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Optimization of growth condition of selected microalga strain for fucoxanthin production

Diploma thesis

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Declaration

I declare that the Diploma Thesis ‘Optimization of growth condition of selected microalga strain for fucoxanthin production’ is my own work and all the sources I cited in it are listed in the References.

Prague, 26.04.2021 Irena Knetlová

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Thus, I am adding my special thanks for the existence of microalgae, the subject of interest of my master thesis.

Abstract:

Microalgae are a large group of predominantly unicellular organisms with wide adaptability to diverse environmental conditions. The adaptation is associated with the production of valuable compounds: pigments, which show a wide range of applications. One of these pigments is fucoxanthin with a positive effect on human health. Fucoxanthin is an especially strong antioxidant. Currently, fucoxanthin is obtained mainly from macroalgae, although fucoxanthin content is higher in microalgae. The obstacle for fucoxanthin production from microalgae is the high costs of production and subsequent processing. Therefore, the optimization of cultivation conditions to reduce production costs is a priority. The master thesis focuses on certain microalga which has shown to have high potential in fucoxanthin yield. The microalga produces besides fucoxanthin also eicosapentaenoic acid and docosahexaenoic acid. The master thesis is introducing the results of microalgal performance during experiments testing various cultivation conditions. All experiments were done during an internship at the Centre Algatech, Institute of Microbiology of the Czech Academy of Sciences.

Keywords: microalgae, cultivation, optimization, pigment, fucoxanthin, biotechnology

Abstrakt:

Mikrořasy jsou početnou skupinou převážně jednobuněčných organismů, vyznačujících se velkou měrou přizpůsobivosti k rozličným podmínkám prostředí. Přizpůsobivost je spojena s produkcí cenných sloučenin: pigmentů, které vykazují širokou škálu užití. Jedním z těchto pigmentů je fukoxanthin, který má pozitivní vliv na lidské zdraví. Fukoxanthin je obzvláště silný antioxidant. V současné době se fukoxanthin získává hlavně z makrořas, navzdory tomu, že obsah fukoxanthinu v mikrořasách je vyšší. Překážkou pro produkci fukoxantinu z mikrořas jsou vysoké náklady na výrobu i na následné zpracování. Prioritou je tudíž optimalizace kultivačních podmínek, která by vedla ke snížení výrobních nákladů. Diplomová práce se zaměřuje na určitou mikrořasu, u níž se prokázal vysoký potenciál výtěžku fukoxanthinu. Mikrořasa produkuje kromě fukoxanthinu také kyselinu eikosapentaenovou a kyselinu dokosahexaenovou. Tato diplomová práce informuje o výsledcích experimentů za různých kultivačních podmínek. Všechny experimenty byly prováděny během stáže v Centru Algatech, Mikrobiologického ústavu AV ČR.

Klíčová slova: mikrořasy, kultivace, optimalizace, pigment, fukoxanthin, biotechnologie

1 Table of Contents

2 Introduction

Microalgae are generally described as a large group of predominantly unicellular microscopic organisms, living in freshwater or marine environments. By estimate, microalgae include 22,000 to 26,000 species (Norton et al., 2019). The significance of microalgae is enormous. Since they are at the bottom of the food chain, they serve as a base support for the ecosystem. This wide and versatile group adopted various life strategies to improve its energy management, such as pigment production (Maeda et al., 2008; Kraan, 2013). Pigments are colorful substances that either improve the light-harvesting capacity of organisms and actively participate in photosynthesis, as collectors and transmitters of electrons, or serve as photoprotection against excessive light radiation (Maeda et al., 2008).

One such example of pigment is fucoxanthin (henceforth FX), which is a yellow-to-brown light-harvesting pigment, classified as a primary carotenoid. FX represents up to 10 % of carotenoids in nature (Kraan, 2013) and poses a unique structure that is associated with a beneficial effect on human health (Maeda et al., 2008; Kim et al., 2012; Kraan, 2013; Zhang et al., 2015; Foo et al., 2017). Due to its health benefits, FX is marketed as a dietary supplement. Currently, FX is obtained predominantly from macroalgae such as *Laminaria japonica*, *Eisenia bicyclist*, and *Undaria pinnatifida*. However, these macroalgae contain a lower amount of FX compared to microalgae, which have not yet been widely utilized in the commercial production of FX (Kraan, 2013). To detect prospective high-yielding microalgal strains is therefore a priority.

Hibberdia magna, belonging to the class of Chrysophycae, is the subject of the research of this master's thesis. This freshwater microalga produces a relatively high amount of FX, thus it can be potentially suitable for use in biotechnology. Besides FX, *Hibberdia magna* produces long-chain omega-3 fatty acids: eicosapentaenoic acid, and docosahexaenoic acid. These long-chain omega-3 fatty acids, referred to as PUFA, are also associated with health benefits for humans, such as the prevention of cardiovascular diseases (stroke or high blood pressure), and a beneficial impact on the central nervous system (Dyall et Michael-Titus, 2008).

Currently, the main source of PUFA for dietary supplements is fish, which are often under threat of overfishing. Therefore, microalgae as an alternative source of PUFA would be beneficial. However, their higher cost in comparison to omega3 fatty acids derived from fish remains the main obstacle (Harwood et Guschina, 2009).

Although *Hibberdia magna* appears to be a promising source of both FX and PUFA, its biotechnological potential has not been researched and described yet. Therefore, to uncover its potential, the precise optimization of its growth conditions is needed, to possibly lower the production cost of prospective large-scale cultivation (Ruiz et al., 2016).

3 Work Objectives

The main objectives of the thesis were defined as follows:

1. To learn how to manipulate, handle and grow *Hibberdia magna* under various physical and biochemical conditions.

2. To broaden the knowledge of *Hibberdia magna*.

3. To evaluate the biotechnological potential of *Hibberdia magna*.

4. To give recommendations for further scientific research.

4 Literature Review

4.1 Microalgae as food

Microalgae in human nutrition lend themselves to a wide range of applications, such as nutritional supplements, or natural food colorants. Various foods, such as wine, pasta, beverages, snacks, cereals, oils, vegetarian food gels, cheese, yogurt, and other dairy products can be enriched by algal extract (Spolaore et al., 2006; Raja et al., 2008; Caporgno et Mathys, 2018).

Caporgno et Mathys (2018) suggest that algae should be considered as a source of protein, carbohydrates, polyunsaturated fatty acids, essential minerals, vitamins, and carotenoids. The study stresses the unsustainability of current protein sources. Currently, around 80 % of the protein for feeding European livestock is imported from abroad. These plant protein sources are usually associated with environmentally challenging practices. They suggest there is potential in large-scale algae cultivation for bulk protein. The idea of utilizing algae for protein production is not recent, it was suggested as early as 1952 at the Algae Mass Culture Symposium. However, the large-scale cultivation of algae poses several drawbacks. For instance, the food safety of algae is not well examined and possible hazards such as allergens should be studied. Another obstacle is the high energy requirements for biorefining the desired substance; for microalgae the subsequent processing accounts for a 50-60 % increase in biorefining costs. According to Ruiz et al. (2016) the commercialization of bulk commodities is not yet profitable. Currently, the market price is low, and the revenue is not able to cover the production costs or bring profit to the producer.

However, algae are still considered to be an interesting source of bulk protein due to their low environmental requirements. Caporgno et Mathys (2018) compared algal protein to traditional meat sources. The land requirements per 1 kg of protein represent <2.5 m^2 for microalgae, 42-52 m^2 for chicken, 47-64 m^2 for pork, and 144-258 m^2 for beef (ibid.: 2).

Besides protein, microalgae produce polysaccharides and oligosaccharides which are considered as prebiotics, helping and improving the growth of desired intestinal microbiota. Among the best utilization of microalgae, Caporgno et Mathys (2018) count the innovative functional food, enriched with high-valuable compounds derived from algae, and thus improving the nutritional profile of products. This practice is economically feasible and beneficial.

4.1.1 Nutraceuticals and dietary supplements from microalgae

Nutraceuticals are nutrients from food that contribute to the prevention and treatment of diseases. Microalgae are generally acknowledged as an important source of bioactive compounds due to their rich chemical profile. They can be a source of 'A (Retinol), B1 (Thiamine), B2 (Riboflavin), B3 (Niacin), B6 (Pyridoxine), B9 (Folic acid), B12 (Cobalamin), C (L-Ascorbic acid), D, E (Tocopherol), and H (Biotin). Also, these organisms concentrate essential elements including Potassium, Zinc, Iodine, Selenium, Iron, Manganese, Copper, Phosphorus, Sodium, Nitrogen, Magnesium, Cobalt, Molybdenum, Sulfur, and Calcium. Algae are also high producers of essential amino acids and omega 6 (Arachidonic acid) and omega 3 fatty acids (docosahexaenoic acid, eicosapentaenoic acid)' (Bishop et Zubeck, 2012 ibid.: 1).
Other significant compounds for nutraceutical utilization include pigments with a high antioxidative capacity (such as beta-carotene, lutein, and astaxanthin). Pigments rank among the most profitable algal commodities. The global market volume is estimated at $1 bn, which represents 1000 tons sold annually. The attractiveness of marketing natural pigments is very high due to customers' tendency to buy products perceived as more natural.
Bishop et Zubeck (2012) expect a continuous demand for nutraceuticals derived from microalgae due to their ability to treat and prevent serious diseases, such as viral infection, cardiovascular diseases, and cancer. Therefore, the importance of microalgae in health-related sectors is most likely to thrive.

4.1.2 Commercially used strains

Commercially established strains include *Spirulina platensis*, *Chlorella vulgaris*, *Dunaliella salina*, *Haematococus pluvialis*, *Phaeodactylum tricornutum*, and *Aphanizomenon flos-aquae* (Benedetti et al., 2004; Bishop et Zubeck, 2012).
The following several examples of microalgal strains serve to illustrate the wide range of microalgal applications. For example, *Spirulina platensis*, a procaryotic cyanobacterium (Bishop et Zubeck, 2012), represents the richest natural source of vitamin B12 and contains vitamins B1 and B2 and carotenoids from the xanthophyll group. Due to its rich chemical profile, *Spirulina* offers a wide range of applications. For example, an extract of *Spirulina* Calcium-Spirulan can inhibit the replication of viruses. *Spirulina* can act as a functional food, beneficially influencing the composition of intestinal flora (Caporgno et Mathys, 2018).
Another microalgal strain worthy of attention is *Chlorella spp*., characterized by a very broad scale of use. It is composed of 45 % protein and 20 % fat, and this molecular composition

belongs to the most important strains for algal biotechnology. *Chlorella spp*. can provide all required essential amino acids for heterotrophic organisms. It has shown the ability to detoxify metals and pesticides, produces lutein, a carotenoid associated with health benefits, such as anti-inflammatory, antioxidant, or antitumor effects. The *Chlorella spp.* extracts lower blood pressure and cholesterol and accelerate the healing of wounds. Despite all the mentioned positive effects, *Chlorella spp.* poses several challenges, such as allergic reactions and digestive issues when not processed properly (Bishop et Zubeck, 2012).

The unicellular microalga *Dunaliella salina* also belongs among important nutraceutically utilized strains. This microalga is characterized by the production of betacarotene, a high content of protein, and a relatively thin cell wall which can be easily disrupted. *Dunaliella salina* is also unique in its tolerance of saline environment, therefore its production does not compete with water potentially utilized for agriculture. Its tolerance of high salinity also excludes a vast majority of pathogens, bacteria, or other algae in terms of prospective contamination.

Due to its production of various types of carotenoids, *Dunaliella salina* is associated with health benefits (Bishop et Zubeck, 2012). One of its unique health benefits is its hepatoprotective effect. In a study by Hsu et al. (2008), mice were intoxicated by carbon tetrachloride and they were treated with the intake of silymarin and *Dunaliella salina*. Both proved to have favorable effects on the liver condition. *Dunaliella salina* showed to reduce peroxidation processes and increase antioxidant enzyme activity. Therefore, Hsu et al. (2008) suggest the use of *Dunaliella salina* in vivo.

Another important microalga is *Heamatoccocus pluvialis*, which is mainly cultivated for its pigment astaxanthin, produced during a resting phase. The content of astaxanthin represents 1.5-3 % of the dry weight of *Heamatoccocus pluvialis* (Bishop et Zubeck, 2012). Astaxanthin is associated with various health effects such as reducing inflammation and oxidative stress, protecting against the impact of excessive UV radiation, improving vision and positively affecting the nervous system. Miki (1991) tested the antioxidative activities of several carotenoids (zeaxanthin, lutein, tunaxanthin, canthaxanthin, and astaxanthin). It was concluded that astaxanthin is approximately 10 times stronger than other tested carotenoids. Astaxanthin is considered to be a safe substance without any significant risks. It has been marketed in the United States and in Europe without any reported case of toxicity (Spiller et Dewell, 2003).

Phaeodactylum tricornutum is a diatom of emerging importance in biotechnology, utilized as a biofuel precursor (Bañuelos-Hernández et al., 2015). It contains a relatively large quantity of FX, therefore it has been considered as a perspective strain for FX production (Kim et al., 2012).

Aphanizomenon flos-aquae is a freshwater unicellular blue-green alga rich in phycocyanin, characterized by antioxidant and anti-inflammatory properties, which are used in the food industry (Benedetti et al., 2004).

These microalgae represent just a small fraction of biotechnologically used strains that positively influence the human health and manifest an unique nutritional profile (Spolaore et al., 2006; Raja et al., 2008).

4.1.3 Carotenoids

Carotenoids are pigments that either improve the light-harvesting capacity of organisms and actively participate in photosynthesis as collectors and transmitters of electrons or serve as photoprotection against excessive light radiation (Maeda et al., 2008).

Carotenoids are produced by numerous plants, bacteria, fungi as well as algae. They are known for their unique properties of bioactive compounds which contribute to neutralizing the free radicals in an organism and act as protection against them (Bendich & Olson, 1989).

Thus, scientific attention is attracted by the antioxidating properties of carotenoids because of their active response to the human immune systems and involvement in the internal biochemical processes.

Carotenoids such as FX or astaxanthin are involved in scavenging ROS (reactive oxygen species), singlet molecular oxygen, and peroxy radicals which are formed in the process of peroxidation. This antioxidant capacity can lead to a decrease in the progression of illnesses associated with oxidative stress (Hosokawa et al., 2009).

They may also have an auspicious effect on skin protection against excessive light radiation. According to Terao et al. (2010), carotenoids seem to have the ability to prevent oxidative stress by an increase in the concentration of antioxidants in the skin layers when reactive oxygen is induced by solar light and photooxidative stress occurs. This mechanism prevents accelerated aging of the skin.

As reported by Bendich et Olson (1989), carotenoids can be even considered as a protection against neoplasm, abnormal and unstructured growth, and multiplication of cell tissues. Finally, they are divided into several groups based on their biological and nutritional activity.

Since the health benefits of carotenoids are recognized, they represent a highly lucrative sphere of trade (Temple, 2010): ß-carotene, lutein, astaxanthin, and FX are marketed as a dietary supplement (McClure et al., 2018).

4.1.4 Fucoxanthin

FX is a light-harvesting pigment, classified as a primary carotenoid, belongs to the xanthophyll group. FX occurs abundantly in microalgae and macroalgae and by estimation, it makes up 10 % of carotenoids in nature (Kraan, 2013).

FX is bound to various proteins, along with chlorophyll, especially in the thylakoids. The molecular structure of FX is unique among other carotenoids due to its unusual allenic bond, a 5,6-monoepoxide, and 9 conjugated double bonds, and its unique structure is associated with a beneficial effect on human health (Kim et al., 2012, Kraan, 2013). It poses properties that have not been described in any other carotenoids. FX is associated with anticancer, antihypertensive, anti-inflammatory, anti-obesity, and anti-diabetic activity and increases the amount of DHA, a crucial polyunsaturated fatty acid in the human body (Maeda et al., 2008, Kim et al., 2012, Zhang et al., 2015, Foo et al., 2017).

The literature (Maeda et al., 2008, Kim et al., 2012, Zhang et al., 2015, Foo et al., 2017) emphasizes its role as support in the prevention of illnesses associated with an unhealthy lifestyle.

If considering FX as a substance with health benefits, the perspective toxicity of FX should be examined. A study by Beppu et al. (2009) examined the toxicity of FX. In the study, the FX was administrated orally in various dosages to mice (1000 and 2000 mg/kg in a single dose study and 500 and 1000 mg/kg in case of a repeated dose study). No abnormalities were observed in either of the studies, therefore FX is considered to be a safe substance.

Research by Maeda et al. (2008) and Woo et al. (2010) suggest that FX can serve as efficient prevention of metabolic syndrome and impact the function of adipose tissue, as well as reduce blood glucose. Thus, FX can serve as a prevention of diabetes mellitus, an illness with the possibility to become the second most important preventable cause of death after smoking.

