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OSMAC approach to identify novel metabolites
from *Spirulina* with anti-obesity activity as
future nutraceuticals

Hugo Ricardo Filipe Amaral

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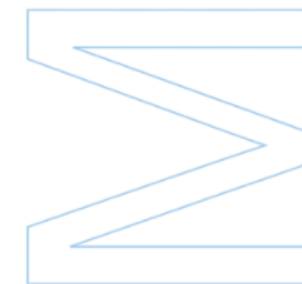


OSMAC approach to identify novel metabolites from *Spirulina* with anti- obesity activity as future nutraceuticals

Hugo Ricardo Filipe Amaral

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Mestrado em Aplicações em Biotecnologia e Biologia Sintética

Departamento de Biologia e Departamento de Química e Bioquímica

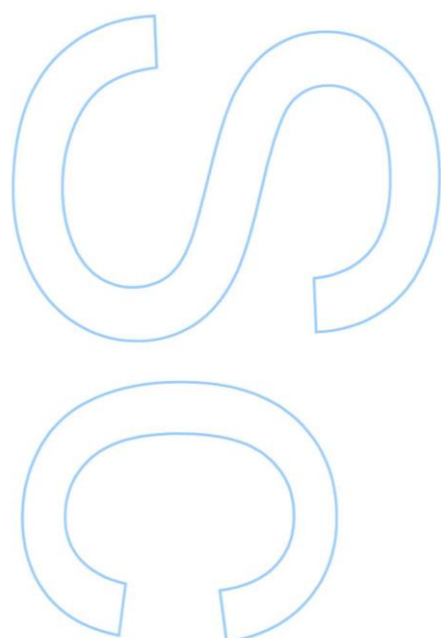
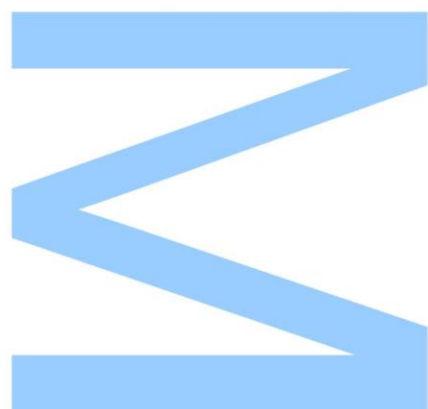
2022

Orientador

PhD Ralph Urbatzka, CSO at Fykia Biotech Lda., Permanent Researcher at Interdisciplinary CIIMAR and Invited Auxiliary Professor at FCUP

Coorientador

PhD Pedro Leão, CSO at Fykia Biotech Lda., Principal Investigator at CIIMAR and Invited Auxiliary Professor at FCUP

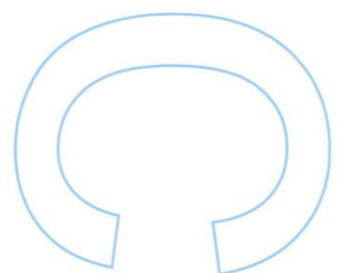
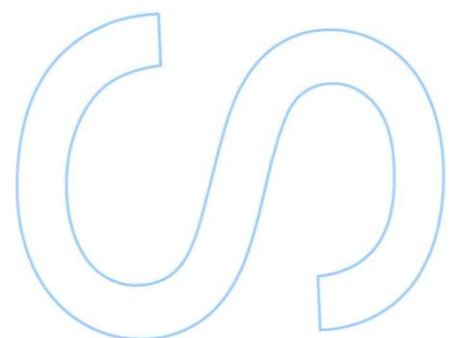
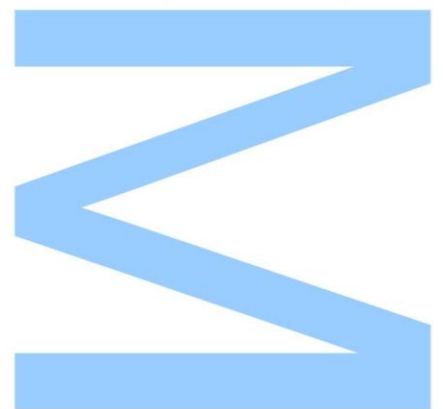




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Sworn Statement

I, Hugo Ricardo Filipe Amaral, enrolled in the Master Degree in Applications in Biotechnology and Synthetic Biology at the Faculty of Sciences of the University of Porto hereby declare, in accordance with the provisions of paragraph a) of Article 14 of the Code of Ethical Conduct of the University of Porto, that the content of this internship report reflects perspectives, research work and my own interpretations at the time of its submission.

By submitting this internship report, I also declare that it contains the results of my own research work and contributions that have not been previously submitted to this or any other institution.

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Hugo Ricardo Filipe Amaral

30/09/2022

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Abstract

Obesity has grown to be one of the leading health issues in the world today, as it exhibits a global growing trend and is related to serious co-morbidities, such as various types of cancer, cardiovascular diseases, type-2 diabetes, and several others. To try and combat this immense issue, the research and development of anti-obesity pharmaceuticals has been immensely focused. However, the pharmaceuticals available on the market present undesirable side-effects, and most of the anti-obesity pharmaceuticals formerly available to patients have been removed from circulation, due to hazardous consequences derived from their intake.

One growing alternative for individuals who are trying to reduce their body fat content is nutraceuticals, since these are natural products with several promoted health benefits. Nonetheless, the efficacy of nutraceuticals is subpar when compared to that of anti-obesity pharmaceuticals. To circumvent these obstacles, researchers have been turning to natural compounds with anti-obesogenic capabilities. Here, cyanobacteria present a huge unexplored potential in the unveiling of novel compounds, which can be applied in the formulation of anti-obesity pharmaceuticals and nutraceuticals. *Spirulina* is a genus of cyanobacteria which has been largely unexplored for its bioactivity, opposing to the *Arthrospira* genus, as its dried biomass is largely sold as a nutraceutical, and shares the name with the unexplored genus, as for many years, these genera were thought to be one, due to their morphological similarities.

Herein, the growth conditions of each of three cyanobacteria strains, two *Spirulina* spp. and one *Arthrospira* sp, were manipulated by employing different light profiles for 21 days in six photobioreactors. The resulting biomass was analyzed for macroscopical differences and then extracted with organic solvents and fractionated by increased solvent polarity extraction, obtaining three fractions from the biomass of each photobioreactor. The 18 fractions obtained from each strain were then tested for their anti-obesity activity in the zebrafish Nile Red fat metabolism assay, where only fractions from the *Arthrospira* strain exhibited any significant bioactivity against lipid accumulation in the zebrafish larvae. Fraction A from photobioreactor 1A demonstrated the highest potency. Following, the fractions obtained from photobioreactor 1A, from all three strains (active and non-active) were analyzed through liquid chromatography coupled to tandem mass spectrometry. The mass spectrometry data allowed for the metabolite profiling of each strain and to assess the differences in their chemical composition, which resulted in the bioactivity in the *Arthrospira* strain, and the lack of it in both *Spirulina* strains.

Several unique compounds were identified in the bioactive *Arthrospira* fraction, with two of them, linoleic acid and phycocyanin a, possessing annotated reports of

bioactivity against lipid accumulation and related diseases. In addition, in all three strains a compound with the m/z of 887.56 for $[M+H]^+$ was present, which is the reported mass for a chlorophyll derivative with potent anti-obesity activity, 13²-hydroxypheophytin a. Through the analysis of the fragmentation data from the compound relating to the m/z of 887.56, it is likely that the compound present in both *Spirulina* strains is an isomer of 13²-hydroxypheophytin a, which does not exhibit the same level of bioactivity, as the chlorophyll derivative, since the fragments resulting from the compound in *Spirulina* were different, than the ones in *Arthrospira*.

Concluding, *Spirulina* does not have anti-obesity activity, which is only present in *Arthrospira*. The chlorophyll derivative hpa, linoleic acid or phycocyanin a could be involved in such activity, as well as several other non-identified metabolites.

Keywords: obesity; anti-obesity compounds; cyanobacteria; *Spirulina*; *Arthrospira*; culture growth manipulation; light profiles; secondary metabolites; lipid-reducing; zebrafish.

Resumo

A obesidade tornou-se um dos maiores problemas de saúde no mundo atualmente, visto que apresenta uma tendência crescente global e está relacionada a comorbidades graves, como vários tipos de cancro, doenças cardiovasculares, diabetes tipo 2, entre outras. Para tentar combater este problema, a investigação e o desenvolvimento de fármacos para o tratamento e prevenção de obesidade, têm sido alvos de um grande foco por parte da comunidade científica. No entanto, estes fármacos, que estão disponíveis no mercado, apresentam efeitos secundários indesejáveis e a maioria deles, que em outrora estavam disponíveis para os pacientes, foram retirados de circulação, devido às consequências perigosas derivadas da sua administração.

Uma alternativa cada vez mais apelativa, para indivíduos que têm interesse em reduzir a sua percentagem de massa gorda, são os produtos nutracêuticos, uma vez que são produtos naturais com vários benefícios para a saúde de qualquer pessoa. No entanto, a eficácia dos nutracêuticos é inferior quando comparada com a dos fármacos para o tratamento da obesidade. Para contornar estes obstáculos, os investigadores têm virado a sua atenção para compostos naturais com a capacidade de tratar a obesidade. Aqui, as cianobactérias apresentam um enorme potencial inexplorado na descoberta de novos compostos, que podem ser aplicados na formulação de fármacos e nutracêuticos para o tratamento da obesidade. *Spirulina* é um género de cianobactéria que permanece bastante inexplorado em relação à sua bioatividade, o que contrasta com o género *Arthrospira*, em que a biomassa seca desta é vendida como um nutracêutico, que partilha o nome com o género inexplorado, *Spirulina*, porque durante muitos anos, estes géneros terem sido considerados como sendo pertencentes ao mesmo, devido às suas semelhanças morfológicas.

Neste trabalho, as condições de crescimento de cada uma de três estirpes de cianobactérias, duas pertencentes ao género *Spirulina* e uma ao *Arthrospira*, foram manipuladas ao utilizar diferentes perfis de luz durante 21 dias, em seis fotobiorreatores. A biomassa resultante foi analisada em relação a diferenças macroscópicas e então extraída com solventes orgânicos e fracionada por extração de polaridade crescente do solvente. Obteve-se três frações derivada da biomassa, de cada fotobiorreator. As 18 frações obtidas de cada estirpe foram então testadas quanto à sua atividade contra a obesidade em bioensaios do metabolismo da gordura do peixe-zebra com Nile Red, onde apenas as frações da estirpe de *Arthrospira* exibiram qualquer bioatividade significativa contra a acumulação de lipídios nas larvas de peixe-zebra, em que a fração A do fotobiorreator 1A apresentou a maior capacidade de redução. As frações obtidas

do fotobiorreator 1A, das três estirpes, foram analisadas por cromatografia líquida acoplada à espectrometria de massa em tandem. Os dados de espectrometria de massa permitiram contruir o perfil de metabolitos de cada estipre e avaliar a diferença que originou a presença de bioatividade na estirpe de *Arthrospira* e a falta desta em ambas as estirpes de *Spirulina*.

Vários compostos únicos foram identificados na fração bioativa de *Arthrospira*, sendo que dois deles, ácido linoleico e feofitina a, possuem bioatividade contra a acumulação de lipídios e doenças relacionadas. Além disso, verificou-se que em todas as três estirpes, estava presente um composto com m/z de 887.56 para $[M+H]^+$, que é a massa anotada na literatura para um derivado de clorofila com potente atividade contra a obesidade, sendo este composto a 13²-hidroxifeofitina a. Através da análise dos dados de fragmentação do composto em relação ao m/z de 887.56, é provável que o composto presente em ambas as estirpes de *Spirulina* seja um isómero da 13²-hidroxifeofitina a, mas que não apresenta o mesmo nível de bioatividade, como o derivado da clorofila, uma vez que os fragmentos resultantes do composto presente na *Spirulina* eram diferentes dos da *Arthrospira*.

Concluindo, *Spirulina* não possui atividade contra a obesidade, algo presente apenas na *Arthrospira*. O derivado de clorofila hpa, ácido linoleico ou ficocianina a podem estar envolvidos nesta atividade, assim como vários outros metabolitos não identificados.

Palavras-chave: obesidade; cianobactéria; *Spirulina*; *Arthrospira*; manipulação do crescimento de culturas; perfis de luz; metabolitos secundários; redução lipídica; peixe-zebra.

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Abbreviations list

4-Di-10-ASP	4-(4-(Didecylamino)styryl)-N-Metilpyridinium Iodide
5-HT	5-hydroxytryptamine
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BBE	Blue Biotechnology and Ecotoxicology
BMI	body mass index
BODIPY FL-C16	4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-s-Indacene-3-Hexadecanoic Acid
BOGA	Biotherium of Aquatic Organisms
CCAP	Culture Collection of Algae of Protozoa
CEO	Chief Executive Officer
CIIMAR	Interdisciplinary Centre of Marine and Environmental Research
COX	cyclooxygenase
CSO	Chief Scientific Officer
DCM	dichloromethane
DMSO	dimethyl sulfoxide
dpf	days post fertilization
EC₅₀	half-maximal effective concentration
EIC	extracted ion chromatograms
EW	egg water
FDA	Food and Drug Administration
FIMT	Fragment Ion Mass Tolerance
FWHM	full width at half maximum range
GLP-1	glucagon-like peptide 1
GNPS	Global Natural Products Social Molecular Networking
GRAS	generally recognized as safe
HDL	high-density lipoprotein
HESI	heated electrospray ionization
hpa	132-hydroxy-pheophytin a
IC₅₀	half-maximal inhibitory concentration
LC-HRESI-MS/MS	liquid chromatography high resolution electrospray ionization mass spectrometry
LC-MS	liquid chromatography – mass spectrometry
LC-MS/MS	liquid chromatography with tandem mass spectrometry
LDL	low-density lipoprotein

LEDs	light emitting diodes
LEGE-CC	culture collection of the Blue Biotechnology and Ecotoxicology group
LPS	lipopolysaccharide
MB	methylene blue
MeOH	methanol
MFI	mean fluorescence intensity
MGDG	monogalactosyldiacylglycerol
MIC	minimum inhibitory concentration
MoNA	MassBank of North America
MS/MS	tandem mass spectrometry
MS-222	tricaine
NADP+	nicotinamide adenine dinucleotide phosphate
NL	normalization levels
NO	nitric oxide
NPA	Natural Product Atlas
OD	Optical density
OSMAC	one strain many compounds
PCC	Pasteur Culture Collection
PI	principal investigator
PIMT	Precursor Ion Mass Tolerance
PPARγ	peroxisome proliferator-activated receptor- γ
PTU	1-phenyl 2-thiourea
PUFA	polyunsaturated fatty acid
REV	resveratrol
RNA-Seq	RNA sequencing
ROS	reactive oxygen species
SD	standard deviation
Si	silica
SPE	solid phase extraction
SQDG	sulfoquinovosyl diacylglycerol
STAT-1	signal transducers and activators of transcription 1
TNF	tumor necrosis factor
USA	United States of America
USD	United States Dollars
VLDL	very low-density lipoproteins
Z8 25	Z8 medium with 25 grams of tropical marine salt per liter

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Introduction

1.1 General overview on obesity

Obesity, at the present day, has become one of the major health issues in the world, as its prevalence can be prominently seen in developed countries. Furthermore, in developing countries it is growing at alarming rates. Since 1975, obesity has tripled worldwide, and 1.9 billion adults were considered overweight in 2016, with 650 million falling into the obese category [1]. Unfortunately, these numbers will only get higher and higher in the upcoming years, with estimations that one billion adults will be living with obesity in the year 2030 [2]. Despite these staggering numbers, the population of some countries already presents even more extreme numbers of obesity prevalence, such is the case for the United States of America (USA). According to data from 2017 and 2018, 42.4% of adults were obese and 30.7 % were overweight, while 19.3% and 16.1% of children and adolescents being obese and overweight, respectively [3]. Across the Atlantic, in Portugal, obesity has become progressively more prevalent, with it being at 22.3% in 2016 [4].

Along with significantly hindering the lifestyle of an individual, obesity is a serious health hazard since it increases the probability of developing obesity related co-morbidities. Type II diabetes, cardiovascular diseases (stroke, hypertension, coronary heart disease, congestive heart failure), cancer (breast, colorectal, endometrial, esophageal), asthma, osteoarthritis, chronic back pain, are all examples of co-morbidities which have a high incidence in obese people [5].

Obesity is generally defined as an abnormal or excessive accumulation of fat in the adipocytes [6]. Adipocytes are the main constituent of adipose tissue, which is divided into two types, firstly white adipose tissue, which is responsible for storing excessive energy as triglycerides, and secondly, brown adipose tissue, which is the fat that aids in the regulation of body temperature, as it is ready to be spent by the organism through the release of energy as heat, and through activity or movement. The excessive accumulation of fat comes from an undesirable positive energy balance, which comes from the imbalance between white and brown adipose tissues [7,8]. A person is considered overweight when their body mass index (BMI) is of 25 kg/m² or more, while someone is categorized as obese when it is of 30 kg/m² or more [5].

1.1.1 Current available solutions for obesity treatment

With the severe impacts of obesity on health, and its growing trend, there has been a high demand for obesity treatments and solutions. Until today, the better option for an individual is a change of its lifestyle, either by dieting or exercising, or both, which

in turn can demonstrate a significant impact in the reduction of the fat levels of an individual. However, there are other solutions to fight obesity.

Bariatric and metabolic surgeries, such as laparoscopic Roux-en-Y gastric bypass, adjustable gastric band, and sleeve gastrectomy, are some of the options, when it comes to the treatment of obesity, despite them being extremely invasive and costly to an individual. These surgeries represent an effective solution, as long as the patient maintains a healthy lifestyle afterwards. For example, for the laparoscopic Roux-en-Y gastric bypass, 85% of patients lose and maintain a 50% weight loss, however, in 5% of cases there were early complications and a mortality rate of 0.1% [9]. Nonetheless, this measure is mostly used in severe obesity cases, as a last resource option for the patient.

A more common anti-obesity strategy is pharmacotherapy, as in the past, there were several pharmaceuticals available on the market that could significantly help anyone reduce their body mass. Another alternative, gaining popularity at the moment, are nutraceuticals and dietary supplements with anti-obesity claims, as these appeal to the public, since most derive from natural sources, which is associated with a lesser risk of complications. One nutraceutical considered a “super-food” with anti-obesity claims is *Spirulina*, the dried biomass of the cyanobacteria, *Arthrospira platensis*.

1.1.2 Anti-obesity pharmaceuticals

Most of the once available pharmaceuticals, for long-term therapy, have been withdrawn due to serious and adverse side effects towards the patient, namely, fenfluramine and dexfenfluramine, two sympathomimetic amines, which work as 5-hydroxytryptamine (5-HT) receptor antagonists, 5-HT is also known as serotonin. The presence of these compounds will increase the production of 5-HT and its release into the bloodstream, increasing the feeling of satiety by the patient. Nonetheless, these two compounds were both withdrawn by the Food and Drug Administration (FDA) in 1997 due to causing valvular heart disease [10]. Lorcaserin was also a 5-HT receptor antagonist anti-obesity medication, once approved by the FDA, which exhibited no correlation to valvular heart disease, but it was also taken out of the market in 2020 due to it being linked with an increased occurrence of cancer.

The drug rimonabant, which acted by blocking the cannabinoid receptor type 1, a receptor that increased in quantity due to pre-adipocyte differentiation and formation of fatty acids, although never approved in the USA, it was sold in Europe, but withdrawn in 2009, owing to it increasing the probability of a patient developing psychiatric disorders [11].

Sibutramine, a compound that selectively inhibits the combined reuptake of serotonin and noradrenaline, therefore suppressing the appetite, was approved in 1997

by the FDA and in 2001 in Europe, but after just a few years, in 2010, it was removed from sale since it increased the probability of strokes and other cardiovascular incidents [12].

The phentermine/topiramate combination drug sold as Qysmia®, presents a topic of controversy, since the European Medicines Agency has never cleared or approved it, as this medication presents a potential for patients to get addicted to it, the lack of long-term information on the impact of phentermine on the cardiovascular system of a patient, and the side-effects presented by topiramate regarding the cognitive capabilities of an individual by affecting their attention and memory. Nonetheless, the FDA has approved its use, as phentermine/topiramate operates by suppressing the appetite and the effectiveness of the medication has been proven [13]. In trials where patients took the phentermine/topiramate, a weight loss of 10.9% was observed, compared to the placebo group with only 1.6%, after 1 year [14]. Therefore, the available pharmaceuticals, for the long-term treatment of obesity, are only a few.

Tetrahydrolipstatin, more commonly known as orlistat is the longest available anti-obesity medication in the market, approved in 1998, it is a pancreatic lipase inhibitor, inhibiting the metabolization of triglycerides into fatty acids, resulting in the lack of fat absorption by the intestine [15]. Although effective, as in a study, individuals who were administered orlistat lost on average 10.6 kg after 1 year, comparing to the control group, that only lost 6.2 kg [16], orlistat presents some side-effects due to its mode of action, as 20% of patients experienced fecal incontinence [17].

Another available long-term anti-obesity pharmaceutical is the combination of the compounds naltrexone and bupropion, sold as Contrave®. This medication was approved by the FDA in 2014 and works as an appetite repressor, by inhibiting the reuptake of norepinephrine and dopamine. Although effective, naltrexone/bupropion has some serious side-effects by increasing the blood pressure and heart rate of a patient, which in turn presents a challenge for people who already suffer from cardiovascular diseases, as such is the case for many individuals dealing with obesity [18].

Lastly, liraglutide, a compound that derives from the glucagon-like peptide 1 (GLP-1), as GLP-1 increases satiety and the feeling of fullness as well as decreasing appetite. Contrary to the aforementioned medications, liraglutide is administered through injection to the patient [19]. Liraglutide presents the highest desistance by patients, between all anti-obesity drugs approved by the FDA, because of its side-effects, namely vomiting, nausea, constipations, and diarrhea [20].

1.1.3 Natural compounds as anti-obesity agents

Due to the immense complications revealed by the drugs used for the treatment of obesity, immense interest emerged in the investigation of safer more effective natural products with anti-obesogenic properties, than the already available pharmaceutical on the market. Numerous compounds, from natural sources, have been found to possess anti-obesity potential. Lipstatin, a compound isolated from the bacteria *Streptomyces toxytricini* is an optimal inhibitor of the pancreatic lipase, as it is selective, irreversible, and extremely potent, moreover, it is the precursor of the aforementioned anti-obesity pharmaceutical, Orlistat [21]. More examples of compounds with bioactivity against obesogenesis are platycodins, extracted from the roots of *Platycodon grandiflorum*, most commonly known as the Chinese bellflower. In a study in hamsters as animal model, platycodins decreased lipid absorption and its digestion by inhibiting the pancreatic lipase. While in another study, through *in vitro* assays, an aqueous extract of platycodins had a half-maximal inhibitory concentration (IC₅₀) of 20 g/L towards the inhibition of the pancreatic lipase [22,23]. Found in the rhizomes of *Curcuma longa*, the phenolic acid, curcumin, it has shown to inhibit 3T3-L1 adipocyte differentiation by reducing the expression of the peroxisome proliferator-activated receptor- γ (PPAR γ), a major transcription factor in adipogenesis, stopping the formation of white adipose tissue [24]. Furthermore, capsaicin, a very well-known compound owing to the heat it provides some peppers with, has shown to inhibit the differentiation of 3T3-L1 cells into adipocytes, by increasing the expression of browning genes in white adipose tissue as well as upregulating uncoupling proteins, responsible for the thermogenesis in brown adipose tissue [25].

Green tea has also been documented to possess a lot of health benefits, due to its rich content in polyphenols. A major constituent of green tea extract are catechins, and in a study where adult zebrafish and larvae were used as animal models, it was shown that green tea extract reduced visceral adipose tissue volume in high-fat diet induced obese zebrafish larvae, while also diminishing the visceral adipose tissue and plasma triglycerides in adult obese zebrafish. After an RNA-seq (RNA sequencing) based transcriptome analysis, it was gathered that the anti-obesogenic effects of the green tea extract were due to the activation of the WNT/ β -catenin pathway, which suppressed adipogenesis [26]. This goes in line with a study, which indicated that the main catechin, 50-70% of all catechins in green tea extract, (-)-epigallocatechin-3-gallate activates the WNT/ β -catenin pathway [27].

