Biotechnological potential of an isolated and a known green microalgal strain and their cultivation in biofilm based reactors

PhD Dissertation

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Abbreviations

AA – arachidonicacid

A_d – surface or footprint area in m^2

ALA – α- linolenic acid

BBM- Bold's basal medium

BLAST- Basic Local Alignment Search Tool

CHP unit – combustion heat and power unit

DHA- docohexaenoic acid

DMSO - dimethyl-sulfoxide

DW – dry weight of biomass in g

EPA- ecosapentaenoic acid

FA- fatty acid

FAME – fatty acid methylester

GC-MS – gas chromatography- mass spectrometry

GLA – γ- linolenic acid

H – height

I_{in}- incident light intensity

ITS – internal transcribed spacer

L – length

M_d – dry weight of biomass in g

MUFA – monounsaturated fatty acid

OD- optical density

PAA - polyacrylic acid

PAH - polyallylamine hydrochloride

PAR – photosynthetically active radiation in µmol photons $(m^2s)^{-1}$

P_{area} – biomass productivity on surface base in g $(m^2day)^{-1}$

PBR- photobioreactor

PET – polyethylene- terephthalate

PI- polyimide

PLL - poly-L-lysine

PMMA – poly (methyl methacrylate)

PP – polypropylene

PS – polystyrene

PSS - poly(styrene sulfonate)

$P_{surface}$ – biomass productivity on surface base in g $(m^2 day)^{-1}$

PUFA – polyunsaturated fatty acid

PVC – polyvinylchloride

PVP - polyvinylpyrrolidone

P_x – biomass productivity in g $(m^2 day)^{-1}$

RAB – revolving algal biofilm

RABR- rotating algal biofilm reactor

RBC – rotating biological contactor

rpm – rotation per minute

SFA – saturated fatty acid

SH- Sueoka medium

T – temperature in °C

t – time

TOC- total organic carbon

W – width

WW – wet weight of biomass in g

$Y_{x/e}$ – biomass yield on light

κ – conductivity

I. Introduction

1.1 Introduction to microalgae

The term "algae" refers to a very wide and diverse group of organisms, including prokaryotic and eukaryotic ones with unicellular and multicellular forms, from µm to m range. They exhibit various carbon utilization ways as well; such as phototrophic, mixotrophic and heterotrophic systems. Their taxonomic classification and the relations between groups are rapidly evolving due to newer and newer molecular studies of the last years. The divisions are presented in **Table 1**.

Kindgom	Division	Class
Prokaryota eubacteria	Cyanophyta	Cyanophyceae
	Prochlorophyta	Prochlorophyceae
Eukaryota	Glaucophyta	Glaucophyceae
	Rhodophyta	Bangiophyceae
		Florideophyceae
	Heterokontophyta	Chrysophyceae
		Xanthophyceae
		Eustigmatophyceae
		Bacillariophyceae
		Raphydiphyceae
		Dictyochophyceae
		Phaeophyceae
	Haptophyta	Haptophyceae
	Cryptophyta	Cryptophyceae
	Dinophyta	Dinophyceae
	Euglenophyta	Euglanophyceae
	Chlorarachniophyta	Chlorarachniophyceae
	Chlorophyta	Prasinophyceae
		Chlorophyceae
		Ulvophyceae
		Cladophorophyceae
		Bryopsidophyceae
		Zygnematophyceae
		Trentepohliophyceae
		Klebsormidiophyceae
		Charophyceae
		Dasycladophyceae

Table 1- Classification of algal groups. Source: Barsanti el al. Algae- Anatomy, Biochemistry and Biotechnology, 2006 [1].

Consequently, this term does not refer to a taxonomic order; however it is commonly used to assemble these mostly photosynthetic organisms. Besides the taxonomic classification,

two major artificial groups are often used to distinguish algae. These categories are microalgae and macroalgae, which are based on the unicellular or multicellular organization. Here, I would like to give details about algae by using these categories.

Macroalgae, often known as seaweeds, are widely present in marine environments and play a great role in the marine ecosystem. Moreover for centuries, they are used for instance as food, feed, pharmaceuticals and fertilizers, both from natural and cultivated sources. Some of the most significant genera are *Ulva, Laminaria*, *Porphyra, Undaria*, *Eucheuma*, and *Gracilaria* (from divisions Chlorophyta, Heterokontophyta, Rhodophyta),which are extensively cultivated and marketed around the world due to their high nutritional values [1].

In the present PhD study, cultivation of microalgae is the main focus, thus in the following paragraphs, a brief description of their taxonomy, metabolic system and the major secondary metabolites with biotechnological use of important species will be described.

The term microalga also refers to a polyphyletic group, including red, brown, green algae and cyanobacteria.

Cyanobacteria are prokaryotic organisms, so they do not contain membrane enclosed organelles, thus photosynthesis takes places on the thylakoids formed by folds of external cell membrane. Their unique and well distinguishable blue-green color is coming from the light harvesting apparatus, the phycobilisome. This mostly contains phycocianin or phycoerythrin (red-brown color), which are open tetrapyrrole molecules [2].

The eukaryotic microalgae have separate organelles surrounded by membranes as it is shown in **Fig. 1**. The photosynthesis happens in the chloroplast where thylakoids membranes can form grana (stack of several thylakoids). In green algae, the light harvesting molecules are chlorophyll *a* and *b*, which have light absorbing capacity in the blue and red light range, giving the green color to many microalgae and plants. The inorganic carbon fixation happens through the Calvin cycle in the C3 pathway, as in most of the higher plants.

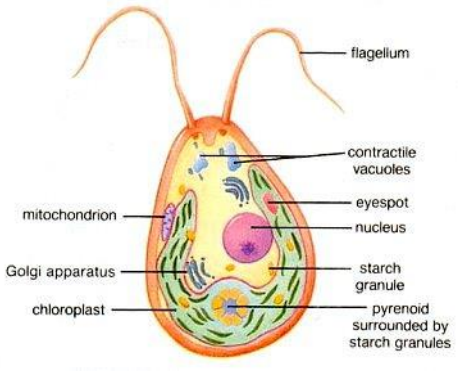

Figure 1- The structure of a typical green microalgal cell. Source: universe-review.ca

The morphology of microalgae ranges in a very wide scale among the species, also within a life cycle of a strain. Shapes can be described as amoeboid, coccoid, filamentous, palmelloid or sarcinoid. Similarly, the size of the cells also greatly varies from 0.5 μm even to 200 μm from strains to strain and from one life stage to another. A typical example for the morphological and size change can be observed by *Haematococcus pluvialis*, when the green motile cells are transitioning to astaxanthin containing akinetes, **Fig. 2**.

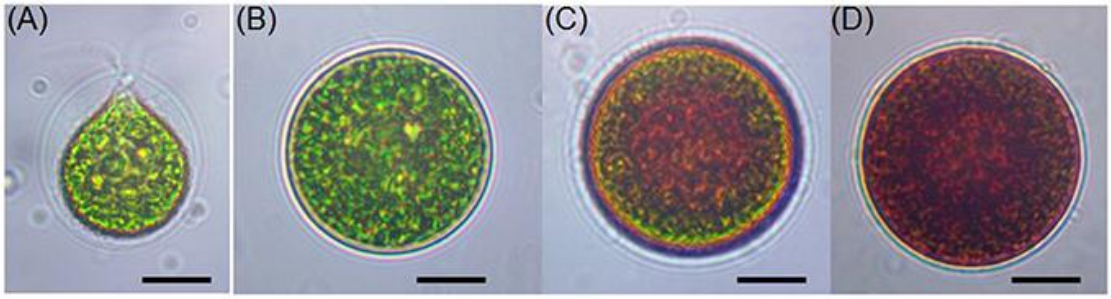

Figure 2- Various forms of *H. pluvialis* during its life cycle. (**A**) a green, vegetative motile cell; (**B**) green palmelloid cell; (**C**) palmella cell accumulating astaxanthin; (**D**) developed akinetes. The scale bar represents 10 μm [3].

When cultivating microalgae in the laboratory or in mass cultivation, the nutrient composition of the growth medium is a major factor for reaching high biomass production. The most important nutrients are carbon, nitrogen and phosphorus. In autotrophic production, inorganic carbons such as CO_2 or HCO_3^- are the main requirements for organic compound production. In case of mixotrophic and heterotrophic cultivation, the most commonly used organic carbon sources are glucose, acetate or glycerol. Besides carbon, nitrogen is the next very important nutrient. It is necessary to for instance protein and nucleic-acid production, photosynthesis (in the light harvesting antenna), thus its absence can result in slow growth and/or stop of reproduction, and consequently in accumulation of secondary metabolic compounds such as carotenoids, polysaccharides and oils. Various forms can be utilized by the microalgae, such as nitrate (NO_3^-), ammonia (NH_4^+) and urea ($CO(NH_2)_2$). However, some cyanobacteria, like *Nostoc* sp., can fix gaseous N_2 from the atmosphere. Phosphorus also plays an important role in the production of nucleic acids and energy transfer for metabolic processes. It is often the most limiting nutrient in algal cultivation as it can bond to other molecules and then becomes unavailable for the cells. On the other hand, when its concentration is sufficient, the cells are able to store it in polyphosphate bodies and use this storage under limiting conditions. Apart from the previously described nutrients, also other elements are necessary for example S, K, Na, Fe, Mg, Mn, Ca and vitamins [4].

Since microalgae are so abundant and represent very diverse metabolic pathways, the application of the biomass is gaining more and more interest in the past decades. In the

following sections, these applications and the microalgae species with biotechnological potential are discussed.

1.2 Importance of microalgae in biotechnology

Algae play a great role in the inorganic carbon fixation and oxygen production as primary producers. In aquatic environments, such as seas, oceans, rivers and lakes, they are considered as the major biomass producing organisms which then accumulates in the global food chain. As it was described in the earlier section, they also represent a great diversity in form, distribution, molecular composition, metabolic processes or reproduction. Studies estimate species number up to 10 million, of which the majority is microalgae [5].

Starting from the 1950's, microalgae were already examined for valuable substances due to their high nutrient content (e.g. protein, starch, vitamins, and essential lipids), their high biomass production capacity and the advantage over crop production that non-arable lands and salt- or brackish water can be also suitable for their cultivation. First commercial production of *Chlorella* and *Spirulina* as food were already started in the 1960s and 70s [6,7]. Later on, interest of researchers moved towards energy production from microalgae as fossil fuels prices drastically increased and availability decreased, along with the recognition of the effects of industrial activity on climate change [8,9]. Due to the diversity of microalgae, energy can be obtained in several forms from the biomass or as a produced metabolite. Energy sources include hydrogen production from photosynthetic activity; methane generation by anaerobic digestion of biomass; fermentation of biomass starch into bioethanol and biodiesel formation from lipids. Among these, biodiesel production from microalgae and its optimization gained the most attention of academic and industrial research groups [10–15]

Several benefits of microalgal based biodiesel production are presented in the literature compared to other oil producer crops, such as soybean, rapeseed, oil palm and jatropha. Besides the previously mentioned favorable characteristics as fast reproduction, ability to growth on non-freshwater and use of lands that are not applicable to agricultural usage; microalgae can accumulate over 70% of lipids on dry biomass basis; can tolerate extreme environmental conditions (light, temperature, salinity, pH); production can be continuous whole year around under optimal conditions; and can sequester CO_2 from industrial sources, in this way reducing greenhouse gas emission [8,16,17].

Calculations have been made to compare oil production capacities of microalgae and oil crops. Authors suggest that microalgae based oil production could reach 10 times higher (considering a 30% lipid content) yield compared to the current best oil crop, palm oil, which also allows to reduce the require land for cultivation significantly [8,11,15]. These calculations,

however, turned out to be rather optimistic (often overlooking the biomass production rates of the species) as current photobioreactor technologies are not capable of such a large scale production of microalgal biomass that would cover a considerable part of fuel consumption, also production cost is considerably higher than of any oil crops [9,18]. On the other hand, promising new technologies in microalgae cultivation are under development and testing, which can increase the biomass producing capacity and overcome several problems of the current large scale cultivation methods.

Microalgae are not only able to produce lipid bodies but thanks to their diversity, a wide range of other secondary metabolites with biotechnological importance are present. These compounds are representatives of groups as proteins, polyunsaturated fatty acids (PUFAs), pigments, carbohydrates etc. Their applications also cover a large variety starting from food and feed additives and ingredients, pharmaceuticals; cosmetics; soil amendments to bioplastic raw materials [7,19–23]. The market for microalgae based products are increasing, especially in the nutraceutical field as the current sources of such compounds as PUFAs (omega-3 and -6); anti-oxidants (astaxanthin, lutein); and pro-vitamins (β-carotene) are often limited [24] and coupled with contaminations (heavy metal, toxins) [25,26], or they require extensive agricultural cultivation which can than interact with the basic food supply [25]. Additionally, chemical synthesis is often not approved for human consumption due to different isomers of a molecule [27]. Autotrophic production of algae, besides providing a clean and natural manufacturing, also eliminates the use of organic compounds compared to fermentation of bacteria and fungi.

Some of the above mentioned compounds are already commercially available from microalgal sources e.g. astaxanthin [28], β-carotene, PUFAs [7], soil amendments and feed for aquaculture [19,29]. *Arthrospira platensis* (whole biomass as dietary supplement) and *Dunaliella salina* (β-carotene, vitamin A pro-molecule) [30] are among the great examples of large scale cultivation of microalgae using the alga's natural characteristic of tolerating alkalinity or high salinity and increased light and temperature [4]. At present, optimization of astaxanthin production from *H. pluvialis* and EPA/DHA (eicosapentaenoic acid and docosahexaenoic acid) production from various microalgal species are under intensive research investigation [26].

EPA and DHA are essential fatty acids for animals and humans and play important role in developing and maintaining a healthy nervous and vascular system. For human consumption the major source is fish and other seafood, consumed directly as food or as fish oil. However fishes are not able to produce them, these only accumulate in fishes through their diet of microalgae and crustaceans. Moreover, due to the increasing pollution of oceans, large consumption of seafood or even fish oil might lead to heavy metal poisoning. This problem could be overcome by direct utilization of microalgae as EPA/DHA sources [25] produced under optimized conditions in photobioreactors and fermenters.

Astaxanthin is well-known for its coloring characteristic in aquaculture (pigmentation of crustaceans and salmonids), and lately more and more studies show its beneficial health effect in humans as a strong anti-oxidant molecule [31,32]. Among the natural astaxanthin sources, such as *Euphasia superba*, *Phaffia rhodozyma* and *H. pluvialis*, the green microalga is so far considered as the best source of natural astaxanthin, as it accumulates around 4% in the biomass under stress conditions. Large scale, commercial production of *H. pluvialis* and astaxanthin are limited to closed and complex cultivation systems. Chemical synthesis is available, however it is not approved for human consumption only as animal feed, as many argues that the isomer composition is not suitable. Furthermore, bioactivity claimed to be different from the natural compound [24].

With further research on strain selection, adaption or molecular engineering and on development of cultivation systems, limitation can be overcome and production can be cost-efficient.

1.3 Selection of microalgae for biotechnological purposes

Strain selection is the first step toward a successful microalgae and high value added compound production. Currently, in the research field, only a limited amount of strains are studied for growth optimization and/or biodiesel and secondary metabolites production [21]. Considering the predicted number of microalgal strains (few millions), a great potential lies in strain selection to isolate species that are highly adapted to certain environments (temperature, nutrients, light etc.), preferably selective to other microorganisms; expressing high photosynthetic activity; suitable for large scale cultivation in photobioreactors (PBRs); and exhibiting extensive accumulation of biotechnologically valuable compounds; moreover harvesting and downstream processing would be rather simple and cost-effective [15,17,29,33] .

Choosing microalgal species for research purposes can be based on the available literature in which a certain strain is characterized and optimal parameters of cultivation are usually given. This type of selection provides the opportunity to compare several cultivation methods and conditions, however some argue that these extensively studied species such as *Chlamydomonas reinhardtii*, *Chlorella vulgaris*, *Nannochloropsis oculate* etc. might not be the most suitable for biotechnological uses. Another commonly used technique is to screen a wider selection of microalgal species from an established culture collection. Different species/strains even from the same genus can greatly differ in biomass productivity and compound production under the examined conditions. The strains kept in a standardized environment and maintained by subcultures, in most cases in axenic form, , might greatly adapt to these conditions, and

might lose their special characteristics. Also mutations are more likely to appear and spread in the culture [34].

In some cases, direct sampling from natural habitats, and locations which are planned to be treated with microalgae or have extreme growth parameters (e.g. municipal, animal, industrial wastewater ponds) can result in a faster and more efficient production optimization, additionally new biotechnologically significant strains can be isolated [35–37].

Nowadays, a new route for selecting the most suitable strains for production is also available due to molecular engineering. Genetically modified microalgae may provide a solution for many current bottlenecks of large scale cultivation of microalgae for instance coupled lipid production and biomass growth. Nonetheless, the common technologies used for yeast and bacteria cells are not well established yet for many microalgae. Studies with *C. reinhardtii* show promising results how to apply these molecular methods on microalgae species [38,39].

1.4 Important microalgal strains for industrial application

1.4.1 *Arthrospira platensis*

Probably the most well-known and most widely produced microalgae are *Arthrospira* (*Spirulina*) *platensis* and *maxima*. These strains belong to the Cyanophyta division and represent a very characteristic morphology of multicellular helix trichomes. The size of these helixes is varying from 6-12 μm in diameter, and 12-72 μm of pitch of the helix. Moreover, the morphology can change to straight rods within one strain as well, depending on the environment. They are abundant in tropical lakes with high alkalinities (pH 9.5-10.5) and high salt concentration (30 g L^{-1} salt). In Africa and Mexico, they were harvested from these natural lakes and sold in dried from as food, for several decades already. Currently, *A. platensis* and *maxima* are produced in about 22 countries across the tropical climate. The total production is claimed to be about 5500 ton per year, which is used as food, feed, dietary supplement and functional food. Due to the highly selective growing environment, the biomass is mostly produced in open raceway ponds, with a biomass density of about 1 g L^{-1}. After the concentration of the cells, the biomass is dried to a powder form. The biomass is rich in protein (up to 70% of weight), carotenoids (phycocyanin and chlorophyll), it contains high amount of γ-linoleic acid, vitamin B_{12} and iron. Based on clinical studies, the consumption of the biomass has a high antioxidant effect and positively regulates the immune system, besides providing iron and vitamins. [4]

1.4.2 *Dunaliella salina*

Another extensively cultivated microalga for dietary purposes is the *D. salina* strain from the Chlorophyceae class. The cell morphology can be described as an oval shape with two flagella, or spherical when containing high amount of carotenoids, in a range of 5-29 μm length and 3-20 μm width. The strain is known by its orange color which is the result of the β-carotene accumulation in the cells, which can reach up to 16% of dry weight. The cells are producing β-carotene, when stress factors are present such as high salt concentration (10-35%) and high light intensity. These also allow cultivating the microalga in open ponds and the use of salt water as growth liquid. The largest producers can be found in Australia, Israel, India and China. Two main cultivation systems are known, the extensive and the intensive one. The extensive cultivation is using unmixed, shallow ponds in a one-stage process. This is mostly used in Australia, where the climate is suitable for all-year-round production thanks to high irradiance and low amount of rainfall. The intensive cultivation is using mixed raceway ponds with two-stage process, resulting in higher biomass density, but also in increased price of production. However, this is necessary due to the higher amount of rain and the larger seasonal changes in Israel, India and China. After the collection of biomass, the produced β-carotene is extracted from the cells and marketed as β-carotene solutions in oil or as water-soluble powder. Considering its health effects, it behaves as an antioxidant and as a source of vitamin A, so it is widely used in cosmetics and as food supplement. [4,30]

1.4.3 *Chlorella species*

In the last decade, *Chlorella* species (Chlorophyceae) are often selected as model organisms for various research experiments testing different culturing techniques and effects of parameters on growth. This is due to their high photosynthetic efficiency, large biomass production, robustness and the simplicity of their cultivation. Besides these, also the composition of the biomass is very attracting, it contains high amount of proteins, lutein and vitamins. Additionally, under unfavorable conditions (such as nutrient limitation, high irradiance and low temperature) the cells can accumulate high amount of starch and lipids. The application of these compounds and the biomass is very diverse starting from human food supplement, feed for aquaculture and poultry (coloring egg yolks) to bioenergy production (biodiesel, biomethane and bioethanol). As a human nutritional material, it is believed to have beneficial effects on immune regulation, tumor suppression and decreasing high blood pressure. Other applications of the cells are also known, which are CO_2 mitigation from flue gases, wastewater treatment for N and P removal and recombinant protein production. Depending on the application purposes, different *Chlorella* species are used, usually, *C. vulgaris*, *C.*

protothecoides or *C. emersonii*. The cell morphology is quite general among the species; cells are spherical with a range of 2-10 μm in diameter. Currently, the main producers are in Japan and Taiwan, where circular ponds are used and the biomass is sold as food supplement. Many strains are also able to grow on organic carbon sources, thus fermentation or mixotrophic cultivation is preferred for the production of high value compounds. [4]

1.4.4 *Haematococcus pluvialis*

One of the main focuses of current biotechnological researches is the cultivation of *H. pluvialis* and its astaxanthin accumulation. *H. pluvialis* exhibits different morphological phases during its life cycle, such as the zoospores (small, spherical or pear-shaped cells with two flagella), palmellas (nonmotile, spherical cells) and the enlarged aplanospores with high astaxanthin content. This change is usually triggered when the environmental conditions are not optimal. These factors are increased light intensity, high salt concentration, nutrient limitation and their combinations. In the published literature, the amount of astaxanthin of cells is around 4-5% of dry weight. This can be reached in a two-stage process. In the first stage, optimal conditions for growth are given, then when the culture reaches the stationary phase, the so called "red phase" is introduced, where the applied stress factor induces astaxanthin production of the cells (meanwhile cell division ceases). The astaxanthin is mostly extracted from the biomass and then dried. Since the optimal conditions for *H. pluvialis* is in the mesophile range, its cultivation in open ponds is not possible. The industrial production happens mostly in tubular photobioreactors, however in some cases the red stage is conducted in open ponds with nutrient limitation or high salinity, which can reduce the chances of contamination. The biggest producers nowadays are in Hawaii, USA, Israel, China and Japan. Synthetic production is also possible, but it is only authorized for agriculture usage, while the natural astaxanthin is only marketable for human consumption. Also, studies suggest that the synthetic astaxanthin has lower bio-availability and health effects than the natural one. The value of the astaxanthin and its need in the market are growing (projected to reach $1.1 billion in 2020), since more and more beneficial effects are shown to be connected to this super- antioxidant compound. Including for example reduced cardio-vascular diseases and inflammation, increased immune-system defense and eye function, skin protection against UV radiation and possible inhibitory effects on several cancer cells. As feed additives, it plays an important role in coloring egg yolks, flesh of chicken, salmon and crustaceans, besides the previously mentioned health benefits. [3,4]

1.5 Microalgal cultivation techniques

1.5.1 Suspension-based photobioreactors

Numerous microalgal cultivation techniques were designed and tested to achieve high biomass production rates in a cost efficient way (**Fig. 3**). One of the first large scale systems are the open ponds or raceway ponds. This shallow, circulated pond technology allows an easy and cheap installation process, maintenance and cleaning are also considered simple with low energy input. However, its application is often limited to microalgal species that are able to growth under extreme conditions, such as high salinity (*D. salina*) or highly alkaline medium (*A. platensis*) as contamination represents a great problem when the culture conditions are within a more general range. Additionally, other disadvantages have to be taken into consideration when applying an open pond system [8,13,15,33,40–44] specifically,

- low biomass productivity, which is coupled with low biomass density in the suspension, generally around 0.5-1 g L^{-1},
- poor mixing, thus light and CO_2 distribution is suboptimal,
- large occupied land area or footprint area,
- increased evaporation, and
- difficult temperature control.

On the other hand, these systems are often considered as control systems to other photobioreactors since the most experimental data of outdoor, large scale cultivation is available from open/raceway ponds.

To overcome the above mentioned bottlenecks of open pond systems, closed photobioreactors were developed, such as tubular, plat-plate, air-lift column and bag PBRs [43]. Biomass production efficiency is significantly increased in these systems, due to

- better light utilization and gas transfer,
- reduced risk of contamination, or even axenic conditions can be maintained,
- decreased evaporation,
- improved mixing and temperature control, and finally
- increased cell density of about 20 g L^{-1} [45].

Nonetheless, application of these cultivation techniques brings up other difficulties, including

- higher cost of installation and maintenance;
- accumulation of O_2;
- surface attachment and growth of cells; and
- scaling up might be limited.

Closed PBRs are also widely used and often optimized to the microalgal species, culture parameters and downstream processes, resulting in very diverse culture systems.

The cultivation methods described in the previous paragraphs are all representing the conventional way of microalgal biomass production, namely the suspension cultivation. All the suspension based productions are facing a crucial problem of cost effective, large-scale algae cultivation, which is the cost of biomass concentration. Various downstream processes were developed to separate the biomass from the culture medium, including chemical and auto-flocculation; gravity sedimentation; flotation; centrifugation and filtration. Based on economic analysis, harvesting is considered as one of the major part of total production cost, which can reach up to 20-30% [33,42,46,47]. This is one of the main reasons why the current photoautotroph suspended setups are struggling to enter the bioenergy market.

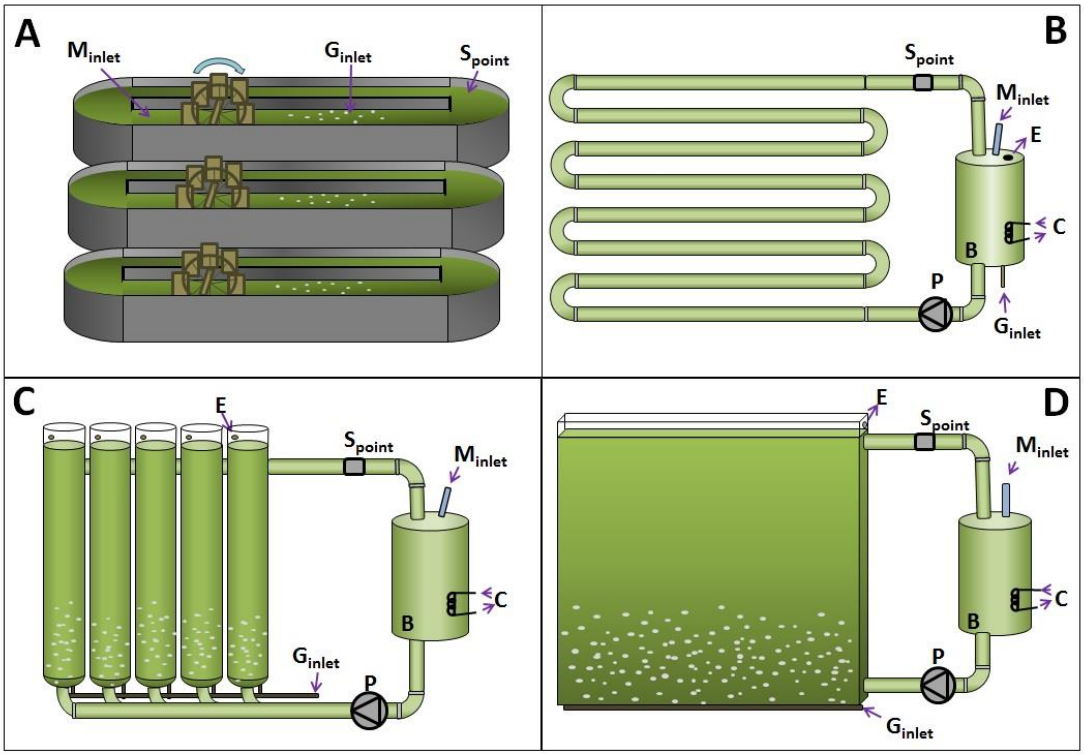

Figure 3- Schemes of the most common suspension based photobioreactor used for microalgae cultivation. **A** Raceway pond; **B** Tubular PBR; **C** Column PBR; **D** Flat-plate PBR. The schemes include the connected buffer tank (***B***) with the nutrient inlet (M_{inlet}), temperature regulation (***C***); the sensoring point (S_{point}) where several parameters can be measured (pH, dissolved oxygen, temperature, nutrient level etc.); gas inlet (G_{inlet}) and exhaust (***E***), and the circulation pump (***P***).

