$MnCl_2 \cdot 4H_2O$	1.81
$ZnSO_4 \cdot 7H_2O$	0.22
$Na_2MoO_2 \cdot 2H_2O$	0.39
$CuSO_4 \cdot 5H_2O$	0.079
$Co(NO_3)_2 \cdot 6H_2O$	0.049

Calibration curve of optical density and biomass dry weight

Biomass DW in g L^{-1} can be estimated based on a calibration curve, which correlates this parameter to the OD at the wavelength of 540 nm. The following figure (Figure A_1) represents the calibration curve used to control culture growth in every assay realized in the present study and both parameters were measured at the same time for the same sample. Cell physiology and morphology were assumed constant. However, this parameter was periodically analysed by a light optical microscope.

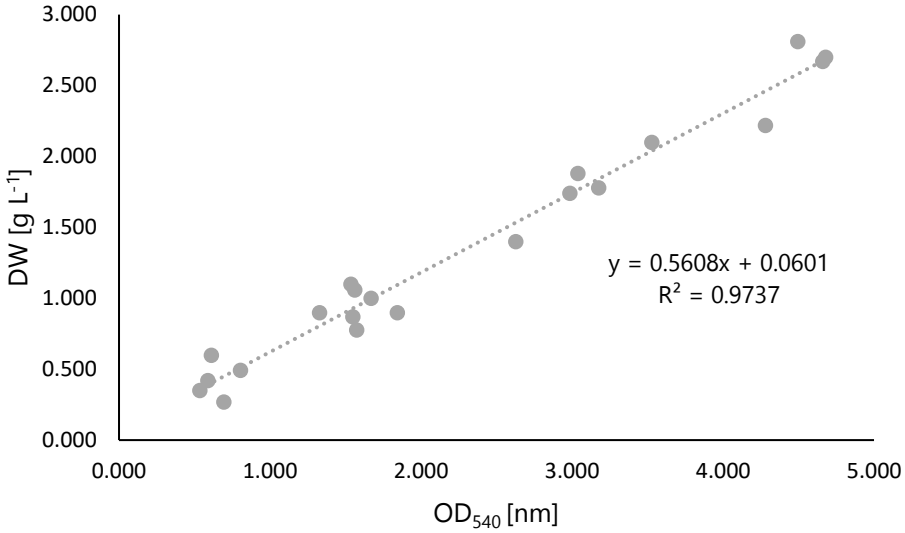

Figure A_1. Relationship between the optical density at 540 nm and the dry weight of an autotrophic culture of *Arthrospira* sp. (0071AN) in Zarrouk medium. The linear regression is [$y = 0.5608x + 0.0601$], with r=0.9868 and n=21.

Calibration curve for nitrate concentration

The nitrate concentration in mM was determine by Equation A_1 linear regression. Where "y" is (OD_{220}-(2×OD_{275})) and "x" the NO_3^- (mM).

$$y = 3.73x + 0.000356 \qquad \textbf{Equation } A_1$$

Trial: Screening of three different culture media

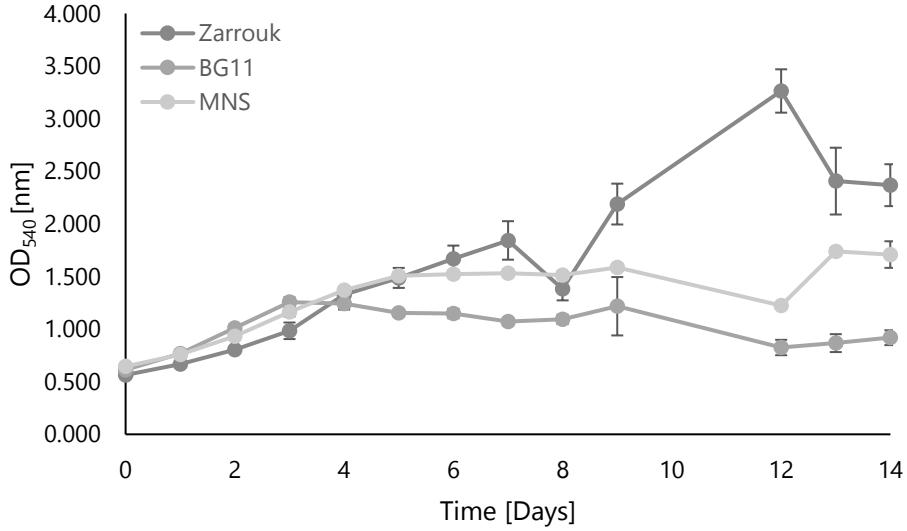

Figure A₂. Optical density at 540 nm variation in three different media (Zarrouk, BG11 and MNS) during the trial. The assay was performed in 1 L flasks, under laboratory-controlled conditions. All conditions were run in triplicates and the values presented are their average and the error bars are the respective standard deviations.