1.2 Cyanobacteria overview

One immense opportunity for the biodiscovery of natural compounds with bioactivity is in aquatic environments, since around 71% of the surface of Planet Earth is covered in water, with around 96.5% being the marine environment of the oceans [28,29]. Despite this, the inaccessibility, and complications in researching the marine environment caused that it has still remained a mostly untapped and underexploited domain [30].

Cyanobacteria are the primordial oxygenic photosynthetic microorganisms on Earth, making them the culprits of the atmosphere altering from anoxygenic to oxygenic conditions. They are gram-negative bacteria, therefore being photoautotrophic prokaryotes, and commonly referred to as blue-green algae [30,31]. Cyanobacteria are found in most places on Earth, from salt and freshwater to most soils and even in rocks, monuments, and walls. This wide array of locations of habitats is due to their adaptability to any environmental conditions, owed to their cellular mechanisms and survival strategies [31,32]. A few of these strategies are the formation of endosymbiosis with sponges, corals, diatoms, and mutual associations with other organisms, which allow cyanobacteria easier ways to get nutrients and, in most cases, these associations offer them a defense against threats. Cyanobacteria can also fixate dinitrogen from the atmosphere, which no other oxygenic photoautotroph can accomplish, using it for their nutrition [31]. Additionally, they can produce different metabolites according to their environment, to better adapt the abiotic stress [32]. Altogether, it is easy to understand why cyanobacteria can proliferate almost everywhere and in a rapid manner, which is why most people associate them with the toxic blooms which are hazardous for the population, since they produce some toxic secondary metabolites [30]. More and more research, has been made on the day-to-day life applications of cyanobacteria bioproducts, ranging from pharmacological compounds to the biotechnological capabilities of these blue-green bacteria [31]. 40% of cyanobacteria compounds are lipopeptides, amino acids constitute 5.6%, fatty acids and macrolides both are 4.2% and amides are 9%. Lipopeptides, were described to have cytotoxicity, antitumoral, antibiotic and antiviral activities [30].

1.2.1 Cyanobacteria as a source for bioactive compounds

Although not as investigated as marine bacteria, marine fungi, sponges, and cnidarians in terms of volume of new compounds unveiled, the research done on cyanobacteria has been fruitful, and even at the same level as macroalgae, with dozens of new compounds having been discovered [28]. This booming field of research is mostly due to the valuable products cyanobacteria can metabolize, and the possible

manipulation of said bioproducts, through the alteration of the growth conditions of cyanobacteria, inducing specific responses, in turn aiding the production of desired metabolites [32].

Janadolide is a compound obtained from the cyanobacterium *Okeania* sp. It is a cyclic polyketide-peptide hybrid with a *tert*-butyl group, and it possesses an extremely strong bioactivity against *Trypanosoma brucei brucei*, the parasite responsible for African trypanosomiasis, better known as sleeping sickness, with an IC₅₀ of 47 nM. Most importantly, janadolide showed no cytotoxicity towards human cells [33].

Portoamides A and B, two cyclic peptides isolated from *Phormidium* sp, are an example of cyanobacteria natural products that demonstrate significant cytotoxicity. The combination of portoamides A and B, acts as a mitochondrial toxin, by disturbing the energy metabolism of the cell. These compounds affected the viability of colon cancer cells as well as normal cells, since the zebrafish larvae used on the study showed system toxicity [34]. Another example of a cyclic peptide with notable cytotoxicity activity against breast cancer cells is the cyanobactin wewakazole B, isolated from *Moorea producents*, as cyanobactins are one of the main classes of compounds isolated from cyanobacteria [32, 36]. The unveiling of such compounds presents an excellent opportunity in the design of anti-carcinogenic drugs, since cyclic peptides possess high membrane permeability and bioavailability, as well as resistance to degradation by enzymes and are very diverse in terms of functions [37].

To date there are three FDA approved anti-carcinogenic pharmaceuticals derived from cyanobacteria on the market. Brentuximab vedotin designed from dolastatin 10, compound extracted from the cyanobacterium *Dolabella Auricularia*, is used in the treatment for Hodgkin's disease and anaplastic large T-cell systemic malignant lymphoma and was approved by the FDA for market distribution in 2011. Brentuximab vedotin is sold under the name Adcetris® by Seattle Genetics. The second compound to be approved by the FDA, this one eight years later in 2019, was polatuzumab vedotin. It is used in the treatment of lymphomas and lymphocytic leukemia, and it sold under the name Polivy™ by Genetech/Roche. Finally, the compound belantamab mafodotin-blmf, sold under the name Blenrep™ by GlaxoSmith was approved by the FDA for the treatment relapsed multiple myeloma in adult patients in 2020 [38].

1.2.2 Anti-obesogenic potential of cyanobacteria

As previously mentioned, most compounds present either a cytotoxic, antiviral, and antibiotic bioactivity, however, the unveiling of new compounds with anti-obesogenic activity is an area of extreme interest due to the weight of the problem in the world today, and the various potential solutions, emanating from cyanobacteria.

Yoshinone A is such an example, extracted and isolated from the cyanobacterium *Leptolyngbia* sp, this compound possesses a γ -pyrone in its structure, followed by a linear side chain. Yoshinone A exhibited potent inhibitory activity towards the differentiation of 3T3-L1 cells into adipocytes and as importantly, revealed to be non-cytotoxic [39]. A similar compound to yoshinone A, kalkipyron, which also possesses a γ -pyrone with a linear side chain, was found to have a more potent activity towards suppressing the adipocytic differentiation, however, it also exhibited potent cytotoxicity. Here lies one of the main challenges in the discovery of anti-obesity compounds from cyanobacteria, in assuring they are effective without any harm [40].

An already known chlorophyll derivative, 13²-hydroxy-pheophytin a (hpa), was shown to possess a potent lipid reducing ability. It was isolated from a *Cyanobium* sp. and tested in a zebrafish Nile red fat metabolism assay, where it exhibited reduction of the neutral fat levels in the zebrafish larvae, with a half-maximal effective concentration (EC₅₀) of 8.9 μ M. The compound was also effective in *in vitro* assays, by decreasing the lipid accumulation in spheroids of 3T3-L1 murine preadipocytes, while revealing no harmful effects in the embryos and larvae development. Although the lipid reducing mechanism was not full cleared, it was noted that lipolysis was not affected, while the expression of PPAR γ was altered by the 13²-hydroxy-pheophytin a. Further investigation noted that, the cyanobacteria commonly known as *Spirulina*, genus *Arthrospira*, possessed high amounts of this chlorophyll derivative [41]. This indicates the biotechnological potential of 13²-hydroxy-pheophytin a, since *Arthrospira* possesses the GRAS (generally recognized as safe) classification from the FDA, making it an excellent candidate in the design of a novel anti-obesity pharmaceutical or even in 13²-hydroxy-pheophytin an enriched supplements or nutraceuticals [30,40]. Since the dried biomass from *Arthrospira* has already revealed in numerous studies, some of which will be mentioned ahead, the ability to ameliorate the symptoms of obesity and its related co-morbidities in humans, after just a few months of supplementation.

1.3 Distinguishing *Spirulina* and *Arthrospira*

Regarding their taxonomy, cyanobacteria have presented innumerable challenges to researchers over the years, since, traditionally, it was executed based on their morphology and not on the analysis of the evolutionary history of cyanobacteria [42]. One case, which exemplifies the lack of uniformity and regulation in naming different cyanobacteria species, is the distinction between the cyanobacteria genus *Spirulina* and the genus *Arthrospira*. Both genera belong to the class Cyanophyceae, while *Spirulina* is in the Spirulinales order, and *Arthrospira* is inserted into the Oscillatoriales order [42,43].

The main morphological differences between the species belonging to these two genera are that *Spirulina* spp. presents unicellular filaments with no septa, whereas the filaments of *Arthrospira* spp. are multicellular, possess septa and are generally 2 to 5 times bigger than that of the ones belonging to *Spirulina*. As stated above, the species are still similar since, the species from both genera are filamentous with unbranched filaments and their trichomes are helical, with most species exhibiting trichomes which are tightly coiled, although, in *Arthrospira* they may vary from tightly coiled to uncoiled and straight [43]. Macroscopically, *Spirulina* generally grows in thin mats, while adhering to a surface or floating at the surface level, and when enough biomass has been gained, it starts to form dense, cluster-like, structures [44]. On the other hand, *Arthrospira* usually floats to the surface, due to the vacuoles in its filaments giving them buoyance, but it is not rare to find filaments at the bottom surface, as long as they get enough luminosity. Therefore, *Arthrospira* grows better in shallow waters, like in lakes and puddles. Contrary to *Spirulina*, when disturbed, *Arthrospira* disperses in the medium, becoming suspended, giving it an almost homogenous look [45]. Both cyanobacteria genera generally demonstrate a green color, with *Arthrospira* presenting a more blue-green than *Spirulina*, however, pinkish-red *Spirulina* has been found in the Baltic Sea [46]. *Spirulina* is mostly found in marine waters along the coast of the Atlantic Ocean but can also appear on land in shallow and brackish salty waters, preferring alkaline, almost neutral (pH of around 8), but some species can grow in slightly acidic mediums. *Arthrospira* prefers highly alkaline waters (pH of 9-11) and water temperatures of around 35°C, as it is found prominently in tropical or sub-tropical areas, such as Central America or countries in East Africa [46,47].

These two genera, in the mid-19th century to early-20th century, were classified as being separate, according to Stizenberger in 1852, who wrote the first taxonomic report on the *Arthrospira* genus, later confirmed by Gomont in 1892, who also reported the existence of the *Spirulina* genus. However, in 1932, Geitler stated the two genera should be put into one, *Spirulina*, due to their similarity in morphology, ignoring the presence of septa in *Arthrospira*. It was only in 1989, due to the reports by Gomont in 1892, that the *Spirulina* genus was separated from the *Arthrospira* genus. This resulted in, for many years, that most of the research made on any of these two genera, was done in the name *Spirulina*, resulting in the marketing and selling of the dried biomass of *Arthrospira* spp. for human consumption to also be referred to as *Spirulina*, which is still accepted until this day, since this misconception would be too arduous to resolve [43,48].

1.3.1 *Arthrospira* as a nutraceutical

Arthrospira has long been documented for its excellent properties for human consumption, as its dried biomass is often referred to as a “superfood”, sold under the name Spirulina. The human consumption of Spirulina has been dated back to the 16th century, as in Mexico, in the city of Tenochtitlán, which today goes by the name of Mexico City, the natives used to bake bread with the blue-green algae [47]. However, it was not only until the 1950s that the cultivation of *Arthrospira* began, both in the USA and Japan, with the research into the health benefits of this microorganism starting in the 1970s in the countries of India, Peru and Thailand, as ordered by West Germany. Nowadays, Spirulina is sold in most countries all over the world with a market size valued at USD (United States Dollars) 393.6 million in 2019, with an estimation of it being valued at USD 897.6 million by 2027 [49,50].

Spirulina is mostly composed of protein (60-70%), making Spirulina an excellent source for amino acids. Spirulina is also rich in β -carotene, a powerful antioxidant, and vitamin B₁₂ making it a good alternative for people who have low hemoglobin levels. Around 13.6% of Spirulina is composed of carbohydrates, and most importantly, of these carbohydrates, none are cellulose, since it is not present in the *Arthrospira* spp. cell walls, which some people have trouble processing and digesting in the digestive tract. Spirulina is also rich in linoleic acid and γ -linolenic acid, which are essential fatty acids, with major health benefits, such as the ability to decrease low-density lipoprotein (LDL) levels [51]. One major compound class of interest present in the *Arthrospira* genus is phycocyanin, a class of phycobiliproteins. Phycocyanin is composed by two bilin chromophores, similar to bilirubins, which are potent antioxidants, degrading reactive oxygen species (ROS), which protects plasma proteins and amino acids [52].

Spirulina has become extremely popular as a dietary nutraceutical owing to its anti-obesogenic effects and by preventing and ameliorating obesity related co-morbidities, as several studies have documented the beneficial impact of Spirulina supplementation. One study reported that obese and overweight patients who were given 2g of Spirulina (*Arthrospira platensis*), once a day, while on a calorie deficit diet, for 3 months, had their body mass, body fat, BMI and waist circumference all reduced, and to a higher degree when compared to the placebo group. In addition, blood triglycerides levels and appetite scores were also lower in comparison to the control [53]. In a different study, patients suffering from hypertension were administered, 2g of Spirulina (*Arthrospira maxima*) daily. The patients on Spirulina supplementation showed reduced LDL-cholesterol and interleukin-6, a tumor necrosis factor, which levels are increased when a person is obese. Along with these improvements, the insulin sensitivity and antioxidant capabilities were both increased by the Spirulina supplementation [54].

To report the effects of *Spirulina* on the blood lipid levels, a study supplemented patients who suffered from hypercholesterolemia and ischemic heart disease, which is when the coronary arteries build up plaque causing their narrowing. The study was carried out for 3 months, three groups were established, with the first and second group being supplemented 2g and 4g of *Spirulina* (*Arthrospira fusiformis*) respectively, and the third group was the control. At the end of the three months, the first and second groups showed significantly decreased levels of total cholesterol, LDL, triglycerides, and very low-density lipoproteins (VLDL), with the second group exhibiting a higher decrease. Moreover, high-density lipoprotein (HDL) cholesterol levels increased in the first and second groups [55].

Type 2 diabetes is a very common obesity co-morbidity, and in a study, individuals with diabetes were given 8 g of *Spirulina* (*Arthrospira platensis*), per day, for 12 weeks. Along with the benefits mentioned in the previous study on blood lipid level, plasma malondialdehyde and plasma adiponectin were decreased, which indicates less oxidative stress [56]. It is clear why *Spirulina*, the dried biomass of *Arthrospira* sp, is so highly regarded as a powerful nutraceutical, as it possesses the ability to help individuals, not only with weight loss, but it increases satiety and ameliorates many parameters related with obesity and its co-morbidities.

1.3.2 The uncertainty of *Spirulina* sp

Opposing to the wide and intense research the *Arthrospira* genus has gone through over these past decades, the research on the *Spirulina* genus has been very scarce.

Spirulina subsalsa, a species belonging to the genus, has been documented for its correlation in causing the disease syndrome, *Penaeus stylirostris*, on the blue shrimp. This disease resulted in necrosis of the epithelium of the midgut, dorsal cecum, and hindgut gland, which results in a hemocytic enteritis. This was due to the waste produced by the shrimp and the leftovers from their feed, providing the needed nutrients for *Spirulina subsalsa* to start growing and form blooms in the tanks. The shrimps would then feed on the cyanobacterium, which was present in the stomach content analysis, resulting in the appearance of the disease in the blue shrimp. Such events did not occur in tanks without cyanobacterium, which led to the hypothesis that *Spirulina subsalsa* produces a toxin that is not potent, but when ingested in substantial quantities, leads to the development of such disease [57].

A more recent study on *Spirulina subsalsa*, tested the cytotoxic abilities of fractions obtained from the cyanobacterium and their inhibitory effects on proteases. They showed that four of the seven fractions had high cytotoxic activity, by reducing the

viability of T47D breast cancer cells. Additionally, only two fractions showed inhibitory effects on the proteases, carboxypeptidase A and chymotrypsin, but these effects were not significant, exhibiting that the compounds present in the cyanobacterium, function in a selective manner. This revealed the potential for *Spirulina subsalsa* to produce possible cytotoxic compounds [58].

Spirulina subsalsa has also been analyzed for its potential of accumulation and removal of lead and triphenyltin from water, as a biotechnological application for bioremediation of water [59,60]. Along with these environmental applications, *Spirulina subsalsa* has also been shown to produce polyhydroxyalkanoates, biopolymers that can be used in the fabric of bioplastics [61].

Nonetheless, contrary to the morphological lookalike, *Spirulina* has not been investigated nor documented for the applications in the treatment of obesity and other metabolic diseases and related co-morbidities.

1.4 Relevance of the zebrafish as a model organism

The research and finding of novel compounds for their use as pharmaceuticals has always been a complicated and expensive path, since on average, the cost of a single drug to get to the market is of around USD 985 million, while taking 12 years to do so, as this is owed to its research costs and failed trials [62,63]. Pharmaceutical companies have, therefore, investigated ways to streamline drug discovery, so it becomes faster and cheaper. One method is the application of High Throughput Screening, which provides hits by drugs on selected targets. This strategy has showed some success, however, it has been slowing down due to the limitations of *in vitro* assays, since they fail to replicate the interconnections that play part between all constituents of an organism, such as cells, tissues, organs, and its diverse systems. These interconnections are extremely important to the way a disease is going to evolve in each specific environment. Therefore, some positive hits in *in vitro* assays will not possess the same activity when put through *in vivo* assays [63-65]. An alternative to this is the testing and screening of compound libraries, extracts, and extract fractions on whole-animal models [66]. One of the most popular animal models in research is *Mus musculus*, the mouse, as it is a mammal, with many similarities to humans and a well-known genetic constitution and anatomy. In spite of this, the screening of drugs in mice presents some complications, with the most important being the cost of maintaining mice for research purposes, the ethical concerns raised by the public, and a low-throughput due to the reduced number of off-spring a female mice can have [66]. Owing to this, some smaller animals have been gaining popularity over the years, as they are cheaper and easier to maintain. Such is the case for the zebrafish, *Danio rerio* (*D. rerio*), which,

although not a mammal, possesses similarities in its development and metabolic pathways as it is a vertebrate and possesses a reasonable genetic homology with humans, of around 80%. Moreover, its small size, 5 mm at 3 days post fertilization (dpf) and 2 to 3 cm at adulthood, and the fact each female can lay 200 eggs per week, allows for a large number of individuals to be screened in one assay. A unique advantage of *D. rerio*, is that the fecundation and development of the embryos, which are durable and are of a large size, when taking into account the size of an adult *D. rerio*, is done externally from the body of the carrier, while its embryos and larvae are transparent under optical conditions [65-68]. These characteristics, along with the fact that the development of the embryos is quick, since only 3 dpf the hatching of the embryo takes place, allow the study and visualization of the early onsets of diseases, certain defects, and mutations. Additionally, it has been shown that certain diseases and mutations, that occur on human organs which are not present in the zebrafish, are possible of being studied in this model organism. An example of this is the anti-carcinogenic compound rosuvasatin, which was unveiled, through genetic screening in zebrafish embryos, to possess potential in the treatment of prostate cancer, as it inhibited angiogenesis in zebrafish [68,69].

Regarding the study and research of metabolic diseases which affect the lipid metabolism, such as obesity, the zebrafish, surprisingly, presents some similarities with mammals, in the way they regulate their energy intake in the form of lipids [64]. Examples of this is the conservation of the PPAR γ in *D. rerio*, as the activation of the PPAR γ pathway results in the decrease of adipocyte inflammation in mammals with obesity and the presence of adiponectin and their receptor genes in zebrafish, as in obesity the levels of adiponectin decrease in mammals [70,71].

These resemblances have allowed for the creation of an *in vivo* assay, which allows to analyze the neutral lipid accumulation in *D. rerio* larvae. This assay is designated Nile red fat metabolism assay, since it uses Nile red, a lipophilic stain, which allows, through fluorescence microscopy to infer the anti-obesity activity of extracts, fractions, or isolated compounds, as it stains the intracellular lipids present in the yolk sac and intestinal region of the zebrafish larvae [72]. This assay allows for a much more inexpensive and rapid intermediate step before pre-clinical assays on rodents. It has been successfully employed in cyanobacterial research in the determination of the lipid-reducing activity of the cyanobacteria derived compounds, as well as their safety. An example of this is the chlorophyll derivative 13²-hydroxy-pheophytin a, mentioned before. In another study, 46 different cyanobacterial strains and their fractions were also tested using this assay, which also permitted the assessment of their lipid reducing activities and toxicity towards the zebrafish larvae [41,73].

1.5 Objectives of the research work

This work had the aim of unveiling the anti-obesity potential of two strains of *Spirulina* sp, and to compare those effects to *Arthrospira platensis*, the “classical” *Spirulina* strain in many studies. The former has not been analyzed for any kind of bioactivity towards lipid reduction, while the multiple health benefits, including anti-obesogenic properties, of the latter have been extensively researched.

This main objective was achieved by employing the OSMAC (one strain many compounds) approach, which is the manipulation of the growth conditions of the three cyanobacteria cultures, namely the alteration of light conditions, with the goal to analyze if such conditions would interfere with the production of valuable, but unknown anti-obesity compounds. After a 3 weeks growth period, the cyanobacteria biomass was retrieved, extracted with organic solvents, and fractionated by polarity. The resulting fractions were employed in the Nile red fat metabolism assay in zebrafish larvae, to assess their bioactivity *in vivo*. Liquid chromatography with tandem mass spectrometry (LC-MS/MS) was then used to characterize selected bioactive fractions, and to infer the compounds which constituted each one. A comparative molecular network was generated from active fractions compared to non-active fractions, to analyze their similarities and differences, and, to identify putative candidate compounds with bioactivity towards lipid accumulation.

Along with the developed work on the anti-obesogenic potential of *Spirulina* spp. and its comparison to the morphologically similar *Arthrospira platensis*, appetite reduction bioassays were also carried out. In these bioassays, fractions extracted from a commercially available dried *Arthrospira platensis* biomass, were used in two different bioassay protocols, which utilize the *D. rerio* larvae to assess the appetite reduction potential of compounds or fractions. The first bioassay evaluates the active food uptake in zebrafish larvae with paramecia, as paramecia is appropriate for fish larvae to feed on, while inducing their appetite [74]. The second bioassay utilizes liposomes created from chicken egg yolk, since the egg yolk is comprised of mostly lipids, and will assess the passive food uptake realized by the zebrafish larvae [75].

1.6 Fykia Biotech

Fykia Biotech Ld. Is the company in which this work was developed and carried through. It is a blue biotechnology start-up focused on the unveiling and development of products derived from cyanobacteria and microalgae. The four founders of Fykia Biotech, PhD João Morais – Chief Executive Officer (CEO), research technician responsible for the inhouse culture collection of the Blue Biotechnology and Ecotoxicology (BBE) group (LEGE-CC); PhD Ralph Urbatzka – Chief Scientific Officer (CSO), permanent

researcher at Interdisciplinary Centre of Marine and Environmental Research (CIIMAR); PhD Pedro Leão – CSO, principal investigator (PI) of the Cyanobacterial Natural Products group at CIIMAR; PhD Vítor Vaconcelos – CSO, Full Professor at University of Porto, President of the Board of CIIMAR, and PI of the BBE group;

Started the company in January of 2021 as a CIIMAR spin-off, as they are all researchers at the interdisciplinary institute and possess extensive knowledge on the natural products from cyanobacteria and microalgae.

Fykia possesses three main areas of research and development, firstly the nutraceutical area, a booming field as the ever-increasing obesity epidemic presents tremendous difficulties to the ones affected by it. The other areas present in the research portfolio of Fykia Biotech are cosmetics, in which many cyanobacteria and microalgae are abundant in the antioxidants and anti-inflammatory metabolites desired for this industry, and lastly, the agriculture department, where natural biofertilizers and bio-stimulants derived from these marine microorganisms as novel and sustainable ways of improving the growth and durability of crops and plants, are being researched.

Fykia Biotech aims to shift the industries of nutraceuticals, cosmetics, and agriculture by tapping to the vastly unexplored marine environment and what lies in it.

1.6.1 Market study and acceleration programs

Fykia Biotech has been inserted in several partnership programs such as Sherpa do Mar, REDEALGAE, and EnhanceMicroAlgae.

As a part of this internship, along with the research work done at CIIMAR, market studies were conducted by formulating datasets comprising of companies which do similar work and operate in the blue biotechnology area, agglomerating then possible direct competitors as well as possible business partnerships.

In addition, acceleration programs were searched and applied for, namely BIP PROOF by U. Porto Inovação, Seedbed Incubator by EIT Food, and HiTech by HiSeedTech, in which, only for the latter, acceptance was granted, and the consequent enrollment was made. The HiTech acceleration program was an 18-week, hands-on experience designed for research-focused teams, as it aimed to provide the skills for a better knowledge in commercialization and learn how to evaluate the different paths to move a certain technology to the market. For the Fykia Biotech team, this technology was the patented anti-obesity bioactive compound hpa (PCT/IB2020/051759). HiTech then allowed for the establishment of some high-value business networks as well as the opportunity to present the technology to a panel of several people from the biotechnology industry.

Methods and materials

2.1 General procedures

The weighing of the freeze-dried biomass was done using an analytical balance (Sartorius Entris™), while for the fully dried extracts and fractions, an analytical balance (Sartorius Secure™) was also used, and both balances had a readability interval of 0.1 mg. For each vial, containing either extracts or fractions, three weight measurements were annotated, and their mean was taken.