In a clinical study by Abidov et al. (2010) an obese woman was treated with brown seaweed extract containing 2,4 mg of FX and it resulted in significant weight loss and reduction in liver fat content.

The ability of FX to prevent obesity might be linked with the dietary habits in Japan, where the obesity rates are unusually low in comparison to the Western countries. The Japanese are the greatest consumers of algae in their diet. They consume mainly seaweed, but the variety is rich, more than 50 kinds of algae are recognized in Japanese cuisine (Murata & Nakazoe, 2001). High intake of seafood and seaweed is also associated with the longevity of Japanese people and unusually low rates of cancer (Zava et Zava, 2011).

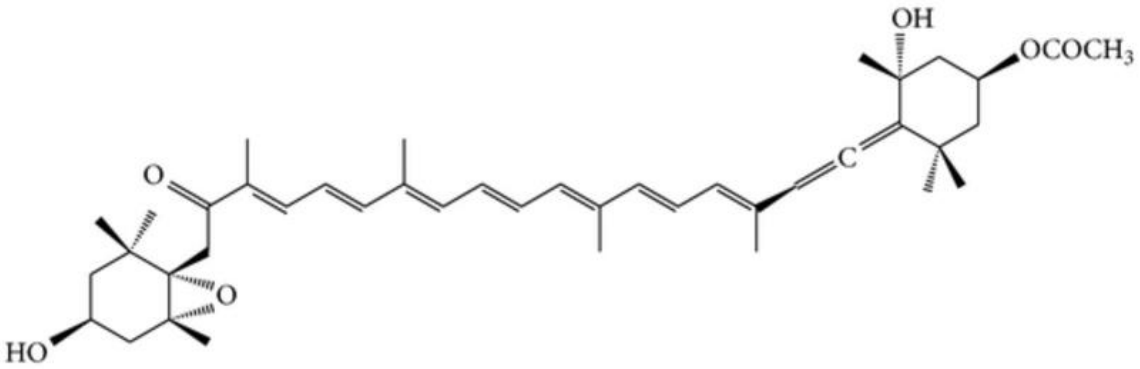

Fig. 1. The chemical structure of FX (Zhang et al., 2015)

4.1.5 Current source of fucoxanthin

The current source of FX for dietary supplements is mainly represented by macroalgae such as *Laminaria japonica, Eisenia bicyclist*, and *Undaria pinnatifida*. The disadvantage of macroalgae as a key FX source is their relatively low amount of FX in comparison to FX yield in microalgae; another disadvantage is the high water content in macroalgae, which have to be dried prior to use (Kim et al., 2012).

In terms of microalgae, the promising source of FX obtained from microalgae is *Phaeodactylum tricornutum*. The study by Kim et al. (2012.) suggests that *Phaeodactylum tricornutum* might be a very convenient source for FX production, due to its high content.

This study compared FX contents in macroalgae versus microalgae. For *Phaeodactylum tricornutum* a relatively constant yield was gained, specifically 15.42 – 16.51 mg/g in freeze-dried sample weight. The amounts of FX obtained from macroalgae were significantly lower, for Eisenia bicyclis the FX content reached maximally 0,26 mg/g, for *Undaria pinnatifida* the content reached 0,87 mg/g, and for the common macroalga *Laminaria japonica* it was just 0,19 mg/g (Kim et al., 2012).

4.1.6 Polyunsaturated fatty acids

Eicosapentaenoic fatty acid (henceforth EPA) and docosahexaenoic acid (henceforth DHA) are long-chain omega 3 fatty acids, often referred to as PUFA. They are valuable compounds occurring in many algal species and that are associated with positive health benefits in the human diet, for instance the prevention of cardiovascular diseases such as stroke or high blood

pressure. They also have a beneficial impact on the central nervous system and their intake is recommended in the treatment of depression (Dyall et Michael-Titus, 2008).

Although they are of great importance, the human body is rather inefficient in synthesizing the omega 3 fatty acids. With aging or in case of illness the enzymatic ability to desaturate and elongate essential linoleic acid and alfa linoleic acid into EPA and DHA decrease. Therefore, it is important to provide an additional source of Omega-3 fatty acids (Harwood et Guschina, 2009).

Besides EPA and DHA, arachidonic acid (henceforth ARA) a long-chain fatty acid is of great importance. ARA is an important precursor for eicosanoids (prostaglandins, leukotrienes, and thromboxanes), biologically active molecules are involved in biochemical processes such as signalization or fight against inflammation. (Khozin-Goldberg et al., 2011)

4.1.7 Current source of PUFA

Despite the fact that microalgae are at the beginning of the food chain, serving as a primary source of omega3 fatty acids for the marine food chain, the main commercial source of PUFA is derived from fish oil (Harwood et Guschina, 2009) (Khozin-Goldberg et al., 2011), mainly fatty fish such as salmon, mullet, or mackerel. The natural habitat of those fish, i.e. seas and oceans, is under continuing threat of exploitation. Therefore, a search for alternative and more sustainable maintained sources of PUFA would be beneficial for the environment. This might open an opportunity for large-scale microalgae cultivation (Adarme-Vega et al., 2012, Khozin-Goldberg et al., 2011). However, the main obstacle to the commercial production of omega3 fatty acids from microalgae is the production cost compared to omega3 fatty acids derived from fish (Harwood et Guschina, 2009).

Currently, the omega3 derived from microalgae is more expensive and is rather an exclusive option for niche markets. However, (Harwood et Guschina, 2009) admit that the advantage of omega3 derived from microalgae in comparison with fish-derived omega3 is the acceptability to vegetarians and vegans, better taste without fish smell, and especially the reduced risk of contamination, since seafood might be associated with a higher risk of methylmercury and organochlorine contamination, which poses a risk of toxicity and carcinogenicity (Khozin-Goldberg et al. 2011; Watters et Edmonds, 2012). suggest that for the large-scale production of PUFA from microalgae, a technological and biochemical bottleneck is needed, which can lead to a reduction in costs. Further, they suggest the cultivation of nutritionally less-demanding strains that can withstand contamination by predatory organism bacteria or fungi and focus on less expensive cultivation practices such as open ponds or low-cost photobioreactors. Besides

microalgae, transgenic plants and yeast are also considered promising sources of fatty acids. ARA is currently produced from the fungus *Mortierella alpina* (Khozin-Goldberg et al., 2011).

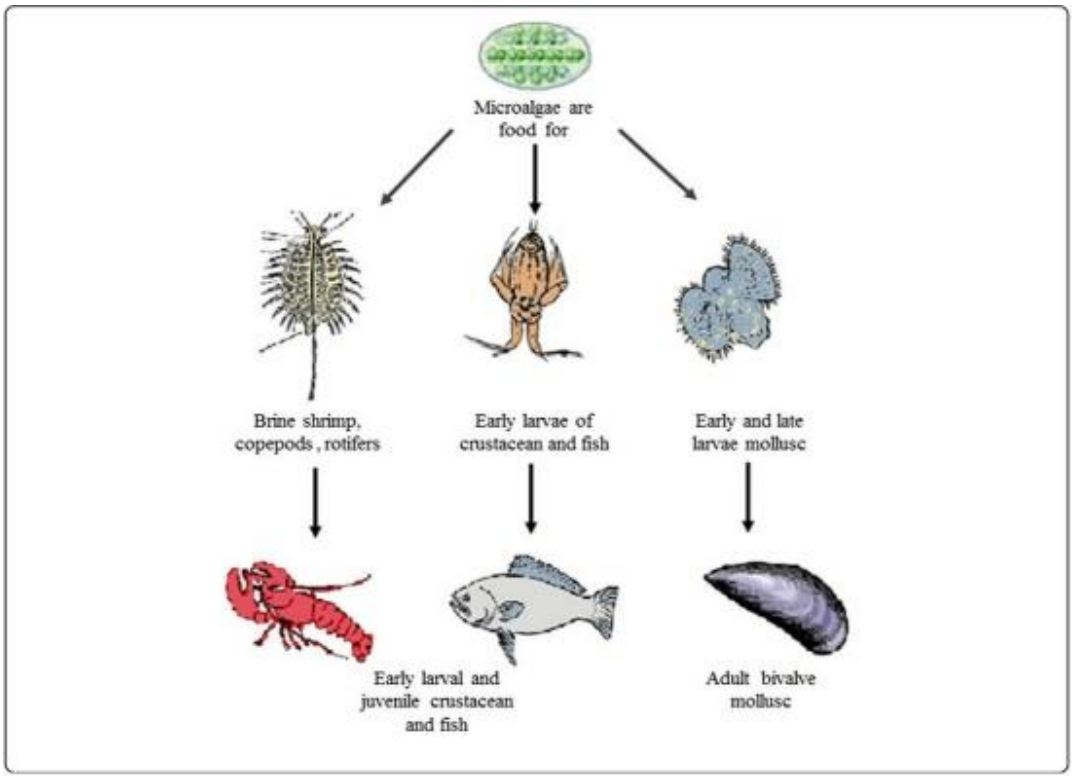

Fig. 2. Microalgae serve as primary food source of marine and freshwater foodchain, (Adarme-Vega et al., 2012)

4.2 Microalgae in agriculture

The application of microalgae in agriculture is at an early development, but a perspective area of microalgae uses. The main areas of emerging microalgae cultivation include the use of microalgal inoculate to impact soil microbiota, biostimulants (production of phytohormones), biofertilizers, and biopesticides (protection against fungi, bacteria nematodes, and other pathogens).

4.2.1 Biopesticides

Biopesticides are defined as substances that are derived from living organisms. They can be divided into three groups based on their origin; biochemical pesticides, botanical biopesticides,

and lastly microbial biopesticides, including pesticides derived from microalgae (Costa et al., 2019). Microalgae and cyanobacteria produce a wide variety of chemical compounds, referred to as secondary metabolites, with a capability of antifungal, antibacterial, and toxic activities against nematodes. Phenolic compounds such as polyphenols and tocopherols, carbohydrates, proteins, oils, saponins, alleo chemicals, nitrogen-rich peptides, and sesquiterpenes can be obtained from microalgae and cyanobacteria (ibid.). These compounds cause a disruption of the cytoplasmatic membrane, and subsequently paralyze the protein synthesis of pathogens (ibid.). Generally, there are two ways of functioning of algal biopesticides: the first one is the stimulation of the promotion of enzyme production in plant enzymes such as catalase, chitinase or peroxidase, enyzmes tend to increase when the plant is exposed to algal metabolites. The second way of functioning is forming a thin layered biofilm (extracellular polymeric substances). Both ways protect the plant. Among the first algae reported to have biopesticide activity was *Chlorella vulgaris,* producing a secondary metabolite called chlorellin which impacts the growth of both gram-positive and gram-negative bacteria and shows antifungal capacity. Other strains which deserve closer attention are *Anabaena laxa* together with *Lyngbya spp*., showing a larvicidal effect, *Nostoc spp*. and *Microcystis aeruginosa*, showing herbicidal effect, and *Spirulina platensis*, showing an antifungal effect (Costa et al., 2019; Fernández et al., 2021). Although microalgae and cyanobacteria strains have a high potential, continuous research is needed to elucidate the pathway of production and their mechanism of action. Costa et al. (2019) also stress there might be several economical obstacles to the commercialization of biopesticides because the production of biopesticides is rather expensive.

4.2.2 Biostimulants and biofertilizers

Traditionally, algae are used as a fertilizer in coastal areas, which results in more efficient water-binding capacity along with improvements in the mineral profile of fertilized soil. The compound derived from microalgae can support the higher plants not only by enhancing water storage and by nitrogen fixation (e.g. through *Anabaena spp*. or *Nostoc spp*.) but can also benefit higher plants by enhancing their growth (Pulz et Gross, 2004). Microalgae can increase the overall soil microbial activity and facilitate better microbial interactions as they support the growth of beneficial microbes (Renuka et al., 2018).

Cyanobacterial inoculation in soil has been reported to benefit the plants in many ways, e.g. enhanced seed germination, plant growth, grain yield, and nutritional value in a variety of crops, and a lower need for nitrogen. Several species of Cyanobacteria are known for their ability to fix the atmospheric nitrogen. This was the subject of research by Osman et al. (2010), who

examined the effect of cyanobacterial inoculation in the form of suspension on pea plants, together with the application of different doses of mineral fertilizers. They came to the conclusion that two cyanobacterial species, *Nostoc entophytum* and *Oscillatoria angustissima*, produce bioactive compounds (such as phytohormones, nitrogen, and carbohydrate fractions). These mixtures of compounds had shown a positive impact on the germination, growth, and metabolic processes. Both of the species have shown better results when they were applied together along with mineral fertilizers, rather than in the inoculation by a single cyanobacterium. This combination of cyanobacteria was capable of reducing the recommended dose of mineral fertilizers by 50 %. The rise in nitrogen after the application of cyanobacterial suspension was most likely caused by several aspects, mainly by nitrogen fixation and nitrate reductase activities of cyanobacteria, uptake of ammonia, and uptake of the amino acids and peptides produced by *Nostoc entophytum* and *Oscillatoria angustissima* (Osman et al., 2010; Renuka et al., 2018).

Cyanobacterial species show an ability to enhance the content of bioavailable phosphorus in the soil. Elevated secretion of organic acids was observed after the application of cyanobacteria. This further impacts the soil pH and influences phosphorus availability. However, the higher availability is attributed not only directly to the cyanobacterial application but to the soil complexity, together with other soil microorganisms which produce chelating substances (Osman et al., 2010; Renuka et al., 2018).

In the opinion of Osman et al. (2010), the use of cyanobacteria as a bioagent along with mineral fertilizers may aid the environment by lowering the adverse environmental impact associated with mineral fertilizer use. They emphasize the potential commercial attractivity of cyanobacteria; however, they concede the requirement for further research.

4.2.3 Phytohormone production

Microalgae produce phytohormones, including auxin, abscisic acid, cytokinin, ethylene, and gibberellins. Their function and physiological effect show similarities to phytohormones in higher plants (Lu et Xu, 2015).

Thus, the isolation of microalgal phytohormone may lead to an enhancement of crop yields in agriculture. This is in compliance with the study by Hussain and Hasnain (2011), which evaluated the impact of cyanobacterial-derived phytohormones on wheat. After inoculation with *Chroococcidiopsis sp*. they observed better parameters in germination, shoot length, tillering, number of lateral roots, spike length, and grain weight which was increased

significantly. Hussain and Hasnain (2011) considers inoculation by cyanobacteria a very efficient way to gain better yield.
However, according to Renuka et al. (2018), more studies are needed for the application of microalgal phytohormones.

4.3 Microalgae in biotechnology

Microalgae are organisms with rather simple nutritional requirements, and are able to yield high production per unit area (Costa et al., 2019). They are fast-growing organisms with a high multiplication rate, which can annually produce up to 100 tons of biomass per hectare and reach photosynthetic efficiency of up to 10 %. Nonetheless, this productive potential is difficult to reach, and the yield is dependent on the production optimization (Fernández et al., 2021).
The appropriate condition is required to maintain microalgal growth and cell division (Raja et al., 2008). The parameters that have a major influence on algal growth include nutrient quality and quantity, such as macronutrients (nitrogen, phosphorus, and silicon if required); subsequently the amount and appropriate ratio of micronutrients (trace elements and vitamins) should be secured. Besides nutrients, appropriate light conditions have to be ensured. Among other parameters affecting algal growth are pH, carbon supply, turbulence, salinity, and temperature (Coutteau, 1996).

4.3.1 Autotrophic and heterotrophic production

Microalgae can be divided into several categories according to the type of their production. They can be produced either autotrophically or heterotrophically. Heterotrophic production requires a higher input of organic carbon sources, mainly in the form of sugars, glycerol and acetic acid. For 1 kg of microalgae 2 kg of glucose are required. Therefore, the heterotrophic production may impose higher resource requirements and therefore reduce its sustainability. Heterotrophic production is common for *Schizochytrium* and *Chlorella*-like genera, which are both mainly used to produce unsaturated fatty acids. Contrary, the autotrophic production of microalgae prevails. The prerequisites for growth are light, carbon dioxide, and other inorganic nutrients that serve as a source of energy for subsequent growth. The microalgae *Spirulina*, *Chlorella*, *Dunaliella*, *Haematococcus*, *Nanochloropsis*, or *Phaeodactylum* are grown autotrophically (Fernández et al., 2021).

4.3.2 Open vs closed system

The microalgal biomass production can be further divided into two main production systems. The first is the closed photobioreactor, usually consisting of tubes or flat panels made of a transparent material such as plastic or glass. Within the photobioreactor the culture is mixed by way of aeration and additional sources of light is often required. This results in high energy demand, therefore microalgae produced in the photobioreactors are focused on human-related application (Fernández et al., 2021).