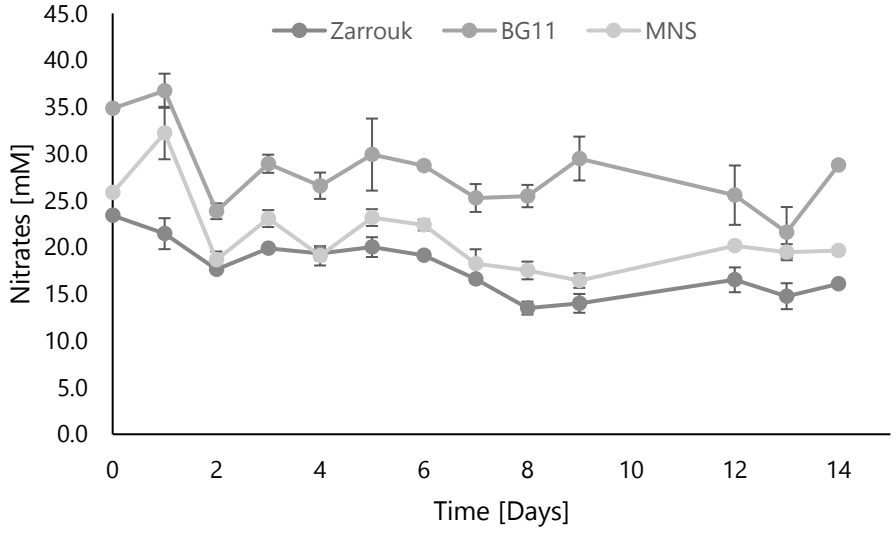

Figure A₃. Nitrate's consumption in mM along the trial by *Arthrospira* sp. in three different media (Zarrouk, BG11 and MNS). The assay was performed in 1 L flasks, under laboratory-controlled conditions. All conditions were run in triplicates and the values presented are their average and the error bars are the respective standard deviations.

Trial: Sodium bicarbonate significance

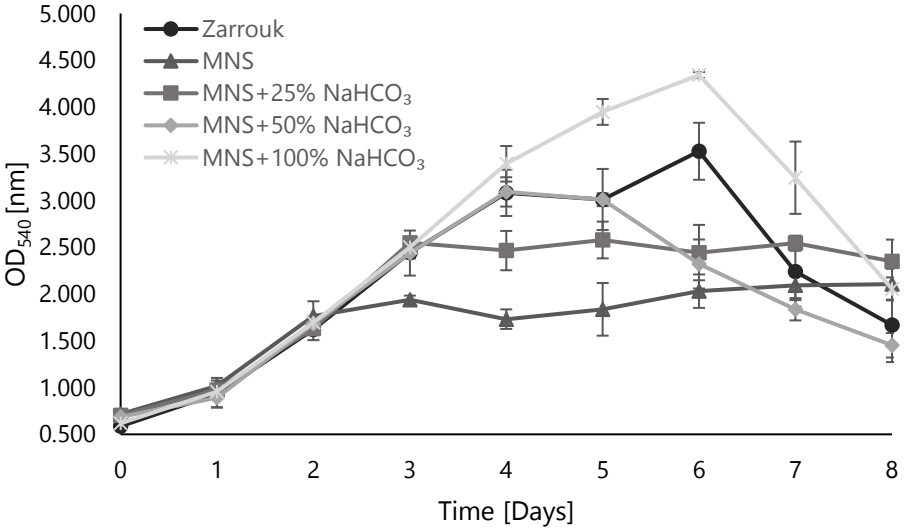

Figure A4. Optical density at 540 nm variation in 5 different media (Zarrouk; MNS; MNS with 25%, 50% and 100% $NaHCO_3$) during the trial. The assay was performed in 1 L flasks, under laboratory-controlled conditions. All conditions were run in triplicates and the values presented are their average and the error bars are the respective standard deviations.

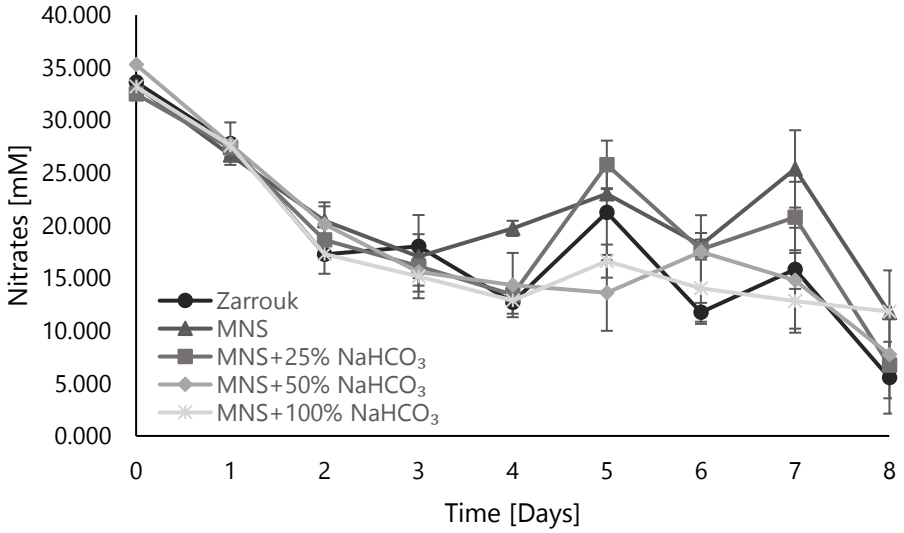

Figure A5. Nitrate's consumption in mM by *Arthrospira* sp. during 8 days in 5 media (Zarrouk; MNS; MNS with 25%, 50% and 100% $NaHCO_3$). The assay was performed in 1 L flasks, under laboratory-controlled conditions. All conditions were run in triplicates and the values presented are their average and the error bars are the respective standard deviations.