The solvents used in the extraction and fractionation steps were analysis-grade, while the ones used in the tandem mass spectrometry (MS/MS) analysis were LC-MS (liquid chromatography – mass spectrometry) grade.

2.2 Culture medium and cyanobacteria culture

Three different cyanobacteria strains were used in this work, *Spirulina* sp. LEGE 11439, *Spirulina* sp. and *Arthrospira platensis* PCC 7345 (Figure 1). Two *Spirulina* spp.

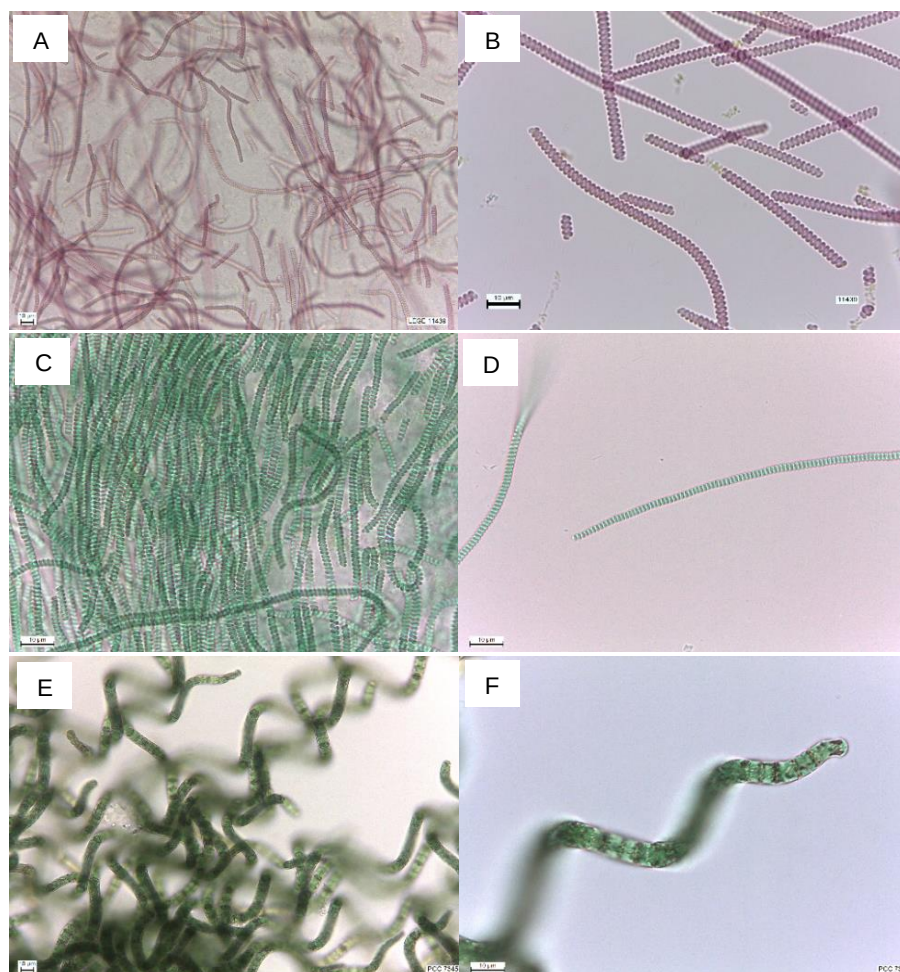


Figure 1 - Optical microscope images of the three cyanobacteria strains utilized in this work. Top – *Spirulina* sp. LEGE 11439 (A – 40X/0.65; B – 100X/1.25); Middle – *Spirulina* sp. (C – 40X/0.65; D – 100X/1.25); Bottom – *Arthrospira platensis* PCC 7345 (E – 40X/0.65; F – 100X/1.25).

strains and one *Arthrospira platensis* strain. The BBE group at CIIMAR possesses its inhouse culture collection, LEGE-CC, which possesses more than 1000 strains, comprising 46 of the known genera of cyanobacteria [76]. The first *Spirulina* sp. strain, which presents a reddish color, was from the LEGE-CC, and it was collected from the Atlantic Ocean, near the Matosinhos Beach in Portugal. The second *Spirulina* sp. was isolated by the BBE team researchers responsible for the LEGE-CC, despite not being included in the collection, as it was retrieved from the Atlantic Ocean in near the Matosinhos Beach, and exhibits a strong green color. The *Arthrospira platensis* was ordered from the Pasteur Culture Collection (PCC), as this culture collection belongs to the Laboratory Collection of Cyanobacteria in the Department of Microbiology from the Institut Pasteur and it was collected from a slough in Del Mar, San Diego, California, USA.

The two *Spirulina* strains were maintained in the same culture medium throughout their growth, Z8 medium (Appendix I) with tropical marine salt at a concentration of 25 grams per liter (Z8-25) supplemented with vitamin B12. However, upon arrival, the *A. platensis* strain was received in a BG11 + ASNIII (1:1, v/v) medium, and the strain was transferred to other culture media since the ASNII could not be prepared, as some of the components for this medium were not available. Four different mediums were tested, BG11 (Appendix II), BG11 with tropical marine salt (25 g/L), Z8 and Z8-25 with vitamin B12. The strain was isolated in 40 mL of each in a 50 mL culture flask and after a month, only the latter one, Z8-25 with vitamin B12, was shown to maintain the morphological conditions it exhibited on arrival. Consequently, the same medium was used for all three strains during this study. The deionized water with the salt already added in and needed glassware were always autoclaved, and each isolation and culture scale-up were done in sterile conditions in Telstar Bio II Advance laminar flow chamber, for minimal chances of contamination. Each cyanobacterial culture started with a small amount of biomass being transferred into 40 mL of medium in a 50 mL culture flask, and once enough biomass had been grown, the culture was upscaled to 400 mL of medium in a 500 mL culture flask and finally to 4 L in a 6 L balloon glass bottle. Once the isolation or scale-up was done, the cultures were placed in the culture room of the Bioterium of Aquatic Organisms (BOGA) at CIIMAR, which kept the cultures at 25°C, with a photoperiod of 16:8 (16 hours of light : 8 hours of dark), and both green strains were kept in places where the light intensity was of 60-100 $\mu\text{mol.m}^{-2}\text{s}^{-1}$, while the reddish strain was kept at 10-50 $\mu\text{mol.m}^{-2}\text{s}^{-1}$, as this strain was more sensitive to light. Aeration was employed on all 4L cultures, as to enhance cyanobacterial growth by renewing atmospheric CO₂ inside the flask and by forcing movement of the medium and biomass.

2.3 Growth manipulation through light profiling

To assess the influence of different light conditions on the growth and metabolite production of the three cyanobacteria strains and their respective bioactivity, the Algem® professional-grade lab-scale photobioreactors, by Algenuity, were utilized (Figure 2). The system from Algenuity consists of two individual photobioreactors that allow for the manipulation of light conditions, the selection of different color profiles, through light emitting diodes (LEDs), as well temperature control. It also possesses the ability to measure the optical density (OD) of the culture, however, due to the nature of the macroscopic morphology of the *Spirulina* genera strains, this option was not employed. Moreover, the pH control system, through the injection of CO₂ into the culture, was not used, for the experiments on the green *Spirulina* sp. and *Arthrospira platensis* PCC 7345, due to hardware problems. At CIIMAR, three Algem® systems are present, with six photobioreactors in total (1A, 1B, 2A, 2B, 3A, and 3B).

After enough biomass had been grown, it was collected from the balloon glass bottles and equally divided into six 600 mL Algem® Erlenmeyer flasks, with 500 mL of new Z8 25 medium, supplemented with vitamin B12. This procedure was done inside the Telstar Bio II Advance laminar flow chamber, and the Erlenmeyer flasks were previously autoclaved, along with their respective bungs, to ensure the minimum chance of outside contamination.



Figure 2 - Algem two photobioreactor system by Algenuity.

To start an assay, the photobioreactors had to be setup individually through the Algenuity software, by selecting the conditions required for each parameter (light intensity, light color, photoperiod, temperature, rotation, and gas flow rate) (Figure 3). Each assay had a three-week duration, where the photobioreactors were kept turned on for the whole period.

Photobioreactor 1A was the one representing the conditions most similar to the ones in BOGA in terms of white light intensity, while for the photobioreactor 1B the utilization of a higher white light intensity was assessed, and in the 2A, the effects of a continuous photoperiod were tested. Photobioreactors 2B and 3A were employed to observe the impact of red and blue light, respectively, on the growth of the strains, while in the photobioreactor 3B, for the first two weeks only blue light was on, while for the final week, the light was switched to red (Table 1-3).

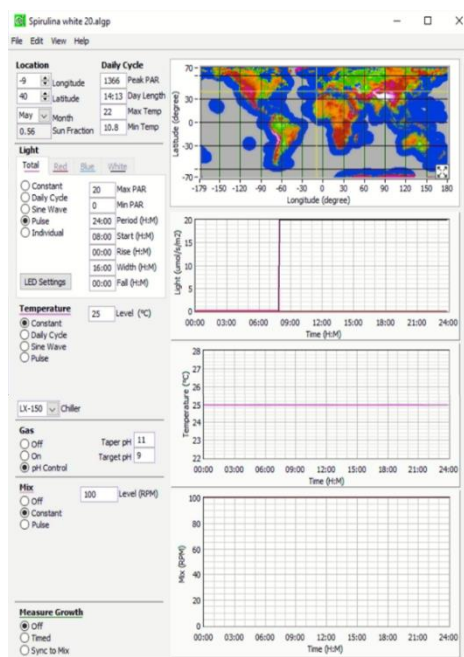


Figure 3 - Algem® inbuilt software. In this image the conditions for the *Spirulina* sp. LEGE 11439 culture in photobioreactor 1A are represented.

Table 1 - Selected parameters for each light profile for all six photobioreactors (1A, 1B, 2A, 2B, 3A, 3B blue light two weeks and red light one week) for the *Spirulina* sp. LEGE 11439 assay.

Bioreactor	Light Profile	Light Intensity ($\mu\text{mol.m}^{-2}.\text{s}^{-1}$)	Photoperiod	Rotation (rpm)	Temperature ($^{\circ}\text{C}$)
1A	White	20	16h ON – 8h OFF	100	25
1B	White	50	16h ON – 8h OFF	100	25
2A	White	20	24h ON	100	25
2B	Red	20	16h ON – 8h OFF	100	25
3A	Blue	20	16h ON – 8h OFF	100	25
3B	Blue	20	16h ON – 8h OFF	100	25
	Red	20	16h ON – 8h OFF	100	25

Table 2 - Selected parameters for each light profile for all six photobioreactors (1A, 1B, 2A, 2B, 3A, 3B blue light two weeks and red light one week) for the *Spirulina* sp. LEGE 11439 assay

Bioreactor	Light Profile	Light Intensity ($\mu\text{mol.m}^{-2}.\text{s}^{-1}$)	Photoperiod	Rotation (rpm)	Temperature ($^{\circ}\text{C}$)
1A	White	80	16h ON – 8h OFF	100	25
1B	White	160	16h ON – 8h OFF	100	25
2A	White	80	24h ON	100	25
2B	Red	80	16h ON – 8h OFF	100	25
3A	Blue	80	16h ON – 8h OFF	100	25
3B	Blue	80	16h ON – 8h OFF	100	25
	Red	80	16h ON – 8h OFF	100	25

Table 3 - Selected parameters for each light profile for all six photobioreactors (1A, 1B, 2A, 2B, 3A, 3B blue light two weeks and red light one week) for the *Arthrospira platensis* PCC 7345 assay.

Bioreactor	Light Profile	Light Intensity ($\mu\text{mol.m}^{-2}.\text{s}^{-1}$)	Photoperiod	Rotation (rpm)	Temperature ($^{\circ}\text{C}$)
1A	White	80	16h ON – 8h OFF	100	25
1B	White	160	16h ON – 8h OFF	100	25
2A	White	80	24h ON	100	25
2B	Red	80	16h ON – 8h OFF	100	25
3A	Blue	80	16h ON – 8h OFF	100	25
3B	Blue	80	16h ON – 8h OFF	100	25
	Red	80	16h ON – 8h OFF	100	25

Once each assay ended, the biomass from each photobioreactor was collected, by filtrating the contents of the Erlenmeyer flasks through a 40 μm nylon mesh sieve, while discarding the medium and rinsing the biomass with distilled water. The biomass was placed in pre-weighted, sterile, and identified 100 mL plastic containers. The biomass in the containers was then left to freeze in the -20°C freezer overnight. The frozen biomass was consequently freeze-dried in a Telstar Lyoquest Freeze Drier, and the resulting dried biomass and container were weighed.

2.4 Biomass extraction

In total, three different experiments (one for each cyanobacterial strain) were carried out, with each assay providing six quantities of biomass grown under contrasting conditions.

The biomass extraction process was similar for all 18 biomasses. Firstly, the dried biomass was grinded into a powder-like consistency, to improve the process of solvent extraction. The biomass was sonicated in an ultra-sonication bath, each time in 24 mL of a 2:1 mixture of dichloromethane (DCM) and methanol (MeOH) (16 mL : 8 mL), which was used as the solvent. The process was repeated three to six times, depending on the amount of biomass and on how much extract was present on the solvent mixture at the end of each bath, as it was repeated until the solvent mixture became transparent after a repetition. A single sonication had the duration of 10 minutes, and after two repetitions in deionized water baths at room temperature, the remaining ones were done at 40°C. The number of deionized water baths at 40°C executed on each biomass from the different strains was dependent on the transparency of the solvent solution at the end of each repetition (Appendix III). After each sonication repetition, vacuum filtration was performed on the solvent mixture with the biomass, so to retrieve the solvent with the extract into a round bottom-flask, leaving behind the biomass to be sonicated again (Appendix IV, Figure IV.1). Finally, the solvent mixture was evaporated from the flask under vacuum on a rotavapor, on constant rotation, while in a partial 30°C distilled water bath. Once only the crude extract was left on the round bottom-flask, the extract was again dissolved in DCM and MeOH (2:1), to transfer the entirety of it into a previously weighted 8 mL glass vial. The solvent mixture of the glass vial was then evaporated under vacuum in a rotavapor. All the resulting vials were left in a desiccator under high vacuum, overnight, to evaporate any remaining solvent from the crude extract, and they were weighed as soon as they were taken out to avoid any condensation.

2.5 Fractionation of the extracts

Fractionation of every single extract was performed, by solid phase extraction (SPE) in 1 and 2 g silica (Si) columns from Phenomenex, inserted in a Visiprep™ SPE vacuum manifold through a step-by-step increased solvent polarity extraction, also known as ABC extraction (Appendix III, Figure III.2). The first solvent used was hexane, which is non-polar, the second and third solvents were ethyl acetate and methanol respectively, which are polar. The size of the Si column was determined by multiplying the mass of the extract by 30, and for some extracts from the LEGE 11439 strain (1A, 1B, and 3B), 2 g Si columns were used. However, due to a shortage in their quantity, 1 g columns were used for the other half of the extracts from said strain (2A, 2B, and 3A). For the following two strains, only 1 g columns were utilized. The amount of solvent used in each step was established at 4 mL, as in the fractionation of extract 1A from LEGE 11439 12 mL were used, which resulted in all the extract having been carried out of the Si column in the first two fractions (A and B).

Before beginning each fractionation, the extract in each 8 mL vial was first dissolved in 2 mL of hexane, and the Si columns were activated with hexane before turning on the vacuum pump, so the Si column would not break.

The amount of solvents used in each step were as shown in Table 4. After the last step of each fraction, the retrieving round-bottom flask inside of the manifold was replaced for another clean one, resulting in three fractions for every extract from each specific light condition, eighteen for each strain (Table 5).

Table 4 - Solvents and the quantities used in each step of the fractionation through increased solvent polarity extraction (ABC extraction).

	Hexane (mL)	Ethyl Acetate (mL)	Methanol (mL)
Fraction A	4.0	-	-
	3.2	0.8	-
	2.4	1.6	-
Fraction B	1.6	2.4	-
	0.8	3.2	-
	-	4.0	-
	-	3.0	1.0
Fraction C	-	1.0	3.0
	-	-	4.0
	-	-	4.0

Each round-bottom flash was attached to a rotavapor so the solvents could evaporate, and once that was done, the fraction was redissolved in a small amount of the solvents used for that specific fraction. Then it was transferred to a pre-weighed 4 mL vial and filtered through tightly packed cotton in a glass Pasteur pipette to remove cell fragments. The solvents in the vial were then evaporated in the rotavapor and placed in the desiccator under high vacuum overnight to fully remove any remaining solvents. The vials were weighed right after being removed from the desiccator and the fraction mass annotated.

Table 5 - Number attributed to each resulting fraction from all photobioreactors. The same attribution was done for all three cyanobacteria strains.

1A			1B			2A			2B			3A			3B		
A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18

2.6 Zebrafish Nile Red fat metabolism assay

For the zebrafish Nile red fat metabolism assays, dimethyl sulfoxide (DMSO) solutions of the fractions were prepared at a concentration of 10 mg/mL.

The experimental procedure for this assay was first described by Jones *et al.* in 2008, as well as by Sara Freitas *et al.* in 2019 [41,72]. The protocol executed in this work was similar to the ones mentioned, with slight modifications to ensure higher zebrafish eggs viability. This work did not require the approval by an ethics committee since the experimental methods done are not considered animal experimentation according to the EC Directive 86/609/EEC for animal experiments. For every strain at least two bioassays were done, each in a different week, to confirm and reduce bias of the results, for LEGE 11439 three bioassays were carried out.

The reproduction of the *D. rerio* was performed by BOGA technicians, as in the day prior to fertilization the male and female specimens are separated into different water tanks. On the day of the start of the procedure, on the late afternoon, they are placed back together in water tanks that simulate the habitat where they would usually reproduce in the wild. On the next morning the fertilized eggs with some of the water from the tanks are retrieved in a flask. The eggs are then cleaned by gently pouring them with the tank water through a drainer, which retains the eggs, these are then rinsed and transferred to a petri dish with egg water (EW), a 60 µg.mL⁻¹ sea salt medium. Any debris, dead or non-viable eggs and feces are discarded, and the eggs are divided into petri dishes with 20 mL of E3 medium (5 mM NaCl, 0.33 mM MgCl₂, 0.33 mM CaCl₂, 0.17 mM KCl) plus methylene blue (MB) (0.0001%) to prevent the growth of fungi around the eggs and embryos [77]. Fifty eggs are placed into each petri dish and then stored in an incubator at 28°C.

On the next day, 1 dpf, 10 mL of the E3 + MB medium is renewed, while adding 2 mL of a working solution of 1-phenyl 2-thiourea (PTU), being at a 200 µM concentration in the E3 medium, to inhibit zebrafish pigmentation and provide a better optical transparency of the *D. rerio* larvae.

At 3 dpf the medium in the petri dishes is cleaned and the embryos and larvae that have hatched are separated according to their stage of development after observation under a stereo lab microscope. In a 48-well plate, six to eight larvae are placed into each well with a plastic Pasteur pipette, and the medium is almost completely removed from the well using a syringe, new E3 medium is added, without the MB. The larvae are then exposed to the fractions pipetted into each well, obtaining a final concentration for each fraction 10 µg.mL⁻¹ in each well. For the baseline control, DMSO (0.1%) was pipetted into three wells, as this was the solvent used for the fraction and

for the positive control, a stock solution of 50 μ M resveratrol (REV) was pipetted into two wells.

The next day, 4 dpf, E3 medium and the fractions and compounds are renewed, while also removing any egg shells or dead larvae. After this, the lipid staining was executed with Nile red in each well, at a final concentration of 10 ng/mL.

At 5 dpf, the *D. rerio* larvae are anesthetized with 20 μ L of 0.03% tricaine (MS-222), and the neutral lipid fluorescence was analyzed in a Leica DM6000 B fluorescence microscope.

2.7 Statistical analysis

The lipid fluorescence from every viable individual zebrafish larva was quantified using the program ImageJ. Significant differences between the solvent control group and exposure groups were examined by the program Graph Pad Prism 8. Firstly, the Gaussian distribution was assessed through Shapiro-Wilk, D'Agostino-Pearson, and Kolmogorov-Smirnov normality tests (P value < 0.05). The One-Way ANOVA test was employed to determine whether there were any statistically significant differences among the baseline control (DMSO), the positive control (REV) and the cyanobacterial fractions. If the data followed a parametric distribution, the One-Way ANOVA was executed with the Dunnett posthoc test (P value < 0.05), while the Kruskal-Wallis with Dunn's posthoc test (P value < 0.05) was chosen if the data followed a non-parametric distribution. The resulting data was presented as box-whisker graph plots with values in 5-95 percentiles, and the fractions which exhibited significant differences in values were annotated with the symbol * (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$).

2.8 Metabolite profiling

2.8.1 Tandem mass spectrometry analysis

Nine fractions, three from each cyanobacterial strain grown in photobioreactor 1A were chosen for MS/MS analysis. Fractions A were dissolved in isopropyl alcohol, while fractions B and C were dissolved in acetonitrile. Fractions were then filtered, through Minisart syringe filters, and transferred into LC-MS certified vials. Blank samples were also made, three for each solvent. The final fraction concentration in each vial was established at 1 mg.mL⁻¹. The vials were then sent for characterization by liquid chromatography high resolution electrospray ionization mass spectrometry (LC-HRESI-MS/MS) in a Q Exactive™ Focus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer with electrospray ionization in positive mode, controlled by the software Xcalibur 4.1 (Thermo Scientific). The UV absorbance of the eluate was set at 254 nm, 300 nm, 455 nm and 600 nm and the MS scan was realized fully at a 70,000 FWHM (full width at half

maximum; range of 150-2000 m/z) resolution and the data-dependent MS² (ddMS², discovery mode) with a resolution of 17,500 FWHM (normalized collision energy was 35 and the isolation window used was 3.0 amu). 5 μ L of each sample was injected and the temperature was kept at 40 °C. The flow rate of the elution of the samples was 0.35 mL.min⁻¹, and these were eluted at a decreasing gradient, starting at 99.5% and going to 10% of the mobile phase A (95% water : 5% MeOH : 0.1% formic acid) with the mobile phase B being at an increasing gradient (95% isopropyl alcohol : 5% MeOH : 0.1% formic acid) until 9.5 minutes, decreasing from then and reaching initial conditions at 17 minutes, each run lasted 20 minutes. The capillary temperature was set at 262.5 °C, while the voltage of the heated electrospray ionization (HESI) was 3.5 kV and the sheath and auxiliary gas flow rates were established at 50 and 12.5 (arbitrary units according to the equipment software).

2.8.2 Molecular networking

The obtained raw spectra files (RAW format) from the LC-MS analysis were initially visualized and interpreted by resourcing to XCalibur (version 4.1) by Thermo Scientific. The RAW files with the LC-MS data were then converted to the mzML format by employing MSConvert present in the ProteoWizard software (version 3.0.22223). The mzML files were then uploaded into the GNPS (Global Natural Products Social Molecular Networking) online servers through the employment of the WinSCP software for Windows. This allowed to run the Data Analysis option in the GNPS server on selected mzML files, as these files were Orbitrap (high resolution) data, the Precursor Ion Mass Tolerance (PIMT) and Fragment Ion Mass Tolerance (FIMT) were both set at 0.02 Da. This allowed to obtain molecular networks, which could be visualized on Cytoscape (version 3.9.1). On the GNPS web server additional programs were run on the MS/MS spectra obtained from the previous Data Analysis, namely MS2LDA, DEREPLICATOR, DEREPLICATOR+ and MolNetEnhancer. These programs allowed for the search and putative identification of the compounds that differ from the active and non-active fractions, as GNPS compared the data to preexisting MS/MS spectrums already uploaded in many databases [78]. As some spectrums present in the server dataset come from low-resolution equipment, the PIMT and FIMT were set at 2.0 and 0.5, respectively.

The GNPS analysis provided hits with previous uploads recorded in the databases and non-identified hits, regarding the metabolites present in the analyzed fractions. For the purpose of identifying the compounds responsible for differences in bioactivity, manual searches were made on several Natural Products databases such as

The Natural Products Atlas (npatlas.org), MoNA – Mass Bank of North America (mona.fiehnlab.ucdavis.edu) and MassBank Europe (massbank.eu).

2.9 Zebrafish appetite reduction bioassays

For the zebrafish appetite reduction bioassays, the *D. rerio* larvae were maintained and prepared in the same way as for the anti-obesity bioassays.