This is consistent with the focus of production of the Czech biotechnological company Algamo, which produces pigment and antioxidant astaxanthin from the microalga *Haematococcus pluvialis*. Algamo uses photobioreactors for its cultivation, with several panels arranged in parallel. According to Martin Dobřichovský, Production Manager of Algamo, the most expensive part of the production is energy, labor, pure carbon dioxide, and subsequent processing of the raw product (personal communication, April 21, 2020).

This high share of energy in closed systems (up to 400 W/m^3) is then reflected in the price of marketed products. Based on Fernández et al. (2021), the price of biomass production cannot be lower than 20 €/kg. Despite the high energy costs, closed systems have certain advantages, such as controllability, which contributes to reaching the highest productivity.

Closed systems are mainly represented by raceway reactors and thin-layer cascades. The culture is exposed to the sunlight and atmosphere, therefore contamination can occur. According to Fernández et al. (2021), 90 % of autotrophic production is produced in the raceway ponds. The production price and energy requirements are significantly lower in open systems. According to Fernández et al. (2021), the production cost can reach 10 €/kg or even decline up to 2 €/kg when the nutrients are supplied in the form of wastewater and waste gases are used as a source of carbon dioxide.

4.3.3 Lighting conditions

When cultivating microalgae, the photoperiod, spectral quality, and light intensity should be ensured. The light régime depends on the design of cultivation and on the density of the culture. When higher densities of culture are achieved the light intensity should be increased to enable the light to penetrate deeper. However, when the light intensity is too high, photo-inhibition may occur (Coutteau, 1996).

Light affects the physiological constitution and chemical composition of microalgae, therefore changes in the lighting parameters affect the rate of growth and metabolite production.

4.3.4 Photosynthesis

Since microalgae are photosynthetic organisms, therefore light is essential for algal cultivation. Photosynthesis is a process of sunlight energy conversion, where inorganic compounds and light are converted into organic matter (Coutteau, 1996, Masojídek et al., 2013). About 3.5 billion years ago the earliest photoautotrophic organisms emerged. They used anoxygenic photosynthesis to utilize light to gain protons and electrons from donor molecules such as hydrogen sulfide to reduce carbon dioxide to incorporate carbon into organic matter (Masojídek et al., 2013). Later on, oxygenic photosynthesis evolved and cause a dramatic change in the atmospheric composition on Earth.

All living creatures are directly or indirectly dependent on the process of photosynthesis (Masojídek et al., 2013). Oxygenic photosynthesis is a redox reaction induced by light energy where carbon dioxide and water are converted into carbohydrates and oxygen. The process of photosynthesis is usually divided into two phases, referred to as light and dark phases. The phases differ in the place of action.

Photosynthesis is dependent on the nature of light. When the irradiance is low, the process of photosynthesis depends linearly on the intensity of light. When the irradiance is elevated the photosynthesis shows lower efficiency. Under continuous supra-optimal irradiance, photosynthesis declines, which is referred to as photoinhibition (Masojídek et al., 2013).

4.3.5 Light and pigments

Light is electromagnetic radiation that is emitted in the form of photons. According to wavelength, the light can be divided into categories displayed in figure n. 3. The visible part of light corresponds to the light utilized for photosynthesis, referred to as PAR, i.e. photosynthetically active radiation.

Three main classes of pigments are recognized: chlorophylls (green pigments), carotenoids (yellow to orange pigments), and phycobilins. The absorption spectrum of chlorophylls represents blue-green (450-470 nm) and red (630-675 nm) light. The absorption spectrum of carotenoids represents light with a wavelength of 400-550 nm. And finally, phycobilins absorb light between 500-650 nm (Masojídek et al., 2013).

Algae can be divided based on their light-harvesting photosynthetic pigments: Rhodophyta (red algae), Chrysophyceae (golden algae), Phaeophyceae (brown algae), and Chlorophyta (green algae) (Coutteau, 1996, Masojídek et al., 2013).

The FX contains two absorption peaks. The broader one lies at 445 nm and the sharper peak is at 663 nm (Wang et al., 2018). FX mainly absorbs blue light of the spectrum.

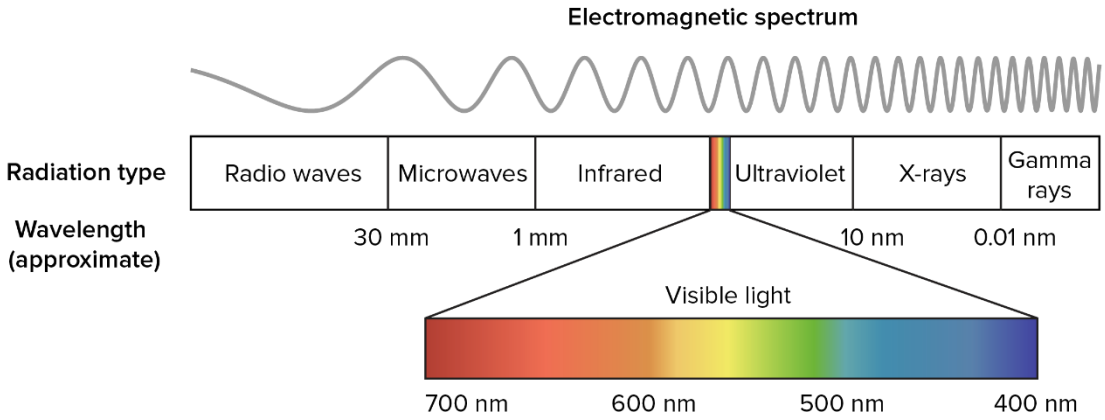

Fig. 3. Electromagnetic spectrum and visible light, Light and photosynthetic pigments

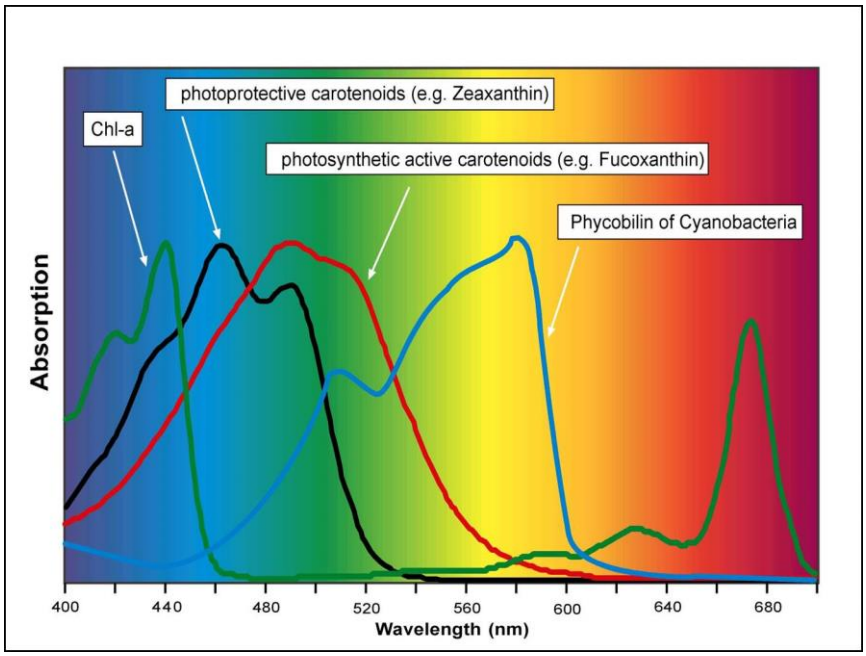

Fig. 4. Absorption spectra of various pigments, FX in red, Phytoplankton pigment analysis by HPLC. GEOMAR

4.3.6 Nutrition

Due to higher cell densities in the culture, there is the need for additional nutrients requirement (Coutteau, 1996). Nutrient supplementation is divided into two main categories: macronutrients (e.g. carbon, nitrate, phosphate, silicate) and micronutrients (trace metals and vitamins) (Coutteau, 1996).

The nutritional requirements depend on the algal energy gain. Photoautotrophic algae requires merely inorganic mineral ions. Although most algae can be categorized as photoautotrophs, they usually require additional supplementation by organic compounds such as vitamins.

Heterotrophic algae require additional supplementation with organic compounds such as glucose. Autotrophy and mixotrophy are other examples of energy gain. According to Grobbelaar (2013) no clear division is possible based on algal tropism because algae can interchange between the various types of tropism. Under unfavorable nutritional conditions, algae are able to produce exudates that inhibit cell division. This can be seen as a strategy to limit competition and protect against predation. The exudate can limit the growth in high cell densities in mass cultures. This may result in a crucial factor preventing the reaching of maximal productivity. It is therefore essential to pay attention to nutritional requirements since they can determine the results of cultivation. The aim is the maintenance of conditions that do not limit growth an obtain high yield of algal biomass.

Most of the culture media are formulated to provide essential nutrients rather in excess; therefore, nutrient deficiency is seldom the case. However, nutrients can become purposefully limiting when aiming to produce special compounds such as carotenoids, PUFA, or polysaccharides (Grobbelaar, 2013).

4.3.7 Carbon

Carbon is an essential element for microalgal growth since it becomes incorporated into biomass during photosynthesis. However, in mass production, the atmospheric carbon dioxide does not meet the demand for high productive microalgae. Carbon is therefore supplied: mostly in the form of CO_2 and HCO_3, or organic carbons in the form of sugars, acids, and alcohol when the culture is mixotrophic. Carbon compounds have a buffering capacity, therefore they help with the maintenance of pH. During photosynthesis, when CO_2 is bound into algal biomass, the pH can continuously rise. On the other hand, in the stage of respiration, when CO_2 is released pH can decrease. According to Grobbelaar (2013) CO_2 is efficient in maintenance of pH of the culture.

4.3.8 Nitrogen

Nitrogen is the second most essential element for microalgal propagation. When nitrogen is limited, the microalgal cells tend to discolorate and accumulate more carotenoids, polysaccharides, and oils, such as PUFA, and decrease the content of chlorophylls.
Nitrogen is added in the form of nitrate, ammonia, or urea. Both ammonia and nitrates contribute to pH maintenance. The effect of pH influence is especially strong when ammonia is used as the main source of nitrogen. When the nitrogen is assimilated from ammonia hydrogen protons are cleaved and acidify the culture medium Conversely, pH grows when nitrate is used as a single source of nitrogen. This knowledge is crucial when optimizing the microalgal growth. Therefore, either ammonia or nitrate can be added during the cultivation to improve the pH balance (Grobbelaar, 2013).

4.3.9 Phosphorous

Although phosphorus represents less than 1 % of the biomass, it should not be neglected, since it is involved in many biochemical processes, mainly in the energy transfer and synthesis of nucleic acids. The obstacle when ensuring appropriate phosphorus supply is its ability to form precipitates with other elements such as C or Fe, which results in a lower bioavailability of phosphorus to microalgae. Naturally, microalgae evolved a mechanism to protect themselves against phosphorus deficiency. The mechanism is called luxury uptake: an excessive uptake of phosphorus and storage for further use when lacking in the environment (Grobbelaar, 2013).

4.3.10 Other micronutrients

Besides C, N, and P other macronutrients such as S, K, Na, Fe, Mg, Ca, and the micronutrients B, Cu, Mn, Zn, Mo, Co, V, and Se are important for proper microalgal growth. Those elements are added in various concentrations with respect to the microalgal strain and lastly, the medium is enriched by vitamins (Grobbelaar, 2013).

5 Methodology

5.1.1 Strain of *Hibberdia magna*

For all experiments involved, the strain of microalgae *Hibberdia magna* was needed. This strain was obtained from the NORCCA collection and it was permanently kept in the algae collection in several Erlenmeyer flasks to prevent loss of a culture. They were covered with a cotton plug and aluminum foil to prevent contamination from the outside environment. The light condition in the room was around 10 μE, the room temperature was stable at 18˚C.

5.1.2 Preparation of inoculum

A 5-liter volume glass bottle with two holes in a silicon plug for outlet tube and inlet tube was sterilized by autoclaving, then a media was mixed with a culture of *Hibberdia magna*, this was done in laminar flow MERCI laminar box OSN 4. Subsequently, a sterile filter was plugged in the inlet tube, which was connected to the carbon dioxide gas mixer for bubbling of culture. The culture was kept in the 5-liter volume bottle in a room at a stable temperature of 18˚C. The continuous light condition was kept between 50-100 μE.

5.1.3 Preparation of media

Several media were used for *Hibberdia magna* cultivation, all media required the preparation of stock solution prior to mixing media solution. The stock solutions were made by weighing a given amount of chemicals and mixing them with a given amount of distilled water. A given amount of concentrated solution or chemical substance was later transferred by pipette or spoon and mixed with the desired amount of distilled water, according to a recipe. Distilled water was prepared by AQUA OSMOTIC type 3. In case the recipe required pH adjustment a diluted HCl was used, pH in the solution was measured by pH meter pH 3310 ProfiLine. Then the media was autoclaved in Tuttnauer autoclave 3870. After autoclaving, they were left to decrease the temperature, and finally, a concentrated vitamin solution was added. The original recipe for WC media which was frequently used was obtained from Andersen (2005) and its subsequent variations were modified according to experiments requirements.

M		WC original		WC+ TRIS		WC+ NO BUFF		2WC+ NO BUFF		2WC_20:15 NO BUFF		WC+ MES 1000mg	
	BUFFER	mg/l	n [mmol]	mg/l	n [mmol]	mg/l	n [mmol]	mg/l	n [mmol]	mg/l	n [mmol]	mg/l	n [mmol]
121,136	Tris - $C_4H_{11}NO_3$	115	0,949	115	0,949								
195,2	MES - $C_6H_{13}NO_4S$											1000	5,123
	N	mg/l	n [mmol]	mg/l	n [mmol]	mg/l	n [mmol]	mg/l	n [mmol]	mg/l	n [mmol]	mg/l	n [mmol]
85	$NaNO_3$	85	1,000	850	10,000	850	10,000	850	10,000	1700	20,000	850	10,000
	P	mg/l	n [mmol]	mg/l	n [mmol]	mg/l	n [mmol]	mg/l	n [mmol]	mg/l	n [mmol]	mg/l	n [mmol]
174,17	K_2HPO_4	8,7	0,050	87	0,500	87	0,500	87	0,500	262	1,504	87	0,500
	rest macro elements	mg/l	n [mmol]	mg/l	n [mmol]	mg/l	n [mmol]	mg/l	n [mmol]	mg/l	n [mmol]	mg/l	n [mmol]
84	$NaHCO_3$	12,6	0,150	12,6	0,150	12,6	0,150	25,2	0,300	25,2	0,300	12,6	0,150
246,36	$MgSO_4 \cdot 7H_2O$	37	0,150	37	0,150	37	0,150	74	0,300	74	0,300	37	0,150
146,98	$CaCl_2 \cdot 2H_2O$	36,8	0,250	36,8	0,250	36,8	0,250	73,6	0,501	73,6	0,501	36,8	0,250
270,2	$FeCl_3 \cdot 6H_2O$	3,15	0,012	3,15	0,012	3,15	0,012	6,3	0,023	6,3	0,023	3,15	0,012
284,07	$Na_2SiO_3 \cdot 9H_2O$	28,4	0,100	28,4	0,100	28,4	0,100	56,8	0,200	56,8	0,200	28,4	0,100
338,224	Na_2EDTA	4,36	0,013	4,36	0,013	4,36	0,013	8,72	0,026	8,72	0,026	4,36	0,013
	micro elements	mg/l	n [µmol]	mg/l	n [µmol]	mg/l	n [µmol]	mg/l	n [µmol]	mg/l	n [µmol]	mg/l	n [µmol]
61,81	H_3BO_3	1	16,179	1	16,179	1	16,179	2	32,357	2	32,357	1	16,179
287,44	$ZnSO_4 \cdot 7H_2O$	0,022	0,077	0,022	0,077	0,022	0,077	0,044	0,153	0,044	0,153	0,022	0,077
249,6	$CuSO_4 \cdot 5H_2O$	0,01	0,040	0,01	0,040	0,01	0,040	0,02	0,080	0,02	0,080	0,01	0,040
237,83	$CoCl_2 \cdot 6H_2O$	0,01	0,042	0,01	0,042	0,01	0,042	0,02	0,084	0,02	0,084	0,01	0,042
233,84	$MnCl_2 \cdot 6H_2O$	0,18	0,770	0,18	0,770	0,18	0,770	0,36	1,540	0,36	1,540	0,18	0,770
241,92	$Na_2MoO_4 \cdot 2H_2O$	0,006	0,025	0,006	0,025	0,006	0,025	0,012	0,050	0,012	0,050	υ,006	0,025

Fig 5. gives information of chemical composition of media used in experiments. WC, WC + TRIS, WC + NO BUFF, 2WC+ NO BUFF, 2WC_20:15 NO BUFF, WC+ MES 1000 mg.

5.1.4 Couter cell counter measurment

It was desirable to determine the number of cells in the inoculum in order to calculate the initial ratio between media and inoculum when starting a new experiment. This was calculated on counter cell counter Multisizer 4e (Coulter Counter).