Trial: Sodium bicarbonate *versus* potassium bicarbonate

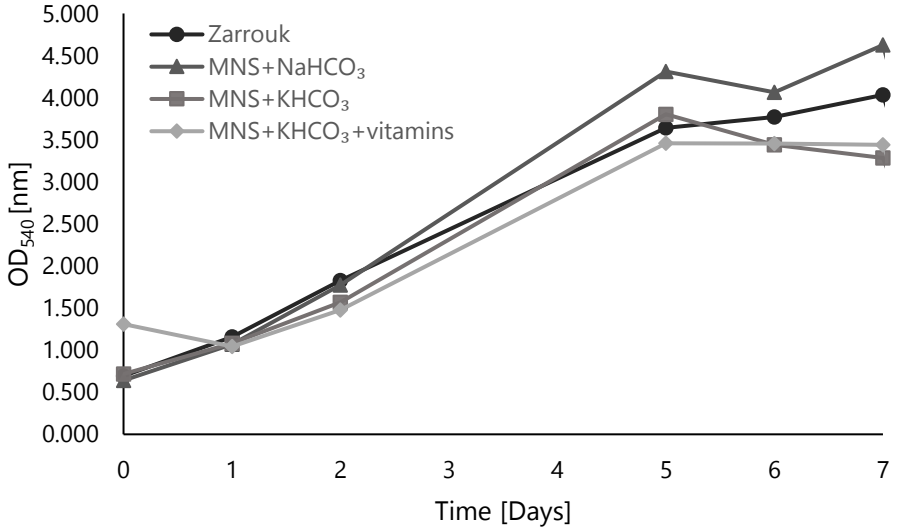

Figure A6. Optical density at 540 nm variation in 5 media (Zarrouk, MNS with $NaHCO_3$ (16.8 g L^1), MNS with $NaHCO_3$ (20.0 g L^{-1}) and MNS with $NaHCO_3$ and vitamins) over one week. The assay was performed in 1 L flasks, under laboratory-controlled conditions. All conditions were run in duplicates and the presented values are their average.

Trial: Sodium source

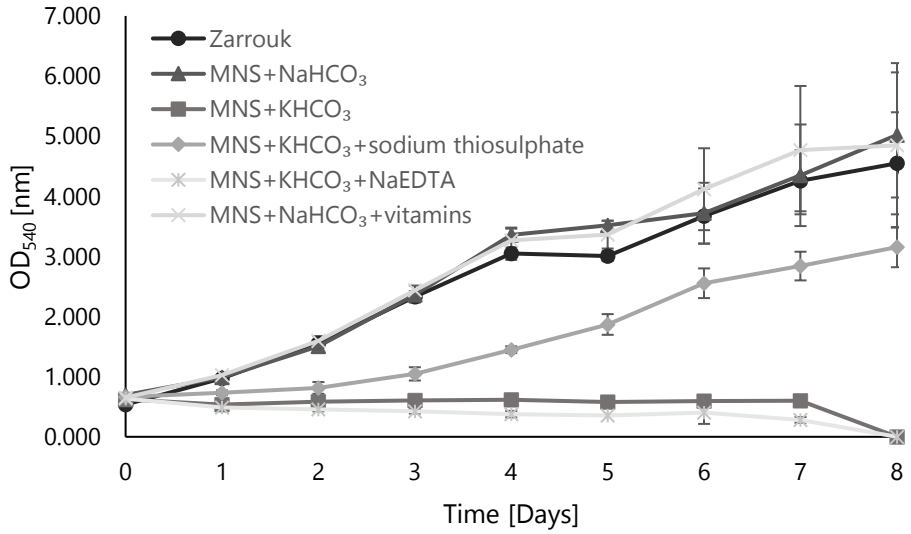

Figure A7. Optical density at 540 nm variation during 8 days in 1 L flasks in 6 media (Zarrouk; MNS supplemented with $NaHCO_3$ at 16.8 g L^{-1}; MNS with $KHCO_3$ at 20.0 g L^{-1}; MNS with $KHCO_3$ and $Na_2S_2O_3$; MNS with KHCO3 and NaEDTA; MNS with NaHCO3 and vitamins). Presented values are the mean and standard deviation of three individual biological replicates.