Two different protocols were employed to assess appetite reduction bioactivity of 9, already prepared, fractions, obtained from a commercially available *Arthrospira platensis* dried biomass (Spirulina pills - ISWARI™) [79]. The fractionation was manually executed through vacuum liquid chromatography, while using MeOH, hexane and ethyl acetate as the solvents. The 9 fractions were tested in two separate bioassays, for each protocol, and they were named from A to I (Fraction 1 – A; Fraction 2 – B; Fraction 3 – C; Fraction 4 – D; Fraction 5 – E; Fraction 6 – F; Fraction 7 – G; Fraction 8 – H; Fraction 9 – I).

The exposure of the zebrafish larvae to the compounds is executed similarly in both bioassays. In a 48-well plate, at 6 dpf, five to seven larvae are placed into each well, using a plastic Pasteur pipette. Consequently, the medium is almost completely removed from each well, with the aid of a syringe. New E3 medium is then added into each larvae-containing well, and the fraction stock solution or compounds are then added, achieving a final exposure concentration of 10 µg.mL⁻¹. DMSO was used as the baseline control, while for the positive control, a stock fluoxetine hydrochloride solution (10 mM) was used. Fluoxetine inhibits the selective reabsorption of serotonin, and its administration results in a decrease in appetite of the individual. Fluoxetine is mostly used in people for the treatment of depression, bulimia nervosa, obsessive compulsive disorder, and a few other psychological complications, while possessing the side-effect of appetite loss [74].

The final exposure concentration of each fraction was at 10 µg.mL⁻¹, and the plate was kept in an incubator with the temperature set at 28°C for 18 hours.

2.9.1 *Paramecium bursaria* appetite reduction bioassay

In this bioassay, cultures of the ciliate *Paramecium bursaria* (*P. bursaria*) were utilized, this eukaryote lives in a mutual endosymbiotic relationship with green microalgae [75]. The *P. bursaria* were acquired from the Culture Collection of Algae of Protozoa (CCAP). The cultures of *P. bursaria* were maintained in 50 mL culture flasks with 40 mL of SES medium, a soil extract medium which is mostly used to maintain protozoa. The medium inside these flasks was renewed every three to four weeks, by equally dividing the contents of each 40 mL culture into two 50 mL culture flasks, and adding 20 mL of fresh medium into each, obtaining two 40 mL cultures.

Once enough *P. bursaria* had been grown, they were transferred into a new growth medium. In a cleaned 2 L Erlenmeyer flask, 10 grains of oats, which were previously boiled in distilled water, 900 mL of distilled water, a full 40 mL *P. bursaria* culture, and 0.5 g of yeast were added. The flask contents were mixed and the *P. bursaria* were allowed to grow at room temperature for around 21 days.

Following the 21 days, while making sure the ciliates were growing well by assessing the state of the culture, the *P. bursaria* were harvested through sequential filtrations. The first filtration was executed through a funnel using a gauze, to retrieve the macroparticles, which mostly resulted from the oats and large clusters of microalgae. The filtrate was retrieved and filtered a second time through gauze and cotton, previously moistened with water, and then a third time using the same material, to ensure a low microalgae density in the resulting filtrate. The third and fourth filtration were done by employing a 40 µm and 1 µm filter, respectively. On the fourth filtration, the paramecia were retained in the filter, therefore, they were washed with EW and collected with a plastic Pasteur pipette, and placed into 50 mL Falcon tubes, this last step was repeated until no *P. bursaria* were left retained on the filter. The zebrafish larvae were fed the collected *P. bursaria* at 5 dpf, to make the zebrafish accustomed to live food.

To calculate the volume of solution needed for the bioassay, so as to ensure the presence of around 2115 *P. bursaria* per well, the number of wells to be tested were counted. Following this, the number of paramecia present in 1 mL of solution was counted with the Sedgewick Rafter Cell S50 counting chamber. Once the needed volume was established (5 mL more than the exact number), the *P. bursaria* were stained with the molecular probe 4-Di-10-ASP (4-(4-(Didecylamino)styryl)-N-Metilpyridinium Iodide) (2.5 mg/mL). 4-Di-10-ASP was added into the paramecia solution in a ratio of 1:500, while protecting it from light, and gently shaken by reversing the Falcon tube. The stained *P. bursaria* solution was then left in continuous agitation for 1 hour, while being protected from light. After the 1 hour, the paramecia from the solution were washed, by filtrating the solution through a 1 µm filter, while washing them with EW.

At 7 dpf, after the 18 hours of exposure to the fractions and compounds, the E3 medium each well was carefully taken out with a syringe, and it was renewed with 750 µL of the stained *P. bursaria* solution. Once the renewal of each well was complete, the plate was protected from light and stored in the incubator at 28°C for 2 hours. Finally, the zebrafish larvae were analyzed under the fluorescence microscope, in the same way as in the zebrafish Nile Red fat metabolism bioassays.

2.9.2 Liposome appetite reduction bioassay

Egg yolk aliquots were prepared, by manually separating the egg yolks from the egg whites and homogenizing them in the vortex. 1 mL of the egg yolk homogenize was stored in 1.5 mL Eppendorf tubes and kept at -20°C.

At 7 dpf, a working egg yolk solution was prepared, by mixing 1 mL of thawed egg yolk and 19 mL of EW, in a 50 mL Falcon tube, resulting in a 5% egg yolk working solution. This solution was then vortexed for 1 minute and 30 seconds, so the EW and the egg yolk could emulsify properly. Consequently, to discard any conglomerates of egg yolk, the working solution was vacuum filtered through a 55 µm filter, twice, switching the filter after the first filtration, while transferring the filtrate onto a new Falcon tube. This resulting solution was then subject to sonication to obtain the liposomes. This step was done by employing the UP200Ht Handheld Ultrasonic Homogenizer (Hielscher Ultrasound Technology™), with its conditions set as follows – P-10W (power); C-50% (pulse); A-20% (amplitude). The sonication repeated 5 times, in 5 cycles, 25 times total, with a 5 second pause between cycles.

The fluorophore BODIPY FL-C16 (4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-s-Indacene-3-Hexadecanoic Acid), a fluorophore, was used to label the liposomes. The BODIPY FL-C16 stock solution (6.4 mM) was diluted at a ratio of 1:1000 in the 5% egg yolk solution, resulting in a final concentration of 6.4 µM. After the BODIPY FL-C16 addition, the solution was vortexed at maximum speed for 1 minute and then left on continuous agitation, while protected from light, for 20 minutes. Consequently, the egg yolk + BODIPY FL-C16 solution was diluted once more at a 1:8 ratio, resulting in a final egg yolk concentration of 0.625%. After confirmation of successful staining, by resorting to the Leica DM6000 B fluorescence microscope, the zebrafish larvae were fed the egg yolk solution.

The feeding was done after the 18 hours of exposure to the fractions and compounds, as the E3 medium in each well was carefully taken out with a syringe, and it was renewed with 750 µL of the 0.625% stained egg yolk solution. Once the renewal of each well was complete, the plate was protected from light and stored in the incubator at 28°C for 2 hours. Finally, the zebrafish larvae were analyzed under the fluorescence microscope, in the same way as in the zebrafish Nile Red fat metabolism bioassays.

Results

3.1 Effects of light conditions on *Spirulina* sp. LEGE 11439, *Spirulina* sp. and *Arthrospira platensis* PCC 7345

Before placement onto the Algem® Erlenmeyer flasks, all the cyanobacteria biomass from the three strains was ensured to be uniform in their respective macroscopic characteristics (Appendix V). After the three weeks in the Algem® photobioreactors with each one being under different light conditions for that duration, some differences regarding the biomass color and the manner in which it aggregated could be discerned.

In normal conditions and when healthy, *Spirulina* sp. LEGE 11439 presents a strong reddish color, while forming strong biofilms mats and aggregating in dense biomass clusters.

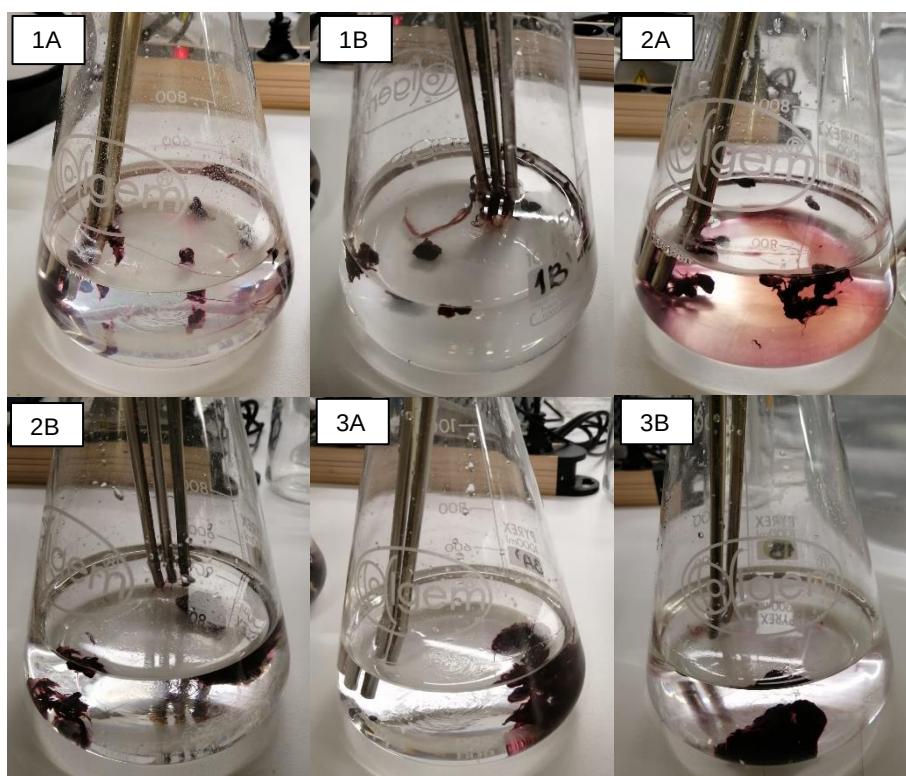


Figure 4 – Images of *Spirulina* sp. LEGE 11439 biomass in each Algem® Erlenmeyer flask (1A, 1B, 2A, 2B, 3A, 3B) after the termination of the growth condition manipulation assay.

In photobioreactor 1A, the biomass color remained the same while aggregating in more but smaller clusters, while in photobioreactor 1B, the biomass presented a much duller red color while aggregating in similar clusters as 1A. In the photobioreactor where no dark period was employed, photobioreactor 2A, the biomass color remained the same, while aggregating in clusters conjoined by filaments and a biofilm mat formed around the inside of the flask. For the red light 2B photobioreactor, the biomass color

remained the same, while aggregating in a larger single biomass cluster. In photobioreactor 3A, the biomass color presented a stronger red color, while aggregating in medium sized clusters. Finally, in photobioreactor 3B, the color of the biomass remained the same, while aggregating in a larger single biomass cluster (Figure 4).

Similar to the LEGE 11439 strain, when healthy and in a fair environment, the *Spirulina* sp. strain exhibits a potent green color and accumulates itself in dense biomass clusters and resistant biofilms.

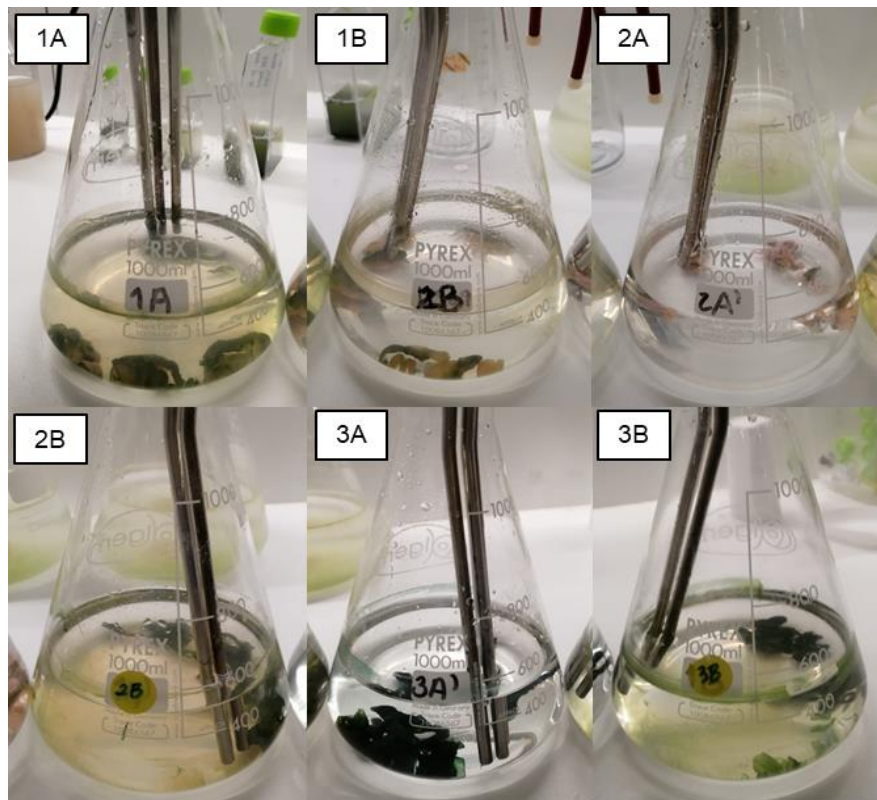


Figure 5 - Images of *Spirulina* sp. biomass in each Algem® Erlenmeyer flask (1A, 1B, 2A, 2B, 3A, 3B) after the termination of the growth condition manipulation assay.

In photobioreactor 1A, the biomass color presented a much duller green, while aggregating in medium sized clusters and the culture medium showed a yellowish color and cloudiness, while in photobioreactor 1B, half of the biomass was without any sort of pigmentation, and it was aggregated in thin, small clusters and the culture medium had a slight cloudiness. For photobioreactor 2A, most of the biomass was without any sort of pigmentation, and it was aggregated in thin, small clusters, with the culture medium being transparent overall. Photobioreactor 2B possessed biomass which showed a slightly duller green color, and it was aggregated in thin and long clusters, as the culture medium showed a yellowish color and cloudiness. In photobioreactor 3A, the color of the biomass was of a strong green color, and it was aggregated in medium sized clusters, and the culture medium was fully transparent. And in photobioreactor 3B the biomass was of a

light green, and it was aggregated in medium sized clusters, and the culture medium showed a green-yellowish color giving it a slight cloudiness (Figure 5).

Contrasting with the latter strains, *Arthrospira platensis* PCC 7345 does not aggregate in clusters and does not form biofilm mats. This genus is generally suspended in the culture medium, which could allow in a future work the automatic OD measurement, in the photobioreactors, and monitor the culture growth. When agitated it almost seems as it forms a homogeneous solution, although the biomass is macroscopically visible, exhibiting a deep green, almost blue-green color. Nonetheless, as it grows, the filaments start to aggregate in larger but still small biomass fragments, and it starts depositing (Figure 6).

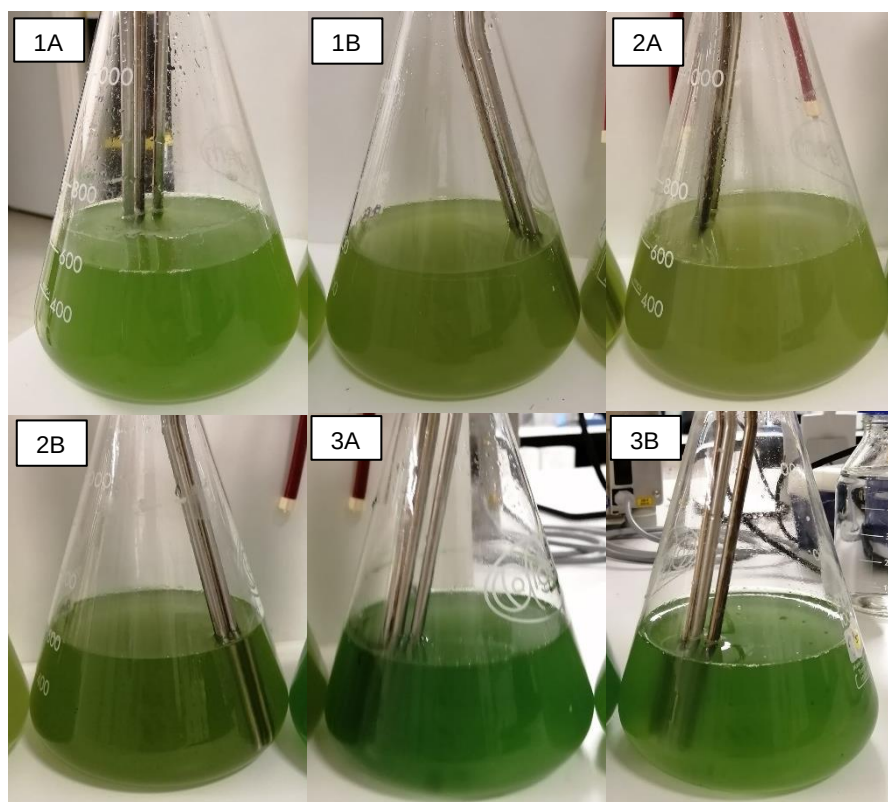


Figure 6 - Images of *Arthrospira platensis* PCC 7345 biomass in each Algem® Erlenmeyer flask (1A, 1B, 2A, 2B, 3A, 3B) after the termination of the growth condition manipulation assay.

The biomass in 1A was of a light green color, while in photobioreactor 1B, it exhibited a slightly dull green color, and in 2A, the biomass was of a very dull green. In photobioreactor 2B, the biomass had a slight green color, and in 3A, the green of the biomass was deep and almost blue-green. Finally, in photobioreactor 3B, the biomass showed a deep green.

3.1.1 Extract production

The amount of biomass of each cyanobacteria strain was not quantified prior to the start of the light manipulation experiments, to gauge if a certain condition would be more propitious to the cyanobacteria growth, as the morphology of the *Spirulina* genera strains did not allow for optical density measurement. Despite this, the amount of extract from each strain, relative to the amount of freeze-dried biomass collected from each photobioreactor was calculated (Figure 7). In general, the extraction efficiency was between 20 to 50% for all strains. The condition with the lowest yield was the continuous white light, for all three strains, while blue light was most effective on the LEGE 11439 strain, and the red light profile showed a good efficiency on the PCC 7345 strain. Nonetheless, two further repetitions of these experiments should be realized to observe if these are consistent.

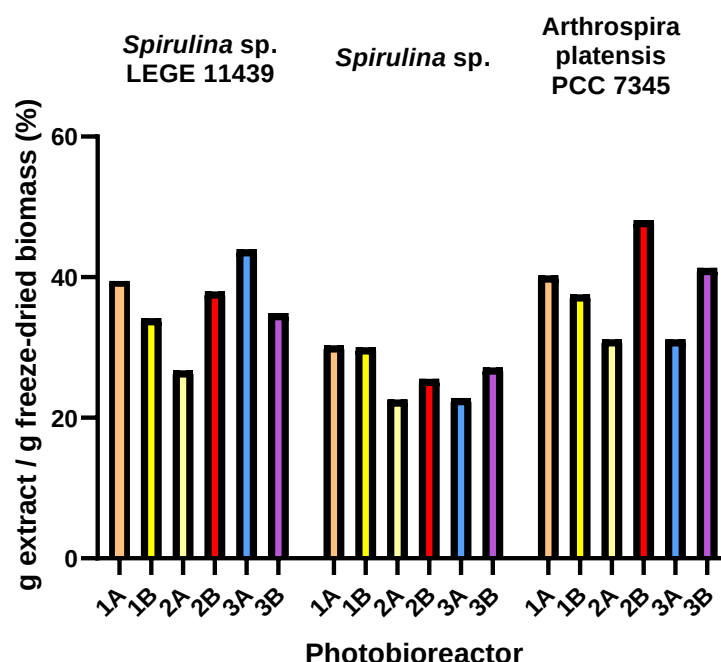


Figure 7 - Percentage of extract amount obtained relative to the weight of freeze-dried biomass from *Spirulina* sp. LEGE 11439 (left), *Spirulina* sp. (center) and *Arthrospira platensis* PCC 7345 (right) collected from each photobioreactor: 1A – normal white light (light orange); 1B – intense white light (yellow); 2A – continuous white light (light yellow); 2B – red light (red); 3A – blue light (blue); 3B – two weeks blue light and one week red light (purple).

3.2 Lipid-reducing bioactivity from the Zebrafish Nile Red fat metabolism bioassay

No fraction belonging to LEGE 11439 showed significant differences ($p < 0.05$), in the ability of neutral lipid reduction in the zebrafish larvae. Nonetheless, zebrafish larvae exposed to fraction 18, fraction C of in the photobioreactor 3B (2 weeks blue light and 1 week of red light), exhibited a neutral lipid reduction of $25.79\% \pm 27.01$ (mean $\pm$ standard deviation, SD), with a p-value of $p = 0.14$ (Figure 8 and 9).

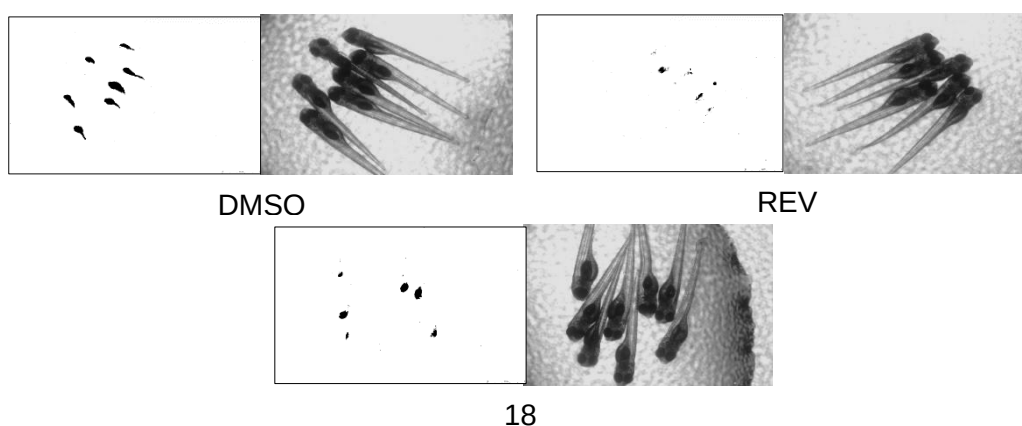


Figure 8 - Representative images of the zebrafish Nile Red fat metabolism assay of fraction 18 from *Spirulina* sp. LEGE 11439. On the left are represented the fluorescence with black contrast on white background and on the right images the bright field images of zebrafish larvae. DMSO, solvent control 0.1%; REV, positive control 50 μ M.

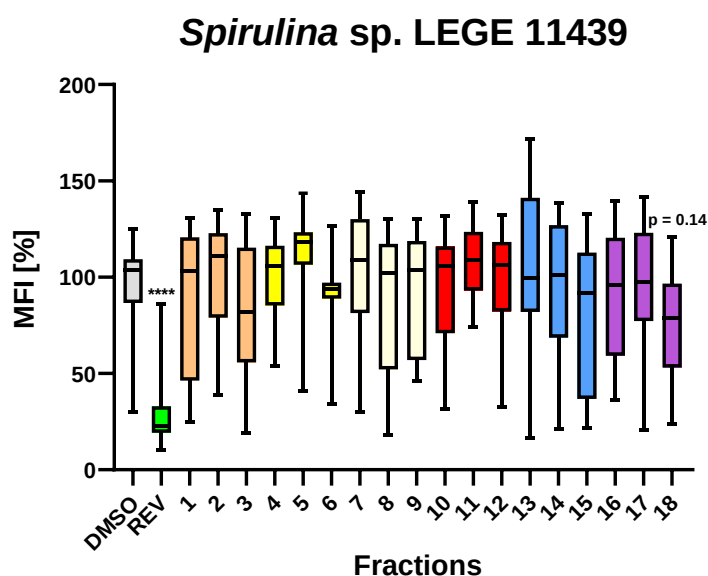


Figure 9 - Lipid-reducing activity in the zebrafish Nile Red fat metabolism assay for the fractions from *Spirulina* sp. LEGE 11439. The solvent control had 0.1% DMSO and the positive control 50 μ M REV. Values are expressed as mean fluorescence intensity (MFI) relative to the DMSO group. The data are represented as box-whisker plots. Statistical differences were analyzed by Kruskal-Wallis with Dunn's posthoc test and are indicated to the solvent control with the symbol * ($* = p < 0.05$; $** = p < 0.01$; $*** = p < 0.001$; $**** = p < 0.0001$).