5.1.5 Experiment set up

In the flow box, a media was mixed with inoculum and culture, the volume of culture added to the media was dependent on the current cell number in the inoculum. Then the media was mixed with culture. A sterile graduated cylinder was used to spread the volume into tubes. For cultivation, in Multicultivator (Photon System Instrument, Czech Republic) the tubes of volume 80 ml were used. For further cultivation in Temperature-controlled cultivators, 170 ml were used. The standard cultivation temperature was set up at 20˚C. The color of the light was white, thus all the colors of the spectrum (red, green, blue) were represented equally. The light intensity of a standard experiment was set to 150 µE. The standard experiment was also bubbled by 1 % of carbon dioxide.

5.1.6 Measurment of optical density

The value of optical density was determined at 750 nm by using the spectrophotometer WWR Spectrophotometer V-1200. The optical density provides information about the absorbance of a sample: it is defined as the ratio of incident light intensity falling upon a sample to intensity of transmitted light through the sample. The absorbance of a sample with *Hibberdia magna* was compared to a cuvette with 2 ml of distilled water used as a reference, the lightpath of cuvette was 1 cm. Initially, a 2 ml sample of culture was taken by plastic syringe, this sample was transferred in a cuvette directly when the value of OD 750 did not exceed 0,5 (a.u.-absorbance units). The optimum values for absorbance measurements are up to 0,5 a.u. If the optical density exceeded 0,5 the dilution with distilled water was needed in order to decrease the risk of error rate at higher densities. The culture was diluted accordingly, either 10 times or twenty times. The values obtained from sampling were converted back, therefore multiplied and recorded both in a notebook and later in an Excel table. Mechanical sampling was conducted in the case of an experiment in a Temperaturecontrolled cultivator. In the case of Multicultivator, the optical density was measured automatically, and the data were recorded in the Multicultivator software. Data gained during the measurement of OD 750 were used to calculate specific growth rate, based on the equation:

$$\mu = \frac{\ln OD\ 750_i - \ln OD\ 750_0}{t_i}$$

i= current day, 0= day zero

5.1.7 Measurment of pH

Since pH turned out to be an important parameter, with the ability to impact the growth of the experiment, it needed to be measured. Ph was measured by pH test strips WWR Chemicals Dosatest pH test strips ph 2.0 – 9.0. When the samples were taken for measurement of OD 750 nm, pH was measured along with. The volume of a sample syringe was poured on the ph test strips. No more than 3 samples of culture were taken, in order to minimize the risk of pH change caused by time delay between measurement

5.1.8 Haevesting

When the OD 750 decreased and growth was no longer observed, the culture was harvested, in order to gain biomass for RP-HPLC (reverse phase high pressure liquid chromatography). Therefore, tubes with culture grown in Multicultivator or Temperature controlled cultivator were unplugged from aeration. The culture was poured into the centrifugation flask and centrifuged in Schoeller UNIVERSAL 320 for 10 min at 3500 rpm, the pellet remaining on the bottom of the centrifuge flask was separated from the liquid supernatant which was discarded. Then the pellet was mixed by pipette with a small amount of distilled water and placed into 2ml vial and centrifuged again for 3 min in Eppendorf-mini spin at 13 400 rpm, the rest of the supernatant was discarded. Pellets in Eppendorf were placed in the freezer and kept in the temperature of -80˚C. Then the samples were lyophilized in SCANVAC CoolsSafe Freeze Dryer and afterward weighed to proceed lipid analysis and HPLC analysis for FX determination.

5.1.9 Dry weight determination

Before harvesting, a 3 ml sample of culture was taken and poured evenly on filtration paper, placed on Vacuum Filtration Distillation Apparatus. Before filtration, the filtration paper was dried in a laboratory oven at 100˚C, placed in the desiccator, and weighed on the analytical balance. After filtration, the filtration paper was placed in the oven for 24 hours to dry perfectly, after removal from the oven the filtration paper was placed again in the desiccator and weighed afterward. The respective weight of empty and full filtration paper was recorded, and the dry weight was determined based on the subsequent equation:

$$\frac{(F_1 - F_0)}{V_{SAMPLE}} = \text{mg/ml} = \text{g/l}$$

F_1-weight of full filter, F_0-weight of empty filter, V_{SAMPLE} – volume of sample

5.1.10 High-performance liquid chromatography

In order to analyze the content of FX Reversed-Phase High-performance liquid chromatography (RP-HPLC) was performed on the Agilent 1100 series. FX content was analyzed based on protocol adapted from (Van Heukelem et Thomas, 2001). Separation of compounds was performed on (Phenomenex Luna, 100 × 4.6 mm, 3 µ, 100A) with a flow of 0.8 ml/min. The

used gradient was: 0 % solvent B, to 100 % solvent B, in 20 minutes. Solvent B was kept at 100 % for 5 minutes. Then solvent B was decreased to 0 % in 3 minutes and kept at 0 % for 3 minutes. Solvent A was 100 % methanol (HPLC grade, VWR) and solvent B was 80% methanol, 20 % demi water. FX reached its peak at 450 nm and between 8 to 10 minutes. The results were both manually and automatically integrated and recorded. These results were compared with the initial weight of the analyzed sample.

Prior analyses of FX content, the extraction of biomass was performed. Although *Hibberdia magna* does not have a silica cell wall unlike many other algae species (e.g. *Phaeodactylum tricornutum*), it was still desired to break the cell walls in order to maximize the yield of FX. However, firstly samples of dry biomass were weighted in 2 ml Eppendorf vial, it was desirable to weigh 2 ± 0.5 mg of biomass. After weighing, and 0.2 ml of beads (0.1 mm glass beads, Biospec Products) were added. Then 1,8 ml of 100 % ethanol was added to each vial and subsequently, all samples were 3 times shaken on bead miller (MiniBeadBeater-16, Biospec Products) each time for 30 seconds). In order to prevent FX degradation since FX is easily degraded by heat and light. The samples were placed in ice for 5 minutes prior to shaking, and all steps of FX extraction were done under dim light conditions. Finally, vial were centrifuged at centrifuge Eppendorf-mini spin for 1 minute at 13 400 rpm. Then 1 ml of the suspension was transferred to HPLC vials.

5.1.11 Lipid analysis direct-transesterification

To evaluate the amount and profile of lipids the method of direct Transesterification was used. 2 ± 0.5 mg of dried biomass was weighed in screw-top tubes (2 ml), in this method methanol is used for hydrolysis of lipids and methyl esters are formed and later are quantified by gas chromatography. Quantitative and qualitative analysis of the FAMEs (fatty acid methyl esters) complement were performed by means of a GC-FID on Trace 1300 (Thermofisher) equipped with a flame ionization detector (FID). A TR-FAME column (60 m × 0.32 mm, df 0.25 μm) was used. Helium was used as a carrier gas, at a pressure of 200 kPa. The temperature ramp was the following: the starting temperature was 140 °C; it was increased to 240 °C, at rate of 4.5 °C min−1 and then maintained at 240 °C for 10 min. The injector was kept at 260 °C and the detector at 250 °C. The retention times of FAMEs were compared to known standards (Supelco 37 Component FAME Mix; PUFA no. 3 Supelco (from menhaden oil)). The amount of individual fatty acids was calculated using internal standards with a known heptadecanoic acid (C17:0) content and corrected by multiplying the integrated peak areas by the correction factors of the FID response.

5.1.12 Statistics and data analysis

As all experiments led to expanding the knowledge of the strain *Hibberdia magna*, the experiments were diverse. Information of optical density, specific growth rate, dry weight, and FX content was recorded in Microsoft Excel and subsequently compared. Statistical analysis of variables was not always performed due to experiment diversity. GraphPad Prism (where SD can be seen) and SigmaPlot were used for a graphical illustration of results. The information gained from the graphical illustration of results was sufficient to help during the design of the next experiment.

5.2 Materials and methodology of experiments

5.2.1 Influence of the aeration

This experiment was conducted in the Temperature-controlled cultivator. In total 9 tubes were cultivated, specifically 3 replicate for each condition. The cultivation temperature was set up at 20˚C, the lightning intensity was set up on 150 μE of white light. Microalgae culture was cultivated in WC+ TRIS media. Measurements of optical density (OD 750) were conducted regularly every 24 hours, data were recorded in the notebook and subsequently the graphs in GraphPad Prism were created. The experiment lasted for 9 days until the harvest.
There were 3 condition of the experiment:

- 3 tubes connected to the airflow without carbon dioxide inlet, this variant was aerated by the air. The tubes were marked as: AIR I, AIR II, AIR III

- another 3 tubes were connected to the airflow enriched by carbon dioxide 1 %. The tubes were marked as: CO2 I, CO2 II, CO2 III

- last 3 tubes were disconnected from the aeration, therefore no aeration was provided. The tubes were marked as: X I, X II, X III

The second experiment was conducted in Temperature-controlled cultivator. The cultivation temperature was set up at 20˚C, the lightning intensity was set up on 100 μE, WC+.

- Aeration enriched with 1 % CO_2
- Aeration enriched with 5 % CO_2

5.2.2 Influence of the light intensity

This experiment was conducted in the Multicultivator. In total 8 tubes were cultivated in monoplicates for each condition. The cultivation temperature was set up at 20˚C, the lightning intensity was set up on 40 μE, 130 μE, 225 μE, 310 μE, 420 μE, 515 μE, 590 μE, and 670 μE of white light, one light condition was assigned to one monoplicate. Microalgae culture was cultivated in WC+ TRIS media. All tubes were connected to the aeration and they were aerated by air. Samples for optical density measurement (OD 680 and 720) were taken every 3 minutes automatically by the software of Multicultivator and the graph was generated automatically after the experiment. The experiment lasted for 9 days until the harvest.

5.2.3 Influence of light intensity and temperature

This experiment was conducted in the Temperature-controlled cultivator. In total 20 tubes were cultivated. in WC+ TRIS media

- 5 different temperatures were tested; 10˚C, 15˚C, 20˚C, 25˚C, and 30˚C.
- 4 different light intensities were tested; 60 μE, 120 μE, 240 μE, and 480 μE

In 5 different cultivation units, the temperature was set up based on the design of the experiment and the intensity of light was set up accordingly for every position. In each cultivating unit, 5 tubes were cultivated. The duration of the experiment differed based on the variant. All tubes were connected to the aeration and bubbled by 1 % CO_2. Measurements of optical density (OD 750) were conducted regularly every 24 hours, data were recorded in the notebook and subsequently, the graphs in GraphPad Prism were created.

5.2.4 Influence of the light spectrum

This experiment was done in the Multicultivator. 8 tubes were cultivated in WC+ NO BUFFER media. The cultivation temperature was set up at 20˚C, the lightning intensity was set up on 100 μE in all spectrum.

The colors of light and their wavelength were set up accordingly:

- white light, all lights (red, green blue LED light equally represented), ultraviolet 405 nm, blue 450 nm, blue 470 nm, green 530nm, red 660 nm and infrared.

All tubes were connected to the aeration and they were bubbled by 1 % CO_2. Samples for optical density (OD 680) measurement were taken every 3 minutes automatically by the software of Multicultivator and the graph was generated automatically after the experiment

5.2.5 Influence of nitrogen and phosphorous concentrations

This experiment was done at the Institute of Botany of the Academy of Science of the Czech Republic. In well plate with (24 holes). In each hole was 2 ml of media and 20 µl of culture were added. Media has different concentration of nitrogen (represented by $NaNO_3$) and phosphorous (represented by K_2HPO_4).

Firstly, WC media without $NaNO_3$ and K_2HPO_4 was prepared, the concentrated stock solution of $NaNO_3$ 5 mol/l and K_2HPO_4 0,5 mol/l, were prepared separately. Subsequently determined amount of stock solutions of $NaNO_3$ 5 mol/l, and K_2HPO_4 0,5 mol/l were tranferred by pipete to the base media WC in order to reach subseqent concentrations:

• Concentration of $NaNO_3$: 0 mM ; 5 mM; 10 mM ; 15 mM ; 20 mM ; 25 mM; 30 mM ; 40 mM

• Concentration of K_2HPO_4: 0 mM ; 0,1 mM; 0,5 mM ; 1,0 mM ; 1,5 mM ; 2,0 mM ; 3,0 mM ; 4,0 mM

The well plates with a culture were cultivated at 20˚C, the lightning intensity was set up on 100 µE of white light and they were measured regularly for OD 680, 750.

6 Results and Discussion

6.1 Influence of the aeration

The aim of the experiments was to examine the impact of aeration on microalgal growth. Three different conditions were tested: without aeration, aeration by air and aeration by air enriched with CO_2. Results of growing abilities are listed in figure n. 6 The variant grown without aeration did not developer throughout the time of the experiment, the dry weight was not determined due to lack of biomass.

Contrarily, the variant aerated with air enriched with 1 % CO_2 has shown the fastest growth without a lag phase. The OD 750 reached its maximum on the 7th day and afterwards the OD 750 started to decline. The dry weight of this variant reached 1,2 g/l.

The variant aerated by air showed slower development but the growth was continuous without any decline till the date of harvest. The final dry weight reached 0,39 g/l.

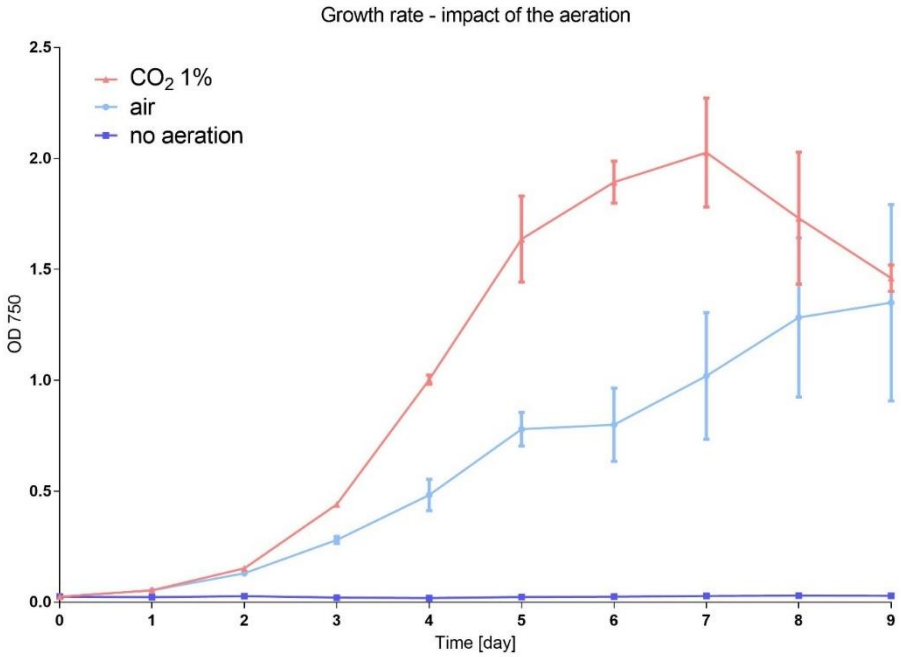

Fig. 6. The optical density of cultures grown under different aeration conditions; aerated with air, no aeration and with air enriched with 1 % CO_2.

After noticing that additional aeration with CO_2 had a positive impact on the development of the culture, another experiment was conducted. The variants were grown under two different concentrations of CO_2. The variant enriched with 1 % CO_2 initially showed a slightly faster growth than the variant enriched with 5 % CO_2; however both variants reached similar maxima at the time of harvest. Measurement of the Ph, which is usually influenced by the CO_2 concentation, was also conducted. It was measured by pH papers. Values for both variants were quite similar during the experiments, the pH of the 5 % CO_2 variant was slightly lower. The dry weight reached 1,79 g/l with 1 % CO_2, and 1,94 g/l with 5 % CO_2.