Trial: Nitrogen concentration optimization

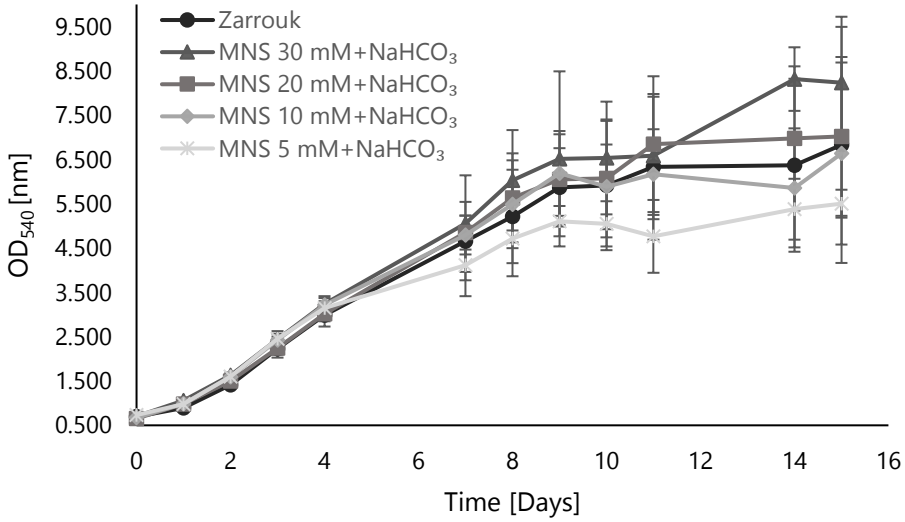

Figure A$_8$. Optical density at 540 nm variation during 15 days in 1 L flasks in 5 media (Zarrouk; MNS with a nitrogen concentration of 30, 20, 10 and 5 mM. All media were supplemented with $NaHCO_3$ at 16.8 g L^{-1}. Presented values are the mean and standard deviation of three individual biological replicates.

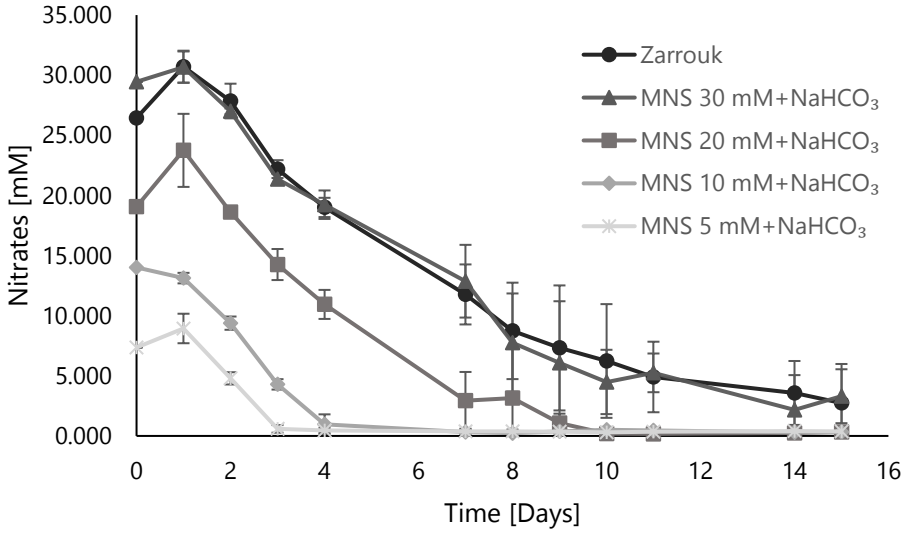

Figure A$_9$. Nitrate's consumption (mM) by *Arthrospira* sp. during 15 days in 5 media (Zarrouk; MNS with a nitrogen concentration of 30, 20, 10 and 5 mM. All media were supplemented with $NaHCO_3$ at 16.8 g L^{-1}. The assay was performed in 1 L flasks, under laboratory-controlled conditions. All conditions were run in triplicates and the presented values are their average and the error bars are the respective standard deviations.

Raceway ponds

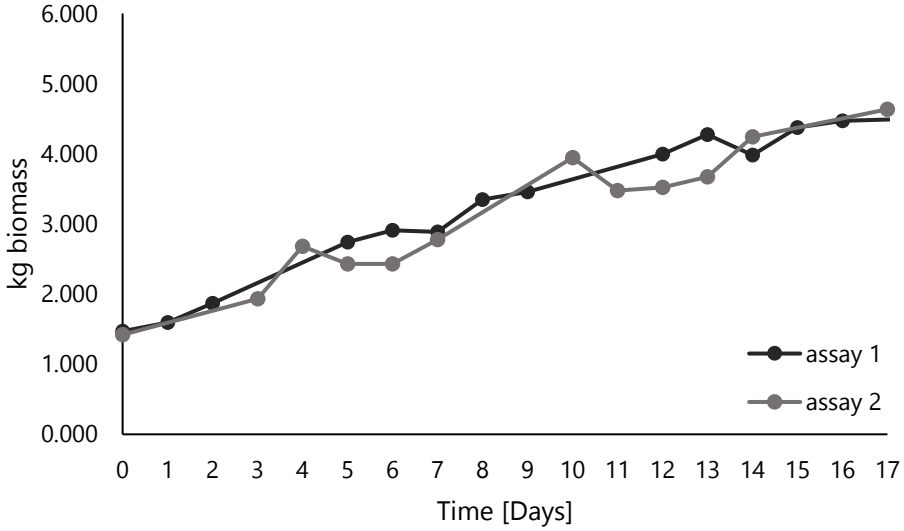

Figure A$_{10}$. *Arthrospira* sp. growth in kg of biomass in raceway ponds with 3500 L in the medium MNS with $NaHCO_3$ at 16.8 g L^{-1}. Growth occurred under outdoor conditions for 15 and 17 days for the assay 1 and 2, respectively.