1.5.2 Biofilm based photobioreactors

In the last few decades, besides suspension based biomass production, attached cultivation of microalgae was gaining more and more interest as a possible solution for a more

cost-efficient microalgal production. Biofilm reactors are using the surface adhesion characteristic of microalgae which is often causing biomass loss in suspension systems, for instance in tubular or flat-plate PBRs. Immobilized microalgae cultivation represents numerous benefits compared to suspension based cultivation methods, such as

- reduced water requirements,
- increased biomass density between 37-200 g kg^{-1}, thus
- decreased harvesting costs,
- better light utilization and reduced light limitation, and
- low maintenance and operation costs [48].

Biofilm based microalgae cultivation systems mostly consist of a substrate layer or surface, where the algal cell can attach to form the biofilm, and a culture medium container which keeps the algal biofilm constantly wet and provides the necessary nutrients. Many different structures can be found in the literature; however their concepts can be divided into two major categories.

A) The surface with the biofilm is fixed and placed horizontally (usually tilted slightly) or vertically. The medium is pumped up to the top of the surface and by gravity flows down meanwhile wetting the biofilm [49]. The medium is usually collected at the bottom of the surface and recycled to the system. Within this group, we can differentiate two other types depending on whether the medium has direct contact with the biofilm or not. In one case, the medium flows on the surface of the biofilm [50–54], while in the other case the biofilm is separated from the medium by a porous membrane [55–60]. The first case is a simpler setup, requiring only one substrate material, while the second one besides the porous membrane, another material is required which supports the porous membrane with the biofilm and leads the liquid through the system.

B) The biofilm supporting substrate material is continuously submerging into and emerging from the medium hence keeping the biofilm wet and supplying the cell with nutrients. The orientation of the surfaces is usually vertical, rotating around an axle. The construction of the systems is more divers compared to the previous group as this concept allow bigger varieties (**Fig.4**) [61–66].

Besides the construction setup, the two categories differ also in the way of inoculation, considering the systems of group A), the initial biomass is applied to the system by spraying, brushing, smearing or filtering onto the surface; while in the reactors belonging to group B) the algal cells forming the biofilm are mostly originated from the medium. Even though, a concentrated biomass culture is usually used and poured or brushed on the surface, due to the submerging, the majority of the cells wash off. The primary biofilm formation is then partly based on the cells remaining on the surface and partly from the cells that reattach to the surface

material from the medium, as a result of suboptimal growth conditions in the reactor tank (low light intensity) [61,62,66].

In both cases, the harvesting process occurs by scraping off the biofilm from the surface material, resulting in a biomass paste, which has dry matter content similar to pastes obtained after centrifugation of suspended cultivations [61–63,66].

A crucial point of biofilm cultivation is the selection of substrate material, as it has to match several parameters to be suitable for application in a biofilm system. Among these parameters, one of the most important is the durability of the material, as it is in contact with the medium and algal cells continuously, it should be non-degradable, not losing its form due to wetting and should be resistant to mechanical stresses caused by regular scraping off [67]. On the other hand, the surface should promote primary algal attachment and biofilm formation and reformation after each harvesting. Numerous materials have been used so far in the biofilm reactors described before, including glass, different plastic materials, metal meshes, cotton based ropes and textiles, printing paper, concrete and filter materials [52,55,61–63,68,69]. However, only few studies addressed the question about the relationship between the type of surface material and the algal growth and what kind of interactions play a role in the primary cell attachment. Surface charge of cells and the surface, the contact angles of materials (tested with different standard liquids) and surface energy were determined to examine whether they correlate with cell growth and biofilm formation [51,53,69–71].

In a recently published study, Gross et al (2016) compared the physico-chemical characteristics of 33 different smooth materials and further tested them for algal growth in a simulated biofilm system. Additionally, selected materials were tested also for surface texture with a wide range of pore sizes in a pilot system [69]. The findings are in correlation with the other published researches and reviews, suggesting that hydrophilic surfaces, based on contact angle measurements with tetradecane (which corresponds to the wettability of a surface; <90° hydrophilic; >90° hydrophobic) and surface roughness are in strong correlation and have a great impact on the initial biofilm formation. Moreover, as Schnurr et al (2015) summarized the observation of several immobilized microalgae cultivation, the biofilm growth can be divided into two phases; in the first phase the surface (e.g. hydrophobicity, surface charge) and cell characteristics are playing a more important role in the initial colonization; while in the second phase (after the first harvesting), the biomass production mostly depends on the surface texture or roughness, which enables the microalgae cells to remain on the surface after harvesting [72].

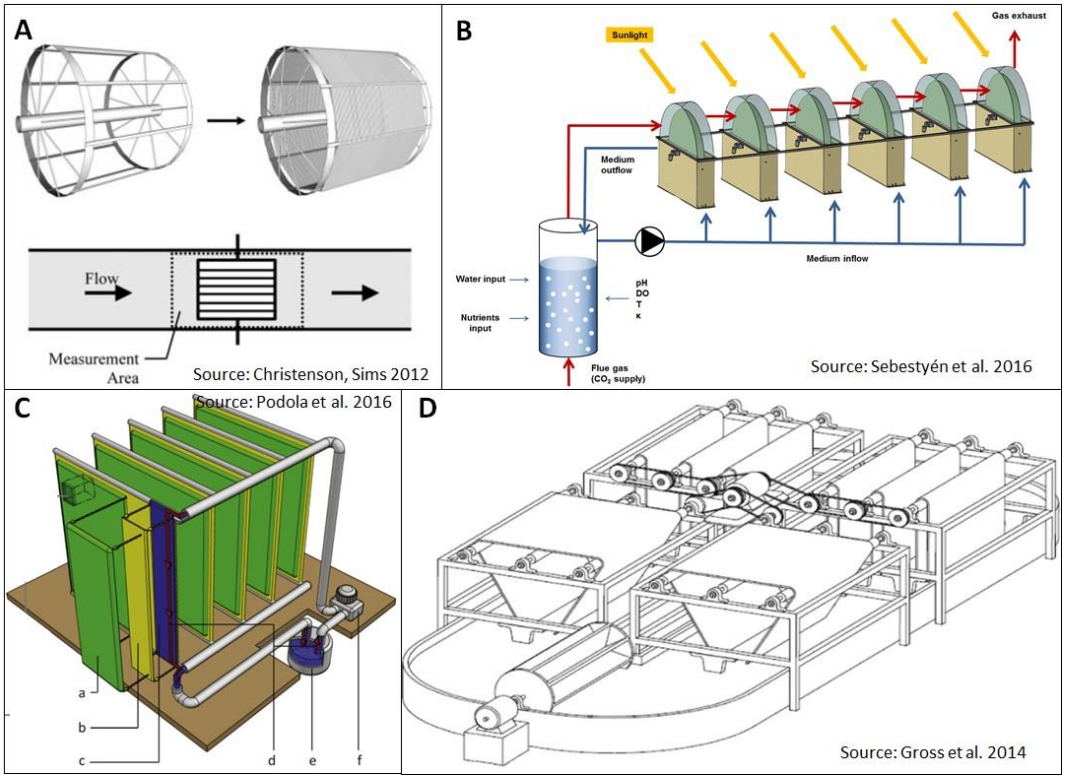

Figure 4- Schemes of the large scale biofilm based photobioreactor used for microalgae cultivation. **A** Revolving Algal Biofilm system (RAB); **B** Algadisk system; **C** Twin Layer system; **D** Rotating Algal Biofilm Reactor (RABR). Within the Fig. **C** the labelling are referring to: (**a**) algal biofilm; (**b**) microporous membrane; (**c**) culture medium; (**d**) direction of medium flow; (**e**) medium reservoir; and (**f**) pump.

II. Objectives

Microalgae cultivation for biotechnological application is promising, but many difficulties should be still addressed and have to be overcome until their full industrial production level can be reached. In order to understand better how microalgae behave in artificial scaled up environments (what kind of parameters influence their production in long term and how their performance can be improved both for biomass and high value added production) new concepts and studies are still necessary.

The importance of such a study was recognized also by the European Union; consequently the Algadisk project came to alive with an international, multidisciplinary consortium. The aim of the research was to develop a biofilm based cultivation system for microalgae cultivation and to test it in laboratory and pilot scale during a long-term operation with several algal species.

The present PhD research is part of this project; of which we focus on the selection of algal strains for cultivation and the tests of the lab-scale Algadisk reactor. However it also goes beyond the objectives of the project by testing an additional biofilm system, the Twin Layer system.

Keeping in mind the goals and the directions of the European project, we set up several steps along which we plan and conduct the experiments.

Firstly, we establish the criteria for the selection of isolated microalgae. One of the main requirements is to find strains that are adapted to the Central European climate, and then they fulfill the following selection criteria:

-surface attachment;

-fast reproduction and biomass production and

-high content of valuable metabolites, namely lipids.

For this reason, natural habitats from Hungary, Germany and Austria are visited to collect samples.

After the strain screening and the establishment of the culture, the surface attachment abilities are examined on numerous specifically designed surface materials. This would allow to choose the most suitable material for the further larger scale experiments, additionally would clarify some relations between surface material and cell attachment.

In the following phases, we aim to operate the laboratory scaled Algadisk reactor with the selected strain and its biomass and lipid production are monitored and compared under different parameters such as low and high irradiation; optimized culture medium and artificial fertilizer. Besides the biomass quantity and quality, it is examined how stable the system is considering contamination, mechanical problems, whether it can operate continuously and how the biofilm formation is affected by the harvest and regrowth cycles.

Not only is the Algadisk system studied for high value added compound production, but also the Twin Layer system. Our aim is to investigate the effects of light intensity and application of stress from the culture medium on the *H. pluvialis* microalga's biomass and astaxanthin production rates, and to demonstrate the benefits over suspension based cultivation of *H. pluvialis* for marketable astaxanthin.

Finally, the two biofilm based cultivation concepts would be analyzed and compared to each other and to other published biofilm systems, based on the experimental data and observations inspected during the PhD research. In every step of the research, by evaluating the results and considering the further steps, the main focus lies in the applicability of the method/process in the pilot Algadisk reactor.

II. Materials & Methods

3.1 Laboratory scaled Algadisk reactor

3.1.1 Microalgal strains and cultivation

Microalgal strains used during the research were maintained in about 50 ml of medium in glass flasks, closed with cotton plugs in order to avoid contamination meanwhile provide sufficient aeration; thin foil was wrapped around the cotton plugs to reduce the evaporation. Cultures were kept at 20-25°C temperature, *Chlorella* cultures were illuminated by 150 µmol photons $(m^2s)^{-1}$, while *Haematococcus pluvialis* species were kept at around 50-60 µmol photons $(m^2s)^{-1}$ in 16:8 hours day: night cycles, manually shaken once a day. The following culture media were used for maintaining the main examined species: Sueoka (SH) medium [73] (Appendix 1, **Table 2**) for *Chlorella* sp #34; M8-a medium [74] (**Table 7**) for *C. sorokiniana* CCAP 211/8K; BBM medium [75] (Appendix 1, **Table 2**) for *H. pluvialis* SAG 44.96 and for *H. pluvialis* CCAC 0125 M0176/1.

3.1.2 Isolation of microalgae from natural water basins in Central Europe

In order to isolate new strains of microalgae, that are able to grow on surfaces, special collecting methodology was designed and conducted. The major sampling places were natural water basins, such as lakes, ponds, rivers, and backwaters around Szeged, Hungary (details in Appendix 2 Table 3.). At these sampling points, pieces of several different materials were submerged under water at around 1 m depth. These materials included glass plates, polycarbonate sheets, polyethylene- terephthalate and polyvinylchloride pieces. This method was used in order to preselect algae species that are able to grow on surface, more specifically on artificial surfaces such as the above mentioned ones. Samples were collected 2 weeks after the installation of sampling materials and placed in two types of microalgal cultivation media, namely A9 [76] and SH for enhancing the cell culture. A second phase of isolation were performed as well, where the main sources of samples were warmer water basins located in Central Europe (see details in Table 3), in which biofilm formation of microalgae was observed. The 50 ml volume cultures in glass flasks were kept at 25°C or 30-32°C (warm water samples). This temperature range was chosen as it is achievable in the lab- scale Algadisk reactor. Samples were illuminated by white fluorescence tubes (Polylux XLr F58W/840, GE Lighting, Budapest, Hungary) at around 150 µmol photons $(m^2s)^{-1}$, in 16:8 hours day: night cycle. As the cultures became denser, algae were plated as well on A9 and SH agar plates for separation and isolation of the different species based on their macroscopic and microscopic characteristics.

From the obtained monoculture strains, a culture collection was established and further maintained under the above described conditions.

3.1.3 Selection of isolated algal strain according to growth and lipid content

Growth characteristic of the isolated algal species were examined and compared by measuring the optical density of cells at 550 nm wavelength. Culture flasks were prepared with 50 ml A9 or SH medium (depending on the strain), closed with cotton plugs and were sterilized prior to inoculation. Initial cell density was set between 0.001 and 0.25 OD_{550nm} and sampling occurred by removing 1 ml sample from each flask and measured optical density by spectrophotometry (Unicam Helios α, ThermoFischer Scientific, Waltham, USA) regularly for about 2 weeks. Due to cell attachment on the glass surfaces, before sampling cell were removed by scraping from the glass walls and the cultures were homogenized.

Lipid accumulation of the strains was detected by staining the cells with the lipid selective Nile Red dye. 50 µl of alga culture was placed on a microscopic glass slide and 2 µl Nile Red dye dissolved in dimethyl-sulfoxide (DMSO, purity 99.9%, VWR International, Radnor, USA) (1 µg mL^{-1}) was added. After 15 minutes of incubation in dark, the samples were analyzed by a fluorescence microscope (Olympus BX51, Tokyo, Japan). The lipid bounded Nile Red dye emits yellow color (570-590 nm) when excited with blue light (470-490 nm). At the same time, chlorophyll content of the cell can be seen in red.

Some samples were further analyzed by GC-MS for total fatty acid content (see Section 3.1.7.2).

3.1.4 Molecular identification of the preselected green microalga

The identification of the isolated SH-34 strain is based on the 5.8S rRNA, 18S rRNA, 28S rRNA genes, and ITS1 and ITS2 genome sequences [77–79]. Axenic monocultures were prepared in SH medium and cultivated at 23°C, illuminated with 150 µmol photons $(m^2s)^{-1}$. Flasks were manually shaken every day. For the extraction process, culture was centrifuged and pellet was further analyzed. DNA was extracted with Power Soil DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, USA). The purified DNA was used in PCR for amplification of the desired sequences based on the method of Wu et al (2001) [79] and Yoshida et al (2006) [78]. Then these sequences were analyzed by SOLiD® sequencer (Thermo-Fischer Scientific, Waltham, USA). The results were compared to available sequences of the BLAST program. This work was done by Dr Edit Szameczné Rutkai and the Laboratory of Microbial Genomics, Biological Research Center, Szeged, Hungary.

3.1.5 Test of different substrate materials and coatings for biofilm formation capacity

Based on the results from selection steps concerning biomass production and lipid accumulation described in Section 3.1.3, a *Chlorella* species, namely *Chlorella* sp. #34 was chosen for further testing the biofilm formation on several surfaces. Also *C. sorokiniana* CCAP 211/8K and *H. pluvialis* SAG 44.96 were tested based on biomass production capacity and/or their high value added compound producing ability.

A special, closed cultivation system was designed in order to test several surface materials with combination of polyelectrolyte layers. These growth surfaces were prepared by Cranfield University, using four plastic substrates that are commercially available and/or were used already for biological purposes, namely

PET-polyethylene terephthalate

PS-polystyrene film

PP- polypropylene

PI-polyimide

Each substrate was covered by different combination of polyelectrolytes in order to gain a negative or positive outer surface charge. The following polyelectrolytes were applied:

PVP - polyvinylpyrrolidone (cationic)

PAA - polyacrylic acid (anionic)

PAH - polyallylamine hydrochloride (cationic)

PSS - poly(styrene sulfonate) (anionic)

PLL - poly-L-lysine (anionic)

In the following orders:

Coatings:

1: PVP/PAA/PVP/PAA/PVP 2: PSS/PAH/PSS/PAH

3: PSS/PAH/PSS/PAH/PSS

4: PLL/PAH/PLL/PAH/PLL

As control, substrates without coatings were also tested. Several layers of the polyelectrolyte solutions were applied to reach a more homogeneous covering. (The physico-chemical characterization (surface charge, contact angle, durability etc.) of the surface materials were done by Ms Tasneem Bhaiji, Cranfield University, as part of her PhD research, which is not published yet, thus data is not available.)

As the main characteristics of the Algadisk reactor is the vertically orientated and rotating disks, a similar concept was applied in order to test these surface materials. From each of the above mention surfaces 1x2 cm pieces were cut and fixed on a plastic tube, horizontally in a

way that fixing and later removing did not cause weight change. The plastic tubes were fixed on the lid of 750 ml glass jars, allowing the pieces of substrates to merge into the culture media when placed horizontally (**Fig. 5**). Gas exchange happened through a hole on the lid, contamination was prevented by a sponge placed in the hole. This set up was laid on a bottle roller apparatus (CELLROLL, IINTEGRA Biosciences AG, Zizers, Switzerland) with a medium volume of 260 ml.

With the selected microalgae strains, *C. sorokiniana*, *Chlorella sp*. #34 algae and *H. pluvialis*, our main aim was to examine the effect of rotation on surface attachment of microalgae cells. Culture media of *Chlorella* species was M8-a medium, while *H. pluvialis* SAG 44.96 was incubated in BBM medium. During this experiment, ambient temperature was applied, light intensity was about 35-45 µmol photons $(m^2s)^{-1}$ due to the set-up. pH was not controlled during the incubation. Initial cell density was set to 0.2 OD_{550nm} and incubation lasted for 7 days. The jars were placed on their sides on the tube roller apparatus, which resulted in vertical orientation of the surfaces. The medium did not cover all the time the surfaces, instead, they emerged and submerged into the liquid regularly. This concept resembles the Algadisk system, presented later in Section 3.1.6. Rotation speed was set to 20 rpm. After 7 days incubation, we measured the dry weight of the biomass attached to the surfaces by drying them at 42°C, overnight. Surface materials were placed in pre-weighted aluminum containers, dried together and then weighted together. Biomass dry weights were calculated from these results.

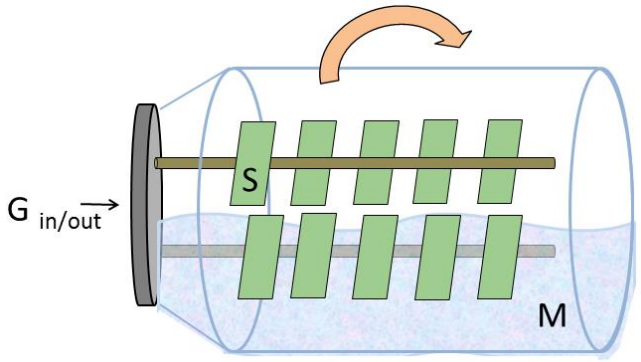

Figure 5- Scheme of the system used for preselection of surface materials; **S**- surface material with algal growth on it; **M**- growth medium; $\mathbf{G_{in/out}}$- ambient gas transfer through a hole on the closing lid. The culturing flaks was placed horizontally and rotated continuously.

3.1.6 Laboratory scale Algadisk photobioreactor set-up and operation

The Algadisk photobioreactor design was based on a Rotating Biological Contractor (RBC) [64] and further modified in order to enhance and examine microalgae growth [62,66]. The main part of the laboratory scale system (**Fig. 6**) consisted of a polypropylene tank, 75 x 35

x 20 cm (L x W x H), and of four disks, each 25 cm in diameter, placed parallel on an axle, 15 cm apart from each other.

The reactor tank was connected to a buffer tank that provided consistent medium level in the reactor tank; continuous circulation and equal distribution of nutrients were supplied by a circulation pump placed in the buffer tank; flow rate was set to 10 L min^{-1}. Total filling volume of the system was 35 L. Disks were continuously rotated via a motor connected to the axle; rotation speed was set to 11 rpm. A half- cylindrical apparatus was designed for the distribution of light over the surface of disks; 6 cool white fluorescent light tubes (Polylux XLr F15W/840, GE Lighting) were used, in a day:night cycle of 16:8 hours. Due to the orientation of disks and light source, the light intensity had deviations between the disks, however the average light intensity on the disk surface (vertically hitting the disk) was 40 ± 15 µmol photons $(m^2 s)^{-1}$. A second type of light panel was built, with which the light intensity was increased to 190 µmol photons $(m^2 s)^{-1}$. The light sources in this case were 8 LED reflectors of 30 W power, 5000-5500 K thermal radiation and cool white color temperature. In order to reduce biomass growth in suspension and enhance biofilm formation, the reactor tank was covered with a stainless steel sheet to decrease the amount of light penetrating the medium.

For the inoculation of the system, dense, pre-cultivated cultures were poured onto the rotating disks and into the reactor tank. Regrowth of the biofilm after harvesting is promoted by remaining cells on the disk surface, thus no re-inoculation was needed during continuous operation. Steps of biofilm formation and reformation are shown in **Fig. 6C**.

Chlorella sp #34 was normally cultivated in M8-a medium with 33 mM urea, except for the lipid accumulation and the fertilizer experiments. During the lipid accumulation phase the medium was replaced by nitrogen free M8-a medium; while a commercially available fertilizer, Agroleaf Power High P (Everris, The Netherlands; see composition in Table 6.), was used in 2 g L^{-1} concentration for the aforementioned experiment. Increased P content fertilizer was chosen for this experiment based on previous suspension studies with other Agroleaf products (data not shown).

Temperature of medium in the system were monitored and controlled: temperature was maintained at 30 ± 1 °C with a heater. The material of the disks was PVC that was roughened in advance with sand-paper of P80 grit size, for initiating a faster and more stable biofilm formation. The used disk materials were different from the tested ones in the Section 4.1.4 which is due to several practical reasons. First of all, the substrate materials were not stable enough to support a 25 cm in diameter disk. Secondly, the price and the availability of materials were also not suitable in many cases. Negatively charged coatings increased the biomass yield of *Chlorella* sp# 34, nonetheless the uncoated ones were also preforming well. Also as Blanken et al [62] reported that the coated PC disks was not stable during a long term operation. Taking into account the cost and the time needed for the preparation of coatings, we have decided to

choose PVC for disk material and leave the surface uncoated. However, as PP with coating #3 performed greatly in the previous experiments, it was also tested under high light conditions.

H. pluvialis was cultivated in BBM medium at the temperature of 23±1°C under low light conditions, 40 ± 15 µmol photons $(m^2\ s)^{-1}$. Four different materials were applied as disks: (1) polypropylene (PP); (2) polycarbonate (PC); (3) polyvinylchloride (PVC); (4) polyethylene (PE); all disks were sanded with P80 sand-paper.

In all of the experiments the pH was kept between pH 6.7-7 by sparging CO_2 gas (purity: 99.5%, The Linde Group, Munich, Germany) into the medium when reaching pH 7.

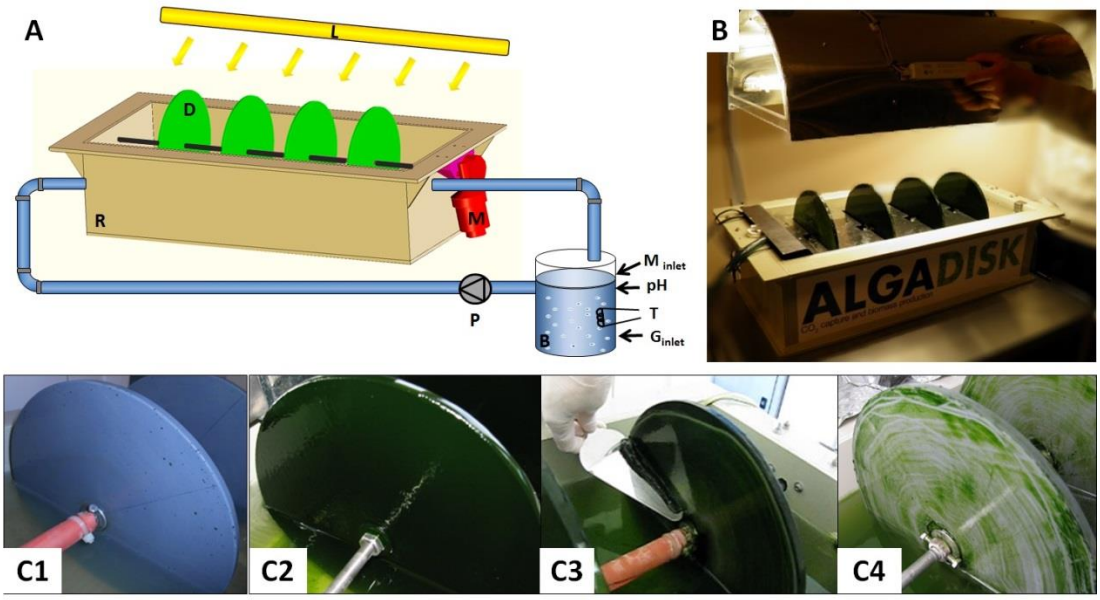

Figure 6- Schematic view of the laboratory scaled Algadisk reactor (**A**), with the sensor and control devices, and a photo of the reactor tank with the light source (**B**). The phases of the biofilm formation is shown on **Fig. 6.C**, including (**C1**) - attachment of cells to surface; (**C2**) - formed biofilm; (**C3**) - harvesting; (**C4**) - remaining cells after harvesting, the source of the new biofilm. The symbols of **Fig. 6.A** are detailed here: ***R***- reactor tank; ***D***- disk; ***L***- light source; ***M***- motor; ***T***- thermostat with temperature sensor; ***B***- buffer tank; ***pH***- pH control unit $\boldsymbol{G_{inlet}}$- CO_2 gas source; ***P***- pump; $\boldsymbol{M_{inlet}}$- medium injection point.

3.1.7 Characterization of biomass growth and biomass composition

3.1.7.1 Harvesting and dry weight determination of the laboratory scale Algadisk reactor

Total dry weight (DW), dry weight content of biofilm and biomass production rates were determined by scraping off the biofilm from each disks with a metal scraper. Wet biomass was measured before drying at 80°C until weight remained constant. The dry mass content of biofilm was obtained from the ratio of dry and wet biomass (WW) and was expressed in unit g DW kg^{-1} WW.

For the calculation of the total biomass yield and the biomass productivity, the biomass harvested from the two sides of the disks was summed up and then average was taken from the four disks. This was used to calculate the biomass productivity in terms of dry weight per disk surface area or footprint area of the reactor. When it is indicated, standard deviation was calculated using at least three replicate disks. The following equation was used to describe productivity, both on disk surface area and footprint area of the reactor tank:

(Eq. 1) $$P_x = \frac{M_d}{t * A_d}$$

where, $\mathbf{P_x}$ is the surface or footprint biomass productivity in $g\ (m^2 d)^{-1}$; $\mathbf{M_d}$ is the average dry weight of biomass in *g*, harvested in a growth-harvest cycle; **t** is the time of a growth-harvest cycle in *days*; and $\mathbf{A_d}$ is the surface area of disks or footprint area of the reactor in m^2.

Biomass yield on light; $\mathbf{Y_X}$ ($g\ mol^{-1}$); for each cycle of laboratory cultivation was calculated based on the incident light on the illuminated fraction of disk surface (50% of total disk surface) $\mathbf{I_{in}}$ ($mol\ (m^2 d)^{-1}$); and the biomass productivity on surface or footprint area, $\mathbf{P_x}$ ($g\ (m^2 d)^{-1}$), according to the following equation:

(Eq. 2.) $$Y_x = \frac{P_x}{I_{in}}$$

3.1.7.2 Fatty acid determination

Dried microalgae biomass were placed in headspace glasses (HS, Chromacol 20-HSV T717), and 10 mL of 5% (v/v) HCl content chloroform: methanol (2:1 v/v) mixture were added to the samples. Gas phases were washed with nitrogen gas (purity 5.0), then glasses were closed and incubated in a ultrasonic water bath (DECON FS1006) at 40°C for 30 minutes. After this step, for transesterification, samples were placed to 100°C for 30 minutes. After cooling to room temperature, 5 mL of each sample were taken and dried. To the residues, 5 mL methanol: water (4:1 v/v) mixture were added and vortexed for 2 minutes. Then 5 mL of n-hexane were added to the samples and they were extracted for 20 minutes. After extraction, n-hexane with C_{12} ISTD (27.2 mg L^{-1} C_{12} on sample bases) was added to the samples. 2µl of each sample was measured by gas chromatography.