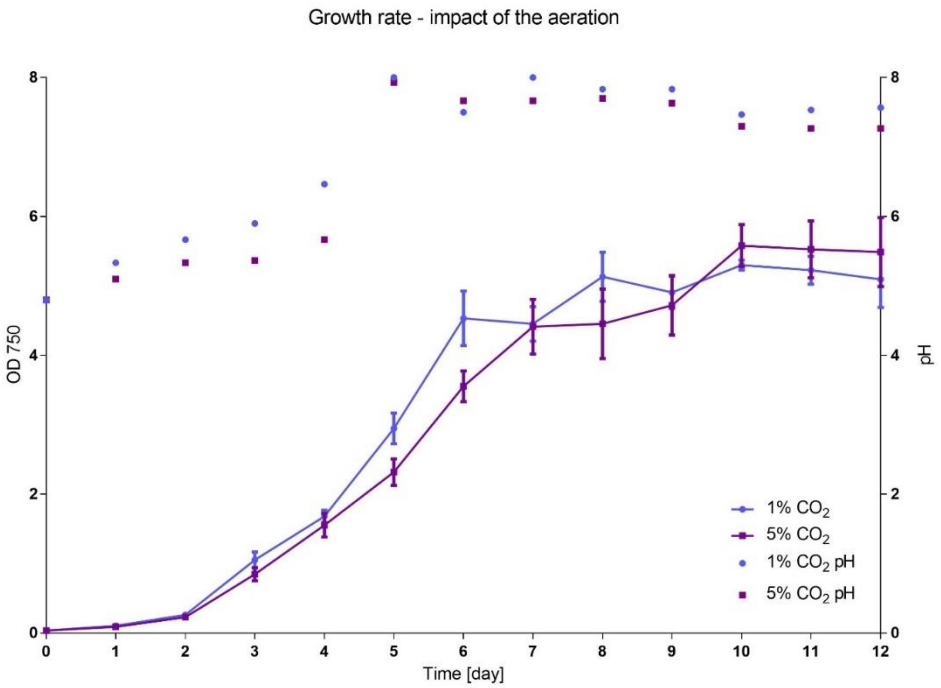

Fig 7. The optical density and pH of cultures grown under different aeration conditions; air enriched with 1 % CO_2 and 5 % CO_2 respectively.

The first experiment brought an interesting result. Since *Hibberdia magna* is an autotrophic organism that requires carbon dioxide to grow, it was predictable that the best conditions and performance would be reached by the variant enriched with 1 % CO_2. The result was consistent with the expectation. A higher concentration of dissolved CO_2 was more readily available in the culture and caused a rapid progression in growth.

The performance of the variant aerated by the air with the addition of 0 % CO_2 was weaker. This result was also in line with the initial assumptions since the concentrations of CO_2 in the atmosphere are notably lower. Similar results were observed by McClure et al. (2018); they also observed the impact of the concentration of CO_2 on the development of Phaeodactylum tricornutum, a microalga that also produces FX. The cultivation conditions in terms of lighting and temperature were very similar to ours. They observed the aeration enriched with 1 % CO_2 performed better than the aeration by air with an addition of 0 % CO_2; the latter variant reached lower density levels.

The last variant tested in our first experiment was without any aeration. The result of this variant was not expected, it had shown a lack of development. However, for maintenance purposes, *Hibberdia magna* was kept in 100 ml Erlenmeyer flasks without mixing or additional aeration

in the collection. Under these conditions, *Hibberdia magna* normally thrives. Therefore, it was expected that no additional aeration would not be an obstacle for further development. Since *Hibberdia magna* is a flagellate organism (Andersen, 1989) it was expected that *Hibberdia magna* can theoretically migrate towards the surface, where the concentration of CO_2 might be higher due to the diffusion of atmospheric CO_2. However, the tubes for cultivation have a smaller surface area exposed to the air in comparison to Erlenmeyer flasks, thus the diffusion of CO_2 might be lower.

In terms of the second experiment, a small difference between the performance of 1 % $CO_2$and 5 % CO_2 was observed. Presumably, the culture was not able to efficiently utilize the excessive 5 % CO_2. The explanation might be in the process of photosynthesis and its rate; the excessive CO_2 was not utilized due to the nature and physiology of microalgal cells.

According to Slade et Bauen (2013) the efficiency of CO_2utilization ranges from 20 % to 90 % in algal biotechnology. The efficiency is dependent on the biotechnological practice: in thin layer cultivation the efficiency can reach 35 %, while in closed tubular photobioreactors, the efficiency can reach 75 %.

In their study, McClure et al. (2018) studied the difference between the impact of aeration with 1 % of CO_2 and 2 % of CO_2 on the performance of *Phaeodactylum tricornutum*. The results showed no significant benefit of an increase in CO_2concentration on the cell concentration (determined by optical density and dry weight). This is consistent with our findings.

However, in the study by McClure et al. (2018) in the variant of 2 % of CO_2 a drop in optical density was observed on day 10, which was most likely caused by a pH decrease and acidification by CO_2. The pH reached 6.9 for the variant enriched with 2 % of CO_2 and 7.3 for the variant enriched with 1 % of CO_2. During our experiment, it was also observed that the pH of the variant with 5 % CO_2 kept lower. The final pH reached similar levels of 7,2 (5 % CO_2) and 7,5 (1 % CO_2). Any significant drop in optical density, possibly associated with acidification in the case of 5 % CO_2, was not observed in our experiment.

Since CO_2 belongs among highest production expenditure in algal biotechnology (Resurreccion et al., 2012), a future focus on CO_2 should be considered.

6.2 Influence of the light intensity

After gaining basic knowledge of aeration optima, the next parameter was examined; the optimal light condition. A broad range of irradiance intensities was chosen because it was desired to examine the survival abilities of the microalgae for further experiments. The intensity of light ranged from 40 μE to 670 μE. Figure 8 shows the OD 720 of all experiments. The

lowest growth activity was noticed at the intensity of 670 µE; this intensity was limiting for the culture. The second-highest irradiance of 590 µ has shown slow development in the beginning, but after 120 hours the rate of growth increased. Almost uniform trends were observed in the case of the lowest intensity of 40 µE and the third-highest 515 µE. However, their OD reached only approximately half of the highest OD of 720. Cultures of irradiances of 130 µE, 225 µE, 310 µE, and 420 µE reached similar OD values. However, the culture grown in 130 µE reached the highest OD of all variants on the final day of the experiment. In terms of dry weight, the greatest was reached for 130 µE 1,44 g/l; next 420 µE – 0,46 g/l, for 225 µE – 0,42 g/l, for 310 µE 0,39 g/l, for 515 µE 0,23 g/l, the same values of 0,2 g/l were gained for 590 and 40 µE, the lowest DW was reached for 670 µE - 0,11 g/l.

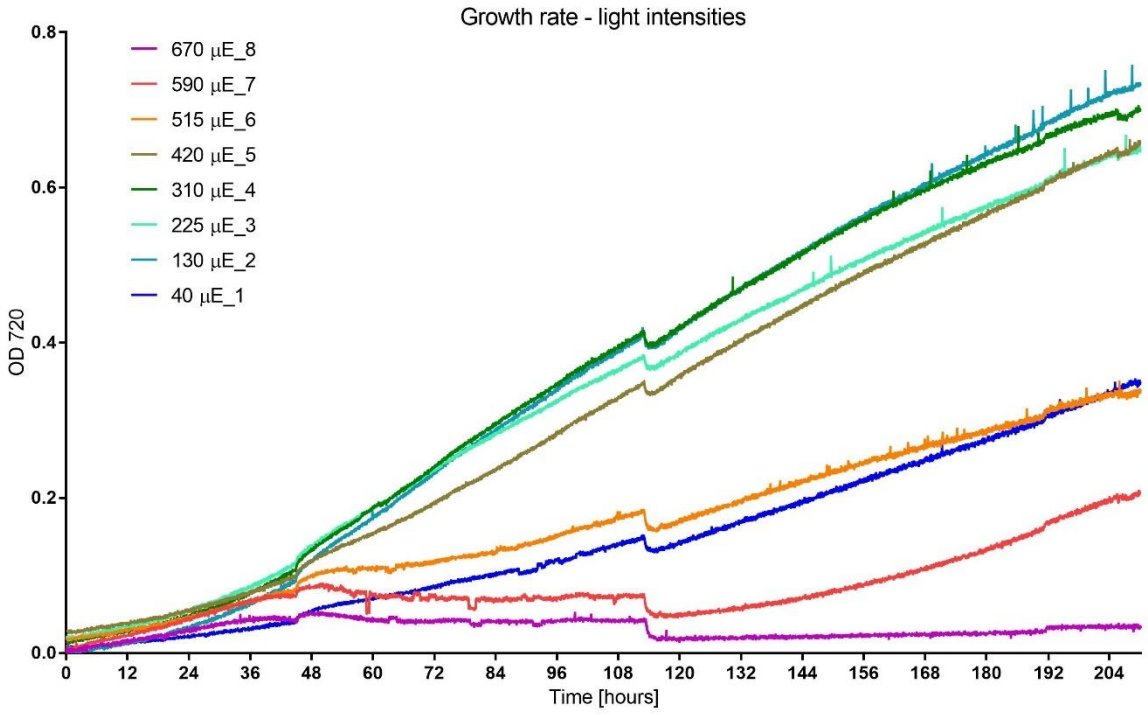

Fig. 8. The optical density of cultures grown at light intensities; 40 µE, 130 µE, 225 µE, 310 µE, 420 µE, 515 µE, 590 µE and 670 µE.

In terms of the biochemical analysis performed, FX showed a decreasing trend with increasing irradiance, with the exception of 420 µE, where the FX was slightly higher than the previous µE, 225 µE, 310 µE. However, the highest yield of FX was noticed at the lowest irradiance of 40 µE, the lowest in the case of 590 µE. The 670 µE was not subject to analysis due to a lack of biomass. The content of EPA was almost stable, the maximal yielded EPA was reached at

310 µE. The minimal yield of EPA was observed at 130 µE. In terms of DHA more distinct results were obtained, the highest accumulation of DHA was observed at 310 µE, the lowest at 130 µE. This can be seen from figure 9.

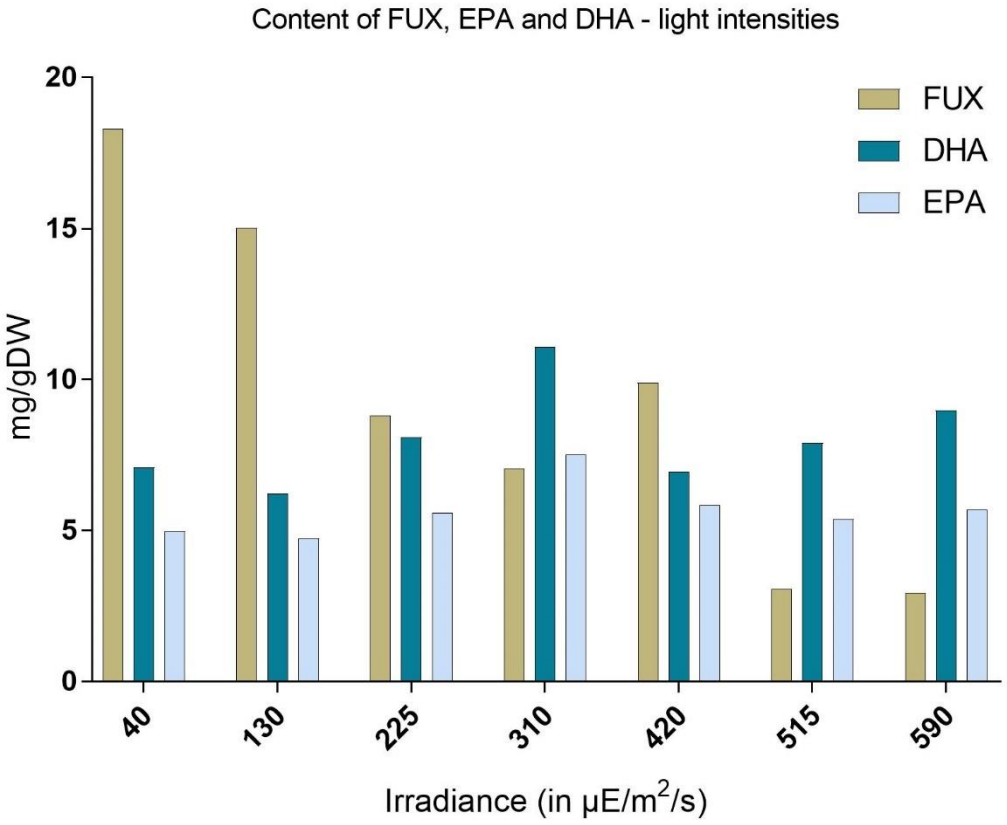

Fig. 9. The content of FUX, DHA and EPA cultures grown at light intensities; 40 µE, 130 µE, 225 µE, 310 µE, 420 µE, 515 µE and 590 µE.

Referring to figure 10, in terms of relative lipid profile, an abundant proportion of C18:4n3 (stearidonic acid) and C14:0 (myristic acid) can be observed. C14:0 tends to be stably represented at all irradiances. It approximately reaches 30 % of fatty acids. In terms of monounsaturated fatty acids (C16:1n7, C18:1n9, C18:1n7, C22:1n9) and in terms of polyunsaturated fatty acids such as (C20:5n3, DHA C22:6n3) there can be observed a tendency to increase the amount with increasing irradiance. Oppositely, C18:4n3 (stearidonic acid) tends to be less represented with increasing irradiance except at 420 µE. C22:5n3 (EPA) tends to be evenly represented with changing irradiances with an exception at 420 µE

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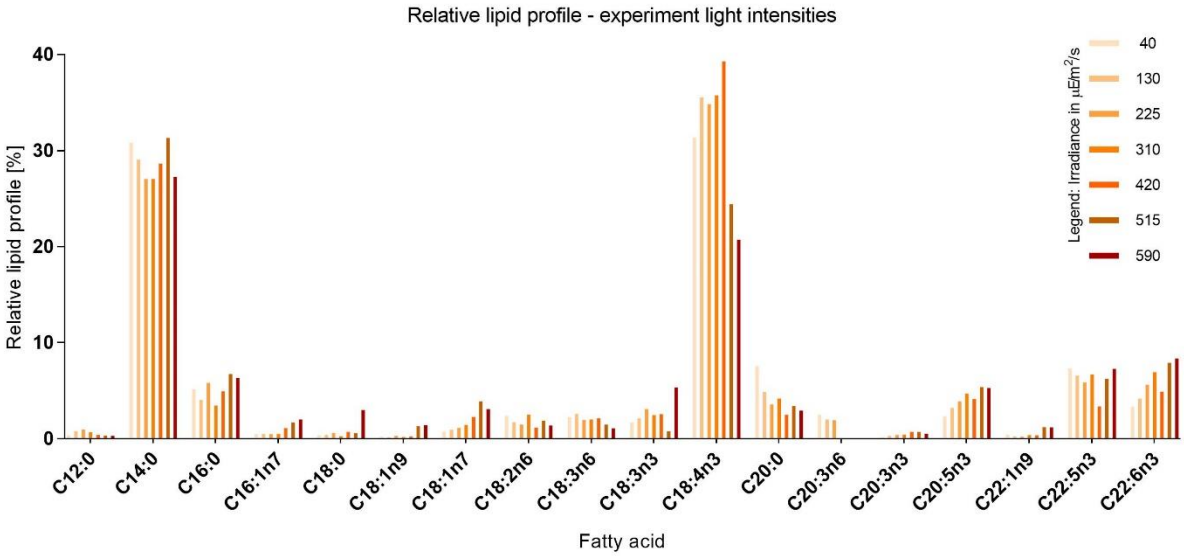


Fig. 10. The relative lipid profile of cultures grown at the following light intensities: 40 µE, 130 µE, 225 µE, 310 µE, 420 µE, 515 µE and 590 µE.

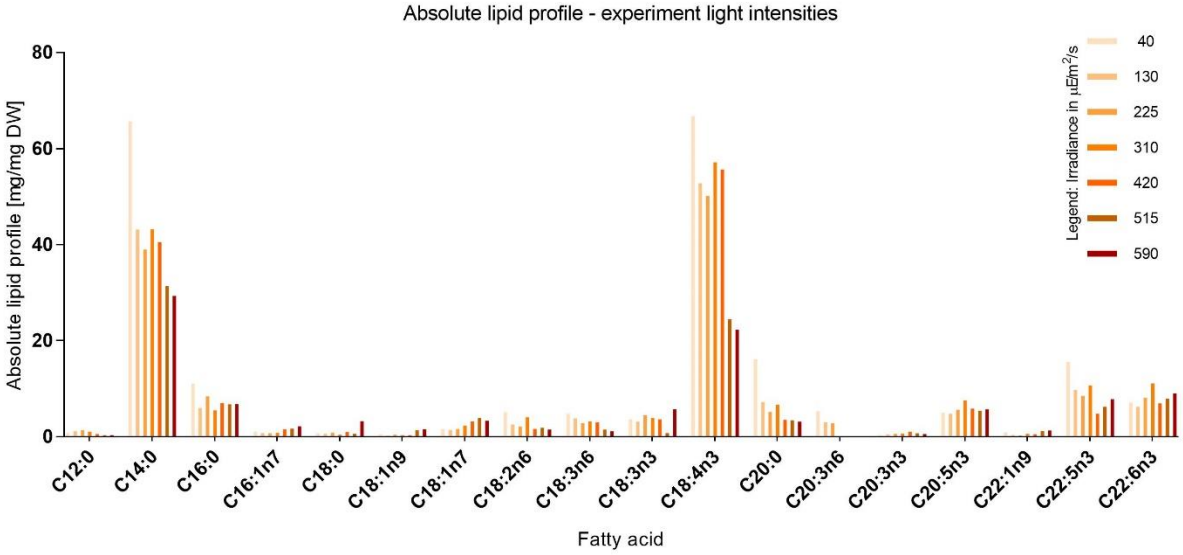


Fig. 11. The absolute lipid profile of cultures grown at the following light intensities: 40 µE, 130 µE, 225 µE, 310 µE, 420 µE, 515 µE and 590 µE.