No fraction belonging to the green *Spirulina* sp. strain showed significant differences ($p < 0.05$), for their ability of neutral lipid reduction in the zebrafish larvae. In contrast, fraction 10 and 16 (both fraction type A) significantly increased the neutral lipid levels (Figure 10 and 11). Fraction 10 was from the photobioreactor 2B (red light) and fraction 16 was from the 3B (2 weeks blue light, 1 week red light).

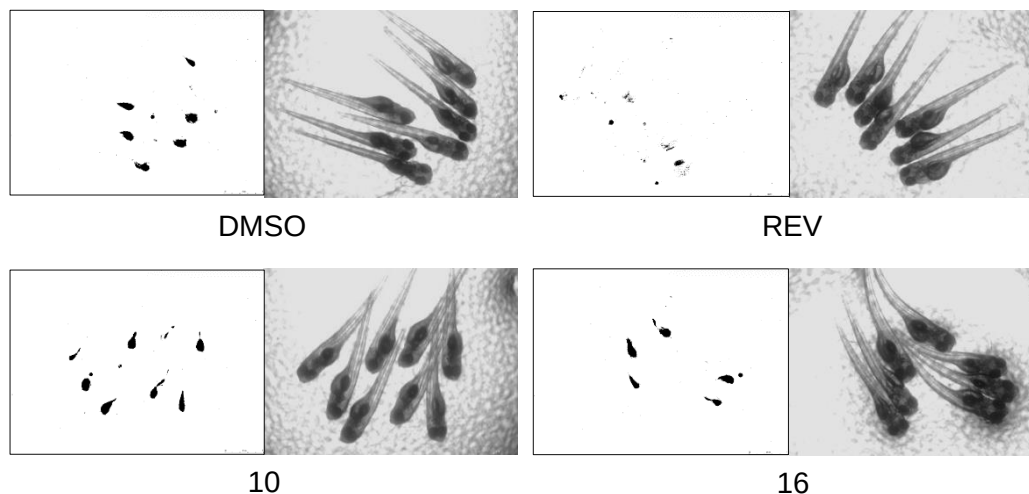


Figure 11 - Representative images of the zebrafish Nile Red fat metabolism assay of fraction 10 and 16 from *Spirulina* sp. On the left are represented the fluorescence with black contrast on white background and on the right images the bright field images of zebrafish larvae. DMSO, solvent control 0.1%; REV, positive control 50 μ M.

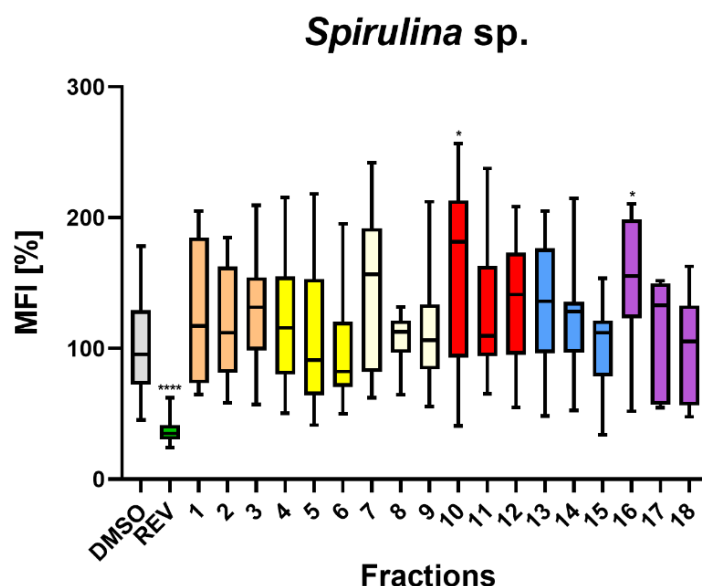


Figure 10 - Lipid-reducing activity in the zebrafish Nile Red fat metabolism assay for the fractions from *Spirulina* sp. The solvent control had 0.1% DMSO and the positive control 50 μ M REV. Values are expressed as mean fluorescence intensity (MFI) relative to the DMSO group. The data are represented as box-whisker plots. Statistical differences were analyzed by Kruskal-Wallis with Dunn's posthoc test and are indicated to the solvent control with the symbol * (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$).

Six fractions from the different biomasses of *Arthrospira platensis* PCC 7345 exhibited significant activity in reducing the neutral lipid levels of the zebrafish larvae (Figure 12 and 13).

Fraction 1, from photobioreactor 1A (white light) showed the highest significance, with a p-value of $p < 0.0001$, while fraction 16, from photobioreactor 3B (2 weeks blue light, 1 week red light), was the second highest, $p < 0.001$. Fraction 13 from the photobioreactor 3A (blue light) had a p-value of $p < 0.01$, and fractions 4, 10, and 14, all had a significance of $p < 0.05$,

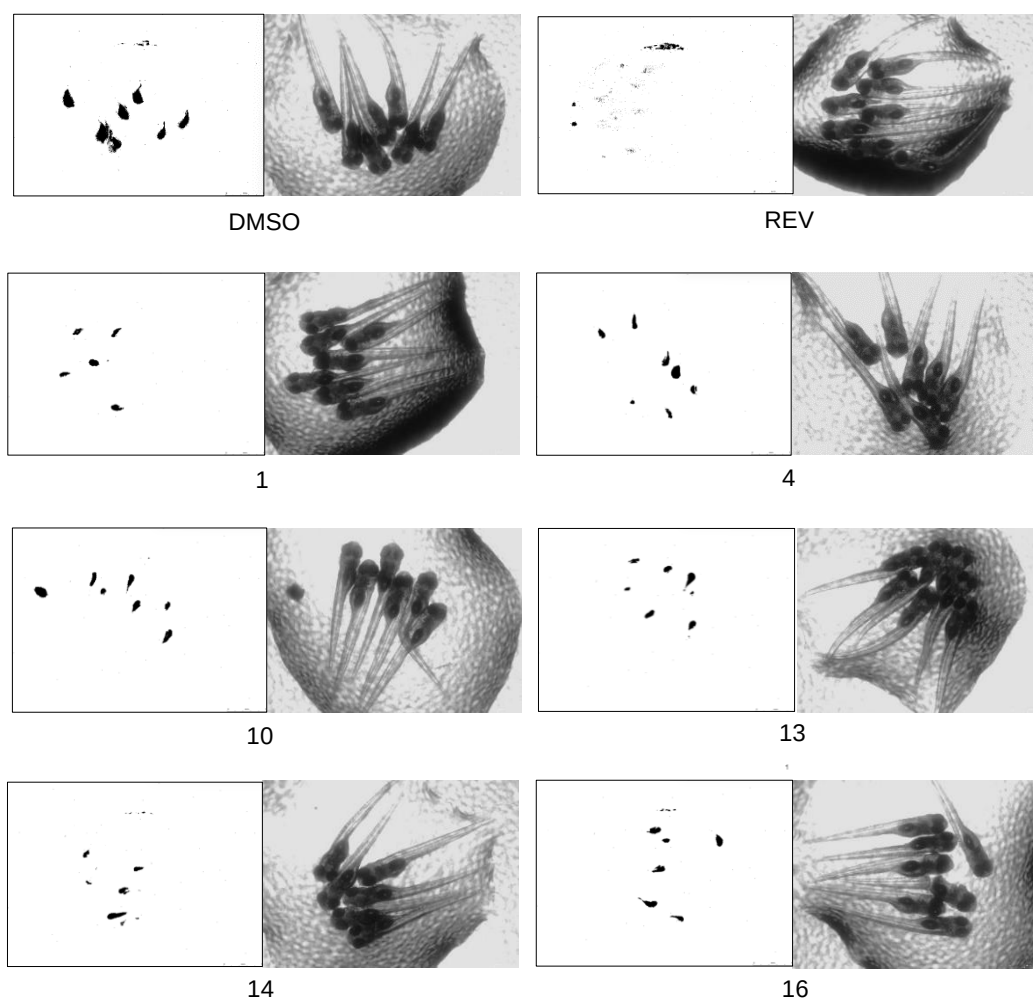


Figure 12 - Representative images of the zebrafish Nile Red fat metabolism assay of fractions 1, 4, 10, 13, 14 and 16, from *Arthrospira platensis* PCC 7345. On the left are represented the fluorescence images with black contrast on white background and on the right images the bright field images of zebrafish larvae. DMSO, solvent control 0.1%; REV, positive control 50 μ M.

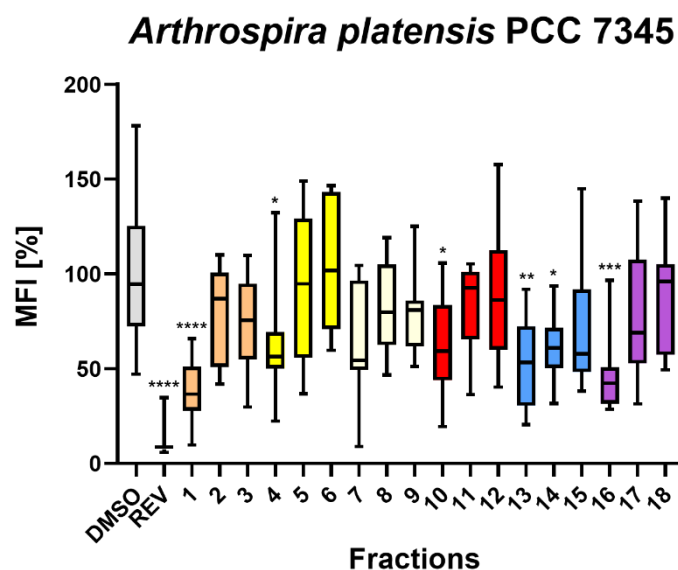


Figure 13 - - Lipid-reducing activity in the zebrafish Nile Red fat metabolism assay for the fractions from *Arthrospira platensis* PCC 7345. The solvent control had 0.1% DMSO and the positive control 50 μ M REV. Values are expressed as mean fluorescence intensity (MFI) relative to the DMSO group. The data are represented as box-whisker plots. Statistical differences were analyzed by Kruskal-Wallis with Dunn's posthoc test and are indicated to the solvent control with the symbol * (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$).

Table 6 - Lipid-reducing activity from fractions with significant bioactivity when compared to the solvent control (DMSO) (mean $\pm$ SD).

Strain	Fraction	Reduction $\pm$ SD (%)
<i>Spirulina</i> sp. LEGE 11439	18 (3B-C)	25.79 $\pm$ 27.01
<i>Spirulina</i> sp.	10 (2B-A)	- 56.64 $\pm$ 65.19
	16 (3B-A)	- 51.33 $\pm$ 50.21
<i>Arthrospira platensis</i> PCC 7345	1 (1A-A)	60.76 $\pm$ 16.37
	4 (1B-A)	39.64 $\pm$ 24.88
	10 (2B-A)	37.31 $\pm$ 24.63
	13 (3A-A)	46.62 $\pm$ 20.84
	14 (3A-B)	39.68 $\pm$ 16.47
	16 (3B-A)	35.56 $\pm$ 21.52

3.3 Profiling of cyanobacterial metabolites

Nine fractions were chosen for the LC-MS/MS analysis, three fractions (A, B, C type) from each cyanobacterial strain, all grown under the normal white light condition in photobioreactor 1A. Only one of the nine fractions showed significant anti-obesogenic activity, fraction 1 (1A - A) from *Arthrospira platensis* PCC 7345. Thus, a comparison of the three A fractions was executed to infer and identify what compounds were unique or in higher presence in fraction A of PCC 7345 compared to the other both *Spirulina* strains, and therefore eventually be responsible for its bioactivity. This comparison was made through the construction of a molecular network by employing the GNPS platform and visualization by the Cytoscape program. The molecular networks are composed of

nodes and edges, the nodes represent spectra found in the fraction, and these can be organized in clusters, connected by the edges, depending on how closely related each compound is to each other. Nodes belonging to fraction A from LEGE 11439 were colored red, the ones of the green *Spirulina* sp. were colored green, the nodes from the PCC 7345 fraction were applied the color blue, while nodes present in two or more of the fractions were colored white. 342 nodes were generated by Cytoscape, while 64 of these were unique to fraction A from PCC 7345. The relative intensities of all the nodes present in the molecular network represented spectra were also analyzed (Appendix VI). Following, these unique MS/MS spectra were manually searched in mass spectra databases, comparing them to already annotated ones present in several libraries (MoNA, GNPS and NPA).

From the 64 unique mass peaks, only 11 presented hits from the databases, with 2 of them forming a duplet (Table 7). Through the employment of the MolNetEnhancer script, it was possible to determine the superclass of certain nodes and clusters of the molecular network, as the ones surrounded by an orange line are lipids and lipid-like molecules, purple represents organic nitrogen compounds, blue are organic acids and derivatives and red are lignans, neolignans and related compounds. The two hits, which formed a duplet, are 1,2-Dioleoyl-sn-glycero-3-phospho-rac-1-glycerol and 1,2-Dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol), both identified, as they were the only compounds with a possible identification with an attributed compound superclass by the MolNetEnhancer script, that being lipids and lipid-like molecules. (Figure 14).

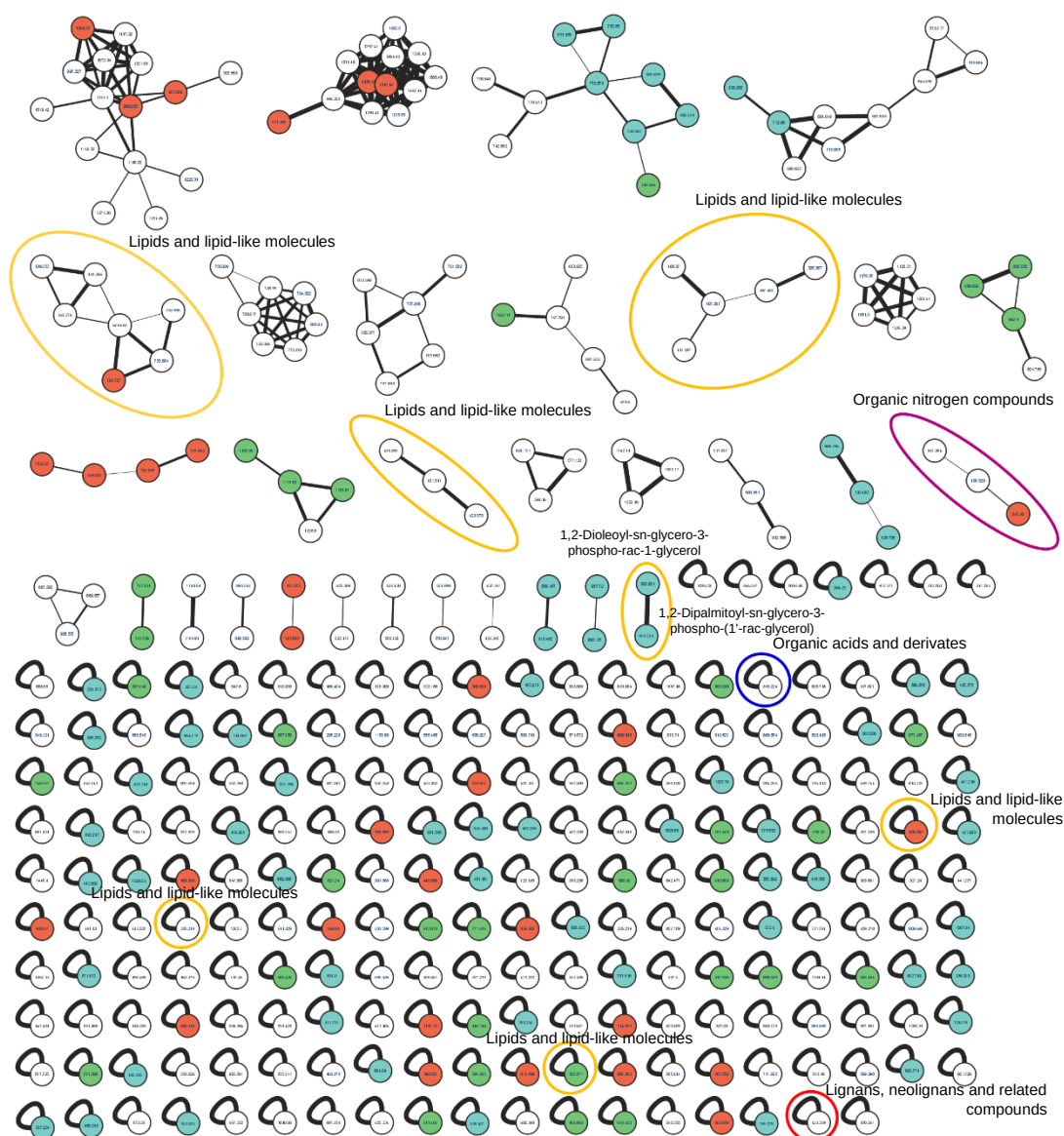


Figure 14 - Molecular network, obtained from Cytoscape through the GNPS server, of fractions 1 (1A-A) from the three cyanobacterial strains: Red – nodes from *Spirulina* sp. LEGE 11439; Green – nodes from *Spirulina* sp.; Blue - nodes from *Arthrospira platensis* PCC 7345; White - nodes from two or more strains. 1,2-Dioleoyl-sn-glycero-3-phospho-rac-1-glycerol and 1,2-Dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) are both identified near their nodes with their respective m/z, 991.288 and 1044.083 for $[M+H]^+$.

Table 7 - Possible identification of spectra unique to Fraction 1 (1A-A) of *Arthrospira platensis* present in the Global Natural Products Social Molecular Networking (GNPS) platform, MassBank of North America (MoNA) and Natural Product Atlas (NPA). GNPS identifications are based on MS/MS fragment identification in databases, while the MoNA and NPA identifications were based on MS1 data, searched for the accurate m/z value within a range of ± 0.002 Da (Dalton).

[M+H] ⁺	RT	Possible Identification	m/z error (ppm)	Compound class	Chemical Formula	Source
281.247	575.845	Linoleic acid	3.71	Fatty acyls	C ₁₈ H ₃₂ O ₂	MoNA
377.195	376.371	8-Hydroxy-2,7,7,11,15-pentamethyl-5,12,16-trioxapentacyclo[9.8.0.0(2),.0,.0(1)(3),(1)]nonadec-13(18)-ene-3,17-dione	3.83	Naphtopyrans	C ₂₁ H ₂₈ O ₆	MoNA
419.206	407.775	2-(2,6-dimethoxy-4-prop-2-enylphenoxy)-1-(3,4,5-trimethoxyphenyl)propan-1-ol	2.49	Lignans and neolignans	C ₂₃ H ₃₀ O ₇	MoNA
		6-[(1E,3E,5E)-6-[(2R,3R,3aR,4R,5R,6aS)-2-ethyl-3,4-dihydroxy-3,3a-dimethyl-2,4,5,6a-tetrahydrofuro[2,3-b]furan-5-yl]hexa-1,3,5-trienyl]-4-methoxy-5-methylpyran-2-one	2.49	Furofurans	C ₂₃ H ₃₀ O ₇	MoNA
421.203	45.179	Isoflupredone acetate	0.86	Steroids and steroid derivatives	C ₂₃ H ₂₉ FO ₆	MoNA
443.206	449.539	Diversolide A	2.35	Carboxylic acids and derivatives	C ₂₅ H ₃₀ O ₇	MoNA
		Picrolichenic acid	2.35	Benzene and substituted derivatives	C ₂₅ H ₃₀ O ₇	MoNA
461.216	451.641	2,20-dihydroxy-13-methoxy-4,7,17,22,22-pentamethyl-5,10,21,23-tetraoxahexacyclo[18.2.1.0 ^{1,17} .0 ^{4,16} .0 ^{6,14} .0 ^{8,12}]tricos-6(14),7,12-trien-11-one	3.35	Benzopyrans	C ₂₅ H ₃₂ O ₈	MoNA
		5H-Furo[3,4-i]oxepino[4,3-a]xanthene-3,12-dione, 1,2,5a,6,7,7a,	3.35	Benzopyrans	C ₂₅ H ₃₂ O ₈	MoNA

		10,14,14a,14b-decahydro-5a,6-dihydroxy-13-methoxy-5,5,7a,9,14b-pentamethyl				
753.550	777.431	Sulfoquinovosyl diacylglycerol				Dereplicator
768.561	774.003	Monogalactosyldiacylglycerol				Dereplicator
811.544	991.288	1,2-Dioleoyl-sn-glycero-3-phospho-rac-1-glycerol				Dereplicator
825.561	1044.083	1,2-Dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol)				Dereplicator
871.572	1001.921	Pheophytin A	2.11	Tetrapyrroles and derivatives	C ₅₅ H ₇₄ N ₄ O ₅	MoNA

3.4 Quantification of 13²-hydroxy-pheophytin a through MS/MS data

To confirm the presence and compare the intensity of the lipid-reducing chlorophyll derivative hpa in all three strains, the MS/MS data was uploaded to the XCalibur software, and through the QualBrowser extension, the spectra of fractions 1, 2, and 3 from each strain were analyzed. This was done by selecting Base Peak as the plot type, while selecting the mass peak of 887.5697 for [M+H]⁺, the precursor mass of hpa but also its isomer (Figure 15). The intensity of mass peaks obtained for fractions 1, 2, and 3, from the three strains are represented in Table 8.

Table 8 – Peak intensity of fractions 1, 2, and 3, from the three cyanobacteria strains, relating to the mass peak of 887.5697 for [M+H]⁺, and their respective retention time.

Strain	Fraction	Peak Intensity	Retention Time Interval (minutes.seconds)
LEGE 11439	1 (1A-A)	1.47E9	14.16 -14.73
<i>Spirulina</i> sp	1 (1A-A)	3.92E8	14.17 - 14.87
PCC 7345	1 (1A-A)	3.45E8	14.12 - 14.66
LEGE 11439	2 (1A-B)	1.61E8	14.12 - 14.75
<i>Spirulina</i> sp	2 (1A-B)	3.77E6	14.12 - 14.69
PCC 7345	2 (1A-B)	1.09E6	14.18 - 14.61
LEGE 11439	3 (1A-C)	3.96E6	14.14 - 14.68
<i>Spirulina</i> sp	3 (1A-C)	1.68E5	14.15 - 14.62
PCC 7345	3 (1A-C)	8.66E4	14.18 - 14.61

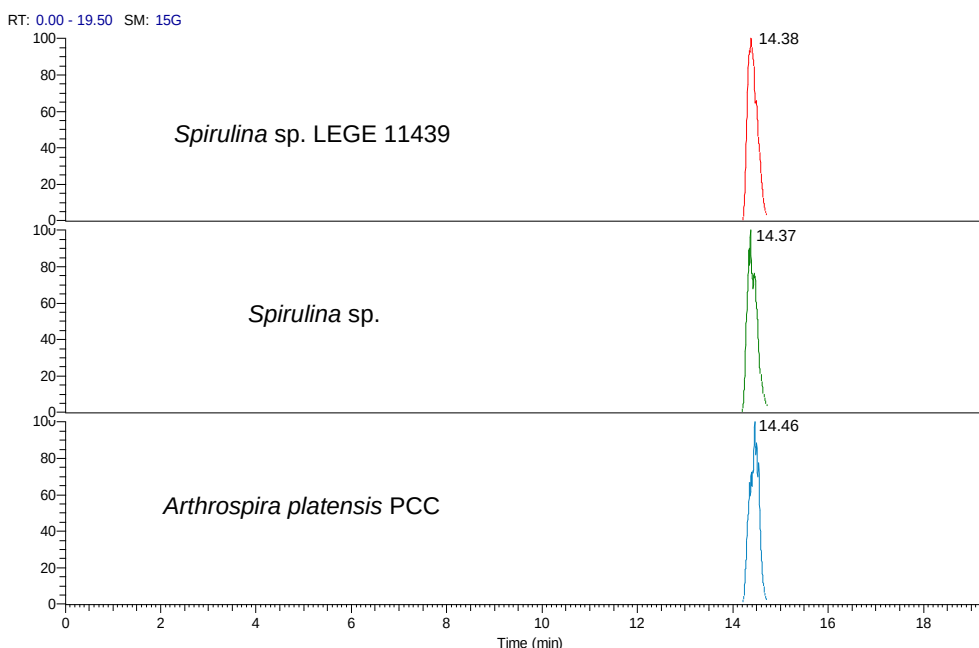


Figure 15 - LC-HRESI-MS analysis of fractions 1 from *Spirulina* sp. LEGE 11439 (top, red), *Spirulina* sp. (middle, green), *Arthrospira platensis* PCC 7345 (bottom, blue), showing the presence of hpa in said fractions. The peaks selected for analysis had a mass range of 887.5647-887.5747 and the following retention times: *Spirulina* sp. LEGE 11439 14.38 min; *Spirulina* sp. 14.37 min; *Arthrospira platensis* PCC 7345 14.46 min.