FAME content and composition of the extracted samples were analyzed by gas chromatography with an Agilent 6890N gas chromatograph (Agilent Technologies, Santa Clara, USA). Parameters of inlet: temperature of 270 °C, splitless mode, 25 mL min^{-1} splitvent. FAME separation happened in a DB-23 column (60 m x 0.250 mm x 0.25 µm) with the following heating program: initial temperature 40 °C for 0 minutes, temperature increased at 7.5 °C min^{-1} to 170 °C, then at 2.5 °C min^{-1} to 215 °C. 215 °C was held for 6 minutes, then at 40 °C min^{-1} to 240 °C and held for 2.04 minutes. Detection was carried out with a 5975 MS detector, used in

auto-tune mode. Carrier gas was helium. Later in the text, the term of total lipid or lipid content is used instead of FAME.

3.1.7.3 Total nitrogen determination of medium

The total nitrogen content was followed in the medium by a Teledyne Tekmar Apollo 9000 TOC Combustion Analyzer (Teledyne Tekmar, Mason, USA), using the built-in function for total N determination, after separating the cells and other particles from the liquid by centrifugation. The concentration was given in mg N L^{-1} medium. Average was taken from three replicates at every sampling point.

3.2 Bench scale Twin Layer system

3.2.1 Microalgal strain and maintenance

The tested *H. pluvialis* M0176/1 strain was chosen from the Culture Collection of Algae at the University of Cologne, Cologne, Germany and was maintained prior to inoculation in 2 L Erlenmeyer flasks with about 1.2 L 3N-BBM medium, aerated with 0.5 % supplemented CO_2 in air at 23 °C with 14 hours of 40 µmol photons m^{-2} s^{-1} light (provided by fluorescence tubes L36W/640i energy saver cool white and L58W/956 BioLux fluorescent lamps; Osram, Munich, Germany). 2 days before the inoculation date, cultures were refreshed with about 500 mL of fresh 3N-BBM medium in order to induce logarithmic growth stage of cells.

3.2.2 Twin-Layer photobioreactor set-up and operation

The Twin-Layer cultivation method is based on a porous membrane technology, where the biofilm is separated from the growth medium [56]. The used Twin-Layer set up was described by Schultze et al [57] and consisted of the following parts: 50 cm long, transparent PMMA (poly(methyl-methacrylate) tubes; standing on a PVC rack, which encloses the liquid medium container (1 L glass bottle) and the inlet for gas and outlet of recycled medium. The PMMA tube is closed with a removable PVC cap with inlet of medium and outlet of gas, moreover holds the 50 x 10 cm (length x width) vertical, glass fiber mat which serves as the source layer for the polycarbonate membrane substrate layer (**Fig. 7**).

The initial biomass of 2 g m^{-2} was immobilized onto the polycarbonate membranes (PC40, 0.4 µm pore size, 25 mm diameter, Whatman, Dassel, Germany) via filtration in a circle area of 2.54 cm^2 [55]. The filters were then placed on the pre-wetted glass fiber sheets in the PMMA tubes. The medium was pumped up from the media bottles to the top of the glass fibers with a flow rate of 4.5-5 mL min^{-1}, and through gravity recirculated into the bottles, allowing to

keep the moisture of the biofilm and provide nutrients. To avoid nutrient limitation, full media (BBM) and stress media in the second experiment (N free BBM, full BBM amended with 0.4%; 0.2% and 0.05% NaCl) were exchanged in every 3-4 days. The experimental tubes were placed in a culture room, where temperature was kept at 23 ℃ and light: dark cycle was 14:10 hours.

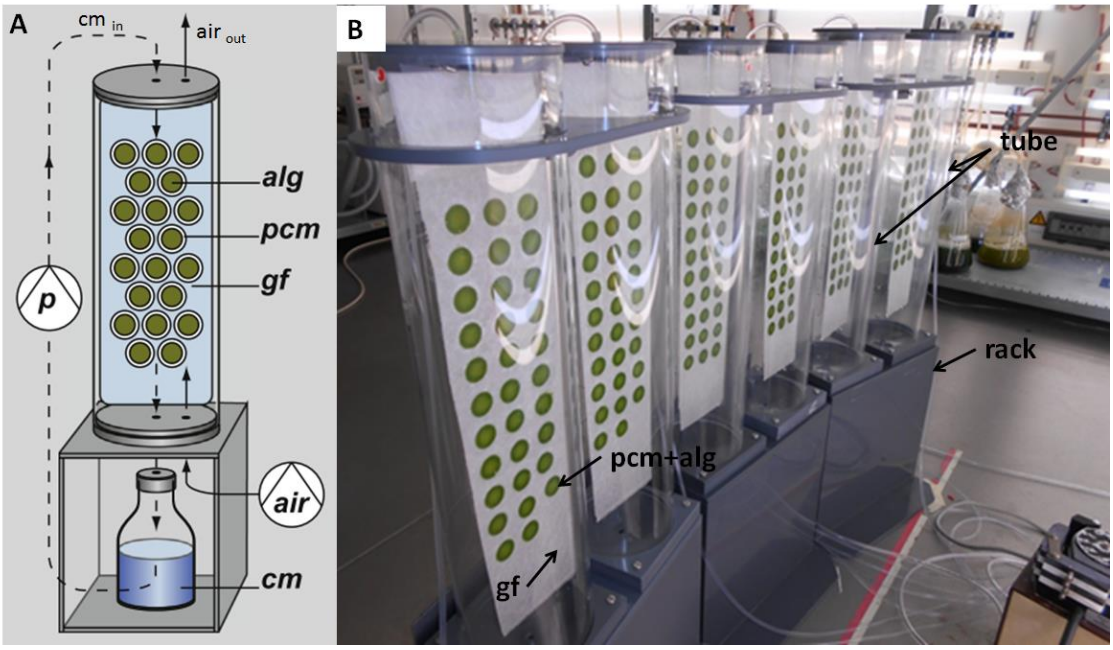

Figure 7– Scheme (**A**) and photo (**B**) of the bench scale Twin-Layer system and the concept of separation of growth medium and biomass. ***cm***- culture medium; ***cm$_{in}$***- culture medium inlet; ***alg***- microalgae biofilm; ***pcm***- polycarbonate membrane; ***gf***- glass fiber; ***air$_{out}$***- outlet of air; ***rack***- PVC rack with the culture medium bottle inside; ***tube***- PMMA tubes; ***pcm+ alg***- polycarbonate membrane with the algal biofilm. The culture medium was circulated via a peristaltic pump from the glass bottle to the top of the glass fiber. Compressed air mixed with pure CO_2 was directly led to the tube.
Source of figure: Schultze et al (2015)

3.2.3 Light intensity screening for optimal growth of *H. pluvialis*

Biomass production of *H. pluvialis* was examined in correlation to light intensity. Six Twin Layer systems (tubes) were set up, each with different light intensities, 26, 44, 85, 119, 135 and 219 µmol photons $(m^2s)^{-1}$ provided by fluorescence light tubes. The total length of the experiment was 18 days; sampling happened in 2 days intervals. At each sampling point three polycarbonate membrane filters with the algal biofilm were removed from each tube (each light intensity) and considered as triplicates for biomass measurements.

3.2.4 Stress induced astaxanthin production in *H. pluvialis*

Based on results of the light screening study, around 210 µmol photons $(m^2s)^{-1}$ light intensity was set in 5 tubes for biomass and astaxanthin measurements. The experiment was designed such way that in the first 8 days of incubation, cells were growing on nutrient-replete BBM medium, supporting cell division and biomass growth. Then in the following 10 days, various stress factors were applied in separate tubes, namely, N free BBM; full BBM with different concentration of NaCl, 0.4%, 0.2% and 0.05%, in order to enhance astaxanthin production and accumulation. In the first phase, sampling only occurred on day 4 and 8, while in the second phase in every 2-3 days. As control, in one system, the biomass was growing on full BBM during the whole 18 days of incubation. At each harvesting point, three filters were removed from each tube and considered as triplicates for biomass and astaxanthin measurements.

3.2.5 Inoculation and characterization of biomass growth and astaxanthin content

3.2.5.1 Inoculation, harvesting and dry weight determination

As previously described pre-cultivated suspension cultures (see Section 3.2.1) were concentrated to about 10 times via centrifugation, and this dense culture was continuously stirred on a magnetic stirrer during inoculation to keep a homogenized culture. The initial cell density of the suspended cultures for inoculation was determined by filtering 1 mL of culture onto pre-wetted polycarbonate filters in triplicates and then dried for 1 hour at 105 °C. After cooling to room temperature in a desiccator, filters were weighted and the volume of inoculation culture was calculated in a way that biomass density on filters was 2 g m^{-2}. Then 27-33 filters were placed on the glass fiber sheet of each tube.

Harvesting was done by removing three filters from each tube, then the biomass that overgrown the inoculation area (2.54 cm^2) were scrapped off and the filters were freeze dried until constant weight. Dried filter were weighted and dry biomass yield on surface base was calculated.

Biomass productivity was calculated using the built-in linear regression function of GraphPad Prism statistical software (GraphPad Software Inc., La Jolla, USA). All the R^2 values were above 0.93.

3.2.5.2 Astaxanthin determination

After determining the dry weight, the freeze dried biomass was further examined for astaxanthin content based on the protocol of Li et al. [80]. Known amounts of biomass, between

1-1.5 mg, were measured into 2 ml Eppendorf tubes and 2 x 1 ml of DMSO was added to each sample, followed by rigorous vortexing, and then samples were incubated in waterbath at 70 °C for 5 min, meanwhile vortexed several times. Finally, samples were centrifuged at 10000 rpm for 5 min. The extraction step was repeated until the cell debris became colorless. In some cases, samples were grinded with sand prior to extraction in order to break akinetes. The supernatant were collected and its absorbance was measured at 530 nm with TECAN plate reader (Infinite M200 plate reader, Tecan, Männedorf, Switzerland). This wavelength was selected as it was previously shown (data not published) to give the most accurate value for the concentration of astaxanthin in a mixture of other pigments. In order to be sure that the extracted samples were not too dense for the analysis, each samples were 2 times diluted (with DMSO) in the 96 well plates (flat-bottom with cover). Astaxanthin concentration was calculated from the equation below obtained from the standard curve of pure astaxanthin (98.6% purity, Dr. Ehrenstorfer GmbH, Augsburg, Germany) in DMSO between the concentrations of 0.1 µg mL^{-1} to 25 µg mL^{-1}, blank was always pure DMSO.

$$y = 0.00665x + 0.0082$$

IV. Results

4.1 Results of biomass growth in the laboratory scale Algadisk reactor

4.1.1 Isolation of microalgae from natural water basins from Central Europe

One of the main aims of this PhD research was to isolate green microalgal species that are able to growth on surfaces and have potential biotechnological benefits, more specifically high lipid content for potential biofuel production. Due to the isolation method, chances were increased to collect biofilm forming microalgae; moreover higher cell number could be taken from the sites. Based on this method, 58 samples were collected, of which 158 monocultures were separated. However on longer terms, not all of the monocultures could be maintained, approximately 50% was discarded from the collection. The list of the remaining 76 species can be found in Table 3. (Appendix 2) with the indication of place of isolation and used culture medium.

In the second phase of isolation, the sampling points were chosen based on the water characteristics, namely increased temperature of water basins and biofilm formation of microalgae. Thermal springs can be good sources for isolating microalgae species adapted to such environmental conditions. Several places in Germany, Austria and Hungary were visited and microalgal biofilm samples were collected. From 60 samples, 26 could be maintained under laboratory conditions, Table 3.

Among these species according to microscopic observations, several different morphological type could be found, however it was not scope of the project to identify all the strains thus no classifications are used for labeling the samples.

4.1.2 Selection of isolated strains based on growth and lipid content

Biomass production is a major selection criterion for selecting microalgae in the present research. Preliminary experiments were carried out in suspension cultures in order to examine the growth curves (data not shown) of the isolated strains. All the 102 isolated samples were grown for about 14 days, meanwhile the optical density at 550 nm of the cultures were followed. The final OD_{550nm} values show high variation between the samples, ranging from 0.1 to 3.8 (**Fig. 8** Appendix 3). In general, samples growing in A9 medium were performing better than those in SH medium, also it can be observed that isolates from the first isolation phase achieved higher biomass production than those from warm water origin. The average density for those species growing in A9 medium was 1.97, while in SH, the average value was 0.95 OD_{550nm}. Some of the best performing isolates were: $OD_{550nm} > 3$: A9-17a; A9-25b; A9-26; A9-44b;

A9-45; OD $_{550nm}$ ~ 1.5: SH-25a and SH-34. From the second isolation phase mainly A9-66, A9-87 and SH-76 can be highlighted, however their optical density remained around or below OD_{550nm} 1 (**Fig. 9**).

Additionally, attachment to the walls of cultivation flasks during growth was noticed and can be seen in Table 3 (Appendix 2.).

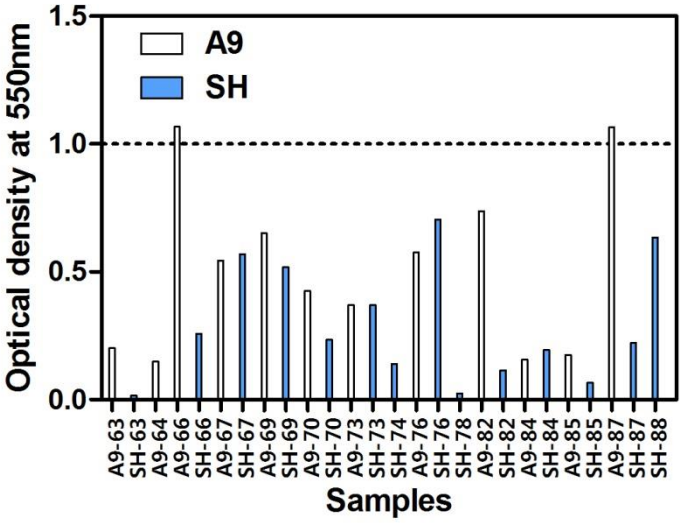

Figure 9- Final optical density (14 days) of isolated samples in the second isolation phase (warm water sources) growing at 30 °C under 150 µmol photons $(m^2s)^{-1}$ light intensity. Blue columns represents samples growing in SH medium, white columns were growing in A9 medium.

Besides the growth characteristics, lipid content was also a major factor to choose an isolate that will be further tested for surface attachment. Due to the high number of samples, first a quick and cost-effective method was applied to pre-screen them. Nile red dying was used to stain lipids in cells and visual analysis of taken microscopic pictures was used to evaluate the results. It has to be noted that the obtained color change cannot determine quantitative values. In Table 3. (Appendix 2) presence or absence of lipid in the cells are marked with (+) and (-). In some cases, the lipid was located in well-distinguishable droplets inside the cell, while in other samples the whole cell emitted yellow light. Some examples of the stained samples before and after using blue light in a fluorescence microscope can be seen on **Fig. 10**.

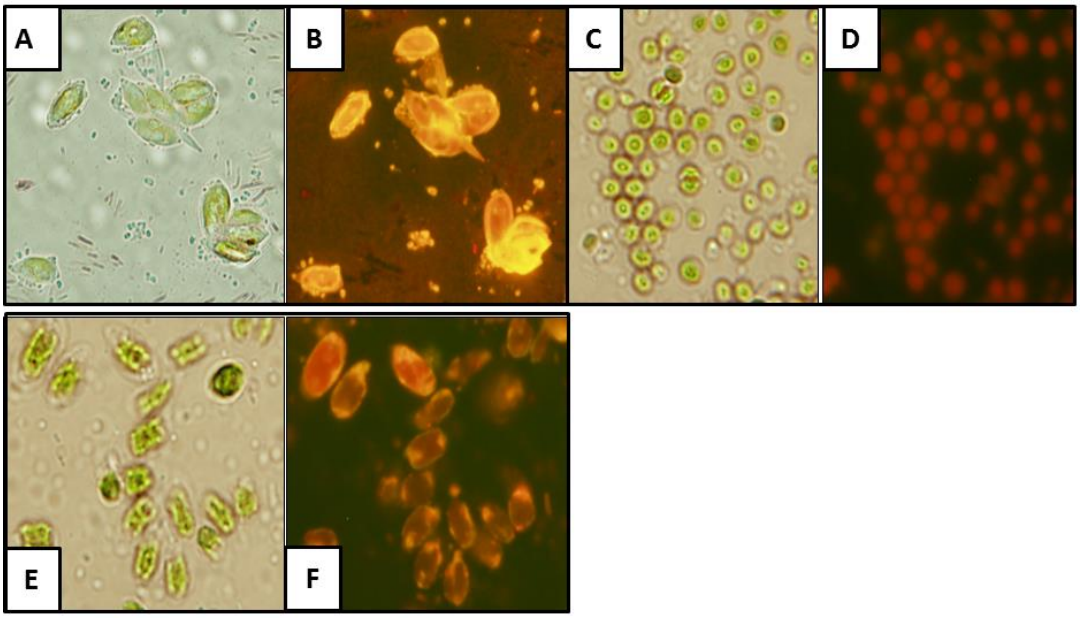

Figure 10- Samples after application of Nile red dye; **A, C** and **E** are pictures from normal light microscopic mode, while **B, D, F** are excited with blue light which causes chlorophyll to emit in red wavelengths and the lipids that were bond to Nile red molecules are emitting yellow light. **A** and **B** are A9-24a; **C** and **D** are A9-16c and **E** and **F** are A9-25c.

Based on the surface attachment ability, the presence of lipid after Nile red staining and growth characteristics, certain samples were chosen for fatty acid analysis by GC-MS. The values of total fatty acid content in dry weight basis are varying greatly from 0.8% to 14.9%, showing not significant differences between the two groups of isolates. Highest value was reached in SH-34, 149 mg g^{-1}, followed by A9-24a with 83 mg g^{-1} ; SH-2 with 75 mg g^{-1} and SH-82 with 65 mg g^{-1}. Hence very slow biomass production was achieved by warm water samples under the applied environmental conditions, they were not considered for continuing examination; SH-82 did not reach 0.15 OD_{550nm} in 12 days of culturing.

For this reason, SH-34 sample was selected to go further with biofilm growth experiments, due to the high fatty acid content, 149 mg g^{-1} and one of the highest cell densities of 1.36 OD_{550nm}.

Based on the molecular identification and alignment search in the available database, the SH-34 sample was identified as a strain belonging to the order *Chlorellales*, however further identification was not possible, as fitting values were 96% compared to *C. sorokiniana* strains and *C. vulgaris* etc. The list of alignment results can be found in the Appendix 4. The isolated strain was named *Chlorella* sp. #34 afterwards.

4.1.3 Results of biofilm formation ability of several surface materials and coatings

During this experiment, four different substrate materials and four different coatings were tested in all combinations. Based on the 7 days growing experiments, the most suitable substrate material and coating type can be selected for each algal species. Evaluation of the surface materials was done separately for three algae species, simulating the concept of the rotating disk reactor, namely that the surface materials periodically submerged to the medium (Table 4).

Surface number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
Substrate	PET	PS	PP	PI	PET	PS	PP	PET	PS	PP	PI	PET	PS	PP	PET	PS	PP
Coating	None	None	None	None	1	1	1	2	2	2	2	**3**	**3**	**3**	**4**	**4**	**4**

Table 4- Surface numbers represents the given substrate materials and coatings combinations. Coating numbers in bold has negative outer charge. For composition of coatings see Section 3.1.5.

After 7 days of incubation, the dry weight of algae attached to the different surfaces was measured (**Fig.11**).

In case of *Chlorella sp*. #34 alga, the most striking difference can be seen between the surface charges. On the negatively charged materials, about double biomass yield was measured as on the positively charged or non-coated surfaces, reaching 2.2 g m^{-2} in 7 days on PS substrate with coating #3. It can be also concluded that between the two negatively charged coatings #3 and #4, there are no significant differences in the total biomass. Among the three used materials, PET produced about 35% less biomass than the PS and PP.

The non-coated substrates and the substrates with coating #1 resulted in similar biomass yield range, with no significant differences between the substrate materials. On the other hand, coating #2 was very unstable; it even resulted in negative values, due to the peeling off of the polyelectrolyte layers, as it can be seen on **Fig 12**.

Such a trend was not observed on the biomass yield results of *C. sorokiniana*. In general, lower biomass production was achieved than in case of *Chlorella sp*. #34. Highest value, 1.2 g m^{-2} was measured on PET with coating #1, on a positively charged surface. Similarly to the *Chlorella sp*. #34, PET also performed worse than the other materials. Coating #2 was just as unstable as in case of *Chlorella sp*. #34 (**Fig. 11.**).

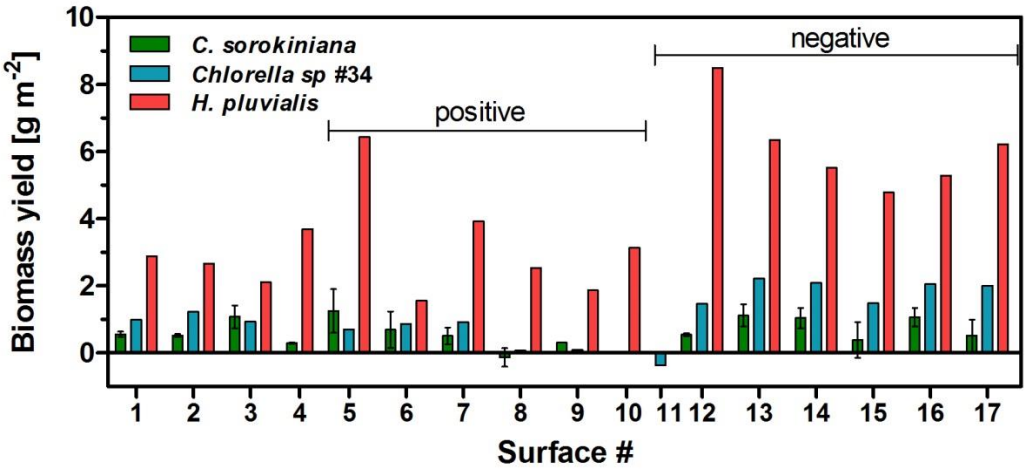

Figure 11- Biomass yield on different surface materials and polyelectrolyte coatings with positive or negative charges of *C. sorokiniana*, *Chlorella sp* #34 and *H. pluvialis*. Cells were incubated in a rotating glass bottle for 7 days, at 25° C and at 35-45 µmol photons $(m^2s)^{-1}$ light intensities.

Among the three tested algae species, *H. pluvialis* showed the highest biomass yield, 8.5 g m^{-2} on PET with coating #3. Overall, the biomass production was about 3.5 times and 7 times higher compared to *Chlorella sp*. #34 and *C. sorokiniana* species, respectively. Negatively charged surfaces had an enhancing effect on biomass yield, however significant differences between the substrates are not present, except for PET with coating #3. On the positively charged surfaces, *H. pluvialis* performed outstanding on PET with coating #1, biomass yield reached 6.4 g m^{-2}. The uncoated substrates resulted in similar values, from 2.1 to 3.9 g m^{-2}. Unlike with the other two alga species, coating #2 did not show the same phenomenon of peeling, hence biomass yield was comparable to the other surface samples, ranging between 1.8 and 3.1 g m^{-2}.

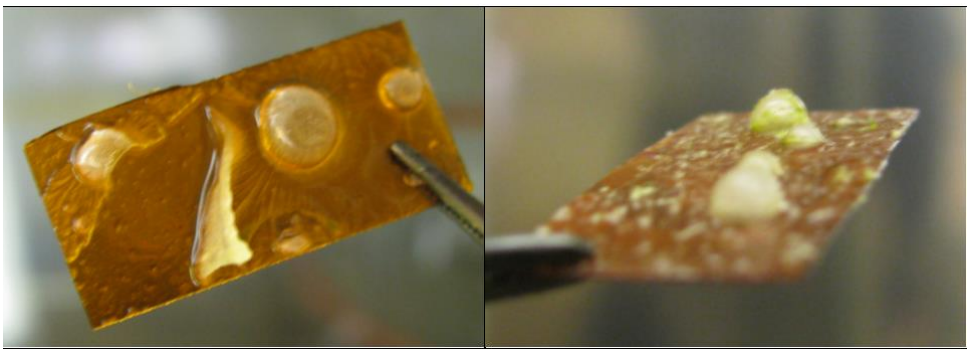

Figure 12- Bubble formation of coating #2 on surface PI (#11) during biofilm formation test with *C. sorokiniana*

Based on these results, the most ideal substrate material and coating combination could be selected. In case of *C. sorokiniana*, highest biomass yield was reached on PET with coating #1, however PP with coating #3 and #4 are performed very similarly. For *Chlorella sp.* #34 alga, negatively charged coating is essential for high biomass production, of which PP with coating #3 and #4 would be a suitable choice. Considering the best material for *H. pluvialis*, PET performed significantly better compared to the other substrates both positively and negatively charged, nonetheless, PET with coating #3 had the highest biomass yield of 8.4 g m^{-2}.

4.1.4 Long term operation of laboratory scale Algadisk reactor under low and high light intensities

This experiment aimed to perform long term operation of the reactor under optimal growth conditions with the preselected *Chlorella* sp. #34. The experiment was running continuously for 98 days, and 7 growth-harvest cycles occurred in different time periods. From the biomass production results (**Fig. 13**), it can be seen that the primary biofilm formation (Harvest #1) required longer growth period, 18 days, due to the slow attachment of cells to disk surfaces. In case of Harvest #2, before the harvesting occurred, a part of the biomass detached from the surface, causing low biomass productivity value. In order to identify whether the length of the growth period (8 days) resulted in detachment of the biomass and to examine the stability of biofilm over a longer growth period (40 days), Harvest #3 took place after 8 days, while Harvest #4 happened after 40 days of growing period. Detachment was not observed in any of these aforementioned cases; however biomass productivity dropped significantly by Harvest #4. For this reason in the following growth-harvest cycles, biomass is scraped off in every 7-8 days. By this regular harvesting, the biomass production shows an increasing tendency between 2.28 g $(m^2d)^{-1}$ and 3.23 g $(m^2d)^{-1}$. The average dry biomass yield is about 17 g m^{-2} during the regular harvests (Harvest #2, 3, 5-7); however it reaches up to 55 g m^{-2} after 40 days.

As it is shown in the diagram (**Fig. 13B**), the biomass density is not necessarily in a strong correlation with the biomass production, more likely circumstances of biofilm formation influenced it. During the cultivation period, the density was varying greatly, however in the last 3 harvests, when the system was the most stable; the density also stabilized around 200 g DW kg^{-1} WW. The extremely outstanding density obtained in Harvest #4 was due to the very long growing period, about 40 days.

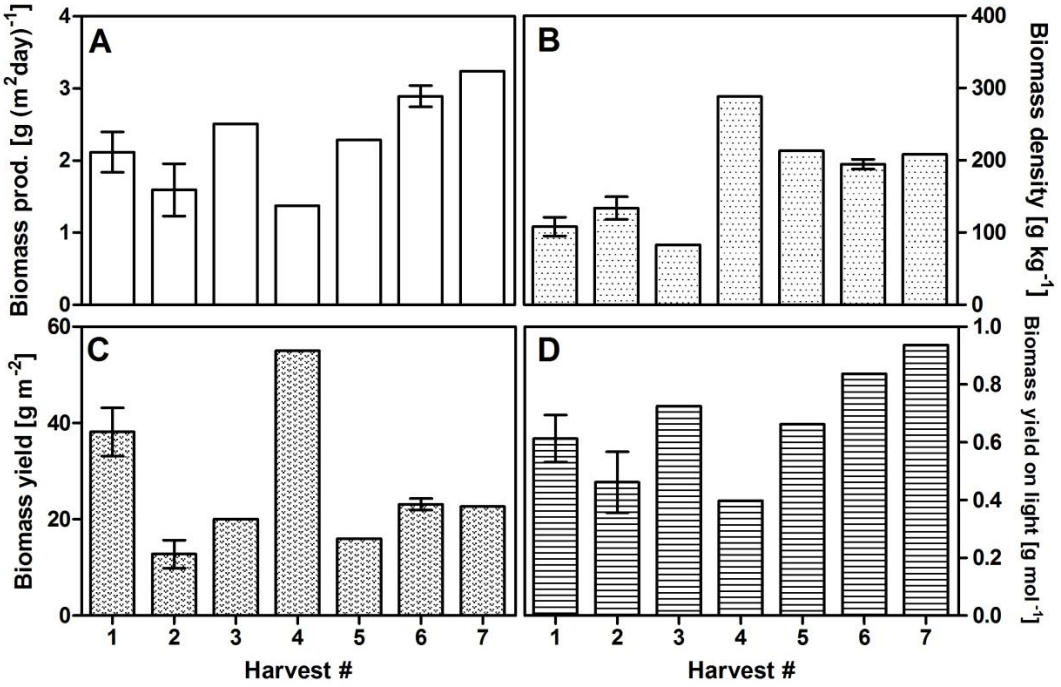

Figure 13 - Results of laboratory scale experiments under low light intensity- (**A**) Biomass productivity on disks surface bases, presented in *g DW* $(m^2_{surface\ area}\ d)^{-1}$; (**B**) dry mass content of harvested biofilm in *g* kg^{-1}; (**C**) biomass yield on surface area base in g DW m^{-2}, and (**D**) biomass yield on light in *g DW* mol^{-1} light for each harvesting under low light (40 µmol $(m^2s)^{-1}$). Growth periods were as following: 18, 8, 8, 40, 7, 8 and 7 days. In case of indicated samples, standard deviations were calculated based on harvested biomass from at least 3 disks.