Referring to figure n, 11 in terms of the absolute profile, there can be again observed an abundant amount of C18:4n3 (stearidonic acid) and C14:0 (myristic acid). Both C14:0 and C18:4n3 tend to be represented more at lower irradiances. As regards monounsaturated fatty acids (C16:1n7, C18:1n9, C18:1n7, C22:1n9) they also increase with irradiance. The

polyunsaturated fatty acids have not shown any unifying trend, they are both rather more abundant at lower irradiances or more evenly distributed depending on light irradiance

This experiment also brought notable results. Within the results of OD 720, the light intensity of 590 μE is noteworthy. A relatively small difference in the light intensities between 670 μE and 590 μE represented the borderline in the performance of both cultures. The culture grown at 590 μE showed a relatively low development initially. After the first 120 hours of cultivation, the rate of development increased. The phenomenon might be explained by two theories. It is possible that initially, the development of the culture was rather slow, however, after reaching higher density in the cultures, the effect of self-shading occurred. Therefore, the intense irradiation posed lower stress on the culture. The second explanation might be seen in physiological adaptation. Initially, during the lag phase with little development, the physiological changes in microalgal cells occurred to perform better at high irradiances. This explanation might find support in the relative lipid profile of the culture since the lipid composition at 590 μE is notably different from the lipid profiles of other intensities. Therefore, a certain change in lipid content might enable adaptation and further development.

A study by Baldisserotto et al. (2019) describes the ability of diatoms to adapt to high irradiance. (Among diatoms belongs also *Phaeodactylum tricornutum*). A diatom employs a wide variety of mechanisms to cope with the excessive irradiance, such as a modulation of the photosynthetic apparatus, change in photoreceptors and signaling proteins, use of ROS scavenging system, modification in lipid and carbon metabolism, and protein synthesis, and modulation of cell wall polysaccharides. Since diatoms pose similar photosynthetic pathways and pigment synthesis, similarities in response to high irradiances by *Hibberdia magna* might be assumed.

In terms of dry weight, the highest was reached by 130 μE - 1,44 g/l. The culture grown at 130 μE had also reached the highest OD 720. However, by comparison, other cultures (225 μE, 310 μE, and 420 μE) reached similar values, but a notably smaller dry weight (0,42 g/l, 0,39 g/l, and 0,46 g/l). Therefore it was assumed that an error had occurred during the determination of dry weight content of 130 μE. Thus, it might be beneficial to repeat the measurement in future experiments.

In a study by Petrushkina et al. (2017) the highest dry weight was reached at high light intensity (226 μE), specifically 3.75 g DW L−1 in *Mallomonas spp*. Although it might also be expected that the optimum for the accumulation of dry weight for *Hibberdia magna* would be also around 225 μE, it might be misleading to make assumptions, due to the lack of replicates and similar performance of cultures of light intensities (130 μE, 225 μE, 310 μE, and 420 μE).

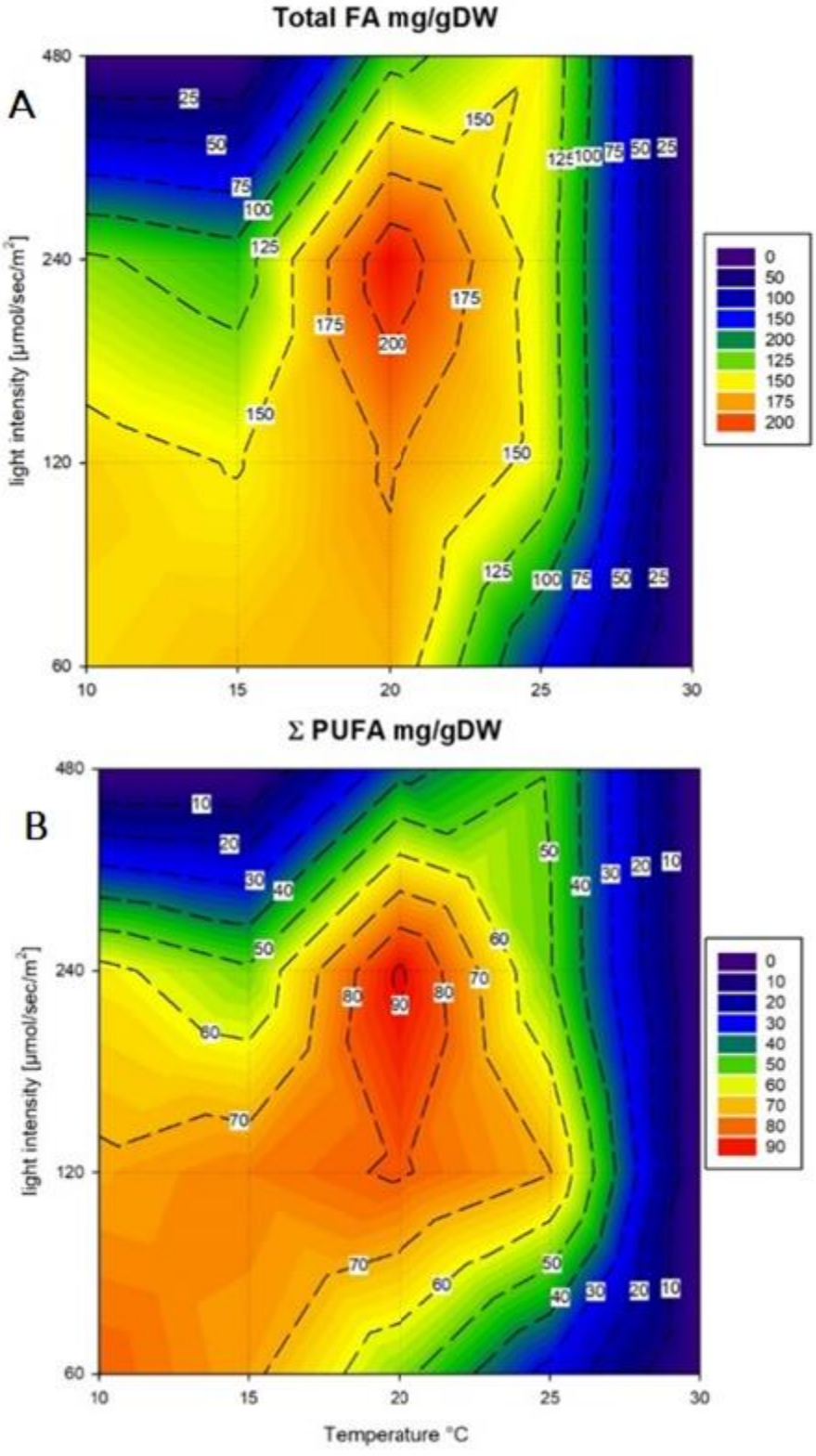
The intensities of (515 μE, 590 μE and 670 μE) reached relatively low dry weight. According to Wang et al. (2018) the photosynthetic machinery became inhibited by excessive light, and therefore, the production of biomass was not enhanced.

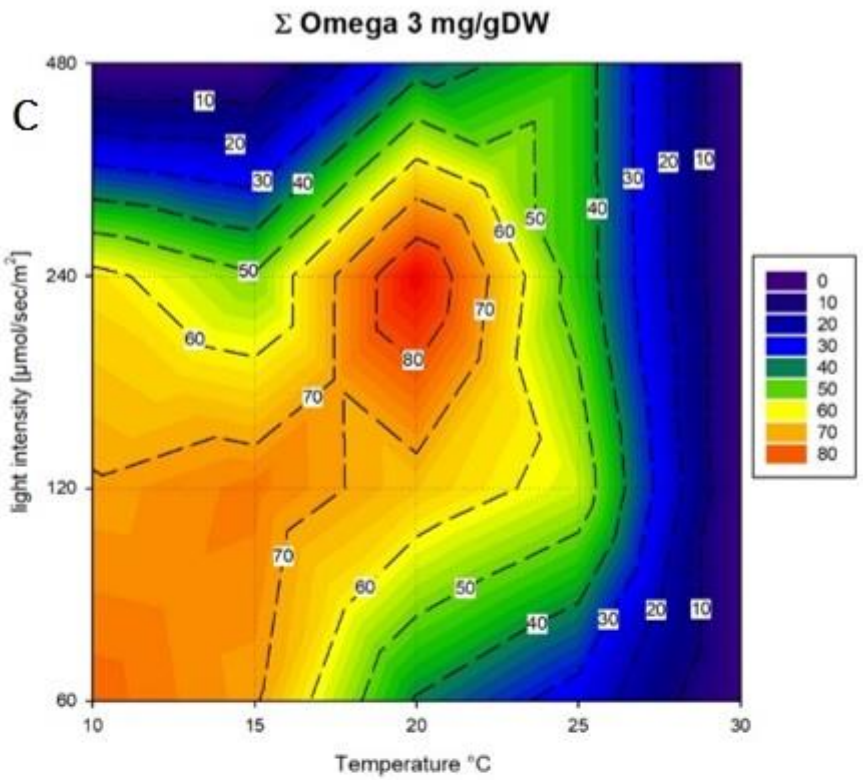
In terms of FX content a tendency for the content to increase with decreasing irradiance was observed. This is consistent with studies by McClure et al. (2018), Baldisserotto et al. (2019) and Gao et al. (2020), all of which observed higher FX accummulation at lower irradiances. However, an exception during our analysis occured at 420 μE, which showed slightly higher values than two previous irradiances. It is premised that this elevation in FX content is caused by an error in the analysis. Since all samples were analyzed in monoplicates, it would be beneficial to conduct another analysis with more samples, to improve the accuracy and to obtain statistically relevant data.

As regards fatty acids, the acummulation of EPA tends to increase with increase in light, then it reaches its maximum at 310 μE; then with increasing intensity there is a continuous decline in EPA accummulation. Wang et al. (2018) observed EPA acummulation in two diatom strains: *Phaeodactylum tricornutum* and *Cylindrotheca fusiformi*s. They also observed a decrease in EPA during an increase in irradiance, however they tested a range of (30 μE – 180 μE).

A study of the effect of light intensities on the accumulation of fatty acids by Conceição et al. (2020) has not observed any significant alteration of DHA contents. The study by Qiao et al. (2016) has observed a notable increase in DHA with increasing light intensity, a range of 50-150 μE was tested. Similar behavior was noticed in our experiment.

6.3 Influence of the light intensity and temperature




Σ Omega 3 mg/gDW
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light intensity [µmol/sec/m²]
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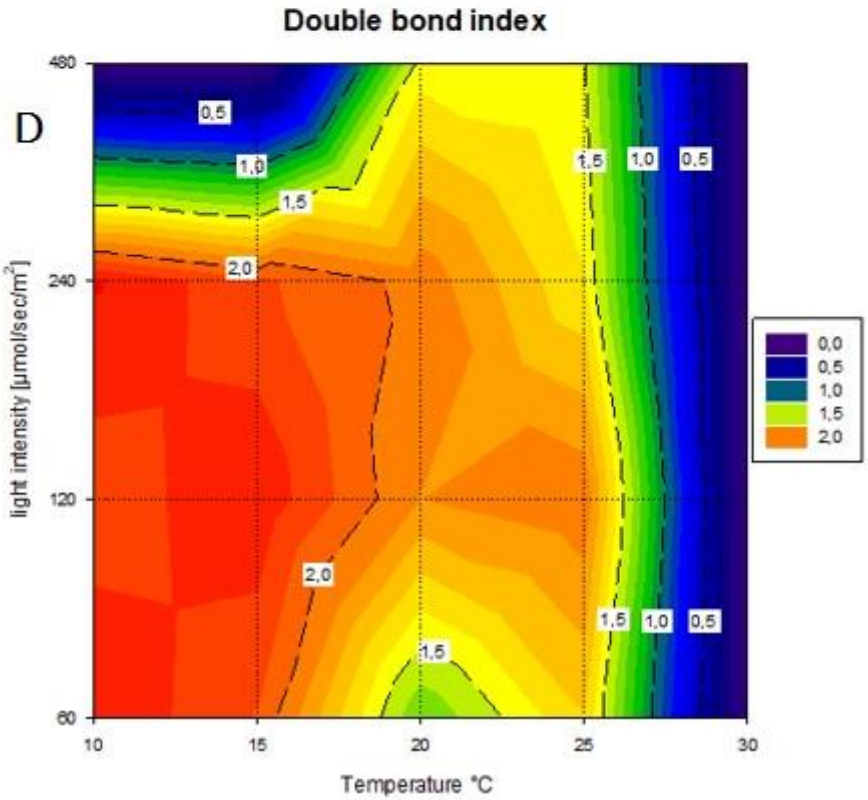
Double bond index
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light intensity [µmol/sec/m²]
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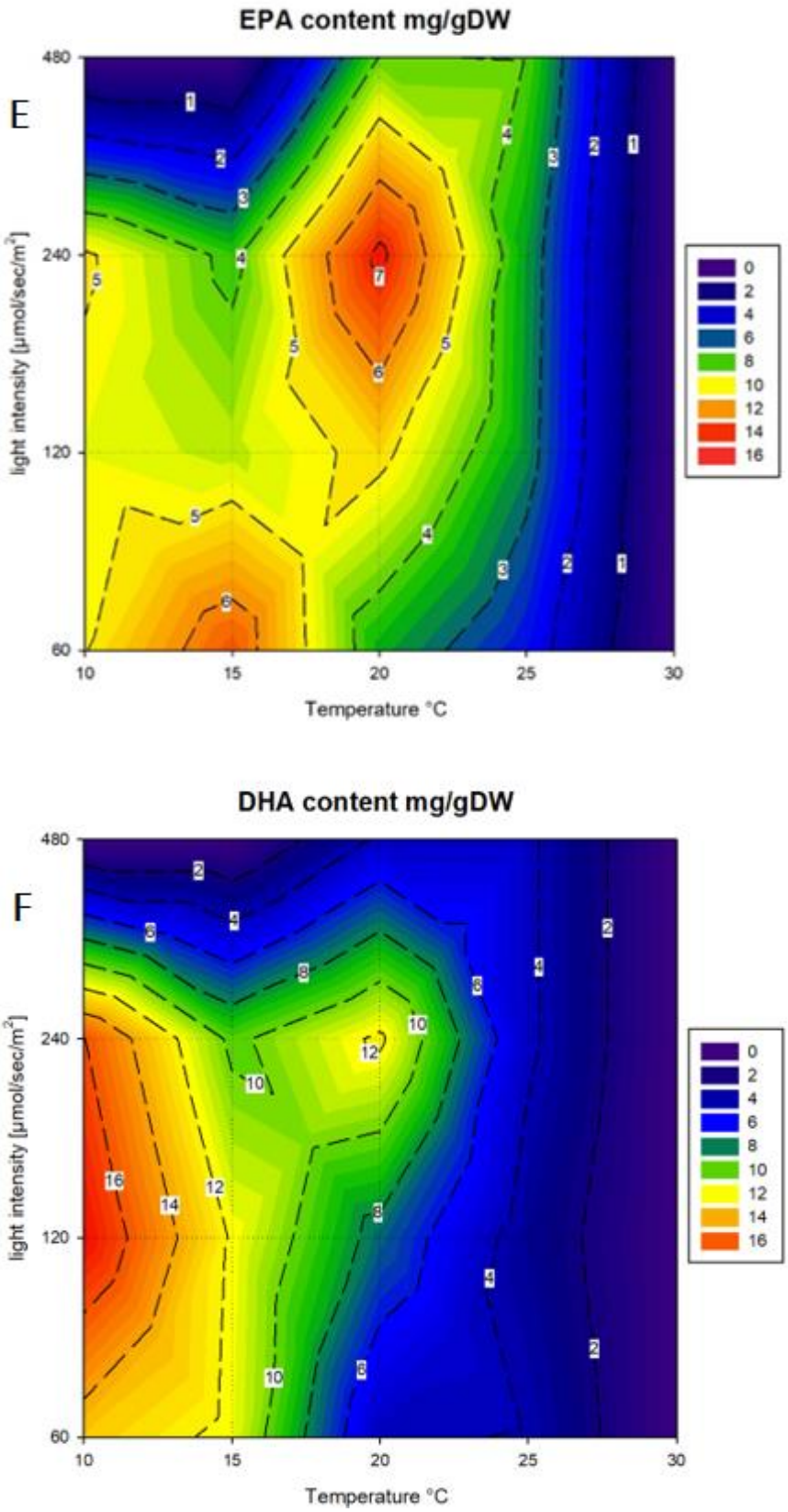

Fig. 12. Absolute content of fatty acids **(A)**, absolute content of PUFA **(B)**, absolute content of omega 3 **(C)**, double bond index (**D)**, absolute content of EPA **(E)**, absolute content of DHA **(F)**

From the figure n. 12. (A) can be seen that he maximum of fatty acid acummulation was observed at 20˚C with irradiance 240 μE, also the range of irradiances (120 μE to aproximately 340 μE) led to high accummulation of fatty acids. The temperature higher than 25˚C at any irradiance led to low or no fatty acid acummulation, also the temperatures in the range of 10-15˚C with irradance higher than 240 μE led to low or no acummulation of fatty acids. Both contents of of PUFA 12 (B) and omega3 12 (C) were very similar to contents of fatty acid 12 (A).