An analysis on the fragmentation of hpa in each fraction was executed, as in another work in *Arthrospira platensis* the presence of an hpa isomer that had reduced bioactivity (approximately 10-fold) than hpa itself was reported. The MS/MS fragmentation of the compounds, despite the similar m/z of 887.5694 for $[M+H]^+$, resulted in different fragment intensities for the two compounds. The higher intensity fragment from hpa was the one with an m/z of 609.27, whereas the higher intensity fragment from its isomer was had an m/z of 531.23, followed by fragments with 559.23 m/z and 591.26 m/z [79].

From the MS/MS data analysis from the two *Spirulina* spp. strains, it was possible to observe that the fragmentation of the compound with a monoisotopic m/z of 887.5694 for $[M+H]^+$, led to the fragment with an m/z of 591.26 exhibiting a higher intensity than the fragment with an m/z of 609.27. In the MS/MS data from *Arthrospira platensis* PCC 7345, the peak intensities for both fragments were similar, with the fragment with an m/z of 609.27 for $[M+H]^+$, exhibiting a slightly higher intensity (Figure 16).

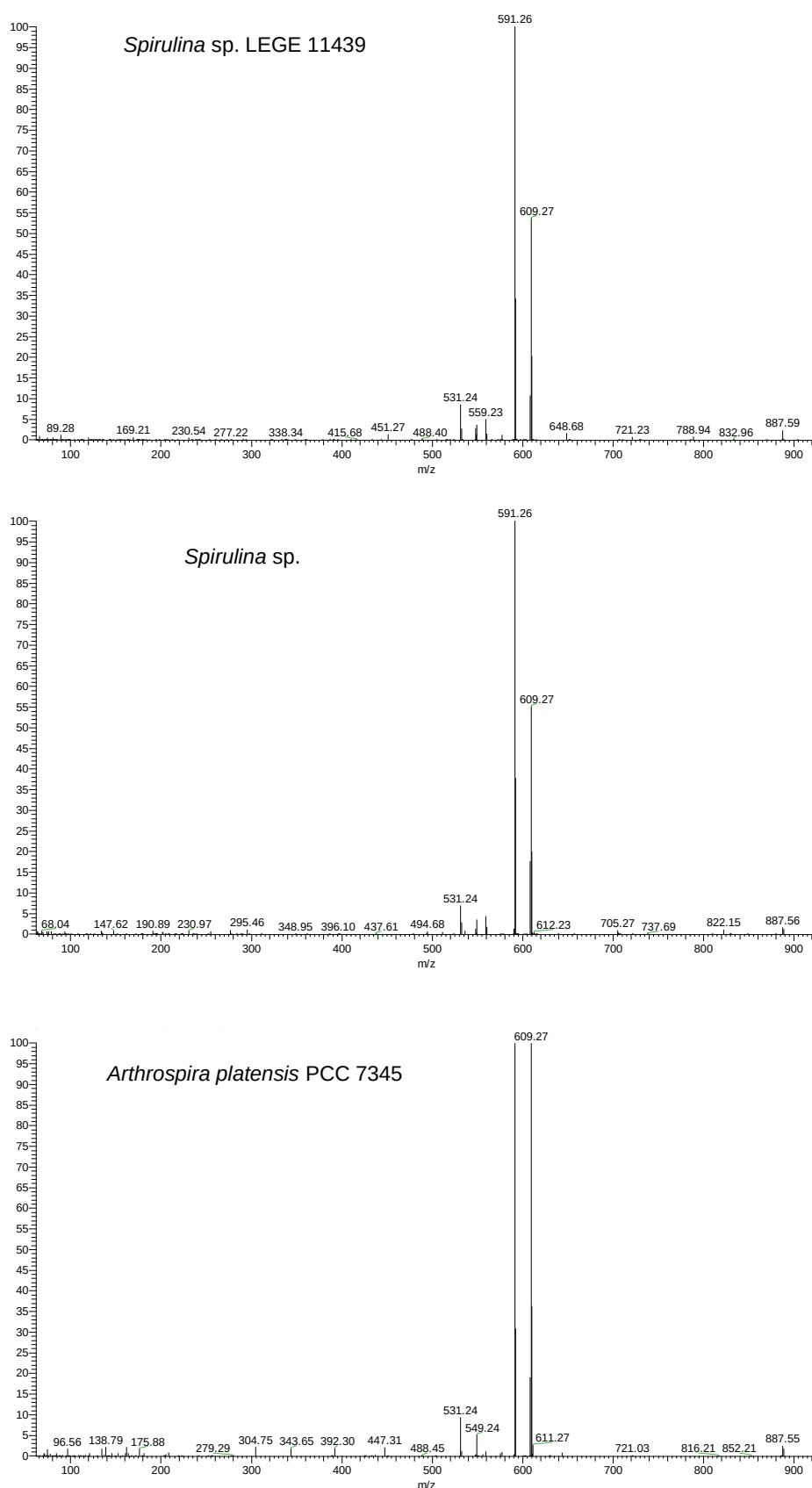


Figure 16 - LC-HRESI-MS spectra of fractions 1 from all three cyanobacteria (top - *Spirulina* sp. LEGE 11439; middle - *Spirulina* sp.; bottom - *Arthrospira platensis* PCC 4735) in positive mode, showing major fragments m/z 609.27, m/z 591.26, m/z 559.23, m/z 549.24, m/z 531.24.

3.5 Zebrafish appetite reduction bioassays

3.5.1 *Paramecium bursaria* appetite reduction bioassay

The results from the appetite reduction bioassay, where stained *P. bursaria* were utilized as the food source for the zebrafish larvae, revealed a single fraction with significant bioactivity towards reducing appetite. Fraction D had a $55.02\% \pm 35.61$ MFI decrease, with a p value of $p < 0.05$, when compared to the solvent control, DMSO (Figure 17 and 18).

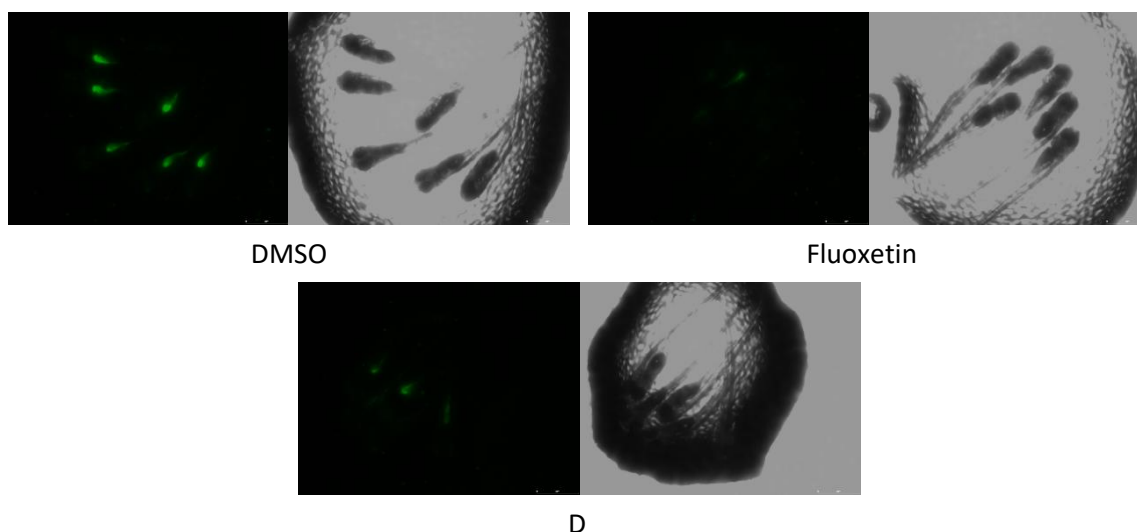


Figure 16 - Representative images of the *Paramecium bursaria* appetite reduction bioassay of fraction D, from commercial *Arthrospira platensis*. On the left are represented the images from fluorescence with GFP (green fluorescent protein) filter and on the right the bright field images of zebrafish larvae. DMSO, solvent control 0.1%; Fluoxetine (FL), positive control 10 mM.

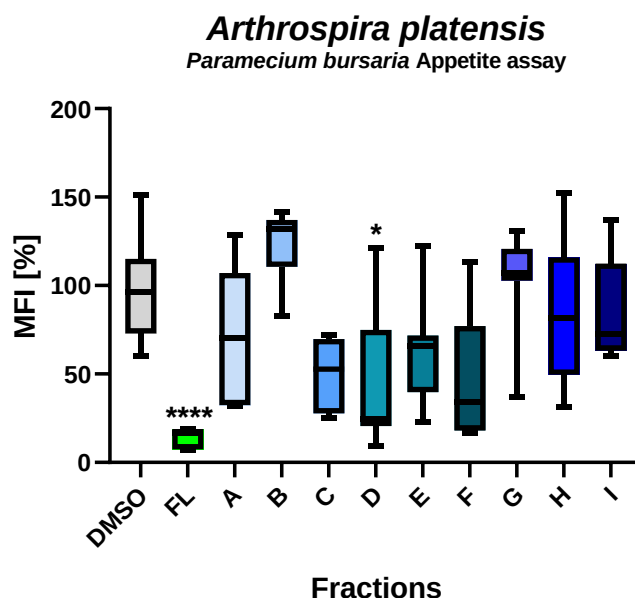


Figure 17 - Appetite-reducing activity results from the *Paramecium bursaria* appetite reduction bioassay, for the fractions from the commercial *Arthrospira platensis*. The solvent control had 0.1% DMSO and the positive control 10 mM Fluoxetine (FL). Values are expressed as mean fluorescence intensity (MFI) relative to the DMSO group. The data are represented as box-whisker plots. Statistical differences were analyzed by Kruskal-Wallis with Dunn's posthoc test and are indicated to the solvent control with the symbol * ($p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$).

3.5.2 Liposomes appetite reduction bioassay

The results from appetite reduction bioassay, where fluorescent egg yolk-derived liposomes were utilized as the food source for the zebrafish larvae, revealed a single fraction with significant bioactivity towards reducing food intake. Fraction D had a $35.52\% \pm 17.36$ MFI decrease, with a p value of $p < 0.01$, when compared to the solvent control, DMSO (Figure 19 and 20). Fractions C, E, and F also exhibited appetite reduction, despite the lack of significance.

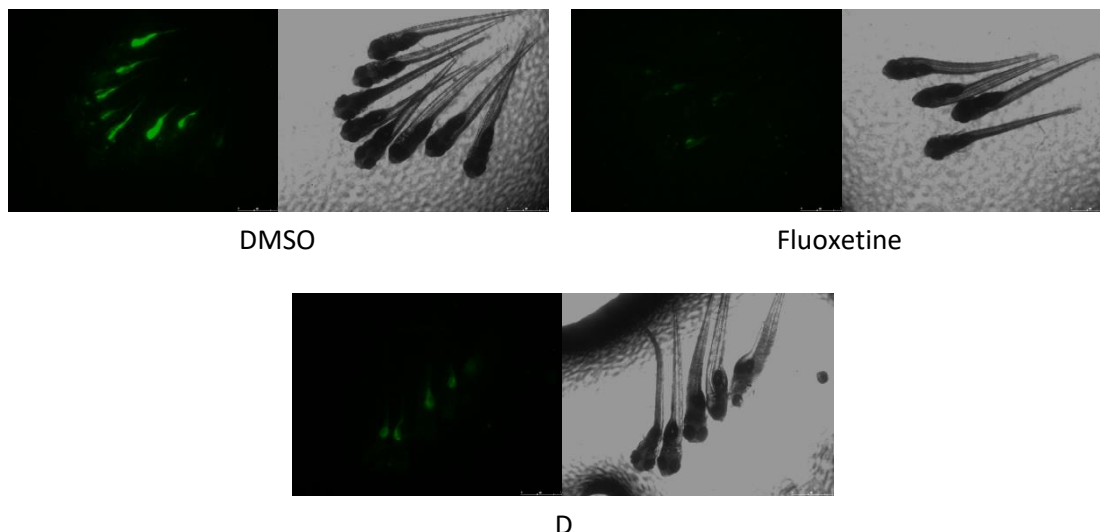


Figure 19 - Representative images of the liposomes appetite reduction bioassay of fraction D, from commercial *Arthrospira platensis*. On the left are represented the images from fluorescence with GFP (green fluorescent protein) filter and on the right the bright field images of zebrafish larvae. DMSO, solvent control 0.1%; Fluoxetine (FL), positive control 10 mM.

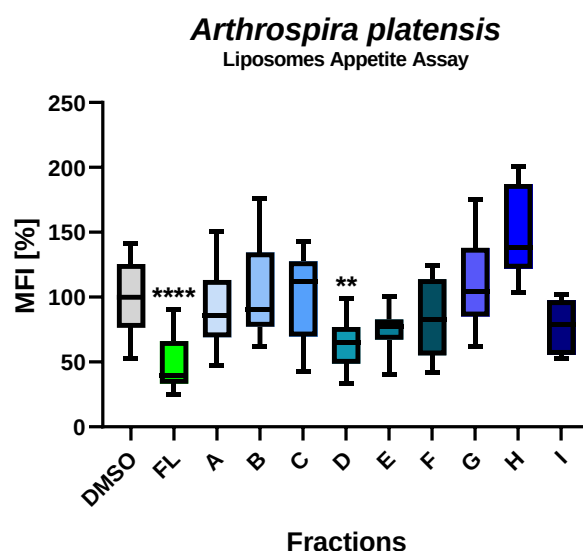


Figure 18 - Appetite-reducing activity results from the liposome appetite reduction bioassay, for the fractions from the commercial *Arthrospira platensis*. The solvent control had 0.1% DMSO and the positive control 10 mM Fluoxetine (FL). Values are expressed as mean fluorescence intensity (MFI) relative to the DMSO group. The data are represented as box-whisker plots. Statistical differences were analyzed by Kruskal-Wallis with Dunn's posthoc test and are indicated to the solvent control with the symbol * ($p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$).

Discussion

4.1 Light conditions and its effects on the cyanobacteria cultures

Most cyanobacteria, possess various mechanisms, allowing them to adapt to sudden changes in their environment and to even survive while in adverse conditions and under abiotic stresses. One manner they can adapt is by altering their cellular mechanisms and metabolic pathways, increasing the production of certain metabolites, or even produce novel and different compounds [80]. Some of these bioproducts may be of biotechnological value, therefore, there is an interest and a need for a better understanding of the conditions, where these products are produced at higher volumes in cyanobacteria. Nonetheless, the stress-induced metabolite production may lead to a higher concentration of certain compounds, but in turn, this can result in a lesser growth of biomass. All these factors have to be considered when looking for the most lucrative strategy for the extraction of high value bioproducts [81]. Hence, the two-stage cultivation strategy is widely used in the biotechnology industry, as in a first phase, the cyanobacteria is grown in a nutrient-rich medium to promote biomass growth, at a faster rate. Once the biomass threshold is hit, the cyanobacteria is placed in a condition where stress responses are induced, therefore increasing the production of the desired metabolite [82].

One parameter in the cultivation of cyanobacteria is light, and the most cost-effective light source is the sun, as most cyanobacteria and microalgae cultivations are done in open ponds since light manipulation through fluorescent lights is costly and the increased secondary metabolites production does not compensate the expenses [81-83]. Nevertheless, LEDs have surged as a low expense alternative to fluorescent lights, when trying to manipulate the light conditions and therefore the growth of cyanobacteria cultures, since with LEDs the energy loss through heat dissipation is minor and they last twice as much as traditional fluorescence lights [84]. One added benefit of LEDs is that they facilitate the selection of more narrow and precise bands of the light spectrum, increasing the light quality and optimizes growth conditions [85].

One crucial factor for an optimal growth of most cyanobacteria species is light, and the most important one for photoautotrophic ones, as through photosynthesis they transform the absorbed photons and transform into chemical energy in the form of ATP (adenosine triphosphate) and NADP⁺ (nicotinamide adenine dinucleotide phosphate) [86]. However, the oxygenic photosynthesis executed by photoautotrophic species of cyanobacteria naturally leads to the damaging of the photosystem II. Photodamage is a consequence of the released electrons from light absorption, which in turn will signal the decrease of photosynthetic rate, to allow for the repairment of photosystem II. If the

photosynthetic rate is not decreased, the ratio of damaged photosystem II to its repair is going to increase, leading to the inhibition of the cyanobacteria growth and to cell death [87]. This imbalance mostly occurs when the cyanobacteria cells are under light of high intensity, overstimulating the electron release from photosynthesis, which will lead to the production of excess reactive oxygen species, harming the cells and consequently the full organism. To try and avoid such harm, cyanobacteria resource to altering their cellular mechanisms and metabolic pathways to produce a wide array of compounds, varying from species to species, which will aid in their protection and survival [88].

The photoperiod of a cyanobacteria also influences its growth, since it regulates their cellular division, occurring during the light period. Therefore, cyanobacteria when under continuous light exposure will have a high biomass growth, which can be fruitful for their cultivation under controlled conditions [89]. Despite such upside, during the dark period is when the cyanobacteria nutrient reserves are going to be produced, namely carbohydrates and to some extent lipids, therefore it is imperative to find a balance for optimal biomass growth and metabolite production [80].

The, mentioned above, selection of narrower color spectra by LEDs, can be applied to test the effects of different light profiles on the growth of cyanobacteria biomass as well as the production of high-value compounds, as the photosystem II, the first protein complex of oxygenic photosynthesis, is stimulated by red light, while blue light affects more the photosystem I [90]. The impact of blue and red light on the growth and metabolite production of cyanobacteria has already been researched and the general conclusion that in cyanobacteria blue light is used with much less efficiency than red light [91]. The reduced blue light usage efficiency of cyanobacteria, is believed to be due to blue light presenting low energy transfer to photosystem II, as chlorophyll a, which can better absorb blue light, is mostly present in photosystem I. In photosystem II, red absorbing phycobilisomes, are mainly present and these do not absorb blue light efficiently, therefore resulting in an energy differential when only blue light is employed in the cyanobacterial culture, with photosystem I retaining all the energy and photosystem becoming energy deficient [91,92].

The effects of the several light profiles utilized in the six Algem® photobioreactors containing the strains utilized in this work, could all be seen as soon as each three-week assay was completed.

The growth of the strain *Spirulina* sp. LEGE 11439 did not present too many noticeable differences, at the start of the experiment and when comparing the six different cultures. The main differences could be seen in photobioreactor 1B, which was under the more intense ($50 \mu\text{mol.m}^{-2}.\text{s}^{-1}$) white light, as the biomass presented clear signs of early cell death, as the color was duller, which could indicate an elevated quantity

of photodamaged photosystem I. The biomass subjected to continuous white light (24 hours of light) in photobioreactor 2A exhibited a biofilm on the bottom of the Erlenmeyer flask, indicating a more rapid growth, as this strain grows by developing a biofilm and forming clusters of biomass. Finally, in photobioreactor 3A, where the biomass was only under blue light, the *Spirulina* biomass showed a much stronger red color, which is most likely due to a higher presence of phycourobilin and phycoerythrin, two phycobilisomes that better absorb blue light.

The growth of the green *Spirulina* sp. after the three-week culture period resulted in a few differences from the moment of commencement and between the cultures themselves. The biomass collected from photobioreactor 1B, which was under the higher intensity white light ($160 \mu\text{mol.m}^{-2}.\text{s}^{-1}$), exhibited signs of cellular death, as around half of the biomass had lost its green color, suggesting a rather fast depletion of the photosystem II. Biomass collected from photobioreactor 2A (24 hours of light) showed extreme signs of cell death, as most of the green color from the strain was replaced by a pale pinkish color, indicating a high dependency of this strain to a dark period in its growth. The cultured biomass in 3A (blue light) exhibited a strong blue-green color, this might be due to a higher quantity of chlorophyll a being produced, as blue light is better absorbed by it. The media in photobioreactors 1A (white light at $80 \mu\text{mol.m}^{-2}.\text{s}^{-1}$), 1B (white light at $160 \mu\text{mol.m}^{-2}.\text{s}^{-1}$), 2B (red light), and 3B (two weeks blue light and one week red light) exhibited varied levels of cloudiness, which is a sign of early nutrient depletion indicating a rapid biomass growth, leading to the belief that in photobioreactor 1B *Spirulina* sp. started to perish due to its rapid growth and early nutrient depletion.

The growth of *Arthrospira platensis* under different light conditions has already been extensively documented, herein, the different PCC 7345 cultures were analyzed based on their color with the biggest difference being exhibited in the culture from photobioreactor 2A, where the typical blue-green color typical from *Arthrospira platensis*, turned into a dull green, which could indicate a worse adaptation to the continuous light period, with the lack of nutrient production.

The efficiency of different light conditions has been largely researched in *Arthrospira platensis*, due to its high biotechnological potential and applications. In *Arthrospira*, it has been shown the optimal photoperiod is 16 hours of light and 8 hours of dark, and that higher light intensities allow for better photosynthetic rate, as long as the rate of photosystem II repair is not overtaken by the rate of photosystem photodamage, increasing its efficiency resulting in a higher biomass growth and elevated CO_2 consumption, increasing metabolite production, at a faster pace [93,94]. Red light utilization in the growth of *Arthrospira platensis* has been showed to efficiently increase the growth of biomass, as the photosystem II of cyanobacteria utilizes light in the orange-

red spectrum much more efficiently than blue light, which is going to result in a much higher protein and carbohydrate concentration. Nonetheless, several studies have demonstrated that the green photosynthetic pigments, such as chlorophyll-a and some chromophores from phycobilisomes, like phycocyanin, their content in *Arthrospira platensis* is greatly increased by light with wavelengths in the green-blue spectrum [95-97].

Hence, the general conclusion, for the optimized growth of *Arthrospira platensis* biomass and its pigment production, is that the culture should be started under red light, which will allow for a rapid growth of the biomass and its machinery, which in turn will result in a faster and more efficient production of high-value compounds, once the culture is placed under blue light, decoupling the pigment production from the biomass growth [95,98].

4.2 Zebrafish Nile Red fat metabolism assay

According to the available literature on the *Spirulina* genus, the nutraceutical value has not been studied, making this work the first evaluation of the anti-obesity bioactivity present in *Spirulina* sp. The zebrafish Nile Red fat metabolism assays done with the fractions obtained from the *Spirulina* sp. LEGE 11439 biomass, revealed no significant bioactivity towards the lipid reduction of *D. rerio* larvae. Similar anti-obesogenic bioactivity results were achieved with the green strain of *Spirulina* sp, as there was no fraction which exhibited any significant lipid-reducing activity.

Contrasting to both strains of *Spirulina*, *Arthrospira platensis* PCC 7345 exhibited excellent anti-obesogenic bioactivity, as expected since the anti-obesity effects of the *Arthrospira* genus have been extensively studied. It has been shown that daily *Spirulina* (dried biomass from cyanobacteria of *Arthrospira* spp.) supplementation can lead to the reduction of the body mass, neutral fat levels, BMI and waist circumference of an individual. Regular *Spirulina* intake has also beneficial effects on the blood lipid levels, as it significantly decreases the levels of total cholesterol, LDL, triglycerides, and VLDL, while increasing HDL quantity. Additionally, along with the improvement of obesity symptoms, *Arthrospira platensis* aids patients with obesity related co-morbidities, such as hypertension, while also increasing insulin sensitivity, to ameliorate type-2 diabetes effects [53-55].

Nonetheless, 6 of the 18 tested fractions on the bioassays revealed significant lipid-reducing activity. These results suggest the production of one or more secondary metabolites by *Arthrospira platensis* PCC 7345 with anti-obesogenic capabilities. As most of the bioactive fractions were fractions A, which resulted from the fractionation by increased solvent polarity, where hexane and ethyl-acetate, non-polar solvents, were

firstly employed. Hence, the responsible compound or compounds are of a non-polar nature.