The second experiment presented here was performed with the light panel containing 8 LED lamps, providing about 190 µmol photons $(m^2s)^{-1}$ light irradiance on the disk surface with the same algal species, *Chlorella* sp #34. The set up was running continuously over 43 days and 4 growth-harvest cycles occurred. Between the two experiments, the reactor was cleaned and freshly inoculated. Similarly to the previous experiment, it can be also observed that the primary biofilm formation takes longer, and then the biomass productivity increases (**Fig. 14**) from harvest to harvest, however it remained lower compared to the previous experiment, 1-1.5 g DW $(m^2day)^{-1}$.

During the 4th growth-harvest cycle, the axis stopped rotating and some part of the biofilm dried out, and very likely the other part under the liquid level, also damaged. The experiment has stopped after this failure.

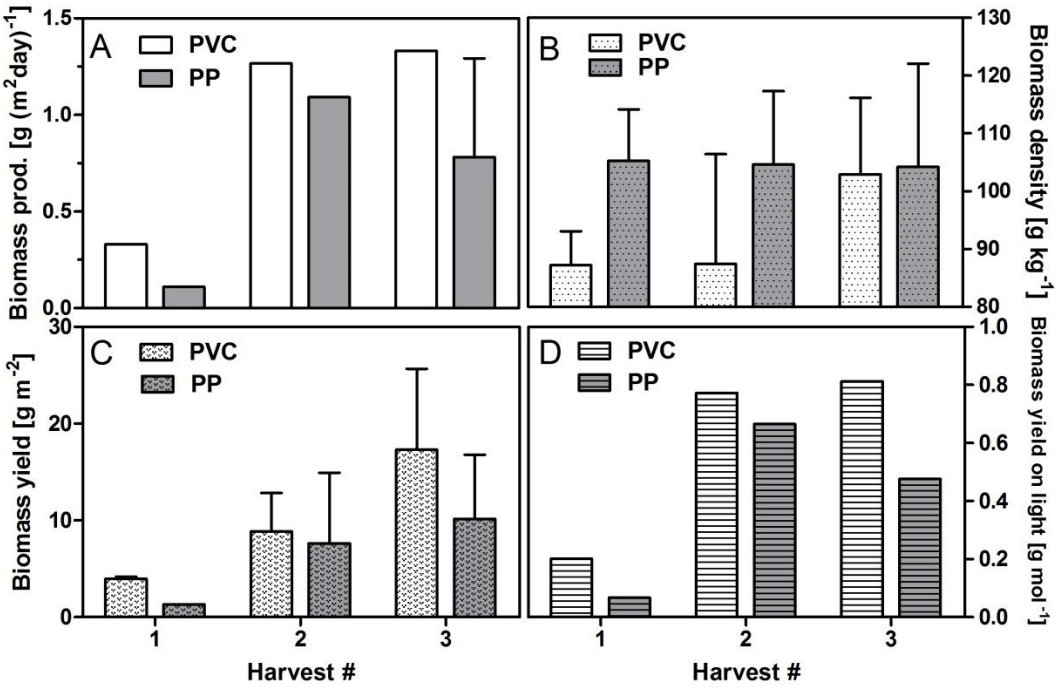

Figure 14 - Results of laboratory scale experiments under high light intensity-(**A**) Biomass productivity on disks surface bases, presented in *g DW* $(m^2\ d)^{-1}$; (**B**) dry mass content of harvested biofilm in *g* kg^{-1}; (C) biomass yield on surface area base in *g DW* m^{-2} and (**D**) biomass yield on light in *g DW* mol^{-1} light for each harvesting under high light intensities (190 µmol $(m^2s)^{-1}$). Growth periods were as following: 12, 7, and 13 days. In case of indicated samples, standard deviations were calculated based on harvested biomass from at least 3 disks.

Under the high light intensity and same growth conditions as described before, besides sandpapered PVC disks, also PP disks with coating #3 were tested. This substrate material and coating performed as one of the best in the surface test experiments with *Chlorella sp* #34, with 2.1 g m^{-2} biomass in 7 days. In the laboratory-scale Algadisk system, biomass production remained under 1.1 g $(m^2day)^{-1}$ and after Harvest #2, it started to decline. On the other hand, biomass density was stable, giving biomass content slightly above 100 g kg^{-1}. Biomass yield on light was very low, in none of the harvests reached 0.1 g mol^{-1}.

4.1.5 Biomass production on a commercially available fertilizer in the lab scale Algadisk reactor

In the laboratory, well defined and optimally composed culture media are used for cultivation of organisms, including microalgae. In our case this medium was M8-a enhanced with urea as nitrogen source. Since these media are developed for providing optimal growth conditions, often by excess nutrient content, using salts from high graded sources, their large scale application may contribute to a costly alga biomass production. Hence, other sources of

macro- and micronutrients should be considered as well with lower commercial price. Artificial fertilizers are widely used in agriculture to enhance crop production and can be a suitable growth medium for fresh water microalgae. In this study Agroleaf Power High P (for composition see Section 5.1.3.1, **Table 8**) was used to test biomass production and lipid accumulation of *Chlorella* sp #34 in the Algadisk system. This fertilizer was selected based on preliminary tests (data not shown), where different mineral compositions were compared in suspension cultures.

The effect of different media can be observed on the biomass productivity that remained between 1- 1.7 g DW $(m^2day)^{-1}$ and also on the biomass density and biomass yield on light values. The highest biomass density was about 130 g kg^{-1}; while the average was 115 g kg^{-1}, excluding the result of the first harvesting. On the other hand, biomass yield on light was varying around 0.4 g mol^{-1} in Harvest #2-5 (**Fig. 15**).

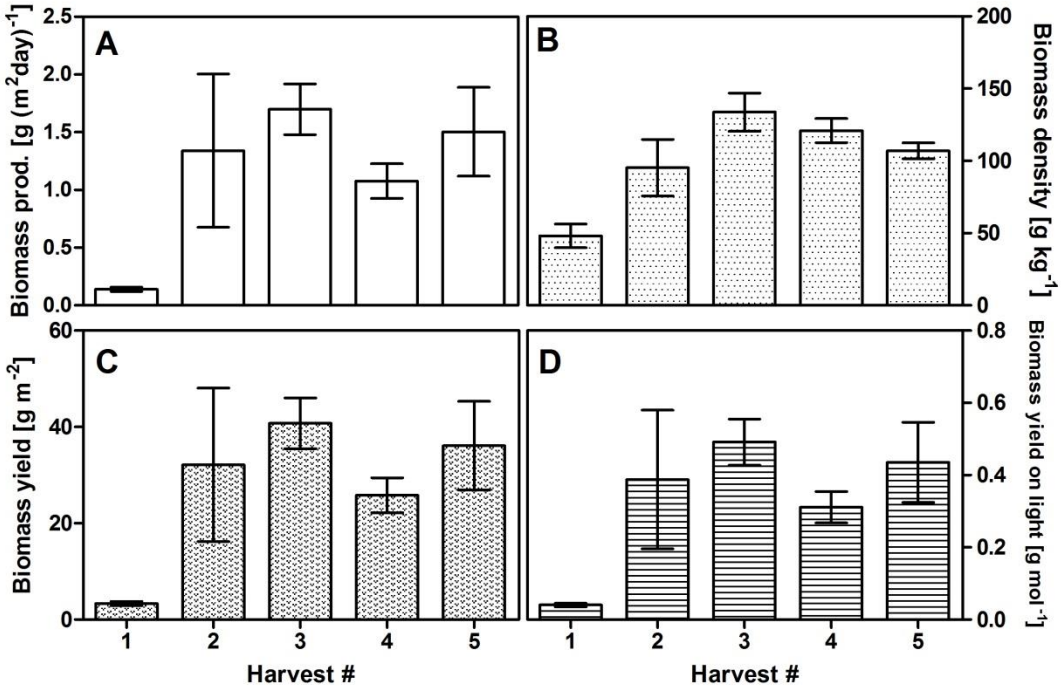

Figure 15- Results of laboratory scale experiments under low light intensity with artificial fertilizer as medium- (**A**) Biomass productivity on disks surface bases, presented in *g DW$(m^2 d)^{-1}$*; (**B**) dry mass content of harvested biofilm in *g kg^{-1}*; (C) biomass yield on surface area base in *g DW m^{-2}*, and (**D**) biomass yield on light in *g DW mol^{-1}* light for each harvesting under low light intensity (40µmol $(m^2s)^{-1}$). Growth periods were as following: 24, 12, 15, 15 and 25 days. In case of indicated samples, standard deviations were calculated based on harvested biomass from at least 3 disks.

4.1.6 Lipid content and productivity under nutrient replete and deplete conditions of biomass growing in lab scale Algadisk reactor

Microalgae can be a source of several compounds that have a potential for biotechnological usage. One of the main research topics of microalgae biotechnology is the biofuel utilization of lipid containing cells.

Besides determining the biomass producer efficiency of the laboratory scale Algadisk reactor with *Chlorella* sp #34, described in the earlier Sections 4.1.4 and 4.1.5, total lipid concentration of biomass and total lipid productivity was analyzed as well in all experiments with low (Harvests #5, 6, 7) and high light intensity with both disk material, PVC and PP and also by using fertilizer as a medium after each harvesting point.

Moreover a new run was designed and commenced. The aim of this set up was to study the effects of nitrogen (N) limitation on biomass and lipid production. In order to have a high amount of biomass on the disks, N limited phase started when a thick biofilm developed (visual determination) on nutrient replete medium, then the reactor was refilled with fresh medium free of N. Stress condition was applied for about 20 days, then biofilm was harvested and biomass productivity was determined. In order to follow the effects of absence of N on total lipid content, samples from medium were taken at three time points of incubation. Since the Algadisk system is designed to operate continuously, the medium was changed again back to full medium after harvesting. In the previous experiment at optimal conditions, biofilm regrowth was faster after harvesting the primary biofilm. It was expected during this set-up as well; however biofilm did not developed even after longer period. To understand it better and be able to exclude some parameters, the same experiment was repeated again, after cleaning the whole system. The same phenomenon was observed as previously: biofilm did not form again after harvesting the stressed biomass.

Total fatty acid contents of the biomass grew on full media are low, around 4 (m/m) % and 5.5 (m/m) % at low and high light conditions, respectively. However considering the biomass production rates; total fatty acid productivity reached 100 mg lipid $(m^2day)^{-1}$ under low light intensity, while lipid productivity remained between 60-80 mg $(m^2day)^{-1}$ at high light intensity in both disk material. However, significant differences cannot be seen between the PVC and PP disk material neither considering lipid content, nor total lipid productivity (**Fig. 16** and **18**). The biomass growing on the fertilizer had the highest fatty acid content of 6.5% in Harvest #4. In average, it also exceeds the values of the other conditions by reaching 5.2%, while 3.9%, 4.3%, and 4.4 % for low light, high light with PVC and high light with PP, respectively. On the other hand, biomass production was limited, thus lipid productivity remained well below the productivities obtained at low light intensity, but increased compared to high light conditions (**Fig. 16**).

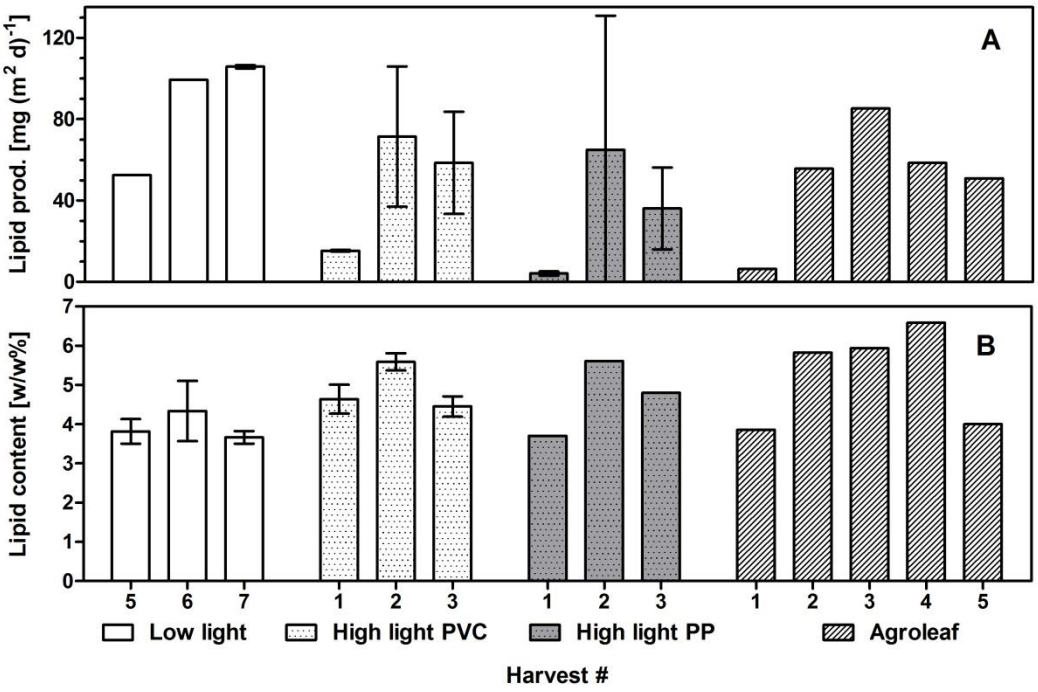

Figure 16- Lipid productivity (**A**) and lipid content (**B**) of dry biomass from each harvesting from experiment with low and high light, and full M8-a medium or Agroleaf fertilizer. Lipid content is presented as percentage of total fatty acid weight in dry biomass and calculated total fatty acid productivities shown as mg $(m^{-2}_{surface\ area}\ day)^{-1}$. Standard deviations were calculated from biomasses of at least three disks.

In contrast with the experiments on full media and on fertilizer, during the first run of the lipid induction experiment, on day 34, normal medium was replaced with N free medium and recirculated as during the previous experiments. Samples were taken for total N (TN) analysis before and after stressing. It can be clearly seen on **Fig. 17**, that nitrogen is not totally absent from the system, nonetheless comparing to the initial concentration of 460 mg l^{-1} N, it is negligible. Nitrogen starvation had an impact on lipid content in cells, the fatty acid content increased more than 2 times compared to low light values, reaching 9.5% of DW. However the accumulation process is quite slow. Moreover, biomass productivity was 0.22 g DW $(m^2 day)^{-1}$ that is about 10 times less than compared to Harvest #1 at low light conditions. Consequently, the calculated lipid productivity was only 21 mg $(m^2 day)^{-1}$.

In case of the repeated run, stress induction happened on day 30, when the full medium was replaced with N free medium. Based on the TN measurements, the N content remained still very high; almost half of the initial concentration, 170 mg N l^{-1}. By the end of the experiment (48 days) the N concentration was reduced to 20 mg l^{-1} and lipid content slightly increased from 2.3% to 2.8%, however this result is significantly lower than in case of the biofilm grew on full medium. This phenomenon could be caused by remaining residues of N containing salts in the biofilm or in the tank that dissolved back after refilling the system or N could also originate

from the dead algal cells that remain in the tank. Biomass productivity was slightly higher than in the previous run; 0.38 g $(m^2day)^{-1}$, although lipid productivity remained very poor, 10 mg FA $(m^2day)^{-1}$.

In each run, only one harvesting could take place, since biofilm did not form again, after changing back to full medium. As it was observed before by the low and high light experiments, primary biofilm formation takes longer than the regrowth in the following cycles, the actual fatty acid capacity of such a two-stage process cannot be evaluated in this case.

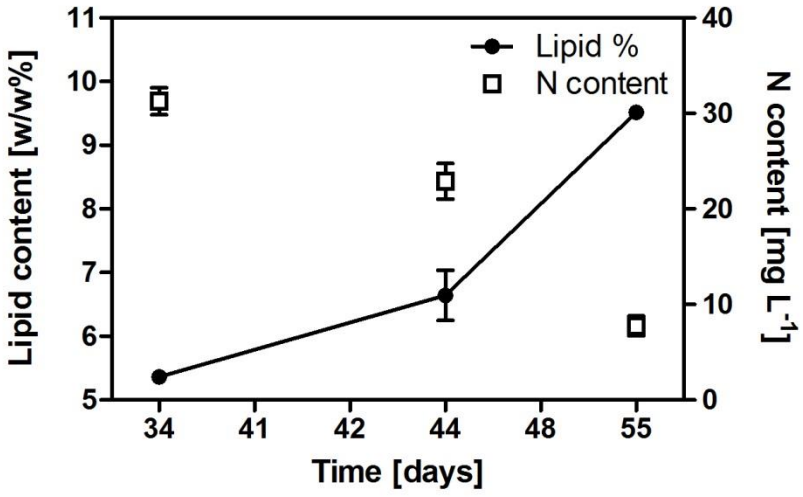

Figure 17- Changes in fatty acid content of biomass (●) and N concentration in liquid (□) of the two phase, stress experiment (first run). On the diagram the *x* axis shows the time of harvest in days passed from inoculation. Left *y* axis shows the scale of total lipid content of biomass in (m/m) %. Right *y* axis shows the measured total nitrogen content of medium. Error bars indicate the standard deviation.

Additionally to the total lipid content, the fatty acid composition of biomass from different experimental setups was also examined. In total, concentration of 36 different fatty acids could be determined and compared. The fatty acids were ranging from short chain length to long chain length molecules, C6 to C24, including several saturated, mono- and polyunsaturated ones. The detailed list can be seen in Table 5 (Appendix 5) with the average mg values of fatty acid content of dry biomass in each growth-harvesting cycle.

Values of saturated, monounsaturated and polyunsaturated fatty acids are grouped together and presented as percentages normalized to 100% for a better comparison. **Fig. 18** shows that the proportion of the different groups is slightly changing between the harvests, and also between the experiments. The saturated fatty acids (SFA) are in the range of 25-35% in all cases; modest increase can be observed under high light, both PVC and PP, in Harvest #4, where the ratio increased from 25% to 30-35% compared to the previous harvest cycles. Along with this, the percentage of PUFA decreased from 40-50% to 35%, which is also represented under low light conditions and with artificial fertilizer. The major variation occurred in the

content of monounsaturated fatty acids (MUFAs), 20%, 30%, 35% and 20% for low light, high light PVC, high light PP and Agroleaf fertilizer, respectively, however within one experimental setup, its ratio remained similar.

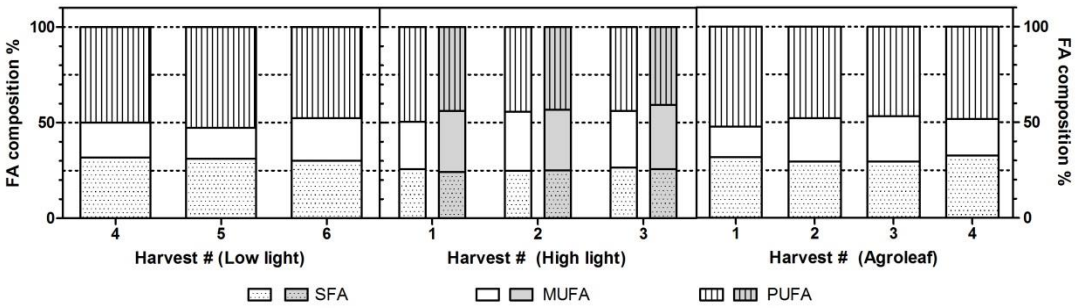

Figure 18 – Proportion of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids in the biomass growing under different conditions. The values are normalized to total lipid content. At High light conditions, results of PVC (white column) and PP (grey column) disk material are shown as well.

Besides the proportional distribution of fatty acid groups, the dominant and biotechnologically interesting molecules were further analyzed and compared.

In all experimental conditions, the dominant fatty acids were myristic acid, myristoleic acid, pentadecenoic acid, palmitic acid, palmitoleatic acid, heptadecenoic acid, stearic acid, elaidic acid, oleic acid, linoleic acid and α-linolenic acid. These fatty acids are medium length chains (C14-C18), with variable saturation level. Among these, palmitic acid, heptadecenoic acid, oleic acid, linoleic acid and α-linoleic acid are the major components, displaying minor differences between low light and high light conditions, as it is presented in **Fig. 19**. The values of biomass grew on PVC and PP disks (under high light) can be considered identical, while at low light, the medium (artificial fertilizer) had a slightly stronger effect on the FA content, especially considering the concentration of the fatty acids grouped in the "Other" category. Growing on artificial fertilizer, these molecules are expressed in several fold compared to M8-a medium; increase from 1% to 3%.

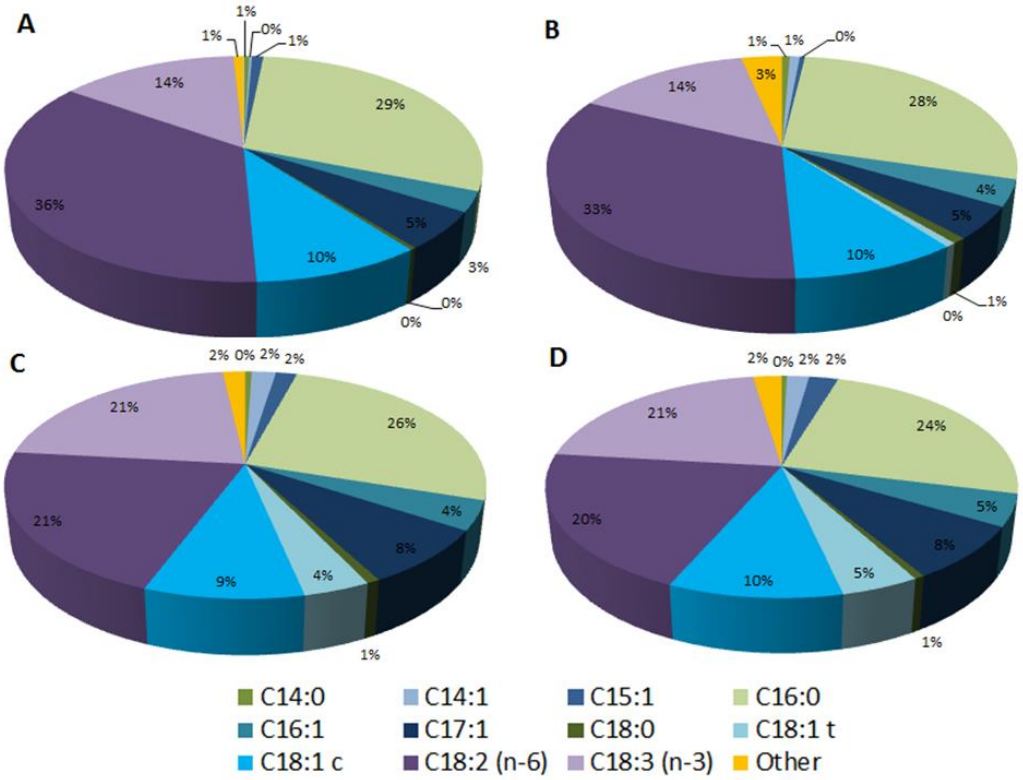

Figure 19- Major FA composition given as % of the total lipid content of biofilms harvested under several cultivation conditions; (**A**) low light intensity; (**B**) artificial fertilizer; (**C**) high light intensity PVC disks; (**D**) high light intensity PP disks.

On **Fig. 20**, the biotechnologically important PUFAs such as EPA, DHA and γ-linoleic acid (GLA) are presented in more details of the biofilm from the high light conditions and using fertilizer. Big variations can be observed among the algal biomasses, EPA is present in all cases, ranging from 0.07% to 0.15% of the total FA, reaching highest content in biofilm grew on PP disks under high light. DHA was only detected in biomasses grew on Agroleaf and high light growing on PVC disks, reaching 0.84% in the earlier case. The amount of γ-linoleic acid was also greatly varying, 0.04% by the PVC biofilm, 0.32 % by the Agroleaf experiment and the highest, 0.9% was by the PP biomass.

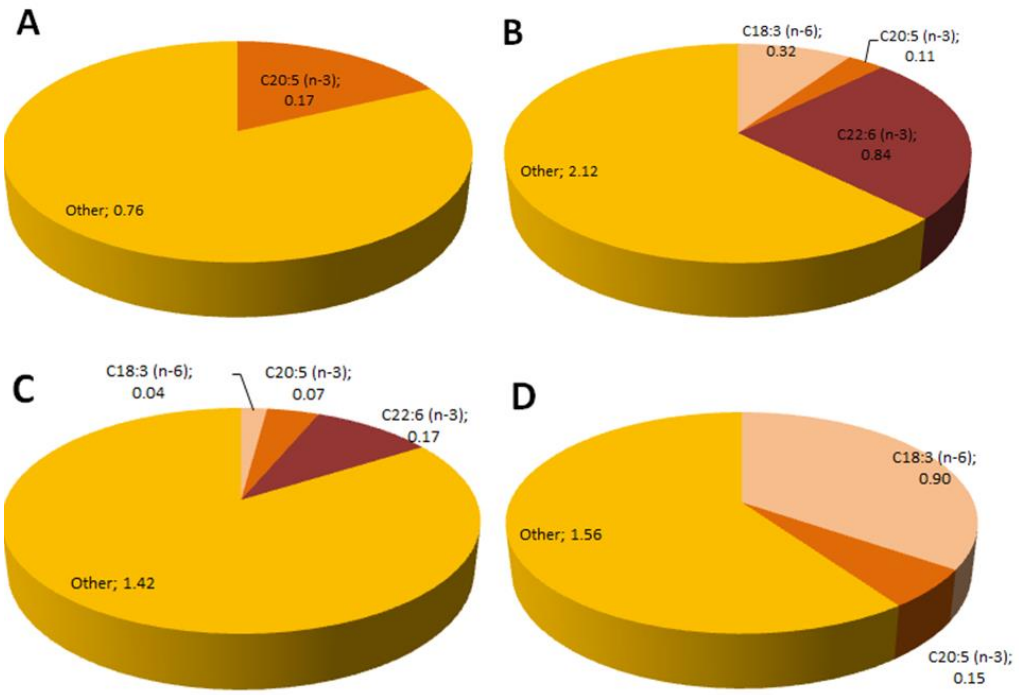

Figure 20- Biotechnologically important PUFA (GLA C18:3 (n-6), EPA C20:5 (n-3), DHA C22:6 (n-3)) composition given as % of the total FA content of biofilms harvested under several cultivation conditions; (**A**) low light intensity; (**B**) artificial fertilizer; (**C**) high light intensity PVC disks; (**D**) high light intensity PP disks.

4.1.7 Cultivation of *H. pluvialis* in the laboratory scale Algadisk reactor

H. pluvialis gains great interest in microalgae biotechnology for its high astaxanthin producing capacity. Cultivation of this species is mostly done in suspension system, applying a two-step process for high biomass production and then high astaxanthin content. Our attempts were to grow *H. pluvialis* in a biofilm system, where the medium and the biomass is separated, thus introducing stress factors of the medium, such as nutrient limitation, can be cost effectively achieved.

The laboratory scale Algadisk reactor was initially set up with four sand-papered disk materials: (1) polypropylene (PP); (2) polycarbonate (PC); (3) polyvinylchloride (PVC); (4) polyethylene (PE), in order to test which would be the most ideal substrate for this microalgae. These materials were chosen instead of the previously tested ones, due to their higher stability, rigidity and better availability on the market, which are major concerns when constructing the pilot Algadisk reactor.