Meteorological conditions

Raceway pond (assay 1)

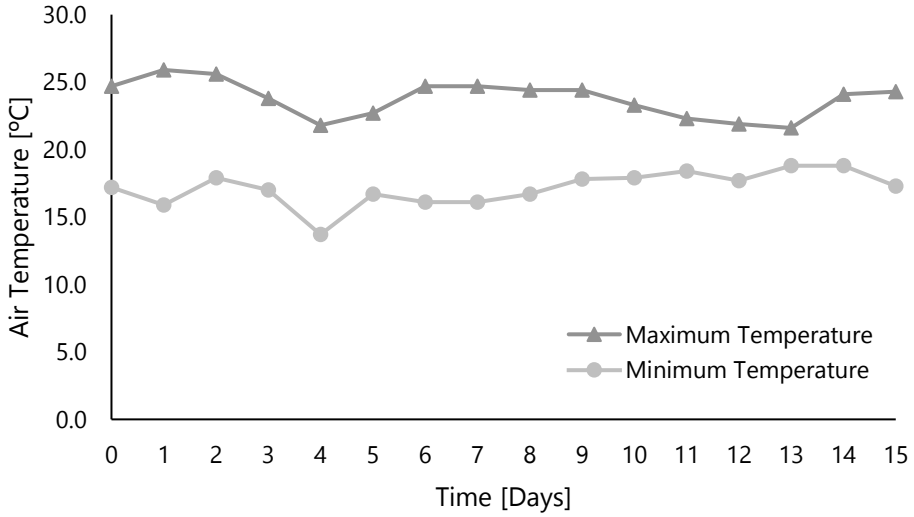

Figure A$_{11}$. Maximum and minimum temperature (ºC) registered on site during fifteen days of the first growth trial in raceway ponds.

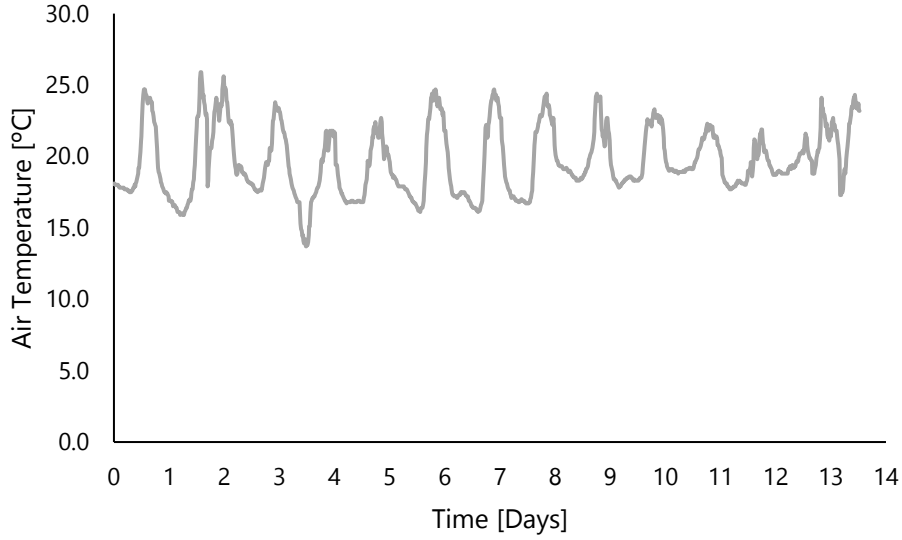

Figure A$_{12}$. Air temperature (ºC) registered on site during fifteen days of the first growth trial in raceway ponds.