Carotenoids and chlorophylls are non-polar pigments, with carotenoids such as β -carotene being less polar than chlorophylls, while chlorophyll-b is slightly less polar than chlorophyll-a [99]. The responsible compounds are most likely chlorophyll-derived, as carotenoid production is more efficient under red light over blue light [100]. From the zebrafish bioassay, the employment of blue light, resulted in fraction 13 exhibiting a higher lipid-reducing activity than that of fraction 10 from the culture grown under red light. One study has already unveiled the potential of chlorophyll derivatives as lipid-reducing agents, as 13²-hydroxy-pheophytin a and 13²-hydroxy-pheofarnesin both showed excellent bioactivity in reducing lipid levels of *D. rerio* larvae, with the former compounds possessing an EC₅₀ of $8.9 \pm 0.4 \mu\text{M}$ and the latter $15.5 \pm 1.3 \mu\text{M}$ [41].

4.3 Comparison of metabolite profiles

To better understand what differs from the *Spirulina* genus strains and the *Arthrospira platensis* strain, and to gauge what may be the reason the latter demonstrates such a potent bioactivity towards the reduction of neutral lipid levels in the *D. rerio* larvae, and the former does not, a comparison of the metabolites profiles was executed.

The MS/MS data of fractions A, from photobioreactor 1A, were then uploaded onto the GNPS platform from both *Spirulina* and the *Arthrospira* strain, as fraction 1 demonstrated strongest lipid-reducing activity from *Arthrospira*, and no bioactivity from *Spirulina*. Consequently, the Cytoscape software was used to analyze and assess which compounds were unique to each strain, and most importantly, which possible compounds were only present in fraction 1 from *Arthrospira platensis* PCC 7345 and could plausibly be responsible for its bioactivity. These compounds were identified by searching the unique mass peaks represented by each node on the molecular network, in selected mass spectrometry databases. 64 unique mass peaks were identified, but only 11 of the unique spectra, revealed positive hits, with 14 possible identifications. The remaining mass peaks, which did not possess any hits on the utilized mass spectrometry databases, could be non-identified novel compounds, with viable anti-obesogenic activity, and great biotechnological potential.

Mass peak $[\text{M}+\text{H}]^+$ 281.247 was identified as linoleic acid, an omega-6 polyunsaturated fatty acid (PUFA). Linoleic acid can be found in a myriad of foods, and it is considered an essential fatty acid as human bodies are not able to synthesize it, as it displays a crucial role in maintaining the impermeability of the epidermis, the outer layer of the skin. Deficiency in the levels of linoleic acid, has been studied in human

infants, as it can lead to scaly skin lesions thrombocytopenia, altered plasma fatty acid levels and growth impairment. Numerous studies on the health benefits of linoleic acid have been carried out, as it has been shown that a higher dietary intake of this PUFA can ameliorate lipid blood levels, decreasing the risk for cardiovascular diseases, such as coronary heart ones, while decreasing high glycemia and improving insulin resistance, aiding in the symptoms of metabolic disorders, especially type-2 diabetes [101]. Moreover, increased linoleic acid intake has been reported to diminish the probability of occurrence of colon and breast cancer [102]. Concerns about linoleic acid have also been raised, has this essential fatty acid can be metabolized into arachidonic acid, a PUFA, and the precursor of eicosanoids. Eicosanoids, such as leukotrienes and prostaglandins, are linked to an increased risk of inflammation leading to the promotion of tumoral growth, as these PUFA derivatives can change epithelial cell proliferation, migration and apoptosis. Nonetheless, recent studies have shown that the typical linoleic acid intake and how much of it is converted in arachidonic acid (0.3 - 0.6 %) is insufficient for any negative impact [103].

Mass peak $[M+H]^+$ 377.195 was identified as 8-hydroxy-2,7,7,11,15-pentamethyl-5,12,14-trioxapentacyclo[9.8.0.02,8.04,6.013,18]nonadec-13(18)-ene-3,17-dione, belonging to the class of naphthopyrans. Naphthopyrans are photochromic compounds, meaning they transform between two structures when they absorb enough energy through radiation, possessing the ability to change which colors they emit. Therefore, naphthopyrans possess value in the research of photo-responsive materials, such as nanoparticles or ophthalmic lens [104], although it is unlikely this putatively identified compound was from the *Arthrospira platensis* strain, as naphthopyrans are generally synthetic and it probably derived from contamination [104].

Two possible identifications were made for mass peak $[M+H]^+$ 419.206. Firstly, it was identified as 2-(2,6-dimethoxy-4-prop-2-enylphenoxy)-1-(3,4,5-trimethoxyphenyl)propan-1-ol, belonging to the class of lignans, neolignans and other related compounds. Lignans are mostly found in plants and are considered phytoestrogens, as they can bind to estrogen receptors. They are polyphenols, a large family of compounds, extremely linked to its antioxidant activity and anti-cancer potential. Different lignans have been found to display a wide array of beneficial properties, such as antiviral, anti-estrogenic, antioxidant, antimutagenic and anti-carcinogenic [105]. An *in vivo* assay on rats showed that lignan exposure can help ameliorate breast cancer symptoms, as it diminished tumor growth rate and the occurrence of metastatic events, while also aiding the decrease in size of already existing tumors and preventing the formation of new tumors [106]. An epidemiological study also revealed that higher lignan intake reduced the risk of premenopausal breast cancer risk [107]. In an *in vitro* study,

four colon tumoral cell lines exhibited growth inhibitory effects, which were estrogen-dependent, after exposure to mammalian lignans [108]. Prostate cancer is also affected by lignans, as these are anti-estrogenic, and the prostate needs testosterone to grow, which will be inhibited, as an *in vitro* study showed, where lignans prevented the growth of prostatic cancer cell lines [109].

The second possible identification of mass peak $[M+H]^+$ 419.206 was avertoxin A, a asteltoxin derivative. Asteltoxin is a mycotoxin extracted from the *Aspergillus versicolor* Y10 fungus, present in firmoss (*Huperzia serrata*). This mycotoxin has potent activity against acetylcholinesterase, preventing the deposition of amyloids in neurons which leads to Alzheimer's disease. Nonetheless, avertoxin A exhibited no inhibitory activity against the human acetylcholinesterase. In the same study, avertoxin A cytotoxicity was assessed on human cancer cell lines (MDA-MB-231, HCT 116 and HeLa), revealing no anti-tumoral bioactivity [110].

Mass peak $[M+H]^+$ 421.203 was identified as isoflupredone acetate, a synthetic glucocorticoid used in veterinarian medicine mostly used in cattle, but also employed on pigs and horses, for the management of various complications. It can be used to treat (i) hypokalemia syndrome in cattle, which is extreme deficiency in potassium which leads to anorexia and gastrointestinal stasis; (ii) inflammation of the breast tissue in cows due to mastitis; (iii) fatty liver disease, the accumulation of fat around the liver; and (iv) ketosis, which occurs when the body and brain resource to ketones for energy, due to a low intake of carbohydrates. Isoflupredone acetate has never been approved for human use, it exhibits applications for the treatment of metabolic complications as well as lipid accumulation in other mammals [111,112]. This glucocorticoid, as it is synthetic, it should not have been produced by the cyanobacteria, nonetheless, glucocorticoids are crucial for the preservation of human metabolism, and their imbalance can lead to obesity by increasing fatty acid and lipid accumulation in the muscle and liver [113].

Two possible identifications were made for mass peak $[M+H]^+$ 443.206. The first possible identification was diversolide A, a sesquiterpene lactone derivative. In a study where diversolide A was extracted from the roots of *Ferula diversivittata*, its microbial activity was assessed, revealing moderate inhibitory activity with a minimum inhibitory concentration (MIC) of 80 $\mu\text{g/mL}$ against *Escherichia coli*. In another study by the same author, diversolide A exhibited activity towards the inhibition of the tumor promoting stage of the Epstein-Barr virus in *in vitro* assays. Therefore, diversolide possesses potential antimicrobial along with anti-carcinogenic bioactivity [114,115].

Lastly, mass peak $[M+H]^+$ 443.206 was possibly identified as picrolichenic acid. The literature on picrolichenic acid is scarce, this compound is mostly isolated from lichens, namely *Lepra amara* and *Lepra truncate* and is said to be the reason these

lichens are of a bitter acidic nature [116]. Nonetheless, this compound is a phenolic acid, namely a hydroxybenzoic acid derivative, which have revealed to possess bioactivity against lipid accumulation and type-2 diabetes. For example, gallic acid intake revealed to lower the levels of total cholesterol, leptin, LDL-cholesterol, and insulin, along with phospholipids and serum triacylglycerol in high-fat diet-induced mice [117].

Two possible identifications were made for mass peak $[M+H]^+$ 461.216. These compounds were 2,20-dihydroxy-13-methoxy-4,7,17,22,22-pentamethyl-5,10,21,23-tetraoxahexacyclo[18.2.1.01,17.04,16.06,14.08,12]tricos-6(14),7,12-trien-11-one and 20,21-dihydroxy-10-methoxy-1,4,14,19,19-pentamethyl-2,7,18-trioxapentacyclo[11.9.0.03,11.05,9.014,20]docosa-3(11),4,9-triene-8,17-dione. Both of these compounds belong to the sub-class of natural compounds of benzopyrans. No annotations of bioactivity against lipid accumulation was available in the literature, for any of these two compounds.

Even so, the sub-class of which it belongs to has demonstrated an immense array of biological activities. Benzopyrans derivatives have demonstrated bioactivity towards antibacterial, antiviral applications, as well as for the treatment of Alzheimer's disease and arthritis. Moreover, some benzopyrans have anti-carcinogenic activity, either by inhibiting the monocarboxylate transporter or by suppressing the P-glycoprotein. Treatment of type-2 diabetes has also been shown to be accomplished by benzopyran derivatives, through the inhibition of the α -glucosaminidase enzyme, as they lower blood sugar levels by altering the manner in which the intestine absorbs carbohydrates or sugars. Ultimately, benzopyran derivatives have shown anti-inflammatory bioactivity, namely a cyclooxygenase (COX)-2 inhibitor. Activation of COX-2 plays a vital role in the kickstart of fat inflammation, which can lead to fatty liver disease, insulin resistance and consequently, obesity. Hence, inhibition of COX-2 aids the treatment and prevents serious obesity co-morbidities [118].

Mass peaks $[M+H]^+$ 753.550 and 768.561 were identified as sulfoquinovosyl diacylglycerol (SQDG) and monogalactosyldiacylglycerol (MGDG) respectively. SQDG is an anionic lipid, while MGDG is a neutral galactolipid, commonly found in photoautotrophic organisms, such as cyanobacteria. These lipids are mostly present in the thylakoidal membrane, as they play a vital role in photosynthesis. SQDG and MGDG are two of the major lipids present in the cytochrome b6f complex, which is present in the electronic transfer chain from photosystem II to photosystem I, originating the energy differential to transform ADP (adenosine diphosphate) into ATP [119].

A SQDG derivative has exhibited anti-inflammatory bioactivity, in a study, where it was extracted from the marine dinoflagellate *Oxyrrhis marina*, it inhibited the activity of lipopolysaccharide (LPS)-induced RAW 264.7 cells, macrophage-like cells, which

produced nitric oxide (NO), a ROS [120]. SQDG has revealed chemo-preventive potential as it showed apoptotic activity and inhibition of proliferation again against the gastric cancer cell line SNU-1. Additionally, MGDG has shown to possess the ability to increase radiation cytotoxicity of human pancreatic cell lines and it also revealed *in vivo* effects by diminish tumor growth in mice [121,122]. Therefore, both glycerolipids exhibit anti-carcinogenic potential, which is most likely due to their inhibition of mammalian replicative DNA polymerases activity [122].

Mass peaks $[M+H]^+$ 281.247 and 825.561 were identified as 1,2-dioleoyl-sn-glycero-3-phospho-rac-1-glycerol and 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) respectively. Two phospholipids that can rearrange themselves into phospholipid bilayers when in aqueous solution, forming a membrane. Relative to their biotechnological applications, they are mostly employed in liposome formulations for drug delivery in the pharmaceutical industry [123].

Mass peak $[M+H]^+$ 871.572 was identified as pheophytin a. Pheophytin a is a chlorophyll derivative which results from the demetallization (removal of Mg^{2+}) of chlorophyll a. This is an extremely important, light-dependent, reaction for the proper maintenance of photosynthesis, as pheophytin a is the primary electron transporter in photosystem II [124]. Most chlorophyll derived compounds possess a wide display of promising biological activities, and pheophytin a is no different. One study demonstrated that pheophytin a, extracted from the garden plant *Ocimum labiatum*, possessed anti-HIV activity, through the inhibition of the HIV-1 protease, with an IC_{50} value of 44.4 ± 1.5 $\mu g/mL$, while exhibiting no cytotoxicity towards TZM-bl (cell line utilized for HIV infection *in vitro* assays) cells [125].

Pheophytin a also has demonstrated anti-inflammatory activity in several studies, such as the inhibition of LPS-induced TNF (tumor necrosis factor)- α expression in HEK293 cells, as TNF- α is a cytokine which activates some of the inflammatory responses [126]. In a different study, with the aim of unveiling potential anti-inflammatory agents for microglia-mediated inflammation, which can lead to several neurological diseases, pheophytin showed to inhibit several cytokines (interleukin- 1β , interleukin-6 and TNL- α), along with some chemokines, which are smaller cytokines, in BV2 cells which were induced by LPS and interferon- γ . This inhibitory activity in turn suppressed the expression of p65 NF- κB and the type I interferon signaling pathway, therefore decreasing the pro-inflammatory response of the cells [127]. Additionally, this chlorophyll derivate has inhibitory activity against NO production along with suppressing the expression of inducible nitric oxide synthase [128]. As a last example of the incredible anti-inflammation potential presented by pheophytin a, in LPS-induced RAW 264.7 cells, pre-treatment with pheophytin a inhibited the production of prostaglandin E2, a principal

mediator of inflammation, NO and interleukin-1 β . Moreover, it also decreased the transcriptional and translational expression levels of nitric oxide synthase-2 and COX-2 and blocked the ERK (extracellular signal-regulated kinase) and STAT-1 (signal transducers and activators of transcription 1) pathways, as both pathways promote inflammation [129].

The inhibition of inflammatory pathways has been linked with the treatment of obesity and its co-morbidities. Adipose tissue hypertrophy and hyperplasia due to excess energy intake enhances the release of cytokines and chemokines, such as TNF- α and interleukin-6, while decreasing adiponectin, an extremely important hormone in the fat metabolism. Therefore, pro-inflammation responses may lead to several co-morbidities related to obesity, such as cardiovascular diseases, atherosclerosis, formation of blood coagulates, increased insulin resistance and consequently, type-2 diabetes. Additionally, adiponectin has been extensively linked with metabolic diseases, coronary heart disease and increased fasting blood sugar levels, increasing the probability of obesity and type-2 diabetes occurrence [130,131]. Pheophytin a and its anti-diabetic effects have been reported. In a study, where pheophytin a was extracted from the Cuban medicinal plant *Allophylus cominia*, it exhibited, concentration-dependent, inhibitory activity towards the PTP1B enzyme, which negatively regulates the insulin signaling pathway, while a subfraction of the plant extract, containing pheophytin a and pheophytin b, suppressed the α -glucosidase enzyme, responsible for the breakdown of starch into carbohydrates. Therefore, the bioactivity towards both enzymes displays a potential role in the treatment of type-2 diabetes [132]. Additionally, in a follow-up study by the same authors, pheophytin increased glucose uptake in differentiated 3T3-L1, L6 and HepG2 cells, through the increase in insulin sensitivity, and it also lowered the accumulation of lipids in the 3T3-L1 adipocytes [133].

4.4 13²-hydroxy-pheophytin a presence and amount

Arthrospira platensis is extremely rich in protein and a myriad of nutrients with the potential to ameliorate the well-being of any individual. The anti-obesogenic capabilities of this cyanobacterium have been well-documented over the years. Nonetheless, the exact compounds and mechanisms of action responsible for its lipid lowering bioactivity are still not well-established. A study by Freitas *et al.* described the chlorophyll derivative hpa (13²-hydroxy-pheophytin a), with potent lipid-reducing activity, along with *Arthrospira platensis* being shown to have high amounts of hpa, when compared to other photoautotrophic organisms, approved for human consumption (*Chlorella*, spinach, and cabbage) [41].

In this work, through the analysis of the MS/MS data in the XCalibur software, the presence and intensity of hpa in each of the three strains was assessed by analyzing peak intensity in extracted ion chromatograms (EIC). The base peak value selected was that of hpa, previously reported by Freitas *et al.*, with a monoisotopic m/z of 887.5697 for $[M+H]^+$. The strain revealing the highest intensity was the *Spirulina* sp. LEGE 11439, while the green *Spirulina* sp. came in second, and, as a surprise, *Arthrospira platensis* PCC 7345 exhibited the lowest intensity regarding the annotated base peak of hpa.

The analysis on the fragmentation of the compound with a monoisotopic m/z of 887.5694 for $[M+H]^+$, in each fraction, was executed to infer if the compound present, was indeed hpa or its isomer. Since in another work, the hpa isomer presented reduced bioactivity (approximately 10-fold), and through comparisons of the MS/MS fragmentation, the less bioactive isomer presented a much lower amount of the fragment with a m/z of 609.27, putatively identified as $[M - \text{phytyl}]^+$, while fragments with a m/z of 591.26 ($[M - \text{OH} - \text{phytyl}]^+$), 559.23 ($[M - \text{phytyl} - \text{OH} - \text{CH}_3\text{O}]^+$), and 531.23 ($[M - \text{phytyl} - \text{COOCH}_3 - \text{OH}]^+$), were present at a much higher degree. For hpa, fragment 609.27, ($[M - \text{phytyl}]^+$), was the one with the highest intensity, while the other three fragments had a much decreased intensity [79].

From the MS/MS data analysis from the two *Spirulina* spp. strains, the compound with a monoisotopic m/z of 887.5694 for $[M+H]^+$, fragmented in a similar way to the, previously reported, less bioactive isomer of hpa, as the fragment with an m/z of 609.27, ($[M - \text{phytyl}]^+$), showed a lower intensity than the fragment with an m/z of 591.26, ($[M - \text{OH} - \text{phytyl}]^+$). The MS/MS data analysis from the *Arthrospira platensis* PCC 7345, the peak intensities for both fragments were similar, with the fragment with an m/z of 609.27 for $[M+H]^+$, exhibiting a slightly higher intensity, which is more similar to what was previously documented, although not to the same level of intensity. MS/MS fragmentation was not similar, since samples were run on different machines with different collision energies, and in the future, the isolated hpa and its isomer will be run under exactly the same conditions and equipment to confirm these statements.

In consequence of these findings, it is proposed that the reason for the great contrast in lipid-reducing bioactivity between these two genera, is owed to the *Spirulina* strains possessing a higher quantity of the less active hpa isomer, while in PCC 7345 the bioactive hpa is the main compound relating to the monoisotopic m/z of 887.5694 for $[M+H]^+$.

Nonetheless, the putatively identified compounds unique to the *Arthrospira platensis* strain, may possess synergistic effects for its efficacy and bioactivity. As both, linoleic acid, picrolichenic acid, pheophytin a, or some of the unidentified compounds, may have bioactivity against lipid accumulation and towards the treatment of obesity and

its related co-morbidities. Additionally, one main discerning aspect between the two genera of cyanobacteria, *Spirulina* and *Arthrospira*, is the latter possesses a much higher amount of linoleic acid and γ -linolenic acid, two PUFAs with marked benefits relative to blood lipid levels and overall health benefits, than the former [134].

4.5 Zebrafish appetite reduction bioassays

Both appetite reduction bioassay protocols revealed that fraction D possesses some bioactivity towards appetite suppression and food intake reduction. Indicating the presence of at least one bioactive compound in this fraction which can suppress appetite in zebrafish larvae. This fraction also exhibited potent anti-obesogenic activity in zebrafish larvae, through the Nile Red fat metabolism assay, the most out of all 9 fractions [79].

Zebrafish have been recently gaining popularity as a model organism for appetite inhibition research, due to the development of high-throughput bioassays, which employ it as the model organism. Compounds with established appetite modulation in humans have been shown to possess the same effects on *D. rerio*, such as nicotine which suppresses it and ghrelin which enhances it [74,75].

The production of the hormone leptin is also a key modulator in the regulation of appetite, as leptin is a hormone produced by the adipose tissue, which suppresses appetite, providing a feeling of satiety. Studies have utilized zebrafish mutants with loss-of-function in the leptin receptor, to exhibit the effects of leptin in the transcription of genes which regulate appetite in zebrafish. Nonetheless, some mechanisms that regulate appetite suppression are still unclear, something that can possibly be unveiled with the aid of zebrafish as a model organism for appetite reduction studies [135,136]

Arthrospira, along with its anti-obesogenic effects, has been documented to improve satiety in patients who have undergone its supplementation. Although, the mechanism is still uncertain, some believe it to be due to an increase in leptin resistance from the supplement intake [137,138].

Conclusion

Upon the commencement of this work, the main objective was to manipulate the growth conditions of three different strains of cyanobacteria, *Spirulina* sp. LEGE 11439, *Spirulina* sp, and *Arthrospira platensis* PCC 7345, and to analyze its anti-obesity activity and metabolite composition.

Only fractions from *Arthrospira platensis* PCC 7345 revealed bioactivity against lipid accumulation in the zebrafish Nile Red fat metabolism assay. Bioactive fractions were fraction 1, 4, 10, 13, 14, and 16. Fraction 1 from normal white light conditions demonstrated the highest lipid-reducing activity with $60.76 \% \pm 16.37$.

The comparison of the metabolite profiles of fraction 1 from the three different strains allowed for the development of a molecular network, which represented the unique compounds present in fraction 1A-A from PCC 7345, which were absent from the *Spirulina* strains. 64 unique mass peaks were obtained, with only 11 of them possessing hits on mass spectra databases. In total 14 annotated compounds were obtained from the manual search. In total, lipid reducing activity may be caused by putatively identified or non-identified, novel compounds. From the documented bioactivity of the 14 compounds, two compounds appear to be the most suitable candidates for the increased lipid-reducing activity observed in *Arthrospira platensis* PCC 7345 and not in any of the *Spirulina* strains. Firstly, linoleic acid, a PUFA, with an extensive array of health benefits, such as anti-tumoral activity, the ability to lower blood lipid levels and insulin resistance, revealing potential for the treatment of metabolic disorders and type-2 diabetes.

Pheophitin a is the other possible candidate, as it has shown to possess a wide array of anti-inflammatory activity as it has inhibitory activities on several pro-immune responsive targets, one of them being COX-2, and its inhibition prevents the development of metabolic diseases, such as fatty liver disease, and type-2 diabetes. Pheophytin a has shown to inhibit lipid accumulation in 3T3-L1 pre-adipocytes.

The presence of two different isomers with the monoisotopic m/z of 887.5694 $[M+H]^+$ for hpa was observed. Fractions A from the *Spirulina* strains exhibited an hpa isomer with a highly reduced bioactivity towards the lowering of lipids in zebrafish larvae. The higher presence of the less bioactive hpa isomer in both *Spirulina* strains, and the higher presence of the bioactive hpa in *Arthrospira platensis* PCC 7345, may have contributed to the lipid reducing activity in *Arthrospira* and the inexistence of any discernible anti-obesogenic from both *Spirulina* sp. LEGE 11439 and *Spirulina* sp.

Finally, appetite reducing activity was shown due to exposure of *D. rerio* larvae to *Arthrospira platensis*.

Future perspectives

Firstly, further fractionation of the three fractions 1A-A, bioassays and chemical isolation would be required, to assess and confirm if there any compounds with synergistic effects towards hpa in *Arthrospira platensis* PCC 7345 or any antagonistic compounds present in *Spirulina* sp. LEGE 11439 and the green *Spirulina* sp. Furthermore, identified compounds with known activity on lipid reduction could be confirmed with exposure to single compounds, e.g. the PUFA linoleic acid.

The results from this work allowed to assess what light profiles are harmful for these cyanobacteria strains, and that light profiles present an increased potential for the efficient production secondary metabolites. Further light manipulation assays could focus on mixing two or more light colors at the same time, like using 50% white light and 50% red or blue light, as the PCC 7345 fraction with the most bioactivity resulted from photobioreactor 1A. Moreover, as it has been shown for *Arthrospira platensis* a better approach to produce photosynthetic pigments would be to start off the assay with red light and finish with blue light.

In future tests, biochemical characterization of the strains would have to be made, to better understand the effects of the different light profiles.

Appetite reduction activities should be followed-up and the responsible compounds identified by bioassay-guided fractionation procedures.

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Appendixes

Appendix I - Macro and micronutrients composition of Z8 medium

Table I.1 - Z8 medium stock solutions and their respective composition.