Based on our observations, in the tank, the algae produced high biomass by the 7th day, while in the buffer tank there was no significant cell growth. Despite the fast initial suspended growth, biofilm was not formed on disks in the Algadisk system by the 14th day after

inoculation; few colonies were only attached to the PP material, 3 days after inoculation. Compared to the experiments done with *H. pluvialis* for surface attachment (see Section 3.1.5), numerous parameters are different in the current research, such as pH control, addition of CO_2, surface materials, presence of bacteria, volume of liquid and settlement of cells. Based on our experiences, the major problem could have been the settlement of *H. pluvialis* cells in the reactor tank. For this reason, some modifications were made to the system to enhance biofilm growth, e.g. mixing up the algae from the bottom of the tank and using air bubbling to keep the cells suspended in the liquid and lowering the speed of disk rotation. Besides the manual re-suspension of cells and continuous air bubbling, the cells settled down, which made the liquid in the tank almost totally clear, except the lowest 1-1.5 cm layer of it. Two days after slowing the rotation of disks, slightly more algae appeared on PP disk (**Fig. 21**.), but further biofilm formation was not observed in the following days and the experiment was stopped. Taking a step back, growth conditions were modified to be similar to the smaller scale surface material test, surface substrates used in the surface material test were applied. Besides these modifications, the *H. pluvialis* cells did not attach to the surfaces and they were mostly settled in the reactor tank.

Figure 21 - *H. pluvialis* primary biofilm formation on PP disk 2 days after slowing down the rotation speed.

4.2 Results of growth experiments in the bench scale Twin Layer system

As it was described in the previous section 4.1.7, the Algadisk concept was not successful for the biofilm cultivation of *H. pluvialis*, besides the increased mixing and several disk materials tested, none or limited amount of biomass were attached to the surfaces and further

biomass growth was inhibited. Thus a different approach for biofilm cultivation of *H. pluvialis* was tested to examine the possibilities of cultivation this microalgae in a biofilm system and to investigate its behavior under various light intensities and stress conditions (see Section 3.2).

4.2.1 Results of light intensity screening with *H. pluvialis* on the Twin Layer system

Based on literature data, the optimal light irradiance for vegetative growth of *H. pluvialis* is relatively low, around 100 µmol photons $(m^2s)^{-1}$ [81]. Increased light intensities trigger the shift from vegetative cells towards resting cells (akinetes) that known to accumulate the valuable, red carotenoid, astaxanthin, meanwhile, cell division is ceased. This leads to strong limitation of large scale industrial production of *H. pluvialis* in outdoor systems [82,83].

Twin Layer system was selected as another promising concept for efficient microalgal biomass production for biotechnological use. The experiment aimed to study whether this approach can support biomass growth at increased light intensity compared to literature values of suspended cultures. A wide range of irradiance was used from limiting (26, 44 µmol photons $(m^2s)^{-1}$) through optimal (85, 119 µmol photons $(m^2s)^{-1}$) to stressing (135, 219 µmol photons $(m^2s)^{-1}$), considering literature data [84–86].

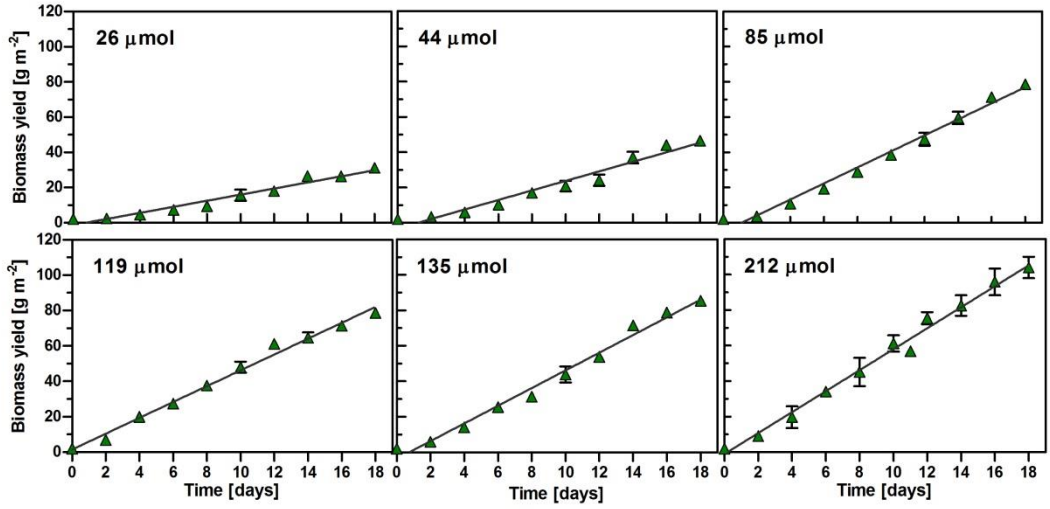

Figure 22 - Biomass yield of *H. pluvialis* under different light conditions in Twin Layer system growing on full BBM medium. Solid line represents the linear regression of data, the slope of these equals to biomass production rate. The goodness of fitting is shown as R^2 value. Error bars represent the standard deviation of at least three replicate filters.

As it can be seen on **Fig. 22**, biomass yield was strongly dependent on the applied light intensity, increasing from 31 g m^{-2} at 26 µmol photons $(m^2s)^{-1}$ to 109 g m^{-2} at 219 µmol photons $(m^2s)^{-1}$. The biomass growth was linear during the entire time of the experiment, 18 days, despite the light conditions. Due to this, biomass productivity was calculated based on linear

regression ($R^2 > 0.93$); reaching 5.8 g $(m^2 d)^{-1}$ at 219 µmol photons $(m^2 s)^{-1}$, while lowest productivity, 1.72 g $(m^2 d)^{-1}$ was gained at the lowest light intensity.

In order to compare the efficiency of the system, as it was also introduced in case of the lab scale Algadisk reactor, biomass yield on light was calculated, as well. Light use efficiency was proportional to light intensity; highest value was obtained at 26 µmol photons $(m^2 s)^{-1}$ being 1.3 g mol^{-1} and decreasing to 0.5 g mol^{-1} by 219 µmol photons $(m^2 s)^{-1}$ (**Fig. 23**).

Since biomass growth was not restricted by increased light intensity (219 µmol photons $(m^2 s)^{-1}$), further experiments were carried out at this condition.

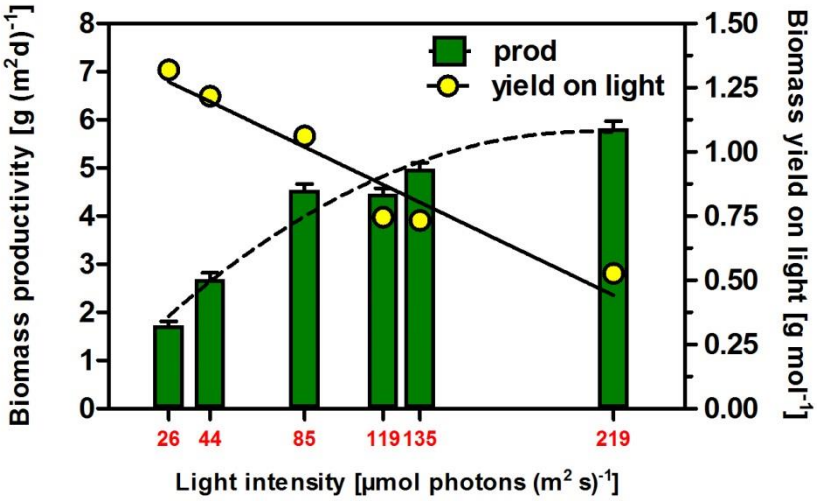

Figure 23 - Biomass productivity and biomass yield on light of *H. pluvialis* under different light condition using full BBM medium. Biomass productivity (left *y* axis, green bars) was calculated by linear regression ($R^2 > 0.93$), and biomass yield on light (right *y* axis, yellow dots) was based on the biomass productivity and the amount of light reaching the surface daily. The values are plotted against the applied light intensities (*x* axis). The error bars are standard deviation gained by the linear regression calculation.

4.2.2 Results of stress induced astaxanthin accumulation of *H. pluvialis*

As it was mentioned before, astaxanthin production is mostly observed when cell division discontinues as a result of a single or several stress factors. The main factors that were extensively studied in suspension cultures are high light intensity, nitrogen limitation and increased NaCl concentration in the medium. As high light was proved not to effect negatively the biomass production in the previous section, in the following experiment, N limitation and different NaCl concentration were applied to induce astaxanthin production and accumulation in cells.

Compared to the control with full BBM medium, biomass yield was lower under stress conditions, except for the tube with 0.05% NaCl. Most prominent decrease was caused by the N deplete medium, reducing the final biomass content with about 10%, to 91 g m^{-2}. Nonetheless, the final values are not significantly different from the control values (**Fig. 24**).

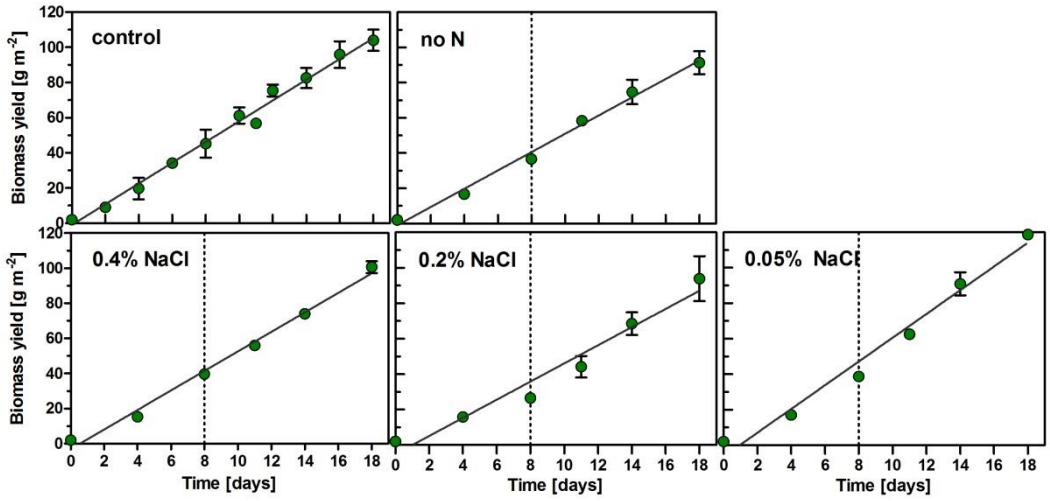

Figure 24 - Biomass yield of *H. pluvialis* growing at 210 µmol photons $(m^2s)^{-1}$ in two phase system, using full BBM medium between day 0 and day 8 (dotted line) for optimal growth, then changed to stress media for astaxanthin production, namely N free BBM medium and full BBM supplemented with 0.4% NaCl; 0.2% NaCl and 0.05% NaCl. Solid line shows the linear regression of data, the slope of these equals to biomass production rate. Error bars represent the standard deviation of at least three replicates.

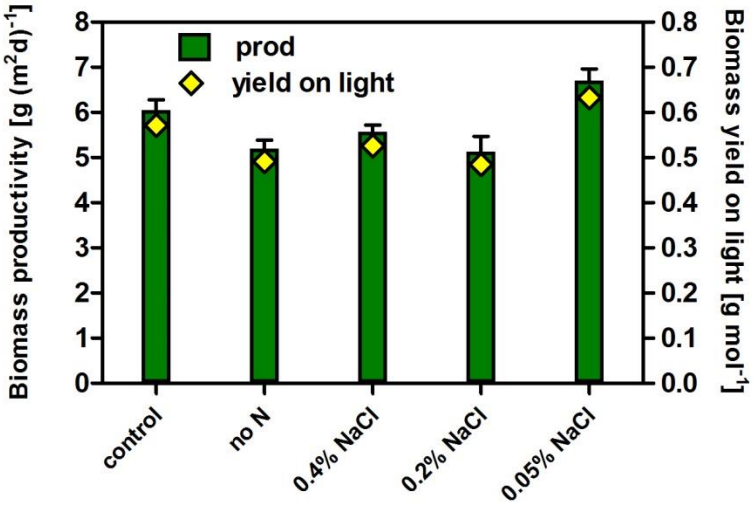

Figure 25 - Biomass productivity and biomass yield on light of *H. pluvialis* under different stress conditions (N free BBM; full BBM with 0.4% NaCl; 0.2% NaCl and 0.05% NaCl, *x* axis). Biomass productivity (left *y* axis, green bars) was calculated by linear regression ($R^2 > 0.93$), and biomass yield on light (right *y* axis, yellow diamond) was based on the biomass productivity and the amount of light reaching the surface daily. The biofilm was cultured on full BBM until day 8; then media were replaced to stress conditions, except for the control where system was running on full BBM during the whole period. 210 µmol photons $(m^2s)^{-1}$ light intensity was used in all cases under a 14:10 hour of light: dark cycle.

Biomass production was also calculated by linear regression, like in the previous experiment, with R^2 values over 0.93. We could conclude that introducing the stress factors to

the system on day 8, the biofilm production did not decrease significantly. Production values are ranging from 5.13 to 6.7 g $(m^2\ d)^{-1}$. Similarly, biomass yield on light values are also in the same range compared to the control, 0.48 to 0.63 g mol^{-1} (**Fig. 25**).

Regarding the astaxanthin content, stress factors did exhibit significant differences, shortly after the stress induction time (day 8) (**Fig. 26**). Astaxanthin content in each tube was the same on day 8, before the stress commenced. The tested NaCl concentrations did not increase the astaxanthin content after 10 days of incubation, varied between 0.5% and 1%, just as in the control. Meanwhile, the effect of N free medium can already be observed 3 days after induction, when the astaxanthin concentration more than doubled from 0.7% to 1.7. On day 18, it reached a plateau phase at the maximum astaxanthin content of 3.5%.

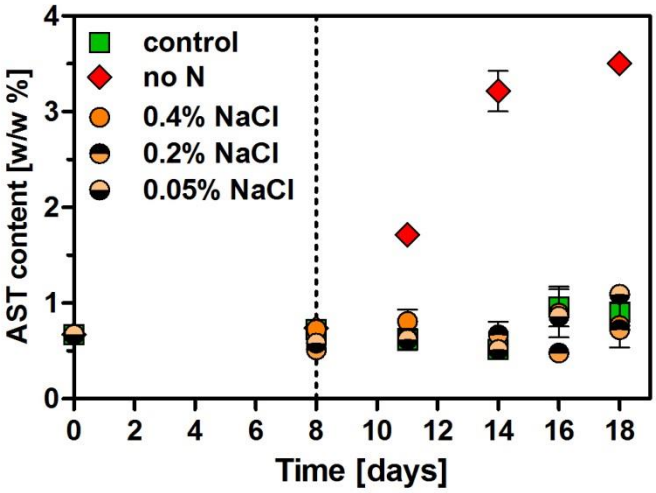

Figure 26 - Astaxanthin content of *H. pluvialis* dry biomass under different stress conditions (N free BBM; full BBM with 0.4% NaCl; 0.2% NaCl and 0.05% NaCl). The biofilm was cultured on full BBM until day 8 (dotted line), then media were replaced to stress conditions, except for the control in which cells were growing on full BBM during the whole period. In all cases 210 µmol photons $(m^2 s)^{-1}$ light intensity was used under a 14:10 hour of light: dark period.

The same trend is followed by the astaxanthin yield (**Fig. 27**). Final total astaxanthin was between 0.67 and 1 g m^{-2}, while under N depletion it reached 3.2 g m^{-2}. Astaxanthin production rates were calculated using linear regression from day 8-18, except for the control where the whole period was considered. The highest, 300 mg $(m^2\ d)^{-1}$ astaxanthin yield was reached under N limited conditions, which presented about a 3-fold increase compared to the control and other stress conditions . Among the stress induced samples, only those treated with 0.05% NaCl had slightly increased astaxanthin productivity, 96 mg $(m^2\ d)^{-1}$, the rest varied around 50 mg $(m^2\ d)^{-1}$ (**Fig. 28**).

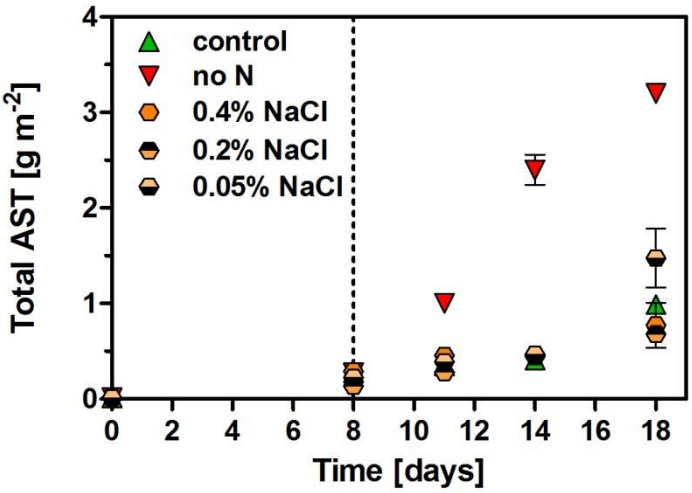

Figure 27 - Total astaxanthin yield in the *H. pluvialis* biomass under different stress conditions (N free BBM; full BBM with 0.4% NaCl; 0.2% NaCl and 0.05% NaCl). The biofilm was cultured on full BBM until day 8 (dotted line), then media were replaced to stressing media, except for the control where system was running on full BBM during the whole cultivation period. 210 µmol photons $(m^2s)^{-1}$ light intensity was used in all cases under a 14:10 hour of light: dark cycle.

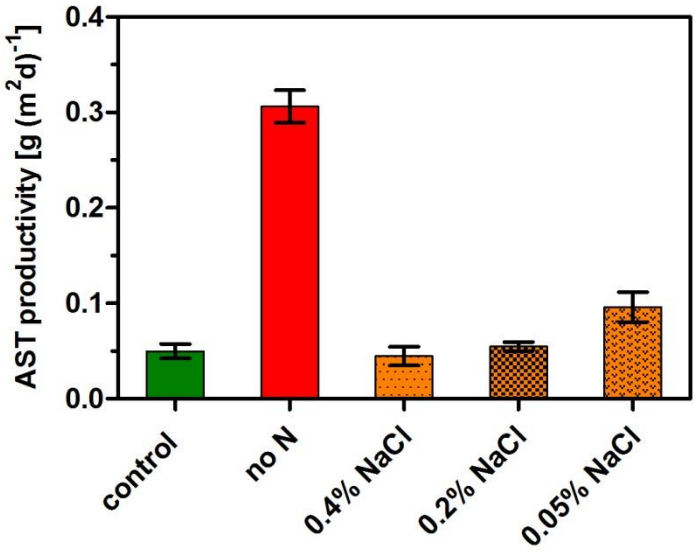

Figure 28- Astaxanthin productivity of *H. pluvialis* in Twin Layer system under different stress conditions (N free BBM; full BBM with 0.4% NaCl; 0.2% NaCl and 0.05% NaCl, *x* axis). Productivity was calculated using linear regression for the stress phase of the experiment (except for the control) which started on day 8. 210 µmol photons $(m^2s)^{-1}$ light intensity was used in all cases under a 14:10 hour of light: dark cycle.

V. Discussion

5.1 Discussion of results from laboratory scale Algadisk reactor

5.1.1 Isolation and selection of microalgae from natural water basins

Microalgae have a great potential for biotechnological application due to their high biodiversity. Compared to the predicted number of microalgae species, only a few has been investigated under laboratory conditions until now [4,5].

Isolation of microalgae from selected environments can results in new strains highly adapted to desired parameters with beneficial characteristics, such as fast growth and high value added metabolite production. In our case, lipid content of cells was one of these criteria, since further use of biomass for biofuel production was set as a goal in the Algadisk project. Microalgae are considered as the novel substitutions for oil crops due to their high lipid content and production rates [8,15].

At the start of this PhD research, no available literature could have been found about lipid production in biofilm systems by green microalgae, thus a large isolation and screening study was carried out. Due to the selected isolation method, using different substrates to induce biofilm formation and collecting naturally formed biofilm samples, about 60% of the isolates showed attachment to culture vessels. Based on the optical density changes of the cultures and the presence of lipid after Nile red dying [87–89], the group of potential isolates could be further reduced. Total lipid content of these samples were determined by GC-MS; the majority had around 3-5% lipid content, which is in the lower range compared to reported values [15,37], while those with 7-8% (SH-2 and A9-24a) are potentially applicable when lipid inducing stresses are applied. SH-34 with 14.5% total lipid content was the most promising strain, considering not only the lipid content but also the surface attachment and the biomass production capacity, since for biofuel application high lipid producing rates are crucial [15].

5.1.2 Different substrate materials and coatings for biofilm formation capacity

Cell adhesion of microalgae onto the surfaces is most of the cases an undesirable characteristic of suspension based biomass cultivation systems such as tubular and thin-layer PBRs; causing for instant shading and loss of biomass. In contrast, biofilm based systems convert this into a beneficial condition and thus cells are concentrated and separated from the medium; volume of water required for cultivation is reduced, allowing a cheaper and more efficient harvesting and dewatering process.

In the present research, three algal species were tested with 17 different substrate and coating combinations, including PET, PS, PP and PI as substrate materials and positively or

negatively charged coatings with different polyelectrolyte solutions. Among the algal species, *C. sorokiniana* CCAP 211/8K does not show any significant differences between positively or negatively charged surfaces and uncoated materials, moreover the lowest biomass production was observed by this alga (see **Fig. 11**). While growth of *Chlorella sp*. #34 was more pronounced when negative outer layer was applied. Highest biomass yield was reached by *H. pluvialis* and similarly to *Chlorella sp* #34, negatively charged surfaces seemed to enhance biomass growth more than positively charged or uncoated ones, however not in all cases.

As Ozkan & Berberoglu (2013) [53] and Irving & Allen [71] suggested before that the surface charges of cells vary according to the pH of the growth medium, and in longer term, cells can adapt to divers environment, which can also modify the surface charge of a certain species.

Interactions between algae cells and surface materials concerning cell attachment and detachment are limitedly studied and results are rather troublesome to compare due to the different algae species, substrate materials and set-ups used. Nonetheless as Genin et al [51] proposed and Schnurr & Allen [72] summarized, algae biofilm formation can be separated into two phases: 1. the adhesion of the first cell layer and 2. the further growth of the biomass; of which only the first phase is influenced by the surface characteristics of cells and substrate. Due to the low sample number and low biomass yield, we could not examine this phenomenon in our experiments; however in the laboratory scaled Algadisk system, a similar tendency was observed (See Section 4.1.4). Moreover, presence of heterotrophic bacteria may greatly increase the biofilm formation due to EPS production [53,69,71]. This might explain the low biomass production rates in the present systems, as the experiments were carried out in axenic conditions.

Among the substrates tested, all were stable and easy to handle; the polyelectrolyte layers however shown differences, especially coating #2, that detached from the substrates in the course of the 7 days incubation. For further application, material price, labor cost and time should be all considered when choosing the substrate for biofilm growth.

5.1.3 Biomass growth of *Chlorella* sp #34 in the laboratory scale Algadisk reactor under different conditions

5.1.3.1 Biofilm forming performance

The concept of the Algadisk reactor design is based on concentration of biomass on certain materials by the formation of biofilm. Biofilm cultivation of microalgae is raising more and more interest in research as several problems and bottlenecks of suspension cultivation can be overcome by such a cultivation method. The main aim of the conducted experiences with the laboratory scale Algadisk reactor was to further confirm the concept that rotating disks are

suitable for biofilm growth and continuous operation is possible without re-inoculating the system. Moreover, different plastic materials were validated as cheaper and lighter disk substrate substitutions to stainless steel metal meshes used by Blanken et al. [62]. Due to the two light panels providing lower and higher light intensities, two light dilution profiles were also compared. An isolated *Chlorella* species was used to test the performance of the reactor, which previously showed high biomass production, attachment to surfaces and high lipid content.

Initially, the strain was incubated at lower light intensity, about 40 μmol photons $(m^2s)^{-1}$ and from the results it can be concluded that the system can operate continuously in a period of almost 100 days without the need of re-inoculation and replacing the media or observing major technical and physical-chemical issues such as failure of rotating motor or pH crashes. Considering the biomass productivity, this set-up is comparable to many other published researches on algal biofilm formation, including horizontal glass plates, vertical twin layer sheets, revolving cotton ducts and a polystyrene rocker system where biomass productivity are in the range of 1.8-3.5 g DW $(m^2day)^{-1}$ [51,52,55,67,90]. However, higher growth rates were already achieved in biofilm system, in some cases it reached up to 31 g DW $(m^2day)^{-1}$ [57], while by Blanken et al. this value was 20 g DW$(m^2day)^{-1}$.

Biomass yield on light or photosynthetic light use efficiency is a more comprehensive way to compare the efficiency of different systems than biomass productivity. In theory, in an optimally working system, 1.5 g DW of algal biomass can be produced on 1 mol PAR photons [91], on the other hand, this value was not obtained experimentally until today; highest biomass yield on light, 1.3 g mol^{-1}, was observed by Cuaresma et al. and Schultze et al. [57,92] in a suspension and in an immobilized system, respectively.

In **Table 6**. biofilm systems are summarized where biomass yield on light values are published or could be calculated based on Equation 2. using the given light intensity, length of light hours and biomass productivity. As it can be seen from the productivity and light use efficiency values, there is not a linear connection between them. In our system under low light intensity, the biomass yield on light (0.94 g mol^{-1}) is among the best performing systems according to the literature data; however the performance of the system might seem limited. In case of the high light experiments, the biomass productivity was about half of the productivity at low light, on the other hand the light use efficiency decreased more than ten times. This indicates that while at low light conditions, the majority of the photons hitting the surface of the biofilm were used to build up biomass, at high light conditions, the photosynthesis and thus biomass formation were hindered and significant part of the photons was lost.

The same phenomenon was published by Schultze et al [57] where the biomass yield on light dramatically dropped from 1.3 g mol^{-1} to 0.5 g mol^{-1} between 22 and 300 μmol photons $(m^2s)^{-1}$.

In the same publication it was also described that the amount of CO_2 available for the photosynthetic processes can greatly influence the biomass production capacity of a system. The main idea behind using CO_2 gas to control the pH of medium comes from the characteristic of the M8-a medium, more precisely when algal cells take up the salts, the pH shifts from 6.7 towards 7 and above. By the regulated addition of CO_2 gas, the pH is reduced due to the formation of carbonic acid. It is in equilibrium with the bicarbonate and CO_2 forms that are both available for cells to take up [93]. Based on the experiments of Blanken et al (2014) with about two-times higher light intensity, this method should be efficient to provide enough inorganic carbon for biofilm production in our system as well, however, it is possible that before the cell could take up the CO_2/HCO_3^- molecules, the CO_2 has diffused from the medium. On few days during the cultivation, it was noticed that the pH could not decrease back to the normal value as fast as during low light cultivation for unclear reason, and since CO_2 gas was used to regulate the pH accordingly, it might cause limitation in the dissolved inorganic carbon concentration in the medium. On the other hand, additional factors might also play a role, however until now; we could not clarify the reasons behind the low productivity at increased light intensities. Nonetheless, the increasing tendency of biomass productivity results of the set-up with 40 µmol photons $(m^2s)^{-1}$ light suggest that the system is suitable for continuous operation and cultivation of microalgae biomass in an efficient way considering biomass yield on light values.

Productivity	Light use efficiency	Light intensity	Duration of light period	Reactor type	Microalgal species	Reference
$g\ (m^2 day)^{-1}$	$g\ mol^{-1}$	$\mu mol\ (m^2 s)^{-1}$	*hours*			
4	1.30	22	14	horizontal PSBR	*H. rubescens*	Schultze et al. 2015 [57]
31	0.60	1023	14			
0.71	0.15[a]	55	24	angled concrete sheet	*B. braunii*	Ozkan et al. 2012 [68]
2.57	0.25[a]	120	24	rocking PS foam	*Chlorella sp*	Johnson & Wen 2010[67]
2.57	0.46	200	24	vertical plastic sheet	mixed	Boelee et al. 2011[94]
6	0.72[a]	96	24	horizontal PSBR	*Pseudochloroccum*	Ji et al. 2014a [95]
9	1.10	100	24	horizontal PSBR	*A. obliquus*	Ji et al. 2014b [96]
20	0.90	422	24	rotating disk	*C. sorokiniana*	Blanken et al. 2014 [62]
5.8	0.10[a]	642	15*	revolving textile belt	*C. vulgaris*	Gross & Wen 2014 [97]
4.74	0.24	404	15*	rotating disk	*C. sorokiniana*	Sebestyén et al 2016 [66]

[a] – the values were calculated based on the given parameters from the publications
[*] - this value is influenced by the natural changes of daylight length

Table 6 – Comparison of different biofilm systems based on biomass productivity and biomass yield on light. In some cases as indicated, the values of biomass yield on light were calculated using the given parameters.