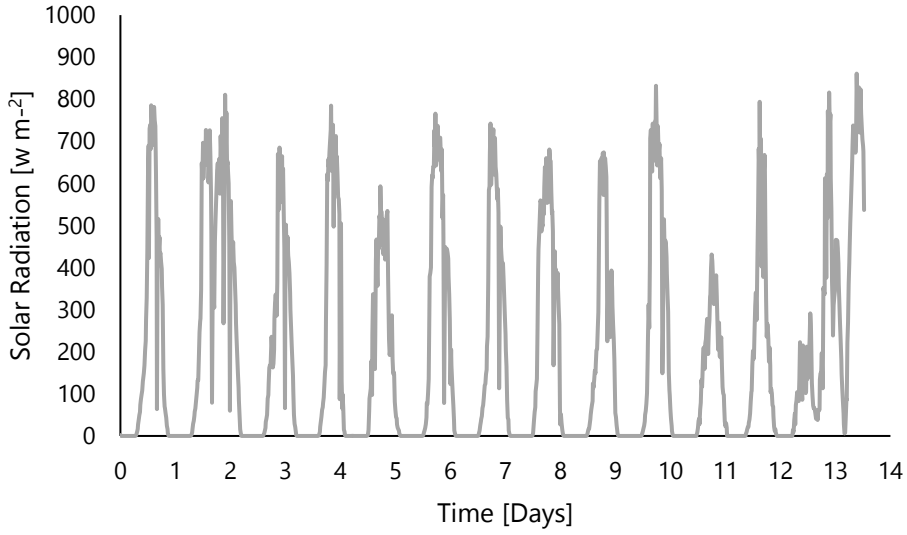

Figure A$_{13}$. Instant radiation (W m^{-2}) registered on site during fifteen days of the first growth trial in raceway ponds.

Raceway pond (assay 2)

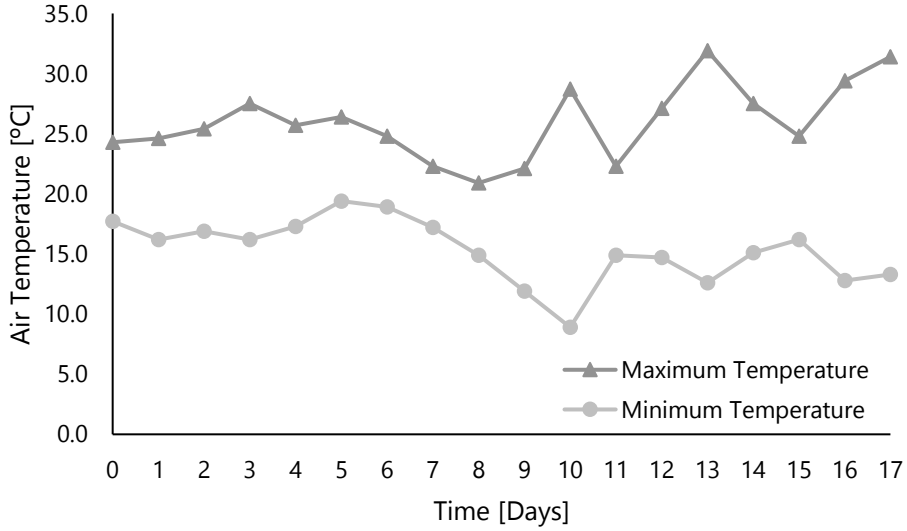

Figure A$_{14}$. Maximum and minimum temperature (ºC) registered on site during seventeen days of the second growth trial in raceway ponds.

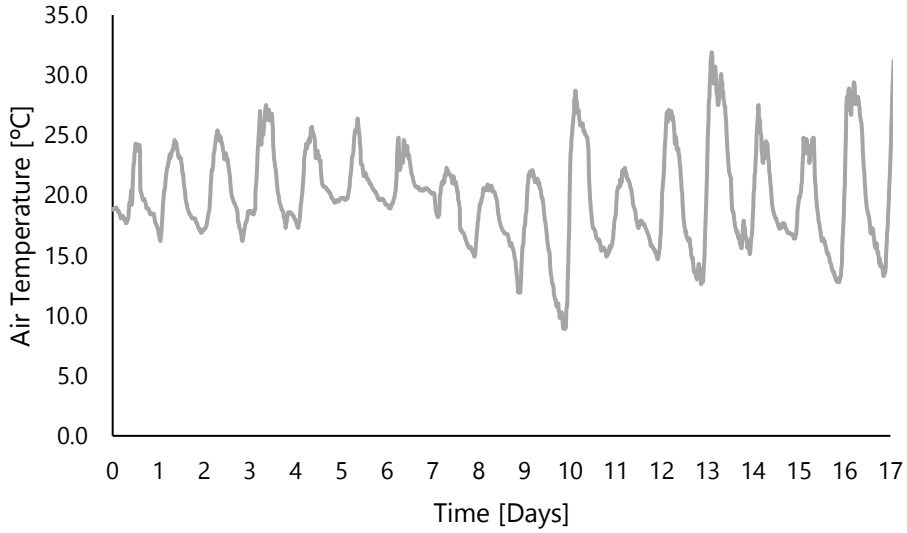

Figure A$_{15}$. Air temperature (ºC) registered on site during seventeen days of the second growth trial in raceway ponds.