From the figure n. 12. (D) can be seen that the double bonds in fatty acids tend to increase with decreasing temperatures. In the range of 10-15˚C the maximal amount of double bond could be find.

The highest accumulation of EPA, figure 12 (E) was observed at 20˚C at the irradiance of 240 μE. The temperature of 20˚C with the combination of any irradiances led to higher DHA accumulation than other temperatures. Another area of high accumulation of DHA was observed at 15 ˚C at the irradiance of 60 μE.

Temperature higher than 25˚C led to low or no accumulation of DHA, figure 12 (D) in the biomass. The combination of low temperatures 10-15˚C led to a decrease in DHA with increasing irradiance, higher than 240 μE.

Favorable conditions for DHA accumulation were observed rather at lower temperatures ranging from 10–15˚C in the combination with irradiance ranging from 60-240 μE. Oppositely, an unbeneficial environment for DHA accumulation was observed with increasing temperature, where the temperature of 15-20˚C represents a borderline, and temperature any higher than 20 ˚C means low or no DHA accumulation, with the exception of combination with 240 μE. The light intensity close to 480 μE has not shown any DHA accumulation.

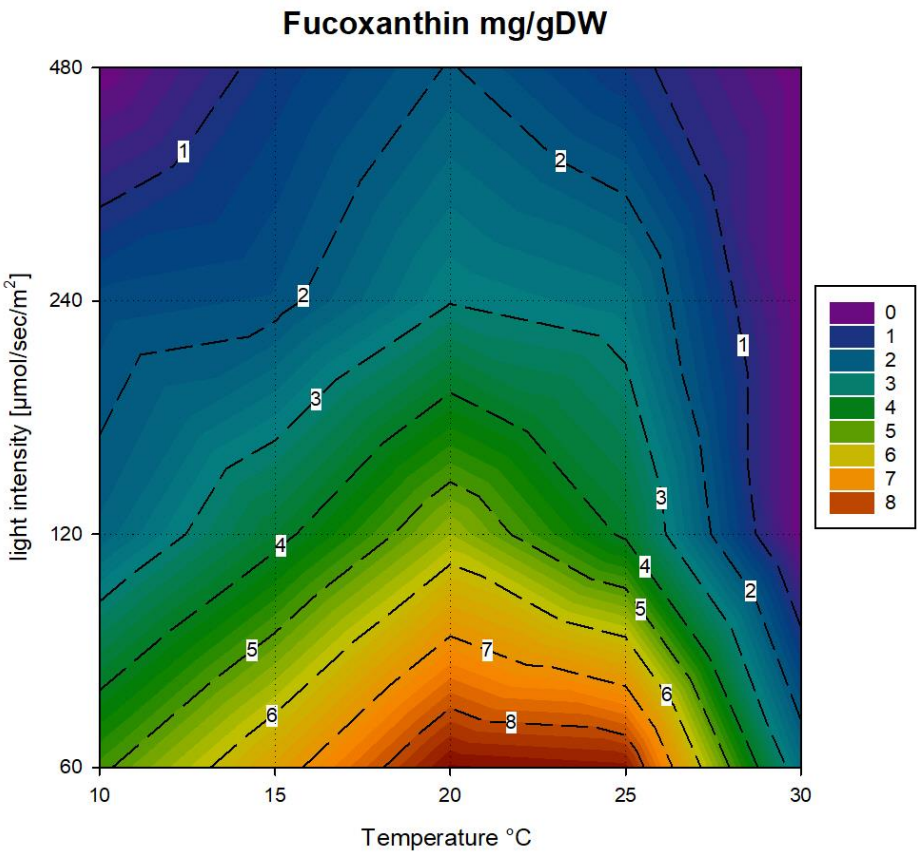

Fig. 13. Content of FX

From the figure 13 it can be seen that more FX is produced at low light conditions, especially at 60 µE, while the higher light intensities are not favorable to FX production. The trend towards FX decrease at higher temperatures is even exponential. In terms of temperature the maximal yield of FX was obtained in the range of 20-25˚C. An obvious trend can be noticed: the higher the irradiance, the lower the FX yields. The lowest level of FX was found at the highest level of irradiance when combined together with both the lowest and highest temperatures of the conducted experiment. However, the production of FX seemed to be affected more by increasing temperature. The combination of 20˚C with any irradiance brought better yield of FX.

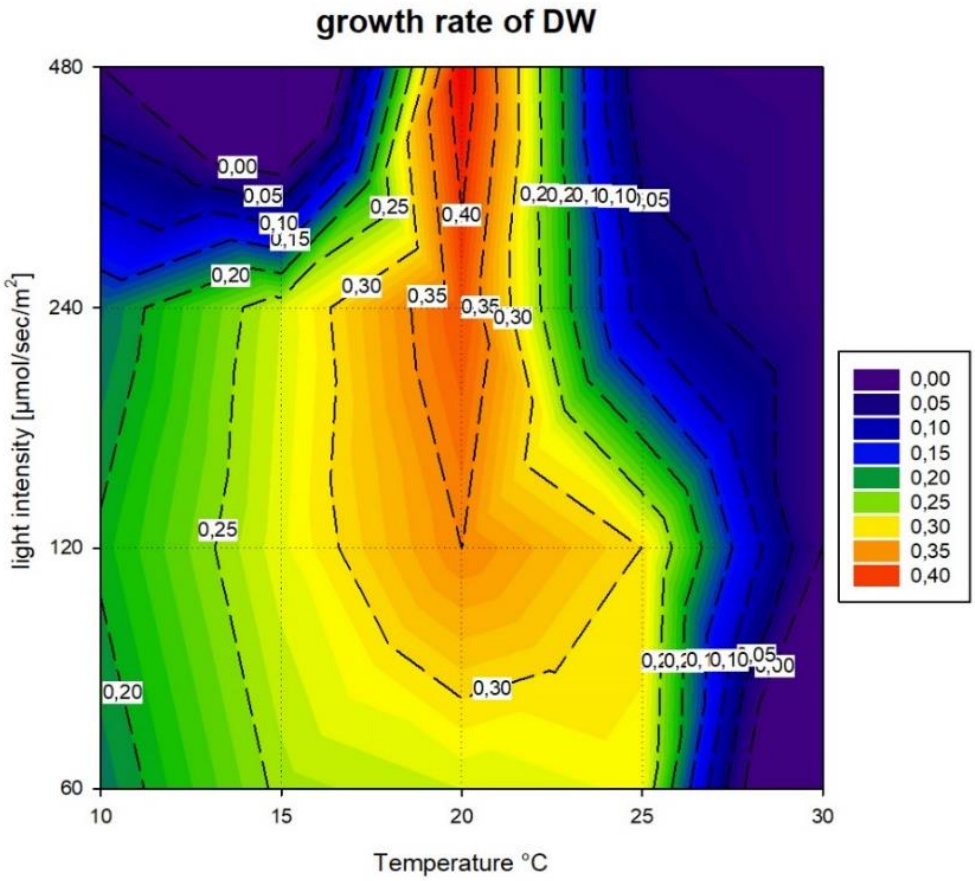

Fig. 14. Growth rate of dry weight (DW)

In terms of the growth rates of DW the most rapid growth was observed at 20 ˚C and at the irradiance ranging from 240 µ to 480 µ, however, all irradiances have shown rapid growth at 20 ˚C. No or little growth was observed at the combination of high intensity (300-450 µE) with low temperature (10-15 ˚C). Also, the combination of high irradiance and high temperature showed to be unfavorable. *Hibberdia magna* tends to withstand higher intensities in combination with higher temperatures (15 to 25 ˚C). The combination of low intensity 60 µE with both low (10˚C) and high (30˚C) temperature was also observed as a growth-limiting.

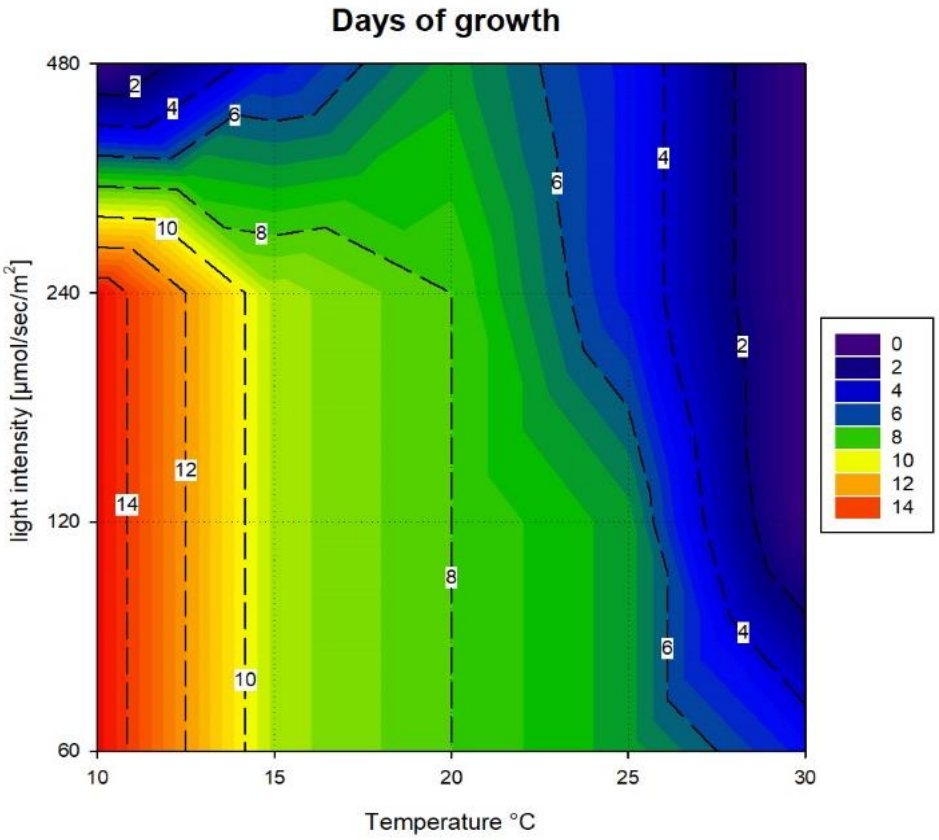

Fig. 15. Days of growth of the experiment

The figure 15. represents the length of the growth period of the experiments. The individual experiments were kept growing until their OD began to decrease. The experiments with the longest growth period were observed at the low temperature of 10 ˚C with the intensity ranging from 60 to 240 µE. The combination of 20 ˚C with any light intensity brought the length of 8 days. In terms of the highest intensity of 480 µE the only development of culture was seen in combination with 20˚C. The duration of 6 days or a shorter period means that the culture has not developed any activity; they were still kept for up to 6 days for observation purposes, therefore all blue areas can be considered as areas with no development.

A study by Guihéneuf et Stengel, (2017) examined the impact of both temperature and irradiance on fatty acids accumulation in *Pavlova lutheri*. They tested temperatures of (8 ˚C, 12 ˚C, 18 ˚C, and 28 ˚C) and light intensities (40 µE and 200 µE). In consent with our findings, they found the optimum for fatty acids production in temperature of 18 ˚C, which is relatively close to our temperature optima (20 ˚C) of *Hibberdia magna*.

However, Guihéneuf et Stengel, (2017) have not observed any differences in relation to light intensity on fatty acid accumulation, but in our case, we did observe.

Guihéneuf et Stengel, (2017) observed the lower fatty acid accumulation in suboptimal temperatures, this was also observed in *Hibberdia magna* at low and high temperatures.

Guihéneuf et Stengel, (2017) recorded the highest yield of PUFA at the intermediate temperature of 12 ˚C at low intensity. They also recorded a decrease in PUFA with increasing irradiance. This is contrary to our results, the ideal condition for PUFA was found at relatively high irradiance (240 μE) and temperature (20˚C).

A study by Jiang et Gao, (2004) suggests decrease temperature to obtain higher yields of valuable PUFA. They observed higher yields of EPA when lowering the temperature from 25°C to 10°C, this might be the subject of further research, however the optimum for EPA was found at a range of temperature (15-20˚C) in *Hibberdia magna*.

In terms of lighting Guihéneuf et Stengel, (2017) also observed the highest content of EPA at low light, this is again contrary to our findings.

Our observation regarding DHA was consistent with a study by Qiao et al., (2016). They observed that DHA tends to decrease with increasing temperature in *Phaeodactylum tricornutum.* This was also observed in *Hibberdia magna*, the temperature higher than 20˚C was not optimal for DHA accumulation.

The increase in double bonds at low temperatures can be explained by the cells' aim to maintain fluidity by of membrane phospholipid layer by reducing saturation. This homeoviscous adaptation is described in the study by Ernst et al., (2016).

6.4 Influence of the light spectrum

This experiment aimed to observe the growth and FX production of *Hibberdia magna* at different values of light spectrum. Two experiments were conducted and they were harvested at different growth phases. Therefore, the accumulation of FX and OD were observed at different growth phases. Experiment A was harvested at the growth phase with a duration of 7 days, the second experiment B of the total duration of 12 days was harvested when the culture reached the plateau stage. Figures 16 and 17 describe the OD 720 of experiments focused on the spectrum. It is obvious that the UV of 405 nm is lethal. As regards similarities, the best performances were reached by blue light (470 nm) (in X together with red 660 nm). Green lights of (530 nm) also kept the similar values. Another similarity might be in the performance of infrared (730 nm), which reached the lowest OD among growing cultures. The differences

were observed in the case of blue (450 nm), wherein the first experiment (figure 16) reaches slightly lower values than the second (figure 17).

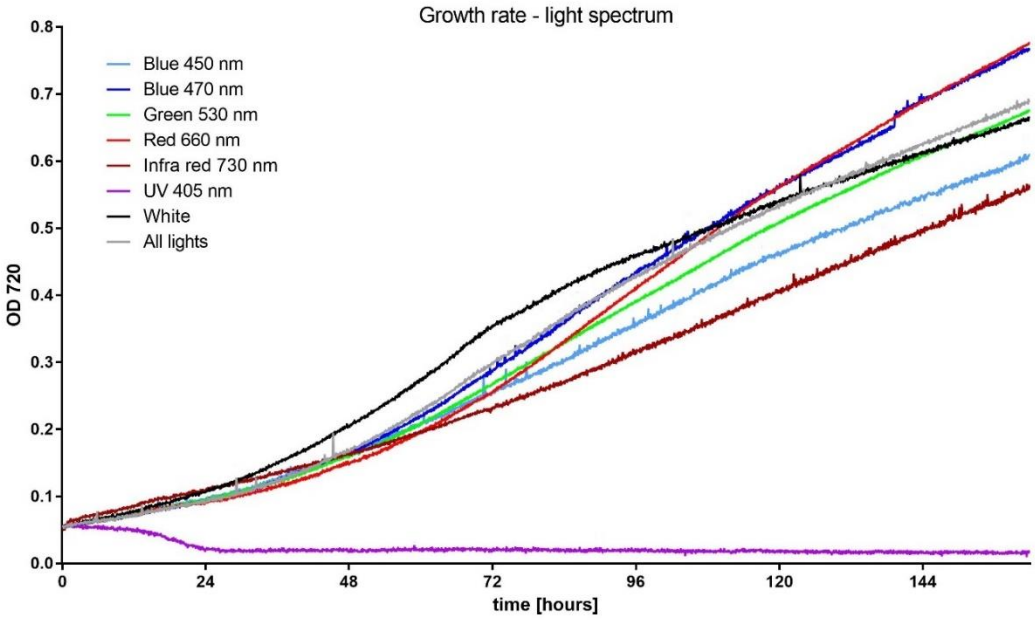

Fig. 16.: The optical density of variants grown at white light; all lights (LED lights: red, green, blue light equally represented), ultraviolet 405 nm, blue 450 nm, blue 470 nm, green 530nm, red 660 nm, and infrared 730 nm.