Considering large scale cultivation of microalgae, one of the major parts of operation costs is the price of inorganic salts for the growth medium. During laboratory scale experiments, optimized, high salt concentration media are used in order to exclude nutrient limitation. However, the preparation of these media are often time and labor consuming due to the low dissolving capacity and precipitation of certain compounds. Moreover, prices of graded chemicals are way too high for large volume consumption. In **Table 7**. composition of M8-a medium is indicated with the amount of salts required for the medium, also including approximate prices from three commercial distributors. In total, 1 m^3 of M8-a medium costs 103€, excluding the cost of labor, water and energy. The price calculated based on Alibaba.com is only applicable for large scale, long term cultivation as usually minimum order is one ton for each chemicals. In this case, this would be the most suitable source of salts.

In contrast, the artificial fertilizer that was applied in the experiment, Agroleaf Power High P (for composition see Table 8.), has significantly lower price, in case of a 2 kg package, the cost for 1 m^3 medium would be around 12.9 € which is only 12.5% of the price of M8-a medium (based on VWR prices).

		VWR		**Acros**		**Alibaba**	
	g/L	**€/kg**	**€/m^3**	**€/kg**	**€/m^3**	**€/kg**	**€/m^3**
1. *P-buffer*							
KH_2PO_4	0.74	35.4	26.1	25.02	20.5	0.9	0.66
$Na_2HPO_4*7H_2O$	0.3073	57	17.5	30.9	9.5	0.54	0.16
2. *Ca-Mg salts*							
$MgSO_4*7H_2O$	0.4	18.5	7.4	25.5	10.2	0.11	0.044
$CaCl_2*2H_2O$	0.013	17.15	0.02	26.6	0.34	0.27	0.0035
3. *Fe solution*							
FeNa-EDTA	0.116	229	11.7	-	11.7*	1.98	0.1
$Na_2EDTA*2H_2O$	0.0372	73.6	2.7	-	2.7*	2.52	1.29
4. *Trace elements*							
H_3BO_3	6.18E-05	63.16	0.004	21	0.0013	0.81	0.00005
$MnCl_2*4H_2O$	0.013	159.74	2.077	35.1	0.45	1.8	0.0234
$ZnSO_4*7H_2O$	0.0032	21.45	0.069	43.7	0.14	0.63	0.0020
$CuSO_4*5H_2O$	0.00183	25.75	0.047	27.1	0.049	1.44	0.0026
5. *N solution*							
Urea	2	17.5	35.0	16.4	32.8	0.25	0.5
	SUM:		**€ 102.9**		**€ 113.8**		**€ 2.8**

*- prices were not available, thus chemical prices from VWR were used to calculate total cost.

Table 7- Salts composition of M8-a medium, and their concentrations in the final medium. The prices are based on a 1kg package from the website of VWR International (www.vwr.com) and Acros Organics (www.acros.com). In case of Alibaba (www.alibaba.com), the prices are based on 1 metric ton.

On the other hand, the performance of biomass production at low light intensity using artificial fertilizer was considerably lower compared to the optimized salt medium, M8-a, however the biomass yield values were comparable to the first harvests at low light intensity with M8-a medium.

As the exact nutrient composition of the fertilizer is unknown, we suspect that some of the nutrients might be limiting. Another disadvantage of using fertilizer as growth medium is the non-ideal composition of buffering salts that would stabilize the system over a longer period. During the experiment, we observed that the pH could not be maintained as before, instead of increasing it often dropped which resulted in insufficient carbon supply. In order to

increase the pH back to normal values, $NaHCO_3$ was added to the medium which increases the cost of the medium.

Besides all the above mentioned difficulties and the lower production rate, such a fertilizer can be an ideal substitution of optimized microalgae media. Further tests would be necessary though to improve the buffering capacity and nutrient ratio of it and thus increase the biomass production. Due to its low price, addition of supplementary compounds would increase the price of the medium, even though it would be still under the calculated price for M8-a medium.

	Agroleaf Power High P		**M8a**	
			m/m%	
Total N		12.00		
	NH_4-N	8.80		
	Urea-N	3.20		10.8
P_2O_5		52.00	**PO_4^-**	15.3
K_2O		5.00	**K**	4.9
Trace elements				
	B	0.030		0.0002
	Cu*	0.070		0.01
	Fe*	0.140		0.4
	Mn*	0.070		0.084
	Mo	0.001		
	Zn*	0.070		0.017

*- chelated by EDTA

Table 8- Composition of Agroleaf Power High P fertilizer compared to M8-a medium; % stands for mass content. Exact chemical composition is not available for the fertilizer.

5.1.3.2 Comparison of disk materials

Surface attachment of algae might be highly triggered or hindered by the characteristics of surface material. Preselection of the materials can result in several fold differences in biomass productivity between the surfaces [61,67,69], just as it was described in Section 3.1.5.

Based on our previous experiments, *Chlorella sp* #34 produced the highest biomass on the negatively charged PP surfaces. However, sandpapered and uncoated PVC disks were chosen to test first in the laboratory scaled Algadisk reactor, due to their similar characteristic and lower price range and better availability. The results proved that sandpapered PVC enhanced the biofilm formation more than the negatively coated PP under high light conditions. Moreover, considering the preparation cost and time of the polyelectrolyte layers on the

surfaces, an uncoated substrate would be more beneficial in a large scale application due to lower production costs.

Limited information is available on the background of surface attachment of microalgae cells. Only few experimental results are published so far on comparing biomass production of different substrate materials. [61–63,67,69]. Blanken et al [62] compared a sand-papered polycarbonate disk with negatively charged polyelectrolyte layer to two stainless steel woven meshes with different particle pass size and found that rougher the surface was the more biomass could be harvested from it.

Based on our observations in the laboratory scaled Algadisk reactor we could draw the following conclusions which might be taken into account when choosing a surface material:

(i) Surface roughness has a high impact on the primary cell attachment to the surfaces, moreover after the harvesting, the regrowth of biofilm also enhanced by the remaining cells.

(ii) Nonetheless surface roughness should be in a μm range, appropriate for the cell size, hence larger pores or sponge like substrate structure can limit the harvesting efficiency and may cause additional costs connected to removing biofilm.

(iii) Stability of the material is crucial in order to have a continuous operation technology and to keep the reactor material costs low.

(iv) The optimal surface material may vary from one system to another, based on the reactor set-up and even the different algae species.

In our case, the PVC disks proved to be a suitable substrate materials, however further improvement of the material might be necessary to create a surface that resembles more a woven texture (however still in μm pore size) and reduce the weight of the disks as it generates force and stress on the rotating motor and axle which may lead to further maintenance costs and occasionally biomass loss (due to dried biofilm). These findings are in alignment with Schnurr & Allen's recent summary [72] where they have compared biofilm systems in order to have a better understanding on correlation between surface substrate and biomass/ lipid production. They concluded that the material does not have significant impact on biofilm formation; however roughness and presence of bacteria enhance the initial microalgal cell attachment.

5.1.3.3 Biomass density of harvested biofilm

One of the most costly process of the cultivation of microalgae is the concentration of the highly diluted biomass from suspension cultures. Among the beneficial characteristics of biofilm based PBRs, the immobilization of cells and thus their separation from water bodies are one of the major ones. By the numerous setup designs and constructions of biofilm-based photobioreactors, biomass density is reported in the range of 37-200 g dry weight kg^{-1} wet weight [50,62,66,68].

In the present study biofilm density was measured from 80 to 300 g kg^{-1}, nonetheless under optimal operation conditions (Harvest #5-7 of low light intensity experiment) it stabilized around 200 g kg^{-1}. In comparison with suspended system, these values equal to the biomass content of algal paste after concentrating [98,99] and presents an increase in biomass concentration of about 200 and 20 times compared to open pond and closed photobioreactors, respectively (Pienkos et al. 2009).

Harvesting technologies are usually optimized individually for the biofilm reactors, however most of the cases they are based on scrapping off the biofilm from the substrate with a knife-like tool. For our bench-scale Algadisk reactor a suction head with a metal blade was designed, that connects to a vacuum cleaner, through a settling cyclone where the harvested biomass accumulates. This method was used only in few cases during the experiments due to the low amount of biofilm. Such a method allows a semi-automatic harvesting with reduced energy consumption and without the dilution of biomass and the need of transporting and storing the growth media. Moreover the medium remaining in the reactor tank can be reused for the following growth-harvest cycles, hence lowering the water demand of cultivation.

For the above mentioned benefits, attached cultivation of microalgae can greatly diminish the expenses of downstream dewatering and concentrating processes, which leads to lower total production cost and thus a more competitive algal biomass production.

5.1.3.4 Lipid producing capacity of Chlorella sp #34 in the lab scale Algadisk reactor

Throughout the experiments, total fatty acid content of harvested biomass was analyzed and compared under the different conditions in order to evaluate whether the used microalgae strain, *Chlorella* sp #34, and the cultivation system were applicable for biodiesel production.

In our case, N limitation was chosen as a lipid accumulating factor, due to its positive effect on lipid content of microalgae and due to the characteristics of the Algadisk system such as the simplicity of replacing the culture medium without removing the biomass from the surface.

Comparing the lipid content of the N replete and N limited conditions, results show that N free medium positively influenced the lipid accumulation. However this is a very slow process; lipid content doubled only after 20 days of incubation, reaching 9.5%. This value is comparable to other reported values in biofilm systems using *Chlorella* species, between 7.7 and 10% FAME [61,67,97]. This value still remained below the content we have observed during the strain selection (Section 4.1.2). Moreover, lipid production rate is only one tenth of the values reported by Johnsons & Wen [67] and Gross et al. [61] in a similar sized reactor.

In contrast, suspension cultivation of *Chlorella* species shows significantly increased lipid content when N limitation is applied; as it was reported by Illman et al. [100] and Ördög et al. . [101]

In the review of Schnurr & Allen [72], several reasons of low lipid content despite the N limitation are proposed such as (i) the metabolic characteristics of used strains, (ii) nutrient storage in the biofilm, (iii) low mass transfer through the thick biofilm layer and (iv) the presence of bacteria mass in open systems, that may mislead the calculations for total lipid content in the dry biomass. These factors might be present in our system as well, and could influence the lipid content and accumulation of the biofilm. Even though the system is open and bacteria are present, massive growth was not observed in any of the experiments (microscopic observations), hence this effect might be negligible in our case.

Besides the slow accumulation process in our system, stressing seems to inhibit regrowth of biofilm after harvesting, which is a major drawback in the Algadisk system. Other approaches of lipid accumulation induction should be developed, in order to reach high lipid content meanwhile preserving the regrowth capacity of the biofilm.

, It can be also concluded from the presented experiments, that without stressing, even though lipid content is lower, due to the high biomass productivity, more lipid can be obtained and production can be maintained continuously, which is a major benefit for industrial microalgae production. This parameter may be enhanced in the future by using other microalgae strains with higher lipid content and parallel high biomass producing capacity.

Besides the quantity of the lipid produced by microalgae, the quality is also a crucial factor to be considered. Depending on the final application of the lipid, different fatty acid compositions are preferred. In order to use microalgal lipids as biodiesel, several criteria have to be considered and parameters should meet the given standards. Such restrictions are the cetane number, cold flow characteristics, cloud point, viscosity and oxidative stability. These depend mostly and strongly on the chain length and level of saturation of fatty acids. The ideal fatty acids that could fulfill all the requirements are C16:1 and C18:1 [102–104]. In our biofilms, these fatty acids are present, however as it is shown on the **Fig. 19**, the contents range from 13% to 20% of total lipid. The other major fatty acids are C16:0, C17:1, C18:2 and C18:3 (n-3) that are also common in other biodiesel feedstocks as well for instance rapeseed, palm and soybean [102] and could provide sufficient parameters for biodiesel usage. Nonetheless, C18:3 might reduce the quality due to the three double bonds. Many studies suggest that the amount of PUFAs should be kept in a low level, similar to other biodiesel sources, in order to avoid oxidative instability, low viscosity and low cetane number [10,104,105]. Other PUFAs with unsaturation level >3 are only present in the biomass in traces, see Table 5. As it can be observed on **Fig. 19** and **20**, the FA composition can vary due to environmental conditions, age of biomass and nutrient availability. Consequently, these have a high impact on the quality of produced biodiesel. A recently, extensively researched technology is the hydrothermal liquefaction, which gives the opportunity to turn algal biomass into bio-crude. By using high temperature, high pressure and a catalyzer, not only the lipid molecules can be utilized but also

carbohydrates and proteins, resulting in a higher yield than normal lipid extraction [106–108]. This can provide a solution for varying and low lipid productivities and unsuitable FA composition.

Apart from the biodiesel production, another main focus of research on microalgal lipid composition are the ω-3 and ω-6 PUFAs, such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), arachidonic acid (AA) and α- and γ-linolenic acid (ALA and GLA). These are essential fatty acids, that vertebrates are not able to synthetize; however they play an important role in the development and maintenance of a healthy cardiovascular and neurological system [25]. Microalgae are one of the primary producers of these molecules and numerous investigations are aiming to replace fish-oil with microalgae based products, due to their various benefits. EPA, DHA and GLA were present in the biomass from high light conditions and growing on artificial fertilizer, however as it can be seen from the measured content they are present in a very low concentration, below 0.01% of the dry biomass and below 1% considering the total FA content. These results and literature data suggest that the used *Chlorella* strain is not suitable for PUFA production. Other microalgae species such as *Nannochloropsis* strains, *Phaeodactylum tricornutum*, *Pavlova* strains and *Isocrysis galbana* are more promising sources, expressing over 20% of EPA and DHA of the total FA [109,110].

The possible biotechnological application of the produced biomass still need to be further examined, taking into consideration the characteristics of the reactor and the species.

5.2 Discussion of results of *H. pluvialis cultivation* in Twin Layer system

Numerous suspension based cultivation methods and systems have been reported for *H. pluvialis* cultivation, and attempts were made to optimize astaxanthin production in these. On the other hand, only few studies are available about the biofilm based cultivation of this microalga and most of them are lacking the examination of increased light intensity over 150 µmol photons $(m^2s)^{-1}$.

5.2.1 Effect of light intensity on *H. pluvialis* growth

Our attempts to growth *H. pluvialis* in the lab-scale Algadisk reactor have been unsuccessful, hence a different biofilm technology, Twin Layer reactor, has been selected that was known to support attached cultivation of this microalga (personal communications with Prof Michael Melkonian, University of Cologne). The first step of our experiments was to identify whether the structure of biofilm can allow exposing cells to stressing light condition without compromising growth. Light intensities above 150 µmol photons $(m^2s)^{-1}$ are considered

as a factor to trigger astaxanthin accumulation and stop of cell division in suspension cultures. However, in the present study in the Twin Layer system, the highest biomass yield and biomass productivity were gained at 219 µmol photons $(m^2s)^{-1}$. Moreover, biomass growth was linear during the whole cultivation period.

The achieved biomass production (about 4-6 g $(m^2 d)^{-1}$) was in the similar range, , as other reported biofilm cultivation of *H. pluvialis* under similar conditions, namely by Zhang et al (2014) and Yin et al (2015) [59,111] and doubled compared to the values reached by Wan et al [112]. Additionally, these values are comparable to other biofilm systems cultivating different green microalgae [61,113].

In all of these cases, continuous illumination was used and initial inoculation biomass density was 5-times higher (10 g m^{-2}) than in our system. In order to have a clearer understanding on the efficiency of these systems, the biomass yield on light was calculated based on their given data. Considering the applied light intensities of 100 µmol photons $(m^2s)^{-1}$ and the biomass production of 6 g $(m^2 d)^{-1}$ (excluding the results of Wan et al [112]) biomass yield on light is about 0.69 g mol^{-1}, which is lower than 0.95 g mol^{-1} what we could expect from our system based on linear regression of biomass yield on light values at different light intensities (**Fig. 23**).

To understand better how biofilm systems can enhance biomass growth of *H. pluvialis* even at stressing light condition, the conclusions of experimental and modelling studies can be applied. These showed that light penetrates only until a certain depth into the biofilm and light intensity decreases steeply in the top layers (100-200 µm) [72,114]. Based on these findings we could suspect that the top cell layers absorbed the major part of light, which caused astaxanthin production and accumulation in these cell layers, while the inner cell layers continue dividing due to the optimal (decreased) light intensities. Based on the findings of Li et al [114], which demonstrated that light penetration depth is independent of the light intensity, consequently further increase of the light would not cause drop in the biomass production either. However, the so called "dark zones" can form when the biofilm thickness is over 400 µm, where respiration and consequently biomass loss can occur. Therefore, biofilm thickness should be kept in an optimum thickness, regulated for instance by frequent harvesting.

Supporting these finding, microscopic observations showed that the biofilm consisted mostly of green, vegetative, rounded cells, known as palmella; and partly red, astaxanthin containing, resting, larger cells, known as akinetes (**Fig. 29.AB**). Additionally, macroscopic inspection indicated that there was a clear separation between the vegetative and resting cells; the red layer was positioned on the top of the biofilm, exposed to the light, while the deeper layers consisted almost exclusively of green cells (**Fig 29.CD**). The astaxanthin value of the biofilm grew at 219 µmol photons $(m^2s)^{-1}$ has increased compared to lower light intensities (macroscopic observation), however did not reach 1% (**Fig. 26**), indicating that application of

further stress factors besides the increased light, is necessary to enhance astaxanthin content of the biofilm.

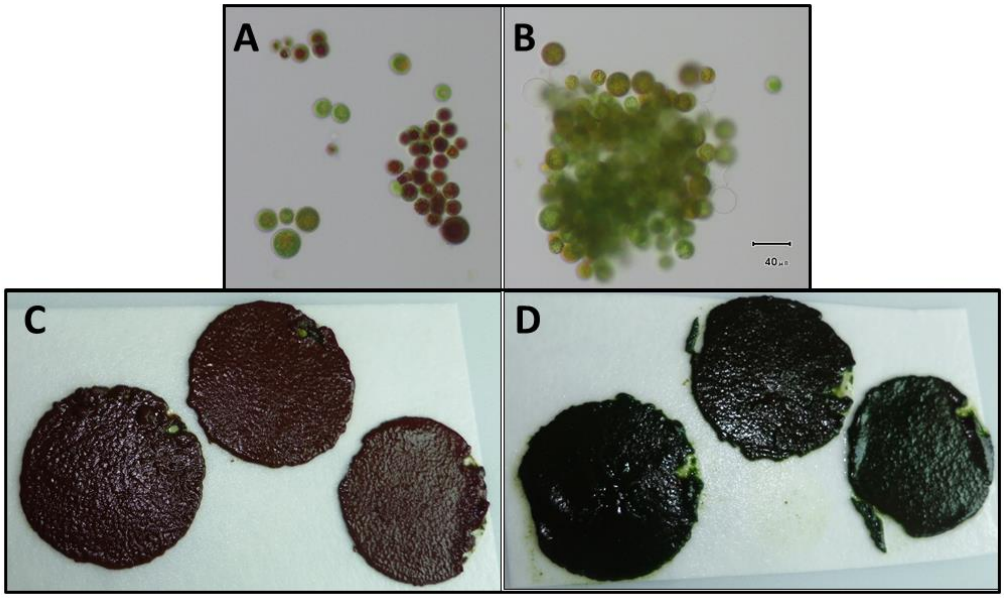

Figure 29- Microscopic (**A, B**) and macroscopic (**C, D**) photos of the *H. pluvialis* biomass after 16 days of cultivation in the Twin Layer system. **A** and **C** pictures are samples illuminated with 219 µmol photons $(m^2s)^{-1}$ showing both green cells and red cells; and **B** and **D** are samples illuminated with 135 µmol photons $(m^2s)^{-1}$ showing mostly green cells and cells that are started to accumulate astaxanthin.

5.2.2 Effects of different stress factors on astaxanthin content of *H. pluvialis* biofilm

It was discovered early which are the main triggering factors of astaxanthin accumulation in *H. pluvialis* [115,116], these were further examined for better understanding of the process [28,117,118].

Based on the literature, the main stress factors (N limitation and added NaCl) were tested in our system, under increased light intensity, 210 µmol photons $(m^2s)^{-1}$. The experimental set up was designed similarly to the Algadisk system, using a two-phase process. This method is also widely used in case of *H. pluvialis*, however often related to costly processes, as cell concentration and replacement of medium. However, in the biofilm systems, due to the separation of medium and biomass, the stressing factor can be easily added by simply replacing the media bottles.

In this experiment, considering the biomass yield, no major differences were observed in any of the stress factors, compared to the control, only slight decrease in biomass productivity can be observed. Moreover, the lowest amount of salt seemed to enhance biomass growth,

reaching 6.7 g $(m^2 d)^{-1}$. Biomass yield on light followed the same tendency and slightly reduced in the N free, and 0.4% and 0.2% NaCl containing media.

On the other hand, astaxanthin content and yield showed significant differences between the applied stress factors. Within the induction time, none of the used salt concentration increased the astaxanthin content; the values remained around the same as in the control samples, 1% astaxanthin of dry weight. In suspension cultures, similar salt concentration have been successfully used to induce astaxanthin production; Harker et al [119] showed that 100 mM NaCl which equals to 0.58% NaCl in the medium, had the most prominent effect of astaxanthin accumulation, however, samples treated with 40 mM (0.23% NaCl) were comparable as well. Kobayashi et al [120] also tested the effect of different salt concentration on the carotenoid/chlorophyll ratio and demonstrated that carotenoid content increased when NaCl content was reduced from 0.5% to 0.1%. In these studies, light intensity was low [119] or cultures were cultivated in dark [120] which allowed the salt stress to express significant increase compared to the control situation, while in our case the applied light intensity, 210 µmol photons $(m^2 s)^{-1}$, already affected the astaxanthin content in a way that the additional salt stress did not have any effect on the cells.

In contrast, the N free BBM medium has caused a 5 times increase compared to the pre-stress value (0.7% to 3.5% astaxanthin in dry weight). N limitation connected to high light is one of the most efficient factors to trigger high astaxanthin content as it was described by several authors reporting astaxanthin contents of 2-5% both in suspension and attached cultivation [59,82,85,112,121–123].

Besides the astaxanthin content, the production rate is a very important parameter considering a large-scale cultivation method. Due to the diversity of published systems and their results (suspended, attached), productivity values are often challenging to compare. Zhang et al [59] recalculated some astaxanthin productivities of suspended systems in order to compare with attached cultivation techniques. Based on these values and the given productivities from other biofilm systems [111], our production rate is among the best ones with about 300 mg $(m^2 d)^{-1}$ under N free condition with increased light intensity.

Nutrient limitation and high irradiance are not only among the most efficient stressing factors, but also economically the most feasible method to increase the astaxanthin content of *H. pluvialis* cells. Nutrient limitation, including nitrogen depletion, occurs naturally in a batch cultivation system. The stressing phase can be planned and controlled by designing the nutrient content of the media [124]. While outdoor cultivation of *H. pluvialis* was so far limited by the irradiance of natural sunlight, the vertically orientated Twin Layer sheets and the structure of biofilm enable an optimal light dilution for vegetative growth and meanwhile support the astaxanthin accumulation in the top layer of the biofilm, which can be extended to the whole biofilm by using another stress factor, such as N limitation.

5. 3 Comparison of Algadisk system to Twin Layer system and their efficiency compared to other biofilm systems

Biofilm based cultivation methods for microalga biomass and high value added compound production are promising technology to provide a cost-efficient, simple and stable solution to substitute suspended cultivation techniques.

In the present study, we employed two different approaches of biofilm based microalgae cultivation in order to produce two green microalgal biomasses. The main objective of this thesis was to test the newly developed Algadisk reactor in laboratory scale with a specifically isolated and selected microalgal strain, *Chlorella sp*. #34, and with a widely examined, biotechnologically important microalga, *H. pluvialis*. As it was discussed before, *Chlorella sp*. #34 was an ideal choice for this technology, on the other hand, cultivation of *H. pluvialis* was unsuccessful. From this result, we suggest that some microalgal strains are not applicable in certain photobioreactors, due to their operation characteristic. The *Chlorella* species, both in this study and in the study by Blanket el al. (2014) showed high affinity to different surface materials and the rotating disk concept was suitable for enhancing biofilm growth and re-growth. On the other hand, we were not able to achieve similar results with *H. pluvialis* besides the several surface substrates and enhanced mixing. For this reason, we have decided to use a different biofilm system, the Twin Layer system, which provided a unique solution for cultivating this microalgae under increased light intensity and also to induce astaxanthin accumulation by nutrient limitation.

The set-up and operation of these systems are considerably different. Moreover the Algadisk technology is already closer to large scale, continuous application than Twin Layer system, due to the regular growth-harvesting cycles, the partly automatized harvesting method and the scalable, competitive disk material. pH control and CO_2 supply were easily regulated, problems only occurred when the non-buffered fertilizer was applied, however this might be overcome with optimization of the fertilizer with some buffering agents. Considering the electricity consumption, besides the artificial illumination which will be excluded in the large scale outdoor system, energy was used for the rotation of disks and for pumping the media from the buffer tank to the reactor tank and some cases for the harvesting. Measurements were not made concerning the used kWhs, however from the technical description of the pump and rotating motor, we calculated an about 0.15 kWh consumption for these. By using vacuum suction to collect the harvested biofilm into a cyclone, the used energy increases, but a 1 kWh consumption of the machine and about 25 mins harvesting time in each growth-harvest cycle, it results in only a 0.04 kWh used energy per harvesting. In this case, the harvesting cost is only 0.02% of the total energy consumption for maintenance within a 7 day growth-harvest cycle, and would be even lower when considering installation, material and labor costs, too. As other

authors calculated and described for suspension cultivations, the harvesting and the efficient mixing (which is essential to prevent loss of light by self-shading etc.) are a significant part (20 % in case of open pond systems) of the total costs [33,125]. Based on this, the Algadisk reactor could compete with the currently used cultivation methods.

Additionally, applying a microalgae strain that besides accumulating high value compounds (such as lipids) is robust, heat tolerant and fast-growing, like *Chlorella* species, will increase the biomass production capacity of the system and reduce the risk of contamination.

The system had limitation when the stress was introduced; namely slow lipid accumulation and failed regrowth of biofilm after harvesting, also it might not be suitable for every microalgae type, as it was observed in case of *H. pluvialis.*

The Twin Layer system is using a different approach for inoculating the surfaces, which enables the use of basically any microalgae strains. In the bench scale system, which was tested in this study, this process is filtration based; however it is not a scalable option. Naumann et al (2012) [55] employed sponge brushing to distribute the concentrated microalga cultures onto the surfaces in the pilot scale Twin Layer reactors; air-brushing and spraying would be also possible inoculation methods. In comparison to the Algadisk system, the major bottlenecks of the Twin Layer concept is the selection of surface substrate material and that repeated growth-harvest cycles were not achieved so far. The polycarbonate membranes are way too expensive for large scale application; nonetheless its characteristic would be ideal for the cultivation, especially in protecting the biofilm from contamination originated from the growth media (due to small enough pore size) and the durability of the material would allow a longer period of use. Consequently, before up scaling the system, an extensive selection should be done to find the most suitable substrate material.

So far, researches with bench-scale and pilot scale Twin Layer system were focusing on optimizing the growth of certain microalgae species [55,57], on removing nutrients from wastewaters [56,126] and on producing valuable compounds by e.g. stressing the culture [127]. As a consequence of the set-up of the bench-scale PBRs, the reformation of the biofilm after harvesting cannot be examined. Data is not available whether the biomass productivity would increase as well as in our Algadisk system, and whether the stress factors would diminish the biofilm formation.

Biomass density of the harvested biofilm was only measured by Naumann et al (2012). It was about 280 and 160 g WW kg^{-1} DW in case of *Isochrysis sp* and *Tetraselmis suecica*, respectively [55]. These values are similar to what we obtained in our Algadisk and to other groups using various biofilm systems. Based on the feature of Twin Layer concept, that the biofilm is not submerged into the media, we suspect that variations of biomass density should be significantly lower than using our Algadisk reactor. .

Energy consumption-wise, the Twin Layer system only requires energy for the circulation of media, harvesting can be done with the same system as in the Algadisk reactor; the energy consumption of electronic devices would be about 0.12 kWh (excluding the harvesting process). Omitting the moving parts from the system, like the rotating disks, besides less energy utilization, also reduces the likeliness of mechanical problems that lead to drying and loss of biofilm.

In the experimental set-up presented in this work, the pH was not controlled due to the regular replacement of the media, however to reduce water requirements, a semi-batch way should be applied with controlled pH by CO_2 gas addition and nutrient concentration.

H. pluvialis is an extremely valuable microalga, it is also known to be rather sensitive to environmental conditions and contaminations. Since both reactors are open, only xenic cultivation is possible. The separation of culture medium and the biofilm by a porous membrane diminishes the chances for a widespread contamination in the Twin Layer system; in most cases contamination would appear in a single spot and if early recognized, it can be removed without disturbing the biofilm formation. This provides a better applicability for such a microalga as *H. pluvialis* than the Algadisk system would do, where contaminations could faster escalate.