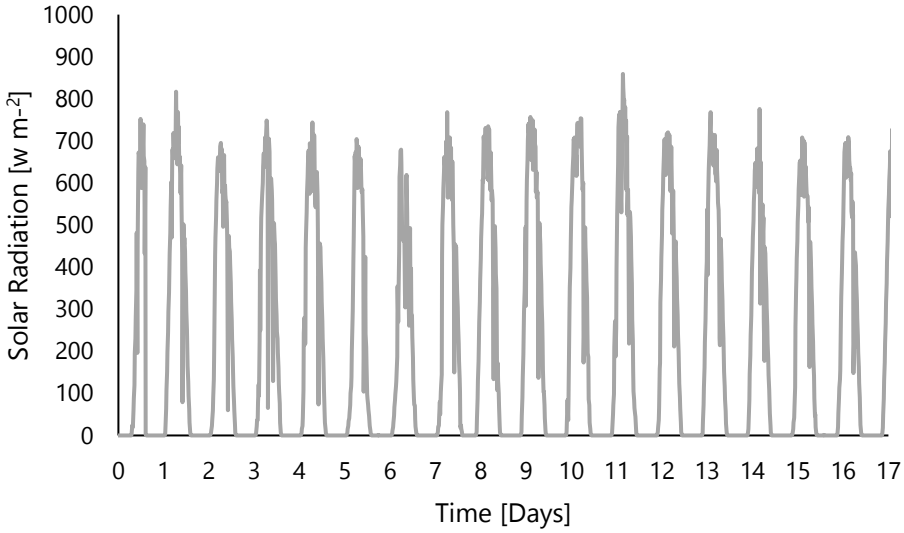

Figure A$_{16}$. Instant radiation (w m^{-2}) registered on site during seventeen days of the second growth trial in raceway ponds.

Harvesting

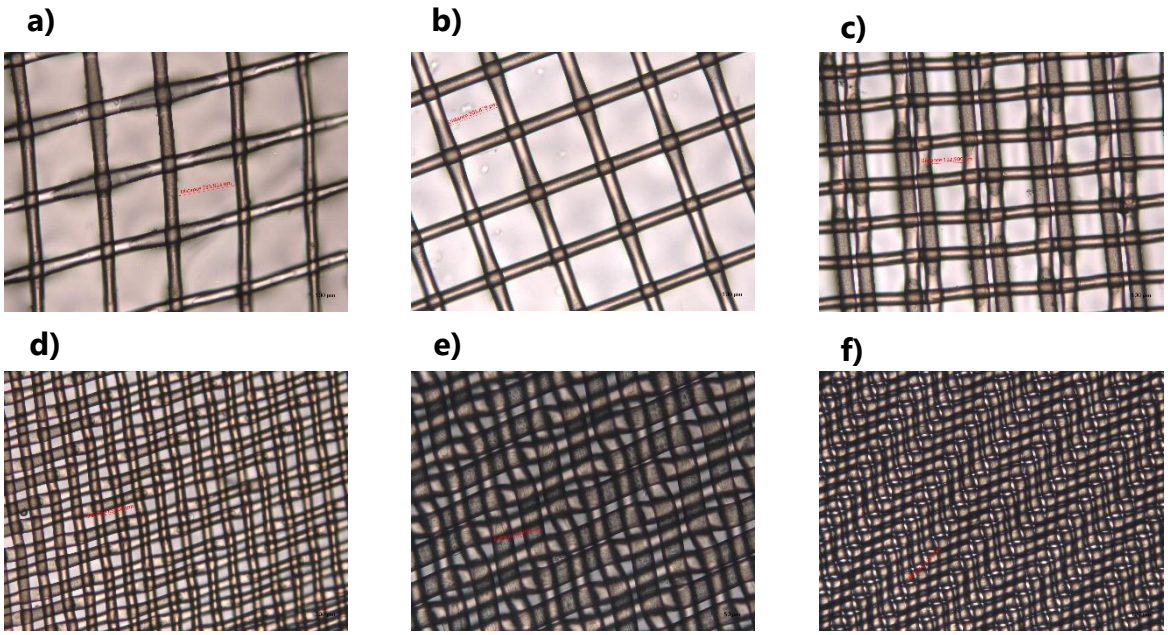

Figure A$_{17}$. Pore diameter of different nets. (a) 233.813 µm, magnification 10×. (b) 205.479 µm, magnification 10×. (c) 122.900 µm, magnification 10×. (d) 45.099 µm, magnification 10×. (e) 18.037 µm, magnification 20×. (f) 6.374 µm, magnification 10×.