Stock solutions		Concentration (mL.L ⁻¹)
Solution A		10
Solution B		10
Fe-EDTA Solution		10
Micronutrients Solution		1

Solution A		
Reagent		Concentration (g.L ⁻¹)
NaNO ₃		46.7
Ca(NO ₃) ₂ .4H ₂ O		5.9
MgSO ₄ .7H ₂ O		2.5

Solution B		
Reagent		Concentration (g.L ⁻¹)
K ₂ HPO ₄		6.2
Na ₂ CO ₃		4.2

Fe-EDTA Solution		
Solution		Concentration (mL.L ⁻¹)
FeCl ₃ *		10
EDTA-Na**		9.5

*FeCl ₃ Solution		
Reagent		
FeCl ₃ .6H ₂ O		2.8 g
HCl (0.1 N)		100 mL

**EDTA-Na Solution		
Reagent		
EDTA		3.9 g
NaOH (0.1 N)		100 mL

Micronutrients Solution		
Solution		Concentration (mL.L ⁻¹)
1 to 12		10
13 e 14		100

Micronutrients 1 - 14		
	Reagent	Concentration (g.L ⁻¹)
1	Na ₂ WO ₄ .2H ₂ O	0.33
2	(NH ₄) ₆ MO ₇ O ₂₄ .2H ₂ O	0.88
3	KBr	1.2
4	KI	0.83
5	ZnSO ₄ .7H ₂ O	2.87
6	Cd(NO ₃) ₂ .4H ₂ O	1.55
7	Co(NO ₃) ₂ .6H ₂ O	1.46
8	CuSO ₄ .5H ₂ O	1.25
9	NiSO ₄ (NH ₄) ₂ SO ₄ .6H ₂ O	1.98
10	Cr(NO ₃) ₃ .9H ₂ O	0.41
11	V ₂ O ₅	0.0089
12	AlK(SO ₄) ₂ .12H ₂ O	9.48
13	H ₃ BO ₃	3.1
14	MnSO ₄ .4H ₂ O	2.23

Appendix II - Macro and micronutrients composition of BG11 medium

Table II.1 – BG11 medium stock solutions and their respective composition

Stock solutions	Concentration (mL.L ⁻¹)
Solution 1	10
Solution 2	10
Solution 3	10
Solution 4	1
Reagent	Mass (g)
Na ₂ CO ₃	0.02
NaNO ₃	1.5

Solution 1	
Reagent	Concentration (g.L ⁻¹)
Na ₂ Mg EDTA	0.1
(NH ₄) ₅ [Fe(C ₆ H ₄ O ₇) ₂]	0.6
C ₆ H ₈ O ₇ .1H ₂ O	0.6
CaCl ₂ .2H ₂ O	3.6
Solution 2	
Reagent	Concentration (g.L ⁻¹)
MgSO ₄ .7H ₂ O	7.5
Solution 3	
Reagent	Concentration (g.L ⁻¹)
K ₂ HPO ₄	4.0
Solution 4	
Reagent	Concentration (g.L ⁻¹)
Na ₂ MoO ₄ .2H ₂ O	0.391
ZnSO ₄ .7H ₂ O	0.222
COCl ₂ .6H ₂ O	0.05
CuSO ₄ .5H ₂ O	0.079
H ₃ BO ₃	2.86
MnCl ₂ .4H ₂ O	1.81

Appendix III – Number of 40°C ultrasonication cycles used in biomass extraction

Table III.1 - Number of repetitions of ultrasonication in a 40°C deionized water bath (#) for the extraction of biomass from each photobioreactor (1A, 1B, 2A, 2B, 3A, 3B) for the three cyanobacteria strains.

<i>Spirulina</i> sp. LEGE 11439	#
1A	3
1B	2
2A	3
2B	3
3A	3
3C	3
<i>Spirulina</i> sp.	#
1A	3
1B	3
2A	4
2B	4
3A	4
3C	4
<i>Arthrospira platensis</i> PCC 7345	#
1A	2
1B	2
2A	1
2B	1
3A	2
3C	2

Appendix IV – Instrument setups used in the extraction and fractionation steps



Figure IV.1 - Instrument setup used in the extraction of the biomass from the three cyanobacterial strains.



Figure IV.2 - Instrument setup used in the fractionation of each extract from the three cyanobacterial strains.

Appendix V – Algem Erlenmeyer flasks before and after the light manipulation assays



Figure V.1 – Images showing the Algem® Erlenmeyer flasks containing the cyanobacterial strains before (left) and after (right) the three-week assay under different light conditions (Top – *Spirulina* sp. LEGE 11439; Middle – *Spirulina* sp; Bottom – *Arthrospira platensis* PCC 7345).

Appendix VI – Molecular network from fraction 1 from all three strains

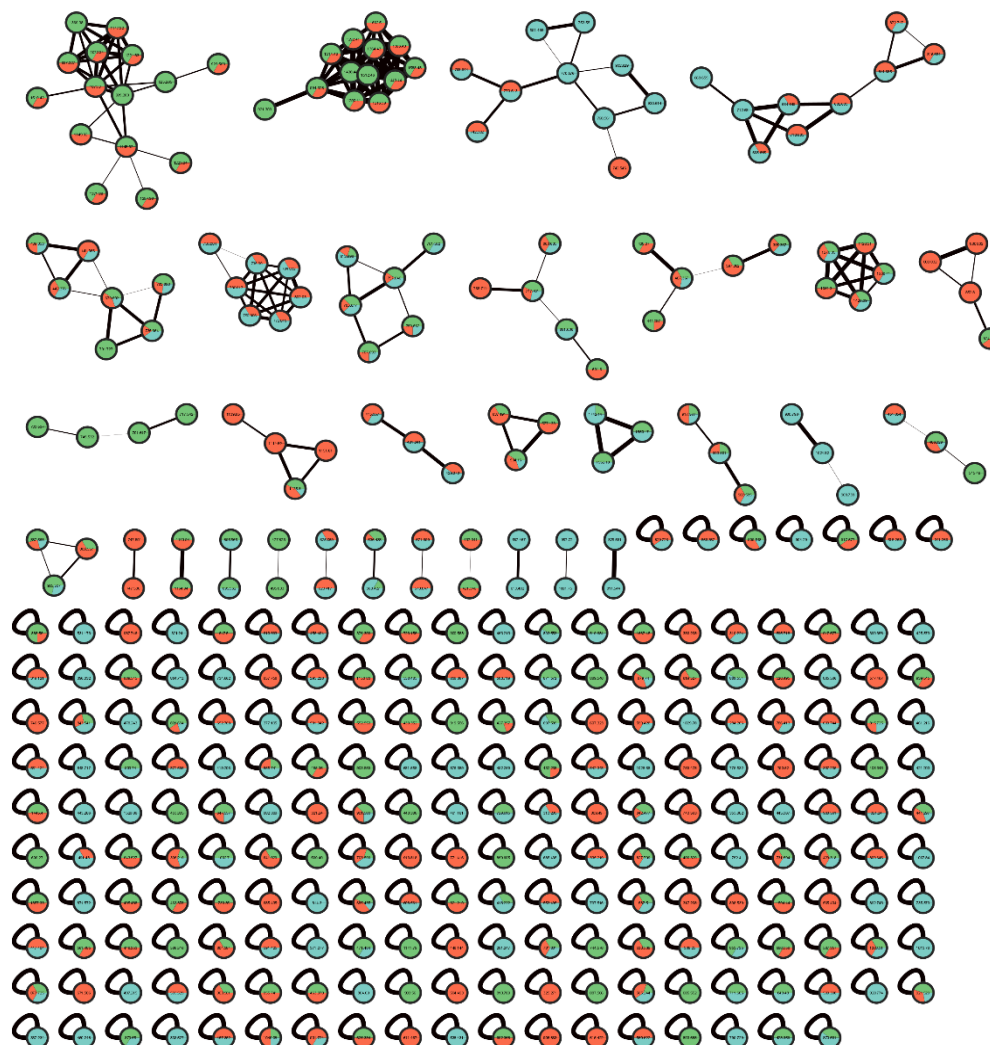


Figure VI.1 - Molecular network, obtained from Cytoscape through the GNPS server, of fractions 1 (1A-A) from the three cyanobacterial strains: Red – relative intensity from *Spirulina* sp. LEGE 11439; Green – relative intensity from *Spirulina* sp.; Blue - relative intensity from *Arthrospira platensis* PCC 7345.

Appendix VII – pH plots obtained from the Algenuity software upon conclusion of the *Spirulina* sp. and *Arthrospira platensis* PCC 7345 light manipulations assays

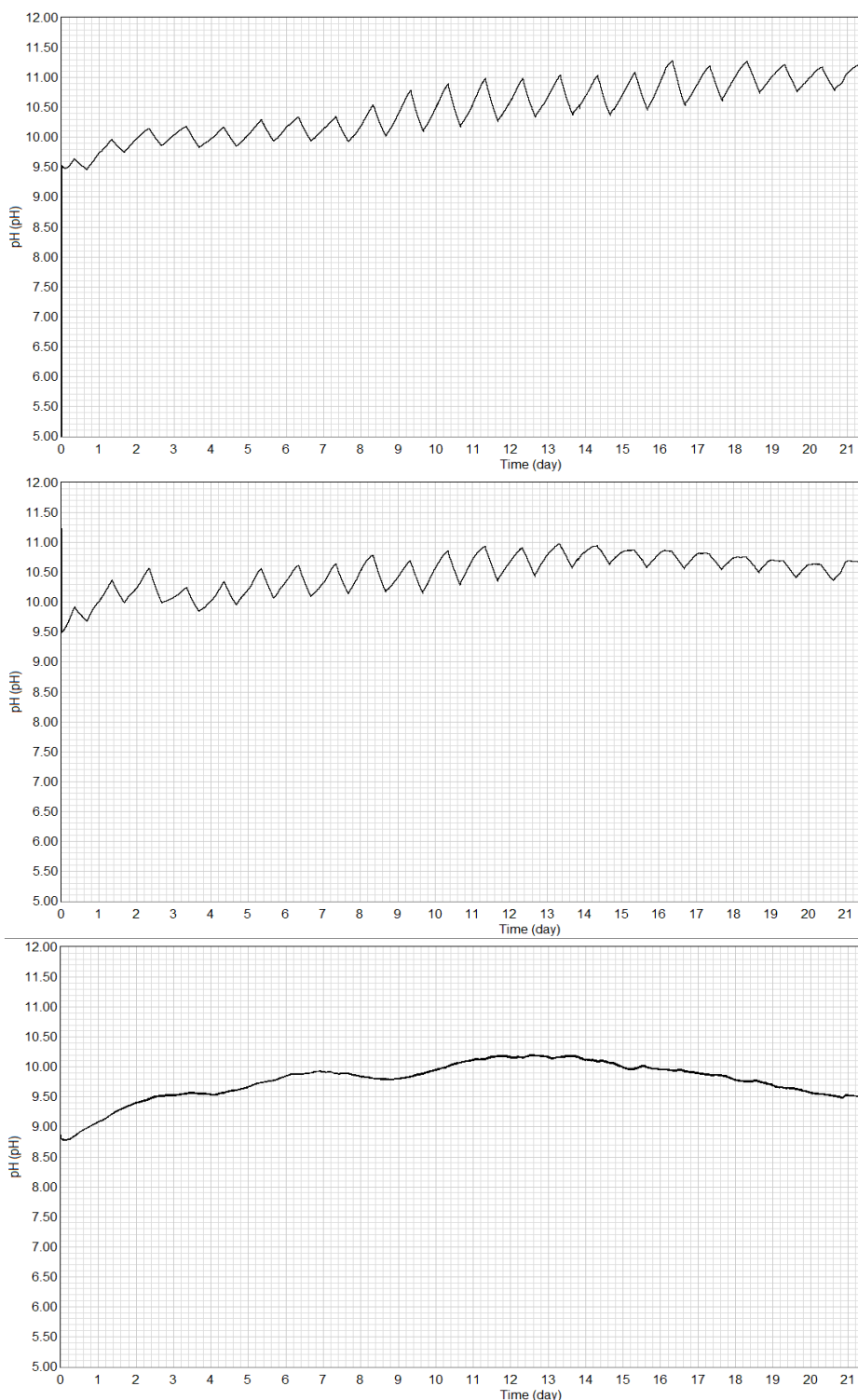


Figure VII.1 - Plots showing pH variation over time during the growth condition manipulation through different light profiles essay on *Spirulina* sp. (top – photobioreactor 1A; middle - photobioreactor 1B; bottom – photobioreactor 2A).

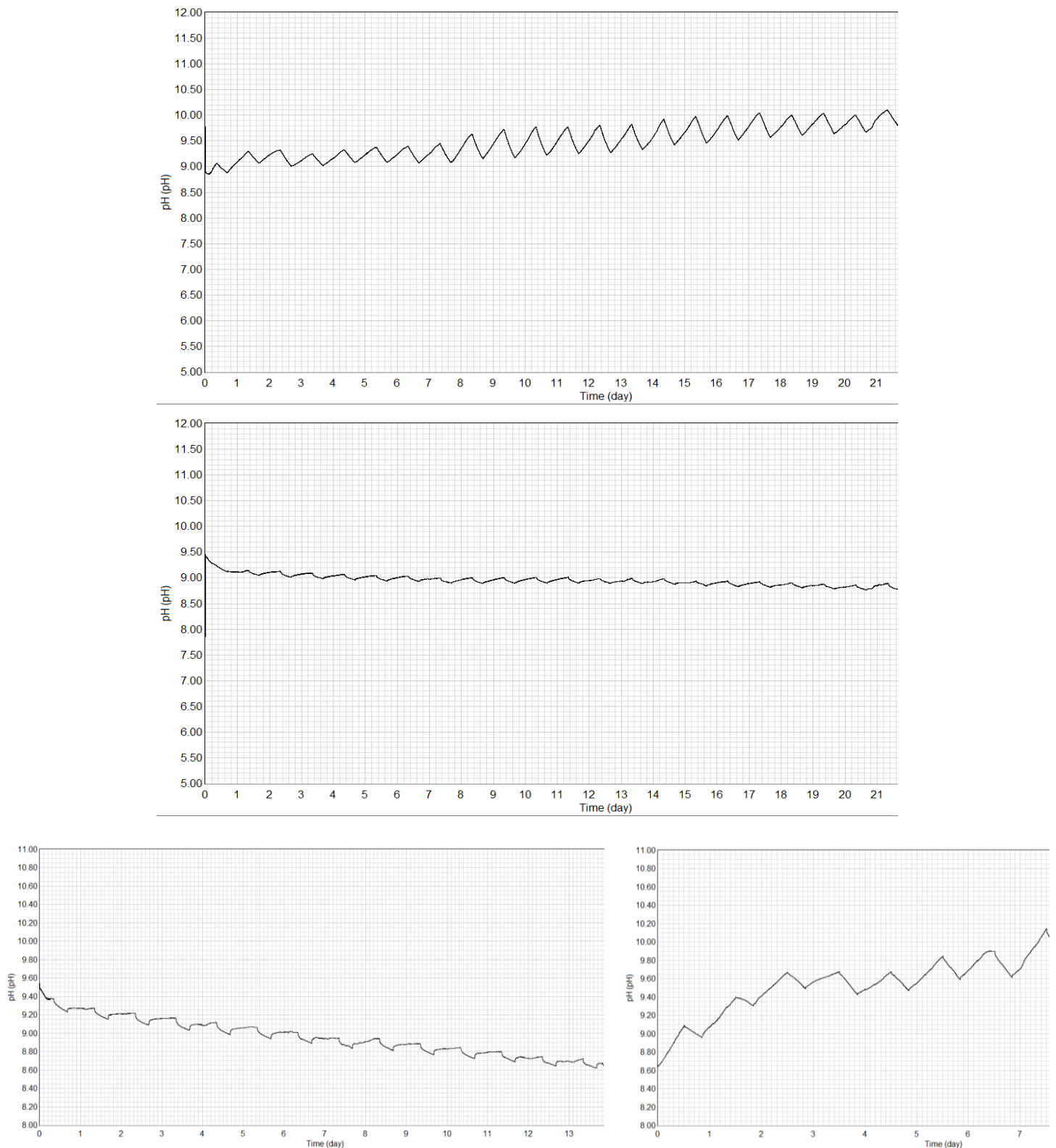


Figura VII.2 - Plots showing pH variation over time during the growth condition manipulation through different light profiles essay on *Spirulina* sp. (top – photobioreactor 2B; middle - photobioreactor 3A; bottom left – photobioreactor 3B on red light; bottom right - photobioreactor 3B on blue light).

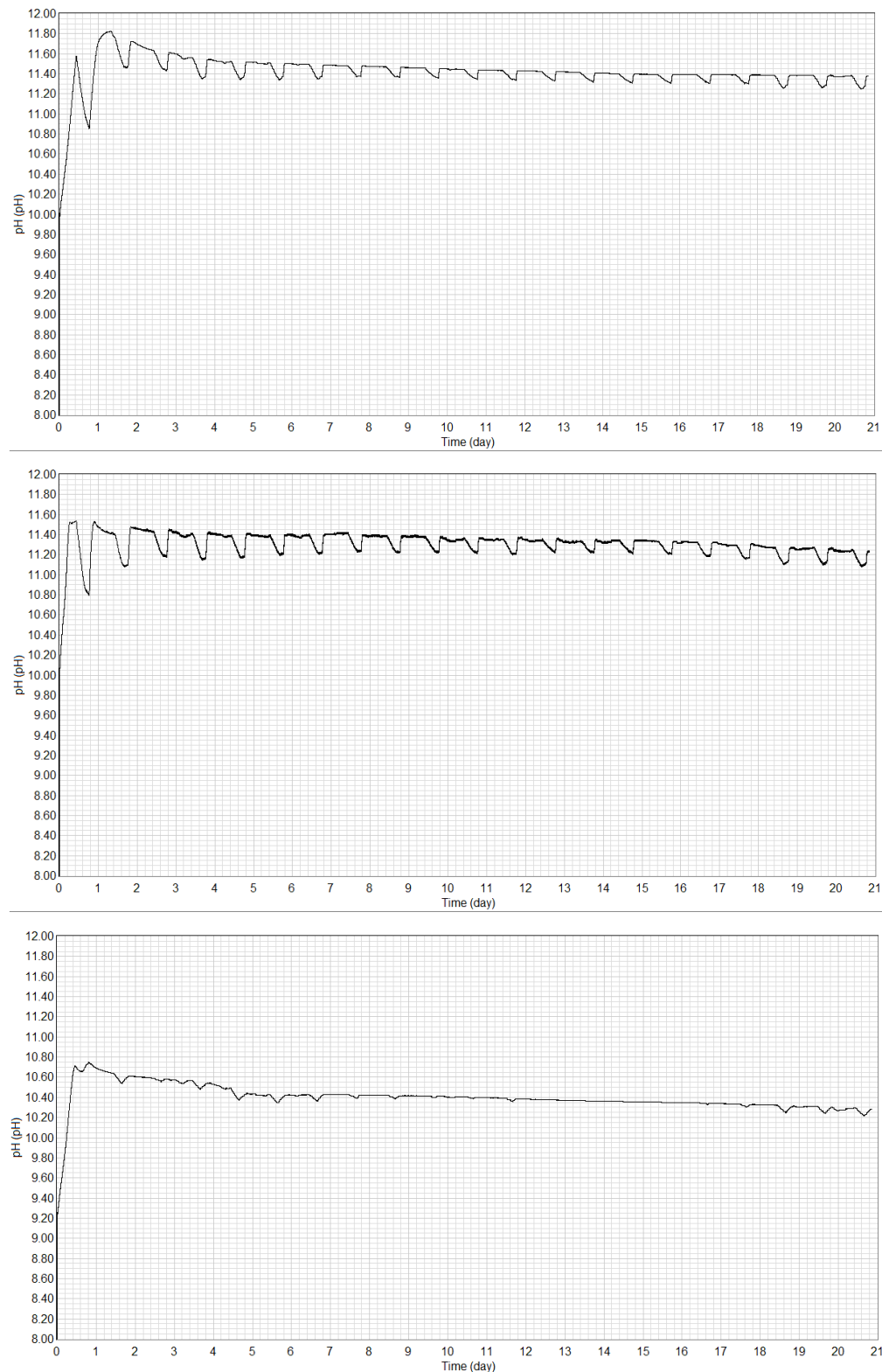


Figure VII.3 - Plots showing pH variation over time during the growth condition manipulation through different light profiles essay on *Arthrospira platensis* PCC 7345 (top – photobioreactor 1A; middle - photobioreactor 1B; bottom – photobioreactor 2A).

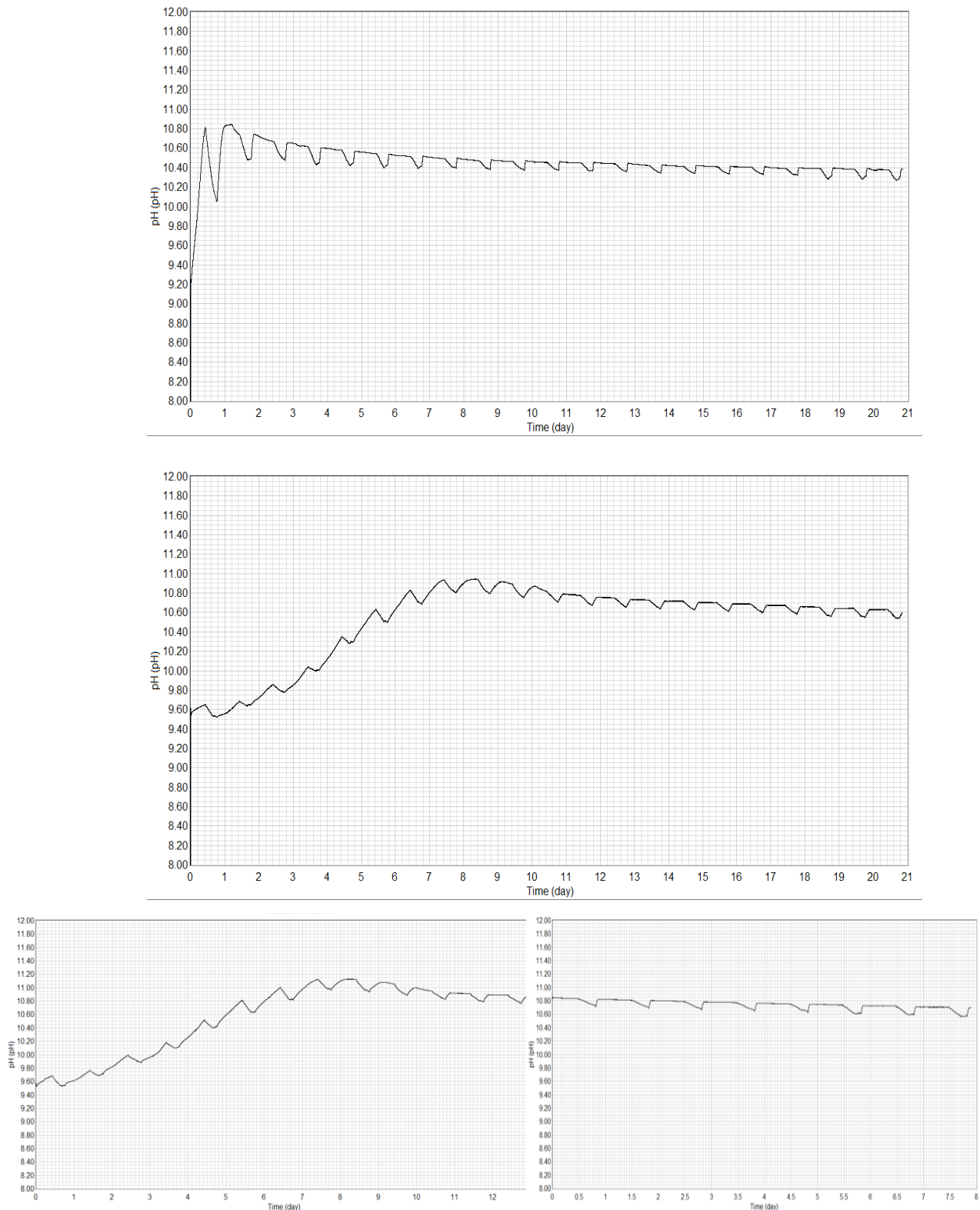


Figure VII.4 - Plots showing pH variation over time during the growth condition manipulation through different light profiles essay on *Arthrospira platensis* PCC 7345 (top – photobioreactor 2B; middle - photobioreactor 3A; bottom left – photobioreactor 3B on red light; bottom right - photobioreactor 3B on blue light).