The objectives of the PhD research such as

- strain isolation for attached cultivation,
- test of different surface materials,
- long-term, continuous operation of the lab scale Algadisk reactor,
- growth of selected microalgal species under several conditions and
- enhancing the production of high value added compounds;

were successfully achieved and the results can be considered valuable to compare with other biofilm systems. Based on these experiments, the large scale, pilot Algadisk reactor was built and operated for about half year in Spain [66]. Both Algadisk and Twin Layer systems have their benefits and drawbacks in comparison with other published attached microalgae cultivations and with suspension based cultivation, as well. In the current state, we can conclude that Twin Layer system needs further development considering upscaling and continuous operation, while the Algadisk system should be further modified to reduce the used materials and increase the stability of the biofilm. Also, it was noted that certain microalgae and/or cultivation purpose might demand different cultivation techniques. They both have potential to compete with currently used industrial scale alga production (suspension based cultivation) and it would worth to conduct more research under several environmental conditions and applying different microalgae species.

VI. Conclusion

In the present work, not only the biotechnological potential of a newly isolated green microalgae strain was examined but also the concept of the Algadisk reactor was proven.

The strain isolation from natural water basins and the preselection based on high biomass productivity and lipid content resulted in a robust, fast growing species that was also suitable for surface attached cultivation in the laboratory scale rotating disk reactor. Surface material and coating tests could not give further insight to the correlation of surface material and cell attachment; on the other hand, we could exclude some materials and coatings that were incompatible with microalgal cultivation.

Biomass production of *Chlorella* sp #34 was tested under various conditions, including low and high irradiance, optimized culture medium and commercial fertilizer. Long-term (about 90 days), continuous operation (7 growth-harvest cycles) was achieved without any major mechanical (e.g. stop of disk rotation) or chemical (e.g. pH irregularities) failures and contamination. The biomass values are comparable to other systems, both suspended and biofilm based; biomass productivity reached 3 g $(m^2\ day)^{-1}$ after a 7 days growing period; while biomass yield on light was about 0.9 g mol^{-1}, which is considerably high value compared to the theoretical maximum of 1.5 g mol^{-1}. The most remarkable feature of biofilm based reactors is the high solid content of the harvested biofilm. In our case, 200 g kg^{-1} was obtained at low light intensity with optimized medium, which is comparable to centrifuged suspension cultures. The increased light intensity and use of commercial fertilizer negatively influenced the efficiency of the system, however with further optimization we are certain that similar or even better biomass values could be reached compared to the low light and rich medium conditions.

Considering the lipid production of the selected *Chlorella* species, content ranged between 3 and 7% and could be increased up to 10 % under N limited conditions. FA composition would be suitable for biodiesel application, presenting high proportion of medium length and mono-, di-unsaturated FAs, nonetheless, the productivity remained below 110 mg $(m^2\ day)^{-1}$, and presence of the stress factor hinder regrowth of biofilm, which are major bottlenecks for application.

Attempts with *H. pluvialis* in the Algadisk reactor showed that this technology might be limited to the cultivation of certain microalgae species. On the other hand, the Twin Layer system was successfully applied for biomass and astaxanthin production from *H. pluvialis*. The biofilm based cultivation allowed to increase applied light intensity without compromising cell division, about 6 g $(m^2\ day)^{-1}$ biomass productivity was measured. Moreover, under the examined conditions, the N limitation enhanced astaxanthin content 7 times (from 0.5 % to 3.5 % (w/w %)) higher than the other stress factors (NaCl in various concentrations). Astaxanthin productivity attained 300 mg $(m^2\ day)^{-1}$, which is in the same range as published data and assures marketable potentials.

Both of the cultivation technologies, Algadisk reactor and Twin Layer system; has great potential for efficient, large scale biomass production. Some problems still have to be solved and parameters should be further optimized for both biomass and high value compound production, in a way that continuous growth can be maintained. Other microalgae species should be taken into consideration to cultivate in these systems (for instance *D. salina*, *N. oculata*) and/or other biotechnological application of *Chlorella* sp. #34 could be the focus of future researches such as starch and protein production. Besides these, the benefits of biofilm based cultivation of microalgae, including high biomass density, better light utilization, reduced water consumption, increased footprint based production and ease of stress induction were demonstrated in this study. We have also proven the efficiency and stability of the newly designed Algadisk reactor. Moreover, results showed that in some cases, the biofilm based cultivation can reduce the negative effects of stress factors as it was presented in the Twin Layer system, where the increased light intensity did not limit the linear growth of *H. pluvialis* cells.

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Summary

Microalgae are a very diverse and large group of photosynthetic microorganisms, including prokaryotic and eukaryotic members as well. They play a significant role in oxygen generation, CO_2 fixation and primary producers of organic matter thus they are inevitable parts of the food chain. Microalgae were observed in all sorts of habitats, including for instance deep sea regions, tropical areas and even under polar ice and snow; tolerating extreme pH, salinity, temperature etc., both in planktonic and benthonic forms [5]. This diversity provides possibilities for a wide range of biotechnological applications, from food and feed industry, pharmaceutical production [22], to agriculture, energy generation from biofuels or via biogas application [8,13,128,129], moreover wastewater treatment and nutrient recovery [99].

In the last decades, interest of researchers moved towards biodiesel production from the accumulated lipid of the microalgae biomass, as fossil fuels prices drastically increased and availability decreased, along with the recognition of the effects of industrial activity on climate change [8,9]. Microalgae are not only suitable for biodiesel formation but thanks to their diversity, a wide range of other secondary metabolites with biotechnological importance are present, as well. For example EPA, DHA, β-carotene and astaxanthin are among the most marketable compounds of the microalgae biomass as human nutraceuticals. However, real large scale, industrial production of microalgae based high value added compounds is restricted and often extremely costly. Some of the main bottlenecks are the low cell density in suspensions and thus high dewatering cost of biomass. Additionally the natural characteristic of most microalgae, that enhanced lipid or astaxanthin accumulation only occur under growth limiting conditions, such as low temperature or nutrient limitation [130], which causing low productivity rates.

Current large scale, commercial microalgae cultivation technologies are exclusively based on suspension cultivation, which consequently coupled with high energy, labour and volume demanding downstream dewatering processes e.g. flocculation, centrifugation, filtration and sedimentation [47,98,99]. The cost of concentrating the biomass from the low cell density medium (between 0.5-10 g L^{-1} dry weight) can reach up to 20-30% of the total production cost, hence reducing the competitiveness of microalgae based products [33,42,47]. Biofilm based cultivation techniques can overcome the exsiting problems of the suspension based methods. Among the beneficial characteristics of biofilm based PBRs, the immobilization of cells and thus their separation from water bodies are one of the major ones. Despite the numerous setup designs and constructions of biofilm-based photobioreactors, biomass density is reported in the range of 37-200 g dry weight kg^{-1} wet weight [50,62,66,68].

Several different biofilm design and set-up have been developed and tested with different algae species for different purposes, however the amount of available information is far below the amount of data and knowledge of suspension cultivation, either of open or closed systems.

Also many of those have not yet been optimized for large scale application yet, which results in lacking of actual efficiency values; and their comparison is often problematic.

As a part of a European project, a newly developed biofilm based system is tested for continuous long term operation with several microalgal strains in lab scale. In the first step of this work, strain isolation and selection were carried out. The strain isolation from natural water basins and the preselection based on high biomass productivity and lipid content resulted in a robust, fast growing species that was also suitable for surface attached cultivation in the laboratory scaled rotating disk reactor and identified as *Chlorella* sp. #34.

With three selected strains, *Chlorella* sp. #34, *C. sorokiniana* CCAP 211/8K and *H. pluvialis* SAG 44.96, 17 different substrate and coating combinations, including PET, PS, PP and PI as substrate materials and positively or negatively charged coatings with different polyelectrolyte solutions were tested for biofilm attachment and biomass growth. Our main aim was to examine the effect of rotation on surface attachment of microalgal cells and to get further insight to the correlation between the surface material properties and cell attachment. Despite the experimental results, the surface material and coating tests could not give further insight to the relationship of surface material and cell attachment; however, we could exclude some materials and coatings that were incompatible with microalgae cultivation.

The newly developed Algadisk system for microalgae biomass production is based on rotating, vertical disks that are halfway submerged into the growth medium. A laboratory scale system was built and tested with the isolated strain to analyze the durability, efficiency of the system, furthermore to investigate the risk of contamination and mechanical/chemical failures. Biomass production of *Chlorella* sp #34 was tested under various conditions, including low and high irradiance, optimized culture medium and commercial fertilizer. Long-term (about 90 days), continuous operation (7 growth-harvest cycles) was achieved without any major mechanical (e.g. stop of disk rotation) or chemical (e.g. pH irregularities) failures and contamination. The biomass values are comparable to other systems, both suspended and biofilm based; biomass productivity reached 3 g $(m^2\ day)^{-1}$ after a 7 days growing period; while biomass yield on light was about 0.9 g mol^{-1}. The most remarkable feature of biofilm based reactors, is the high solid content of the harvested biofilm, in our case, 200 g kg^{-1} was obtained at low light intensity with optimized medium, which is comparable to centrifuged suspension cultures. The increased light intensity and use of commercial fertilizer negatively influenced the efficiency of the system, however with further optimization we are certain that similar or even better biomass values could be reached compared to the low light and rich medium conditions.

The FA production of the selected *Chlorella* species, considering application as biodiesel, were monitored in all the above mentioned set-ups with the lab-scale Algadisk system, additionally a two-step N limited experiment was conducted, as well. In general, lipid content ranged between 3 and 7% and could be increased up to 10 % under N limited conditions. The

determined fatty acid composition would be suitable for biodiesel application, presenting high proportion of medium length and mono-, di-unsaturated FAs, nonetheless, the productivity remained below 110 mg $(m^2\ day)^{-1}$. Additionally, presence of the stress factor hinder regrowth of biofilm, which are major bottlenecks for industrial applications. Apart from the biodiesel production, valuable PUFAs such as EPA, DHA, AA, ALA and GLA were also monitored in the harvested biomass. Even though, they were present in most cases, their values remained below 0.01%, which makes the strain *Chlorella* sp. #34 unsuitable for their production.

Another microalgae strain with biotechnological potential was also in the focus of biofilm growth, namely *H. pluvialis*. *H. pluvialis* is of great interest of microalgal biotechnology research due to its high value added compound, astaxanthin. Accumulation of astaxanthin in this alga is related to unfavorable environmental conditions, such as increased light intensity, nutrient limitation, increased salt concentration or presence of organic carbon sources. Numerous suspension based cultivation method and system have been reported for *H. pluvialis* cultivation, and attempts were made to optimize astaxanthin production in these systems.

The presented results in this thesis showed that the Algadisk reactor was not suitable for *H. pluvialis*. Consequently, cultivation of other microalgal species might be limited, too. On the other hand, a different approach, the Twin Layer system was successfully applied for biomass and astaxanthin production from *H. pluvialis*. This type of cultivation allowed to increase applied light intensity without compromising cell division, about 6 g $(m^2\ day)^{-1}$ biomass productivity was reached. Moreover, under the examined conditions, the N limitation enhanced astaxanthin content 7 times higher (from 0.5 % to 3.5 % (w/w %)) than the other stress factors (NaCl in concentrations of 0.05%, 0.2% and 0.4% (w/w %)). Astaxanthin productivity attained 300 mg $(m^2\ day)^{-1}$, which is in the same range as published data and assures marketable potentials.

Biofilm based cultivation methods for microalgal biomass and high value added compound production are promising technology to provide a cost-efficient, simple and stable solution to substitute suspended cultivation techniques. The main objective of this thesis was to test the newly developed Algadisk reactor in laboratory scale with a specifically isolated and selected microalga strain for biomass and lipid production, additionally to compare it to other biofilm systems, such as the Twin Layer system.

The set-up and operation of these systems are considerably different; moreover the Algadisk technology is already tested in large scale, continuous operation , with regular growth-harvesting cycles, partly automatized harvesting method and scalable, competitive disk material [66]. In case of the Algadisk system, pH control and CO_2 supply were easily regulated, problems only occurred when the non-buffered fertilizer was applied, however this might be overcome with optimization of the fertilizer with some buffering agents. Both of the cultivation technologies, Algadisk reactor and Twin Layer system have great potential for efficient, large

scale biomass production. Some problems still have to be solved and parameters should be further optimized for both biomass and high value compound production, in a way that continuous growth can be maintained. Other microalgal species should be taken into consideration to cultivate in these systems (for instance *D. salina*, *N. oculata*) and/or other biotechnological application of *Chlorella* sp. #34 such as starch and protein production could be the focus of future researches. Besides these, not only the benefits of biofilm based cultivation of microalgae were demonstrated in this study, including high biomass density, better light utilization, reduced water consumption, increased footprint based production and ease of stress induction . But also, we have proven the efficiency and stability of the newly designed Algadisk reactor. Moreover, results showed that in some cases, the biofilm based cultivation can reduce the negative effects of stress factors as it was presented in the Twin Layer system, where the increased light intensity did not limit the linear growth of *H. pluvialis* cells.

Összefoglaló

A mikroalgák egy nagyon diverz és számos fotoszintetizáló mikroorganizmust magába foglaló csoport, ideértve prokarióta és eukarióta élőlényeket is. Fontos szerepet játszanak a légköri oxigén előállításában és a CO_2 megkötésében, emellett elsődleges termelők a táplálékláncban, így alapvető fontosságúak.

Előfordulásukat tekintve, szinte minden élőhelyen megtalálhatóak, , mélytengeri, trópusi régiókban, a sarkköri jég és hó alatt, extrém pH-n és hőmérsékleten, sós környezetben, szabadon úszó és telepes formában is. Ez a sokféleség jó lehetőséget nyújt arra, hogy az alga biomasszát biotechnológiai célra használják fel az élelmiszer vagy takarmányiparban, a gyógyszergyártásban, a mezőgazdaságban, bio-üzemanyagként vagy biogáz alapanyagként , illetve akár szennyvízkezelésnél és esszenciális tápanyagok kinyerésénél.

Az utóbbi évtizedekben, a kutatók figyelme a mikroalga biomasszában felhalmozott lipidek biodízellé történő átalakítására irányult. . Ez az érdeklődés annak köszönhető, hogy a fosszilis üzemanyag árak drasztikusan megemelkedtek és ezzel egy időben felismerték az összefüggést az ipari tevékenységek és klímaváltozás között. A mikroalgák ugyanakkor, nem csak biodízel előállításra használhatóak, hanem egyéb biotechnológiai fontosságú másodlagos metabolitokat is termelnek. Ezek közül néhány, a többszörösen telítetlen zsírsavak, mint EPA, DHA, illetve karotenoidok, mint az asztaxantin és β-karotin, melyek a legkeresettebb humán táplálék kiegészítők közé tartoznak. Ennek ellenére, a valós, nagy léptékű, ipari termelése a mikroalgákból származó termékeknek még korlátozott és gyakran rendkívül költséges. Ennek oka többek között, az optimális körülmények között is alacsony sejtszám, a magas sűrítési költségek, továbbá a fentebb említett anyagcseretermékek előállítási módja. Ezek általában csak valamilyen sejtosztódást limitáló tényező esetén halmozódnak fel a biomasszában, így alacsony termelékenységet okozva.

A jelenleg ismert, kereskedelmi célú, mikroalga termelő technológiák szinte kizárólag szabadon-úszó folyadék kultúrákon alapulnak, melyekből a biomassza kinyerése olyan nagytérfogatú, energia és munkaerő igényes, downstream biomassza koncentráló folyamatokkal lehetséges, mint pl. a flokkulálás, centrifugálás, szűrés és ülepítés. Ezeknek a költsége akár a teljes termelési költség 20-30%-át is kiteheti, ami jelentősen csökkenti a mikroalga alapú termékek versenyképességét. Ezzel ellentétben, a biofilm alapú technológiák megkerülik ezt a problémát. Ezen rendszerek egyik legmeghatározóbb paramétere, a sejtek immobilizálása, így elkülönítve a biomasszát a tápoldattól. Annak ellenére, hogy nagyon eltérő felépítésű és működésű biofilm rendszerek ismertek, a biomassza sűrűsége mindig magas, 37 $g_{száraz\ anyag}\ kg^{-1}{}_{nedves\ anyag}$-tól 200 g kg^{-1}–ig terjed, az eddigi irodalmi adatok alapján.

Számos publikáció található a különböző biofilm rendszerekről, melyeket különféle algák termesztésére használnak, viszont az ismeretek még így is elmaradnak a folyadék alapú rendszerekről fellelhető adatoktól és tudástól. Továbbá, sok közülük, még nem alkalmas nagy

léptékű alkalmazásra, így a valós hatékonysági értékek még hiányoznak és gyakran az összehasonlításuk más rendszerekkel is problémás.

Felismerve az ebben rejlő potenciált egy Európai Uniós projekt keretében vizsgáltuk az újonnan kifejlesztett Algadisk rendszer hatékonyságát labor méretben az itt bemutatott doktori munka során. Az egyik legelső lépés ehhez a törzsizolálás és szelekció volt. A természetes vizekből izolált törzseket magas biomassza hozam és lipid tartalomra vizsgálva, kiválasztottunk egy ellenálló, gyorsan növő törzset, amely alkalmas volt a felületen való növesztésre is a laboratóriumi forgó korongos rendszerben. Ezt a törzset molekuláris azonosítás után *Chlorella* sp #34-nek neveztük el.

Ezt követően, három kiválasztott törzzsel, *Chlorella* sp. #34, *C. sorokiniana* CCAP 211/8K és *H. pluvialis* SAG 44.96 végeztünk kitapadási teszteket, 17 különböző anyag (PET, PS, PP és PI) és borítás (polielektrolit oldatok rétege, negatív vagy pozitív külső töltés) kombinációjával. A vizsgálatok legfőbb célja az volt, hogy elemezzük a forgatás hatását a sejtek kitapadására és a kapcsolatot a különböző összetételű és töltésű anyagok és a biofilm képzés között. Az így kapott eredmények nem adtak további információt erről a kapcsolatról, viszont néhány felületet és borítást ki lehetett zárni a további vizsgálatokból.

Az újonnan kifejlesztett Algadisk rendszer fő eleme a forgó, vertikális elhelyezésű korongok, melyek félig a tápoldatba merülnek. Ezen laboratóriumi rendszert az általunk izolált *Chlorella* sp #34 törzzsel teszteltük, annak érdekében, hogy megvizsgáljuk a tartósságát, hatékonyságát, emellett megfigyeljük, hogy milyen valószínűséggel fordulnak elő fertőzések, és/vagy mechanikai/kémiai hibák. A *Chlorella* sp #34 törzs biomassza hozamát változatos körülmények között tanulmányoztuk, úgy mint alacsony és magas fényintenzitáson, illetve optimalizált tápoldaton és műtrágyán. A rendszer hosszú távú (90 nap), folyamatos üzemeltetése, komoly mechanikai (a korongok forgásának leállása) vagy kémia (pH ingadozások) probléma és fertőzés nélkül kivitelezhető volt. Az elért biomassza hozamok összevethetőek más szuszpendált és biofilm alapú rendszerek eredményeivel. A legmagasabb értéket, 3 g $(m^2\ nap)^{-1}$, egy 7 napos növekedési szakasz után mértük, míg a fény mennyiségére vonatkoztatott biomassza hozam 0.9 g mol^{-1} volt . A már korábban említett kiugróan magas biomassza sűrűsége a biofilm rendszereknek itt is megfigyelhető volt, bizonyos esetekben elérte a 200 g kg^{-1}-t, ami centrifugált biomassza sűrűségéhez hasonló. A magasabb fényintenzitás és a műtrágya használata negatívan befolyásolta a hatékonyságot, habár úgy gondoljuk, hogy további vizsgálatok és fejlesztések után ezek az értéket is megközelíthetik a korábbi eredményeket.

A kiválasztott *Chlorella* sp #34 törzs zsírsav termelését is nyomon követtük a labor méretű Algadisk kísérletek során (alacsony, magas fényintenzitás, gazdag tápoldat, műtrágya), tekintettel a biodízel felhasználásra. Továbbá egy két-lépcsős rendszerben is vizsgáltuk a N hiány hatását a teljes lipid tartalomra. . A lipid tartalom 3 és 7 % között ingadozott a növekedési

kísérletek során, míg a N limitált rendszer esetében ezt az érték 10%-ra nőtt. A zsírsavak összetétele megfelelne a biodízel előállítási feltételeinek, vagyis magas volt a közepes lánchosszúságú, egyszeresen vagy kétszeresen telítetlen zsírsavak aránya, viszont a zsírsav hozam 110 mg $(m^2\ nap)^{-1}$ alatt maradt, ami nem elegendő nagyüzemi termeléshez. Ezen kívül, a szüretelés után a biofilm nem nőtt vissza a korongokon, ami komoly akadályt jelent folyamatos működtetés esetén. A biodízel célú felhasználás mellett, az értékes többszörösen telítetlen zsírsavakat is meghatároztuk a biomasszában, úgy mint EPA, DHA, arachidonsav, α- és γ-linolénsav. A legtöbb esetben kimutathatóak voltak a biomasszában, noha annyira kis mennyiségben (0.01%-a a száraztömegnek), ami alkalmatlanná teszi a *Chlorella* sp #34-et a termelésükre.

Egy másik biotechnológiai potenciállal rendelkező mikroalga törzs is a biofilm alapú termesztés központjában áll, nevezetesen a *H. pluvialis*. A *H. pluvialist* intenzíven kutatják az asztaxantin termelésének köszönhetően. Ennek a pigmentnek az előállítása és felhalmozása a sejtekben valamilyen külső, kedvezőtlen körülmény hatására indul el, mint például a magas fényintenzitás, tápanyag hiány, megemelkedett só tartalom vagy szerves szénforrások jelenléte. Számos folyadékalapú rendszer ismert az alga termesztésére és az asztaxantin termelés optimalizálására.

Az általunk elvégzett vizsgálatok alapján elmondhatjuk, hogy a *H. pluvialis* nem alkalmas az Algadisk rendszerben való növesztésre. Ezek alapján azt feltételezzük, hogy a módszer nem megfelelő minden mikroalgatörzs termelésére. Másrészről egy eltérő megközelítés, a Twin Layer rendszer megfelelőnek bizonyult a *H. pluvialis* termesztésére, ezen kívül a biofilm lehetővé tette, hogy magas fényintenzitás mellett se csökkenjen le a sejtek osztódása. Ezt bizonyítja, hogy a legmagasabb biomassza hozamot (6 g $(m^2\ nap)^{-1}$) a legmagasabb használt fényintenzitás esetén értük el. Ezt követően a különböző stressz faktorok hatását vizsgáltuk, ahol is a N mentes tápoldat hétszer magasabb asztaxantin értéket produkált, mint a többi stressz faktor (NaCl eltérő koncentrációban, 0.05%, 0.2% és 0.4%). A pigment produktivitása is jelentős, 300 mg $(m^2\ nap)^{-1}$, ami más publikált adatokhoz hasonló és megközelíti a piacképes értékeket.

A biofilm alapú technológiák ígéretes helyettesítői a szuszpendált rendszereknek, köszönhetően a költéshatékonyságuknak, egyszerűségüknek és stabilitásuknak. A doktori dolgozat fő célja az volt, hogy teszteljük az újonnan kifejlesztett Algadisk reaktort laboratóriumi körülmények között, egy erre a célra izolált és szelektált mikroalgával és megvizsgáljuk a növekedési és zsírsavtermelési képességeit. Ezen kívül, hogy összehasonlítsuk más hasonló rendszerekkel, mint a Twin Layer technológia.

Az Algadisk és Twin Layer rendszerek felépítése és működése jelentősen eltér, ráadásul az Algadisk technológia nagy léptékű, folyamatos működtetése már megtörtént [66]. Ennek során rendszeres növekedési-szüretelési ciklusok valósultak meg; a részben automatizált

szüretelő rendszer és a lépték növelhető, versenyképes korong anyag pedig hozzájárultak a rendszer hatékonyságához. Mindemellett, a pH szinten tartása és CO_2 adagolás is könnyen szabályozható, problémák csak a műtrágyás kísérlet során fordultak elő a megfelelő puffer hiánya miatt. Mind az Algadisk mind a Twin Layer technológiában, hatalmas lehetőségek rejlenek, melyek hatékonyabbá és olcsóbbá tehetik a nagyméretű, mikroalga biomassza termelést. Néhány nehézséget még le kell küzdeni és további vizsgálatok szükségesek, hogy mind a biomassza mind a termeltetni kívánt anyagcsere-termék hozama ideális legyen, figyelembe véve a folyamatos üzemelést. Más mikroalga törzseket (pl. *D. salina, N. oculata*) is érdemes lenne termeszteni ezekben a rendszerekben és/vagy más biotechnológiai felhasználását tanulmányozni a *Chlorella* sp #34-nek, úgy mint keményítő és fehérje termelés. Végül összegezve a tapasztalatainkat, ebben a munkában, nem csak szemléltetni tudtunk a biofilm rendszerek előnyeit, úgymint a magas biomassza denzitás, jobb fényhasznosítás, csökkentett vízigény, megnövelt területi hozam és a stressz faktorok egyszerű hozzáadása a rendszerhez, de bizonyítottuk az újonnan kifejlesztett Algadisk rendszer hatékonyságát és stabilitását is. Illetve, hogy a biofilm alapú növesztés bizonyos esetekben csökkenteni tudja a sejteket érő stressz faktorok hatását is, mint ahogy az a Twin Layer rendszer esetében is történt, ahol a megemelt megvilágítás nem gátolta a *H. pluvialis* sejtek növekedését.

IX. Appendix

Appendix 1

Table 2- Salt composition of the used growth media, Sueoka, A9 and Bold's basal media.

	Sueoka medium (SH)	A9 medium	Bold's basal medium (BBM)
	mM		
Nitrogen			
NH_4Cl	9.348		
CH_4N_2O	13.320		
$NaNO_3$	2.940		
$MgSO_4*7H_2O$	0.081	1.217	0.304
$CaCl_2*2H_2O$	0.068	0.005	0.170
K_2HPO_4	8.268		0.431
KH_2PO_4	5.291	3.865	1.290
Na_2EDTA	0.149	0.168	0.171
$ZnSO_4*7H_2O$	0.077		0.031
H_3BO_3	0.184		0.185
$MnCl_2*4H_2O$	0.026		0.007
$CoCl_2*6H_2O$	0.007		
CuSO4*5H2O	0.006		0.006
$(NH_4)_6Mo_7O_{24}*4H_2O$	0.001		
$FeSO_4*7H_2O$	0.018		0.018
$FeCl_3*6H_2O$		0.071	
$MnSO_4*4H_2O$		0.014	
$ZnSO_4*7H_2O$		0.013	
$CuSO_4*5H_2O$		0.003	
$Na_2B_4O_7*10H_2O$		0.018	
NH_4VO_3		0.008	
$NiCl_2*6H_2O$		0.001	
MoO_3			0.005
$Co(NO_3)_2*6H_2O$			0.002
KOH	0.553		
H_2SO_4	1 mL		

Appendix 2

Table 3- List of sampling point and samples taken during the isolation phase of the work. Strain number refers to the enrichment culture (SH/A9), sample number and the subculture of that sample (a; b; c..). Samples in Italic were isolated during the second isolation phase.

Place of isolation is also listed, along with the major selection criteria such as the presence (+) or absence (-) of lipids after Nile red dying; surface attachment onto the cultivation flasks (+/-). Samples showing both lipid and surface attachment, moreover expressed high growth were analyzed for total lipid content.