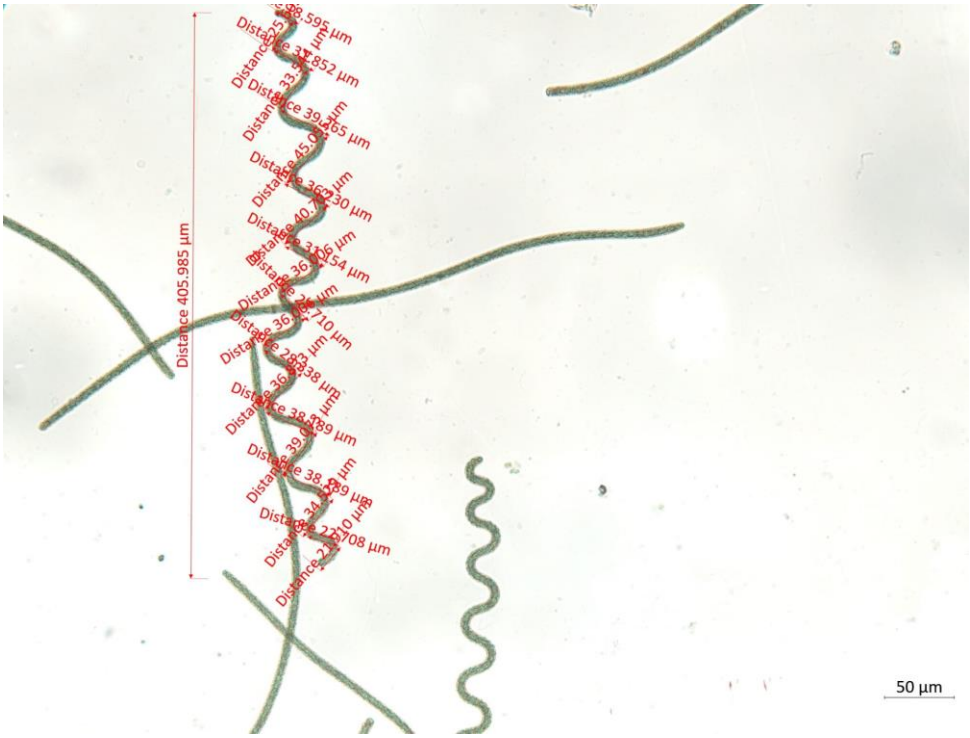

Figure A$_{18}$. Length of a spiral cell of *Arthrospira* sp. (405.985 µm if measured in a straight line or 661.492 µm when all bends where considered).

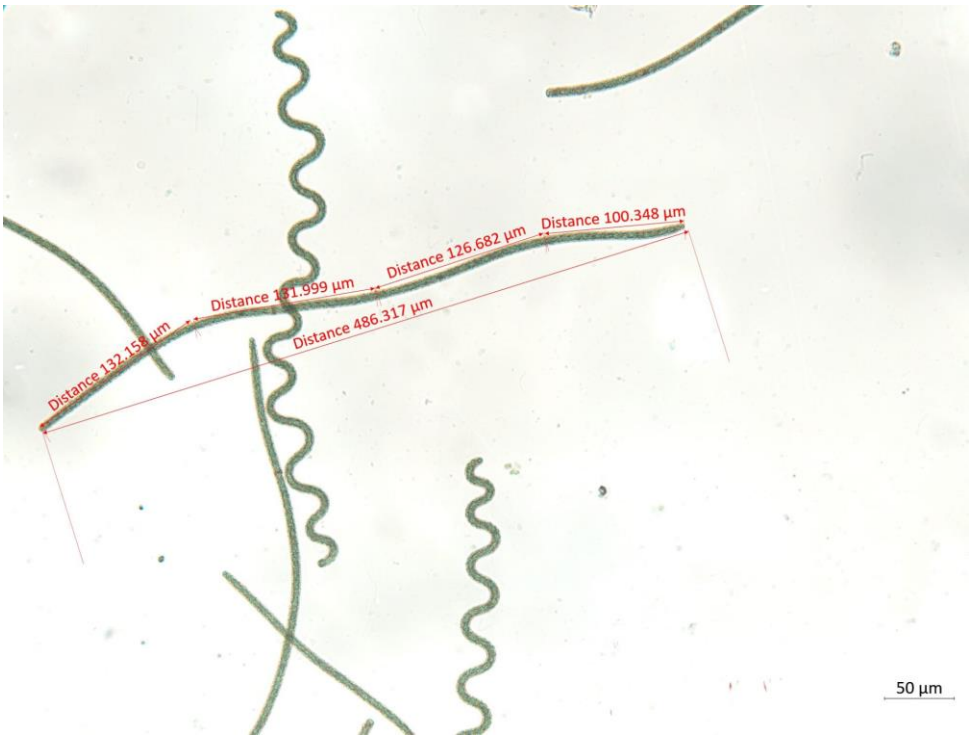

Figure A$_{19}$. Length of a linear cell of *Arthrospira* sp. (486.317 µm if measured in a straight line or 491.187 µm when all bends where considered).

Pigments determination

Table A_5. Composition of the 0.1 M sodium phosphate buffer (pH 7).

Reactants	Concentration (g L^{-1})
$Na_2HPO_4 \cdot 7H_2O$	15.487
$NaH_2PO_4 \cdot H_2O$	5.827

Protein content

Table A_6. Necessary reactants to prepare 50 mL of the reagent 1 used to extract proteins by the Lowry *et al.* (1951) method.

Reactants	Volume (mL)
Sodium carbonate (5% m v^{-1})	48
Potassium sodium tartrate (2% m v^{-1})	1
copper sulfate solution (1% m v^{-1})	1

Note: This solution must be stored in cool temperatures until utilization.

Table A_7. Necessary reactants to prepare 50 mL of the reagent 2 (Folin-Ciocalteau) used to extract proteins by the Lowry *et al.* (1951) method.

Reactants	Volume (mL)
Folin-Ciocalteau	25
Distilled water	25

Note: This solution must be stored in a dark place until utilization.