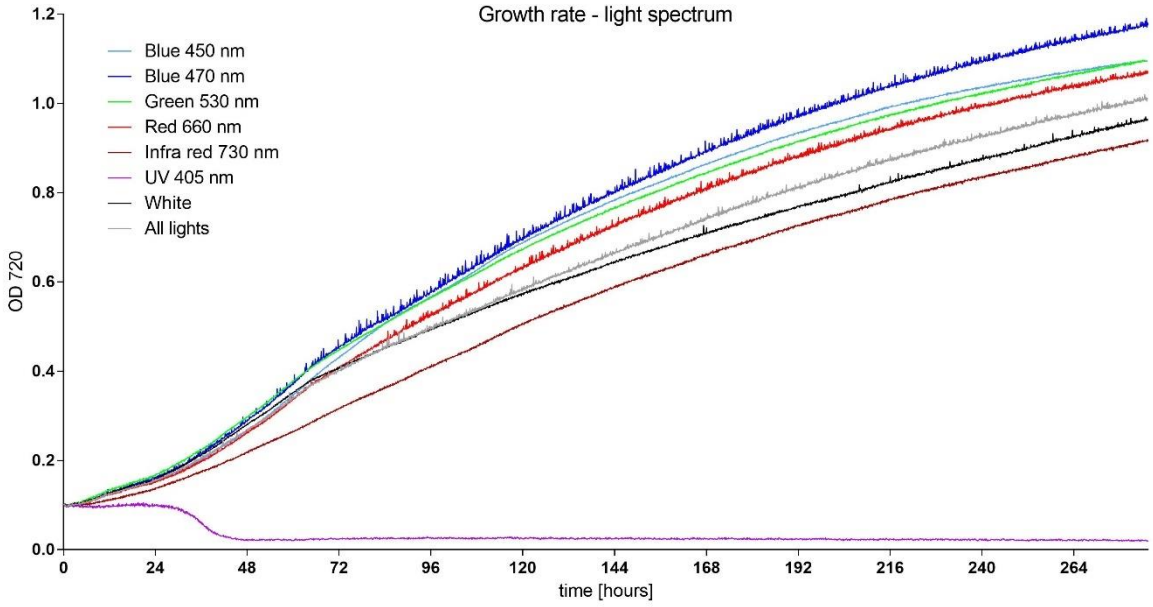

Fig. 17: The optical density of variants grown at white light; all lights (LED lights: red, green, blue light equally represented), ultraviolet 405 nm, blue 450 nm, blue 470 nm, green 530nm, red 660 nm, and infrared 730 nm

The first experiment (figure 18) showed a lower amount of FX and the yields owere more evenly spread in comparison to the second experiment (figure 19). This might be caused by harvesting in different states of growth. The highest FX content was gained at infrared (730 nm), then (660 nm) and (470 nm), which have shown equal results; then all lights, white, green (530 nm), and the lowest content was determined at (450 nm). The FX content at the UV 405 nm was not determined since no biomass was produced, as can be seen in figure n. 9. The highest dry weight 0,43 g/l was recorded in the case of red light (660 nm), while the lowest in case of white light 0,31 g/l.

In the second experiment (figure 19), the contents reached higher values. The FX content was reached accordingly; The highest was gained at blue 470 nm, blue 450 nm, then infrared 730 nm, full lights, white lights, then red 660 nm. The lowest amount of FX was observed in green 530 nm. The UV 405 nm was not determined due to a lack of biomass. The dry weight of this experiment was not determined.

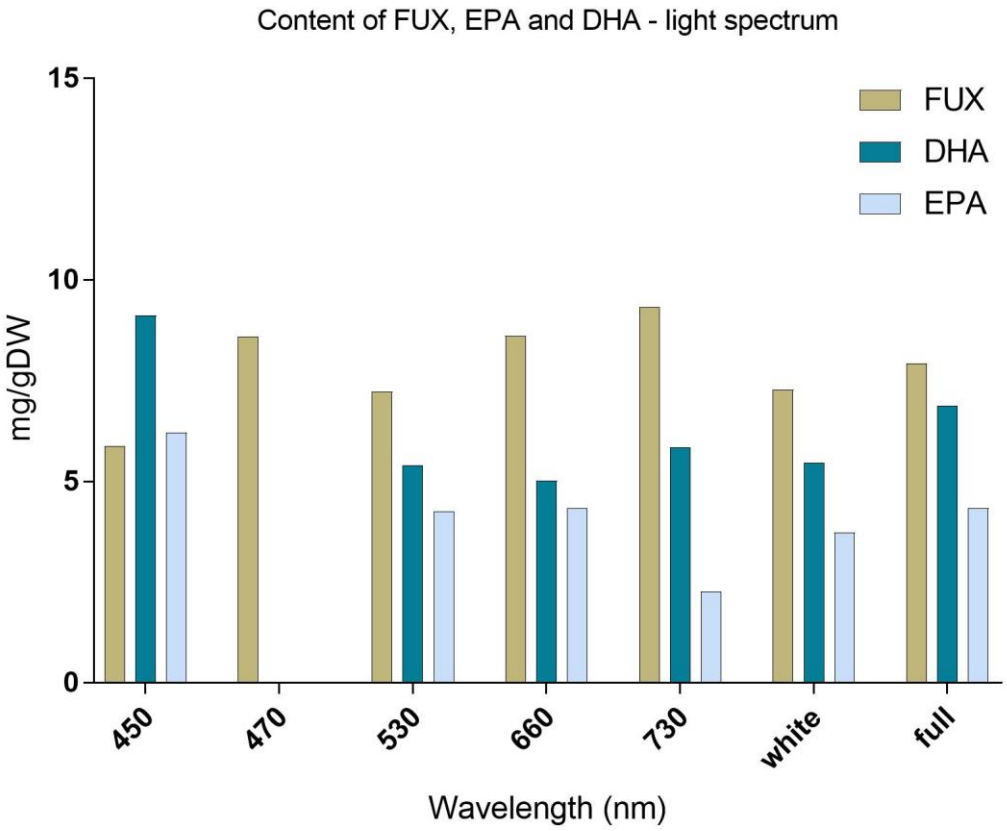

Fig. 18. FX, DHA and EPA content in variants grown at white light; all lights (LED lights: red, green, blue light equally represented), ultraviolet 405 nm, blue 450 nm, blue 470 nm, green 530nm, red 660 nm, and infrared 730 nm.

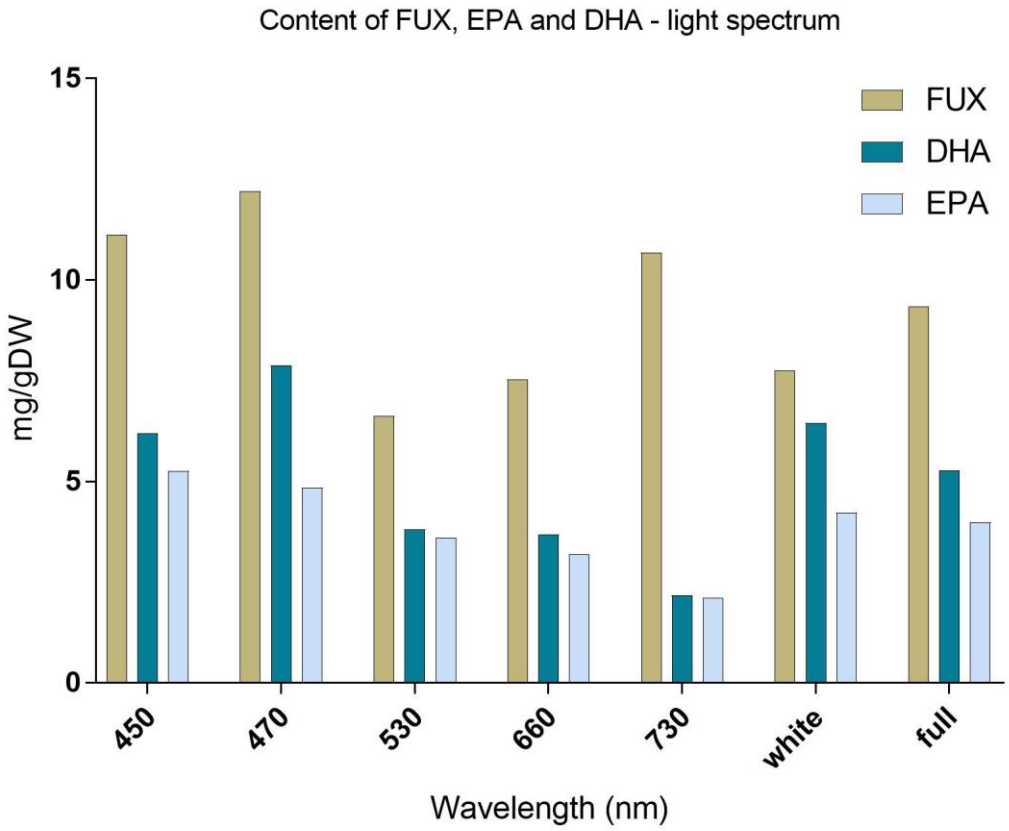

Fig. 19. FX, DHA and EPA content in variants grown at white light; all lights (LED lights: red, green, blue light equally represented), ultraviolet 405 nm, blue 450 nm, blue 470 nm, green 530nm, red 660 nm, and infrared 730 nm

Both the quality and quantity of the light spectrum have a significant impact on algal growth. Diatom, which is used as the main pigment, chlorophyll c and FX can effectively harvest and utilize blue-green radiation. Therefore, due to light-harvesting similarities it might be assumed that *Hibberdia magna* benefits from these spectra. As seen above, the results of OD and FX content of cultures grown in both blue light and green light performed well in the second experiment. A study by Gao et al. (2021) focused on the FX production of Tisochrysis lutea at various light spectra, specifically red + green + blue, blue, red, green, blue + red, and blue + green. They suggest a combination of blue and green light led to the highest FX production; on the other hand, red light caused a decline in FX production. In our experiment, the combination of blue and green light was not tested. However, in terms of wavelength, the spectra of (470 nm) are closest to the combination of green and blue and this wavelength reached the highest FX content in the second experiment. FX absorbs a spectrum of wavelengths in the range from

(480 to 560 nm). However, some FX tends to absorb the light in the range of (420 to 470 nm), where the absorption of FX chlorophyll a/c binding protein is rather high (Papagiannakis et al., 2005). FX also has its small peak of light absorption at (663 nm).

The combination of blue-green light to enhance FX production is widely suggested in the literature (Papagiannakis et al., 2005, Wang et al., 2018, Baldisserotto et al., 2019, Wang et al., 2019).

6.5 Influence of nitrogen and phosphorous concentrations

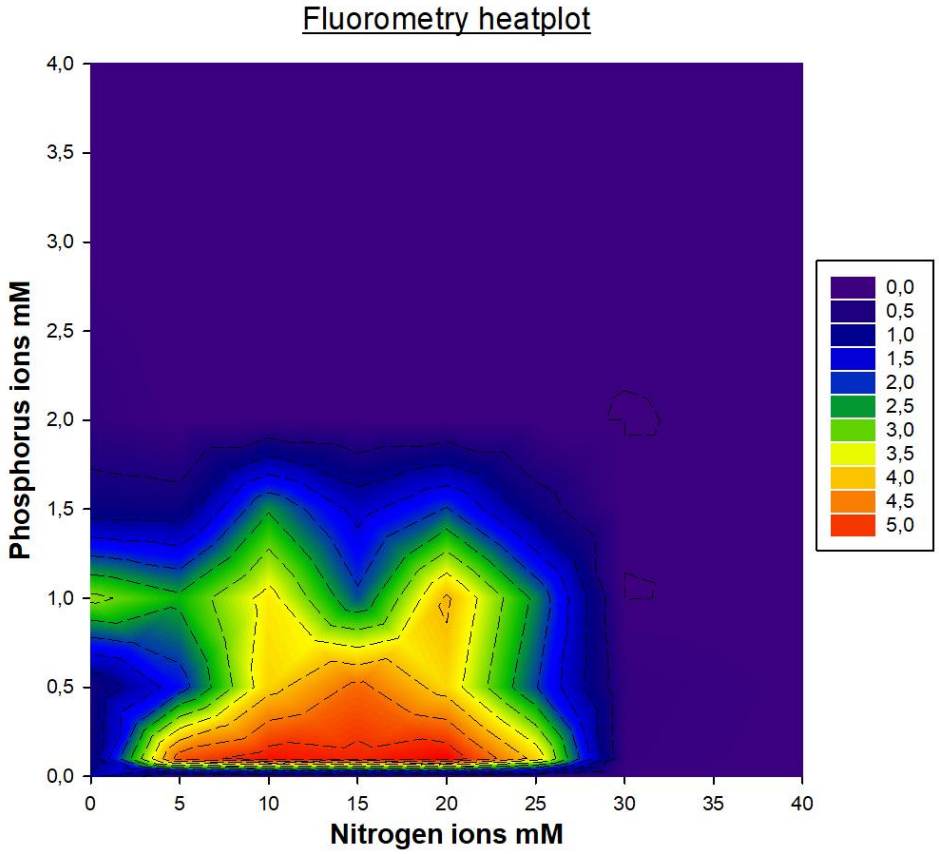

Figure 20. Experiment 5. Nutrients – nitrogen and phosphorus – The graph represents the fluorimetry of cultures grown in different concentrations of ions; $NaNO_3$: 0 mM, 5 mM, 10 mM, 15 mM, 20 mM, 25 mM, 30 mM and 40 mM, and concentrations of K_2HPO_4: 0 mM, 0,1 mM, 0,5 mM, 1,0 mM, 1,5 mM, 2,0 mM, 3,0 mM and 4,0 mM.

As nitrogen and phosphorus are often limiting biogenic elements, it was fundamental to examine the influence of their concentration on the development of culture, examine the optima as well as the possibly lethal concentration. For this purpose, a cross gradient of nitrogen and phosphorus ions concentrations was conducted.

As can be seen from figure n. 20 representing the fluorescence of chlorophyll a, the unfavorable conditions are reached when the concentration of both phosphorus and nitrogen ions is 0 mM. When the concentration of phosphorus is higher than 1,5 Mm and the concentration of nitrogen ions is higher than 25 mM the conditions are likewiseunfavorable. The optima were observed at the combination of the range (10 mM to 20 mM) for nitrogen ions with the range of (0,1 mM up - 0,5 mM) for phosphorus ions. Worth notice is also the combination of 0 mM of nitrogen with 1 mM of phosphorus: despite the lack of nitrogen ions, the culture development was not inhibited. Another notable performance was observed at 1,5 mM of phosphorus: when combined with 10 mM and 20 mM, the results were better than in the combination with 15 mM of nitrogen.

Generally, microalgae tend to thrive in environments with lower nutrient concentrations, due to lower osmotic pressure. Contrary, when the culture reaches a certain density, the insufficient ion concentration can be limiting to further development. Both low and high concentrations may act as stressors for their growth, and low concentrations of nutrients can limit the growth and contribute to lower lipid content. In the literature, much attention is dedicated to nitrogen levels and the prospective alteration in the lipid content. Therefore further research and analysis of high-value compounds should be conducted.

A study by Rodolfi et al. (2017) describes how nitrogen starvation led to lipid accumulation and anincrease in saturated and monounsaturated fatty acids. However, the content of valued PUFA and EPA tended to decrease with increasing deficiency of nutrient. Thereforthe THE sufficient amount of nutrient should be maintained, if aiming on cultivation for dietary supplements (production of PUFA).

A study by Xia et al. (2018) tested different levels of nitrogen concentration in FX production by the diatom Odontella aurita. They suggest that for FX production the plausible conditions are rather higher concentrations of nitrogen. This is consistent with a study by McClure et al. (2018), which suggests that elevated nitrogen levels led to increased FX production by *Phaeodactylum tricornutum*. Therefore the modification of WC + media used for microalgal cultivation might be considered for future experiments and the levels of FX should be analyzed. However, the phosphorus level should be also balanced when targeting the best cultivation conditions. A study by Nur et al. (2019) suggests that the optimum ratio between nitrogen and phosphorus should be evaluated carefully when aiming at FX production. They stress that a high FX yield is not conditioned by enhancement in phosphorus.

7 Conclusion

- Although the majority of experiments were not conducted in replicates, *Hibberdia manga seems* to be a promising microalgal strain with biotechnological potential. The yield of FX, EPA and DHA were high, therefore further research might be considered.

- This master thesis should be seen as an initial step toward the potential biotechnological utilization of Hibberdia manga. Therefore, the information gained by experiments should be considered in the future design of experiments.

- In terms of aeration, it is convenient to enrich the culture with carbon dioxide, since the air has proven to be insufficient for further growth enhancement. Since carbon dioxide represents a significant cost in biotechnology, the optimal concentration should be determined.

- The original WC media was shown to be convenient for maintaining the algae collection, however for the possible biotechnological applications more nutritious media would be more suitable. Therefore, the influence of ion concentration on valuable compounds (FX, EPA, and DHA) should be evaluated.

- In terms of temperature the most frequently used cultivation temperature of 20˚C, has shown to have a positive effect on optical density, therefore the temperatures close to this optima might be evaluated.

- In terms of FX content, future research might focus on low light intensity and blue-green spectrum of the light.

8 Literature

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