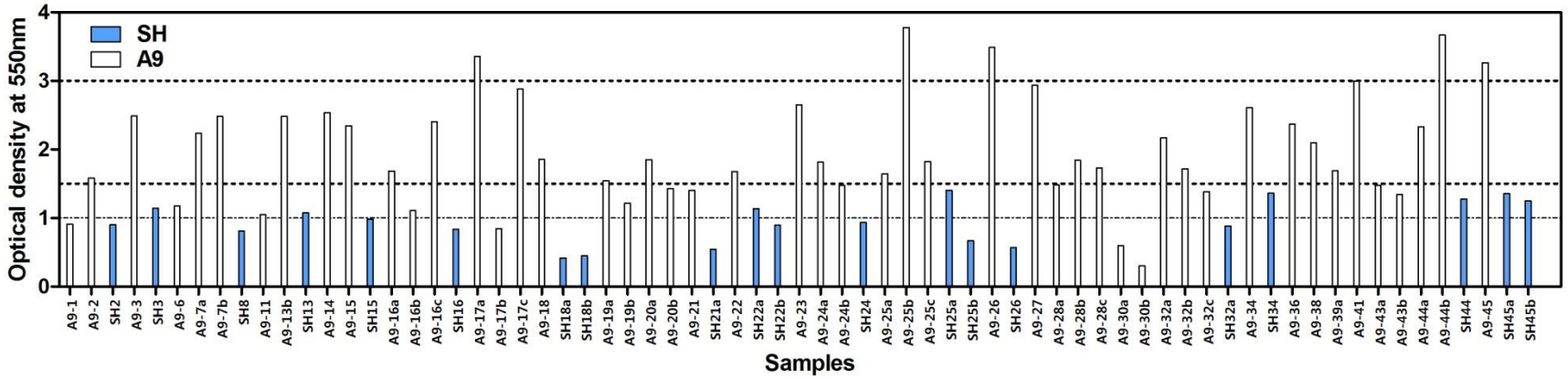
Strain #	Isolation place	Lipid observed after Nile red dying	Attachment to surface	Lipid mg g^{-1} DW
A9-1	Zápor Lake, Szeged, Hungary	+	-	
A9-2	Zápor Lake, Szeged, Hungary	+	+	**26.08**
SH2	Zápor Lake, Szeged, Hungary	+	+	**75.33**
A9-3	Zápor Lake, Szeged, Hungary	+	+	**33.32**
SH3	Zápor Lake, Szeged, Hungary	+	+	**55.02**
A9-6	Buvár Lake, Szeged, Hungary	-	+	
A9-7a	Buvár Lake, Szeged, Hungary	-	+	
A9-7b	Buvár Lake, Szeged, Hungary	-	+	
SH8	Buvár Lake, Szeged, Hungary	+	-	
A9-11	Új Téli kikötő, Szeged, Hungary	+	+	
A9-13b	Új Téli kikötő, Szeged, Hungary	-	+	
SH13	Új Téli kikötő, Szeged, Hungary	+	+	**20.30**
A9-14	Holt-Maros, Szeged, Hungary	-	+	
A9-15	Holt-Maros, Szeged, Hungary	+	+	**48.46**
SH15	Holt-Maros, Szeged, Hungary	-	-	
A9-16a	Zápor Lake, Szeged, Hungary - geotextile	-	-	**33.45**
A9-16b	Zápor Lake, Szeged, Hungary - geotextile	+	+	**25.91**
A9-16c	Zápor Lake, Szeged, Hungary - geotextile	-	+	
SH16	Zápor Lake, Szeged, Hungary - geotextile	+	-	
A9-17a	Zápor Lake, Szeged, Hungary - PET	-	+	
A9-17b	Zápor Lake, Szeged, Hungary - PET	-	-	
A9-17c	Zápor Lake, Szeged, Hungary - PET	-	-	
A9-18	Zápor Lake, Szeged, Hungary - PC	+	+	**35.06**
SH18a	Zápor Lake, Szeged, Hungary - PC	-	-	
SH18b	Zápor Lake, Szeged, Hungary - PC	-	-	
A9-19a	Vértó, Szeged, Hungary - geotextile	+	+	
A9-19b	Vértó, Szeged, Hungary - geotextile	-	+	
A9-20a	Vértó, Szeged, Hungary - PVC	-	+	
A9-20b	Vértó, Szeged, Hungary - PVC	-	+	
A9-21	Vértó, Szeged, Hungary - PET	+	+	**24.75**
SH21a	Vértó, Szeged, Hungary - PET	+	-	
A9-22	Szentmihály, Fehérpart-Szilvás, Hungary- geotextile	-	+	
SH22a	Szentmihály, Fehérpart-Szilvás, Hungary- geotextile	+	-	
SH22b	Szentmihály, Fehérpart-Szilvás, Hungary- geotextile	-	-	
A9-23	Szentmihály, Fehérpart-Szilvás, Hungary - PC	+	+	**36.13**
A9-24a	Szentmihály, Fehérpart-Szilvás, Hungary - PC	+	+	**83.54**

A9-24b	Szentmihály, Fehérpart-Szilvás, Hungary - PC	+	+	**53.99**
SH24	Szentmihály, Fehérpart-Szilvás, Hungary - PC	+	-	
A9-25a	Új Téli kikötő, Szeged, Hungary- PET	+	+	**34.17**
A9-25b	Új Téli kikötő, Szeged, Hungary- PET	+	+	**19.58**
A9-25c	Új Téli kikötő, Szeged, Hungary- PET	+	-	
SH25a	Új Téli kikötő, Szeged, Hungary- PET	+	+	**40.35**
SH25b	Új Téli kikötő, Szeged, Hungary- PET	-	-	
A9-26	Holt-Maros, Szeged, Hungary - geotextile	-	+	
SH26	Holt-Maros, Szeged, Hungary - geotextile	-	-	
A9-27	Holt-Maros, Szeged, Hungary -PVC	-	+	
A9-28a	Holt-Maros, Szeged, Hungary -PET	-	+	
A9-28b	Holt-Maros, Szeged, Hungary -PET	-	+	
A9-28c	Holt-Maros, Szeged, Hungary -PET	-	+	
A9-30a	Komárom, Hungary	+	-	
A9-30b	Komárom, Hungary	+	-	
A9-32a	Lough Leane, Ireland	-	+	
A9-32b	Lough Leane, Ireland	+	+	
A9-32c	Lough Leane, Ireland	+	+	
SH32a	Lough Leane, Ireland	+	+	
A9-34	Maros, Makó, Hungary	-	+	
SH34	Maros, Makó, Hungary	+	+	**149.54**
A9-36	Tápé-Télikikötő, Szeged, Hungary	+	+	
A9-38	Fancsika tavak, Debrecen, Hungary	-	-	
A9-39a	Fancsika tavak, Debrecen, Hungary	+	+	
A9-41	Vekeri-tó, Debrecen, Hungary	-	+	
A9-43a	Bányató, Algyő,Hungary	+	+	
A9-43b	Bányató, Algyő,Hungary	-	-	
A9-44a	Berettyó, Szeghalom, Hungary	-	+	
A9-44b	Berettyó, Szeghalom, Hungary	-	+	
SH44	Berettyó, Szeghalom, Hungary	-	-	
A9-45	Berettyó, Újfalu, Hungary	+	+	
SH45a	Berettyó, Újfalu, Hungary	+	-	
SH45b	Berettyó, Újfalu, Hungary	+	-	
A9-63	Baden-baden, Germany	+	+	**32.78**
SH-63	Baden-baden, Germany	+	+	**13.37**
A9-64	Baden-baden, Germany	+	-	
A9-66	Bad Wildbad, Germany	+	-	
SH-66	Bad Wildbad, Germany	+	+	**34.75**
A9-67	Bad Buchau, Germany	-	+	
SH-67	Bad Buchau, Germany	-	+	
A9-69	Bad Buchau, Germany	+	+	**34.81**
SH-69	Bad Buchau, Germany	+	+	**41.82**
A9-70	Bad Saulgau, Germany	+	-	
SH-70	Bad Saulgau, Germany	+	+	**36.33**
A9-73	Bad Saulgau, Germany	-	+	
SH-73	Bad Saulgau, Germany	-	+	
SH-74	Bad Saulgau, Germany	+	-	
A9-76	Heilingenkreuz, Austria	+	+	**39.92**
SH-76	Heilingenkreuz, Austria	+	+	**54.35**
SH-78	Baden bei Wien, Austria	-	+	
A9-82	Kehidakustány, Hungary	+	+	**42.4**
SH-82	Kehidakustány, Hungary	+	+	**65.6**
A9-84	Hévíz, Hungary	+	-	
SH-84	Hévíz, Hungary	+	-	

A9-85	Hévíz, Hungary	+	+	**43.86**
SH-85	Hévíz, Hungary	+	-	
A9-87	Hévíz, Hungary	+	+	**41.39**
SH-87	Hévíz, Hungary	+	-	
A9-88	Hévíz, Hungary	+	+	**21.65**
SH-88	Hévíz, Hungary	+	-	

Appendix 3

Figure 8- Final optical density (14 days) of isolated samples in the first isolation phase growing at 23°C under 150 µmol photons $(m^2s)^{-1}$ light intensity. Blue columns represents samples growing in SH medium, white columns were growing in A9 medium. Dashed lines show OD_{550nm} at 1; 1.5 and 3.



Appendix 4

List of the alignment results of the molecular identification of SH-34 sample

Locus version	Reference	Fitting
GQ122327.1	Chlorella sorokiniana isolate BE1 18S ribosomal RNA gene, partial sequence	96% 0.097%
X62441.2	Chlorella sorokiniana 18S rRNA gene, strain SAG 211-8k	96% 0.097%
FM205860.1	Chlorella sorokiniana 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain CCALA 260	96% 0.097%
FM205859.1	Chlorella sorokiniana 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain CCAP 211/8K	96% 0.097%
FM205834.1	Chlorella sorokiniana 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain SAG 211-8k	96% 0.097%
AB260898.1	Chlorella sp. IFRPD 1018 genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, partial and complete sequence	96% 0.097%
AB260897.1	Chlorella sp. IFRPD 1014 genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, partial and complete sequence	96% 0.097%
AM423162.1	Chlorella sorokiniana 18S rRNA gene, strain UTEX 2805	96% 0.097%
AB080308.1	Chlorella vulgaris gene for 18S rRNA, partial sequence	96% 0.097%
AB080307.1	Chlorella sorokiniana gene for 18S rRNA, partial sequence	96% 0.097%
JF767012.1	Chlorella sp. NMX37N 18S ribosomal RNA gene, partial sequence	96% 0.097%
FM205884.1	Actinastrum hantzschii 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain CCAP 200/3	96% 0.097%
FM205877.1	Micractinium sp. CCAP 211/11F 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain CCAP 211/11F	96% 0.097%
FM205861.1	Chlorella sp. CCAP 211/90 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain CCAP 211/90	96% 0.097%
EU402596.1	Chlorella sorokiniana strain GXNN 01 18S ribosomal RNA gene, partial sequence	95% 0.097%
AY197628.1	Chlorella sp. NDem 9/21 T-13d 18S ribosomal RNA gene, partial sequence	96% 0.097%
AY197624.1	Chlorella sp. MDL4-1 18S ribosomal RNA gene, partial sequence	96% 0.097%
AY591515.1	Chlorella vulgaris strain CCAP 211/11F 18S ribosomal RNA gene, complete sequence	96% 0.097%
X72854.1	Chlorella sp. (Ssh) gene for ribosomal RNA, small subunit	96% 0.097%
X72706.1	Chlorella sp. (Esh) gene for ribosomal RNA, small subunit	96% 0.097%
AY323840.1	Didymogenes palatina 18S ribosomal RNA gene, partial sequence	96% 0.097%
FR865695.1	Micractinium sp. CCAP 248/2 genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, culture collection CCAP 248/2	96% 0.097%
JF834706.1	Chlorella sorokiniana strain MIC-G5 18S ribosomal RNA gene, partial sequence	96% 0.097%
FJ946885.1	Chlorella sp. VI4 18S ribosomal RNA gene, partial sequence	96% 0.097%
FJ946884.1	Chlorella sp. VI11 18S ribosomal RNA gene, partial sequence	96% 0.097%
FJ946883.1	Chlorella sp. VI2 18S ribosomal RNA gene, partial sequence	96% 0.097%
FM205876.1	Micractinium sp. CCAP 248/2 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain CCAP 248/2	96% 0.097%
FM205875.1	Micractinium pusillum 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain CCAP 248/3	96% 0.097%
FM205874.1	Micractinium pusillum 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain CCAP 248/1	96% 0.097%
FM205838.1	Micractinium sp. SAG 48.93 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain SAG 48.93	96% 0.097%
DQ377324.1	Chlorella sp. NJ-18 18S ribosomal RNA gene, partial sequence	96% 0.097%

AB058372.1	Chlorella sp. MBIC10595 gene for 18S rRNA, partial sequence	96% 0.097%
AF364102.1	Micractinium pusillum strain SAG 48.93 18S ribosomal RNA gene, partial sequence	96% 0.097%
X74001.1	Chlorella sorokiniana 18S rRNA gene, strain Prag A14	96% 0.097%
FN298923.1	Chlorella variabilis 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain CCAP 211/84	95% 0.097%
FM205849.1	Chlorella variabilis 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain SAG 211-6	95% 0.097%
AB260893.1	Chlorella variabilis gene for 18S ribosomal RNA, partial sequence	95% 0.097%
DQ057341.1	Chlorella sp. NC64A 18S ribosomal RNA gene, partial sequence	95% 0.097%
AB219527.1	Chlorella sp. MRBG1 genes for 18S rRNA, internal transcribed spacer 1, 5.8S rRNA, internal transcribed spacer 2, partial and complete sequence	95% 0.097%
AB206550.1	Chlorella variabilis genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, partial and complete sequence, strain: Syngen 2-3	95% 0.097%
AB206549.1	Chlorella variabilis genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, partial and complete sequence	95% 0.097%
AB206546.1	Uncultured Chlorella genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, partial and complete sequence, isolated from Paramecium bursaria Cs2	95% 0.097%
AB191207.1	Chlorella vulgaris genes for 18S rRNA, ITS1, 5.8S rRNA, strain:takaP-3-A2	95% 0.097%
AB191206.1	Chlorella vulgaris genes for 18S rRNA, ITS1, 5.8S rRNA, strain:shiP-7-A4	95% 0.097%
AB191205.1	Chlorella vulgaris genes for 18S rRNA, ITS1, 5.8S rRNA, strain:HB2-2-1	95% 0.097%
AB162917.1	Chlorella variabilis genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, strain: Bnd1-ZK	95% 0.097%
AB162916.1	Chlorella variabilis genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, strain: Dd1-ZK	95% 0.097%
AB162915.1	Chlorella variabilis genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, strain: KM2-ZK	95% 0.097%
AB162914.1	Chlorella variabilis genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, strain: F36-ZK	95% 0.097%
AB162913.1	Chlorella variabilis genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, strain: So13-ZK	95% 0.097%
AB162912.1	Chlorella variabilis genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, strain: OK1-ZK	95% 0.097%
FR865678.1	'Chlorella' luteoviridis genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, culture collection CCAP 211/5B	96% 0.097%
GQ867590.1	Hindakia tetrachotoma strain CCAP 222/69 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	95% 0.097%
GQ507371.1	Dictyosphaerium sp. CB 2008/108 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	95% 0.097%
GQ507369.1	Dictyosphaerium sp. CCAP 222/9 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	96% 0.097%
FM205881.1	Micractinium sp. CCAP 248/14 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain CCAP 248/14	96% 0.097%
FM205873.1	Micractinium pusillum 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain CCAP 248/15	96% 0.097%
FM205872.1	Micractinium pusillum 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain CCAP 248/6	96% 0.097%
FM205871.1	Micractinium pusillum 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain CCAP 248/12	96% 0.097%
FM205870.1	Micractinium pusillum 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain CCAP 248/10	96% 0.097%
FM205869.1	Micractinium pusillum 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain CCAP 248/9	96% 0.097%
FM205868.1	Micractinium pusillum 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain CCAP 248/4	96% 0.097%
FM205866.1	Micractinium pusillum 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain SAG 13.81	96% 0.097%
FM205865.1	Micractinium sp. CCAP 248/13 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain CCAP 248/13	96% 0.097%
FM205864.1	Micractinium sp. CCAP 248/16 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain CCAP 248/16	96% 0.097%

FM205840.1	Didymogenes palatina 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain SAG 30.92	95% 0.097%
FM205836.1	Micractinium pusillum 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain CCAP 248/5	96% 0.097%
FM205835.1	Micractinium sp. CCAP 248/7 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain CCAP 248/7	96% 0.097%
AM231740.1	Micractinium pusillum partial 18S rRNA gene, strain CCAP 248/7	96% 0.097%
AM231739.1	Micractinium pusillum partial 18S rRNA gene, strain CCAP 248/6	96% 0.097%
AM231737.1	Micractinium pusillum partial 18S rRNA gene, strain CCAP 248/4	96% 0.097%
AM231738.1	Micractinium pusillum partial 18S rRNA gene, strain CCAP 248/5	96% 0.097%
AY195980.1	Chlorella sp. JL 2-5 18S ribosomal RNA gene, partial sequence	96% 0.097%
AF237662.1	Micractinium pusillum 18S ribosomal RNA gene, partial sequence	96% 0.097%
AF364101.1	Micractinium pusillum strain SAG 13.81 18S ribosomal RNA gene, partial sequence	96% 0.097%
X72707.1	Chlorella sp. (HvT) gene for ribosomal RNA, small subunit	96% 0.097%
FM205879.1	Micractinium sp. TP-2008a 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain SAG 42.98	96% 0.097%
DQ377321.1	Chlorella sp. FACHB31 18S ribosomal RNA gene, partial sequence	96% 0.097%
AY197620.1	Chlorella sp. Mary 9/21 BT-10w 18S ribosomal RNA gene, partial sequence	96% 0.097%
AY323837.1	Diacanthos belenophorus 18S ribosomal RNA gene, partial sequence	96% 0.097%
FJ946889.1	Chlorella sp. EO5-4C 18S ribosomal RNA gene, partial sequence	95% 0.097%
FJ946886.1	Chlorella sp. WO10-1 18S ribosomal RNA gene, partial sequence	95% 0.097%
JN090876.1	Uncultured eukaryote clone KRL01E16 18S ribosomal RNA gene, partial sequence	96% 0.097%
HQ191364.1	Uncultured Chlorophyta clone PA2009C7 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	96% 0.097%
FM205867.1	Micractinium pusillum 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain CCAP 248/11	96% 0.097%
FM205878.1	Micractinium sp. CCAP 231/1 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain CCAP 231/1	96% 0.097%
FM205833.1	Lobosphaeropsis lobophora 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain SAG 37.88	96% 0.097%
FR865683.1	Chlorella vulgaris genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, culture collection CCAP 211/79	95% 0.097%
DQ377322.1	Chlorella sp. YEL 18S ribosomal RNA gene, partial sequence	96% 0.097%
AY323838.1	Dictyosphaerium pulchellum 18S ribosomal RNA gene, partial sequence	96% 0.097%
FM205880.1	Micractinium sp. TP-2008a 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain CCAP 271/1	96% 0.097%
X63504.1	Chlorella lobophora 18S rRNA gene, strain Andreyeva 750-I	96% 0.097%
FR865697.1	Neochloris aquatica genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, culture collection CCAP 254/5	96% 0.097%
FR865696.1	Marvania coccoides genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, culture collection CCAP 251/1A	96% 0.097%
FR865682.1	Chlorella vulgaris genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, culture collection CCAP 211/74	96% 0.097%
FR865660.1	Chlorella vulgaris genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, culture collection CCAP 211/11S	96% 0.097%
FR865589.1	Chlamydomonas chlamydogama genomic DNA containing 18S rRNA gene, ITS1, culture collection CCAP 11/48B	96% 0.097%
FN298918.1	Chlorella vulgaris 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain CCAP 211/110	96% 0.097%
FN298917.1	Chlorella vulgaris 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain CCAP 211/109	96% 0.097%
FM205856.1	Chlorella sp. ACOI 856 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain ACOI 856	96% 0.097%

Appendix 5

Table 5- Average FA content of biofilm from each harvest of the laboratory scaled Algadisk reactor, under the following conditions: **low light** irradiance; **high light** irradiance with **PVC** and **PP** disks; and **Agroleaf** fertilizer as growth medium. During the **stress** induced lipid production since no regrowth was observed after the harvest, samples were taken on day 34; 44 and 55. Lipid content is presented as mg FAME g^{-1} DW biomass. The fatty acids are given with their common name, CAS-number and lipid numbers. Standard deviation (±) was calculated from biofilm from at least 3 disks.

	Exp	Low light						High light PVC						High light PP					
	Harvest #	4		5		6		1		2		3		1		2		3	
	mg g^{-1}	AV	±	AV	±	AV	±	AV	±	AV	±	AV	±	AV	±	AV	±	AV	±
Caproic acid 142-62-1	C6:0	0.003	0.004	0.003	0.001	0.003	0.001	0.012	0.023	0.023	0.027	0.037	0.025	0.013	0.027	0.036	0.019	0.047	0.002
Caprilyc acid 124-07-2	C8:0	0.001	0.002	0.004	0.001	0.005	0.001	0.005	0.006	0.029	0.037	0.029	0.036	0.006	0.007	0.024	0.027	0.025	0.037
Capric acid 334-48-5	C10:0	0.006	0.008	0.011	0.001	0.009	0.001	0.035	0.069	0.035	0.069	0.145	0.004	0.106	0.071	0.068	0.070	0.134	0.006
Undecanoic acid 112-37-8	C11:0	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.021	0.041	0.021	0.042	0.043	0.044	0.041	0.047
Lauric acid 43-07-7	C12:0	0.035	0.001	0.048	0.013	0.040	0.002	0.072	0.104	0.082	0.103	0.082	0.100	0.070	0.102	0.130	0.102	0.127	0.111
Tridecanoic acid 638-53-9	C13:0	0.002	0.003	0.003	0.001	0.001	0.002	0.003	0.005	0.005	0.005	0.011	0.002	0.007	0.005	0.008	0.004	0.008	0.005
Myristic acid 544-63-8	C14:0	0.178	0.009	0.193	0.033	0.163	0.009	0.286	0.179	0.223	0.007	0.219	0.010	0.154	0.103	0.224	0.009	0.217	0.011
Myristoleic acid 544-64-9	C14:1	0.092	0.016	0.089	0.121	0.083	0.030	0.800	0.242	1.028	0.119	0.789	0.054	0.499	0.333	0.925	0.073	0.809	0.124
Pentadecanoic acid 1002-84-2	C15:0	0.052	0.002	0.050	0.015	0.032	0.002	0.077	0.081	0.042	0.018	0.045	0.010	0.029	0.023	0.054	0.017	0.054	0.017
Pentadecenoic acid 84743-29-3	C15:1	0.330	0.010	0.442	0.074	0.322	0.028	1.064	0.205	1.111	0.114	0.865	0.245	1.781	1.623	1.212	0.208	0.845	0.157
Palmitic acid 57-10-3	C16:0	11.556	0.724	12.772	2.280	10.444	0.469	11.071	2.601	12.810	1.014	10.914	1.386	8.442	4.233	12.897	1.182	11.026	1.395
Palmitoleatic acid 373-49-9	C16:1	0.782	0.093	0.614	0.126	1.870	0.032	1.294	0.615	2.451	1.687	0.438	0.195	1.185	0.728	2.233	0.982	2.602	1.547
Heptadecanoic acid 506-12-7	C17:0	0.057	0.019	0.072	0.011	0.031	0.043	0.067	0.045	0.064	0.043	0.084	0.023	0.084	0.005	0.088	0.006	0.085	0.005
Heptadecenoic acid 29743-97-3	C17:1	1.679	0.132	3.119	0.520	1.687	0.095	4.340	0.320	5.094	0.543	3.490	0.514	3.430	2.293	5.049	0.707	3.485	0.444
Stearic acid 57-11-14	C18:0	0.081	0.096	0.229	0.048	0.104	0.021	0.367	0.245	0.251	0.067	0.199	0.053	0.217	0.159	0.312	0.090	0.211	0.048

Elaidic acid 1937-62-8	**C18:1 trans**							1.17	0.098	2.57	0.265	2.42	0.735	1.12	0.758	2.78	0.398	2.86	0.729
Oleic acid 112-80-1	**C18:1 cis**	4.148	0.528	2.823	1.585	4.234	0.163	2.57	0.438	5.03	0.464	5.17	1.202	2.09	1.454	5.52	0.665	5.69	1.260
Linolelaidic acid 506-21-8	**C18:2**	0.000	0.000	0.008	0.011	0.000	0.000	0.091	0.114	0.134	0.139	0.164	0.158	0.342	0.617	0.135	0.135	0.168	0.112
Linoleic acid 60-33-3	**C18:2 (n-6)**	14.126	1.050	14.992	1.684	12.774	0.583	10.910	1.699	11.851	0.834	9.871	1.210	7.791	5.197	11.528	0.634	10.001	1.325
γ-Linolenic acid 506-26-3	**C18:3 (n-6)**							0.017	0.028	0.013	0.026	0.008	0.016	0.000	0.000	0.000	0.000	0.000	0.000
α-Linolenic acid 463-40-1	**C18:3 (n-3)**	4.882	0.428	7.665	1.001	4.658	0.199	11.862	0.627	12.658	0.847	9.411	1.165	9.349	5.148	12.552	1.648	9.215	0.616
Arachidic acid 506-30-9	**C20:0**	0.038	0.054	0.043	0.018	0.028	0.000	0.072	0.092	0.087	0.086	0.033	0.022	0.021	0.024	0.022	0.023	0.071	0.087
Gondoic acid 5561-99-9	**C20:1**							0.109	0.212	0.002	0.002	0.002	0.002	0.002	0.002	0.006	0.006	0.004	0.004
Eicosadienoic acid 5598-38-9	**C20:2**	0.012	0.017	0.002	0.003	0.006	0.008	0.013	0.015	0.007	0.014	0.003	0.004	0.023	0.007	0.012	0.009	0.005	0.005
Heneicosanionic acid 2363-71-5	**C21:0**							0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
DGLA 1783-84-2	**C20:3 (n-6)**							0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Arachidonic acid 506-32-1	**C20:4 (n-6)**							0.001	0.001	0.021	0.043	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Eicosatrienoic acid 17046-5-2	**C20:3 (n-3)**	0.000	0.000	0.024	0.033	0.000	0.000	0.000	0.001	0.000	0.000	0.001	0.002	0.002	0.003	0.000	0.000	0.000	0.000
EPA 10417-94-4	**C20:5 (n-3)**	0.000	0.000	0.072	0.040	0.000	0.000	0.035	0.027	0.000	0.000	0.000	0.000	0.101	0.124	0.000	0.000	0.011	0.023
Behenic acid 112-85-6	**C22:0**	0.026	0.037	0.000	0.000	0.002	0.003	0.013	0.015	0.014	0.016	0.032	0.001	0.020	0.013	0.028	0.003	0.034	0.000
Erucic acid 112-86-7	**C22:1**							0.004	0.004	0.004	0.005	0.015	0.004	0.004	0.005	0.000	0.001	0.003	0.003
13.16-Z-Docosadienoic acid	**C23:2**							0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Tricosanoic acid 2433-96-7	**C:23**							0.000	0.000	0.000	0.000	0.008	0.006	0.001	0.001	0.000	0.004	0.007	0.003
Lignoceric acid 557-59-5	**C24:0**	0.057	0.080	0.081	0.072	0.109	0.003	0.000	0.000	0.242	0.162	0.000	0.000	0.084	0.168	0.178	0.184	0.166	0.192
DHA 6217-54-5	**C22:6 (n-3)**							0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Nervonic acid 506-37-6	**C24:1**							0.004	0.003	0.001	0.003	0.007	0.006	0.002	0.002	0.002	0.003	0.003	0.004

Exp.		Agroleaf												Stress					
Harvest #		1		2		3		4		5		6		Sampling points	1		2		3	
		AV	±	AV	±	AV	±	AV	±	AV	±	AV	±	AV	±	AV	±	AV	±
Caproic acid 142-62-1	C6:0	0.014	0.002	0.013	0.004	0.030	0.046	0.063	0.102	0.014	0.004	0.257	0.013	0.017	0.428	0.016	0.003	0.014	0.011
Caprilyc acid 124-07-2	C8:0	0.019	0.002	0.017	0.005	0.021	0.010	0.066	0.126	0.015	0.021	0.032	0.020	0.009	0.002	0.010	0.004	0.010	0.001
Capric acid 334-48-5	C10:0	0.017	0.001	0.061	0.114	0.093	0.132	0.101	0.138	0.401	0.014	0.168	0.138	0.006	0.000	0.009	0.001	0.013	0.001
Undecanoic acid 112-37-8	C11:0	0.008	0.006	0.006	0.006	0.007	0.008	0.009	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Lauric acid 143-07-7	C12:0	0.034	0.003	0.037	0.005	0.134	0.177	0.340	0.154	0.153	0.003	0.618	0.292	0.033	0.007	0.056	0.011	0.082	0.004
Tridecanoic acid 638-53-9	C13:0	0.015	0.002	0.012	0.006	0.036	0.076	0.012	0.006	0.123	0.004	0.062	0.066	0.000	0.000	0.000	0.000	0.000	0.000
Myristic acid 544-63-8	C14:0	0.245	0.070	0.282	0.143	0.420	0.118	0.475	0.119	0.459	0.041	1.424	0.085	0.286	0.005	0.539	0.043	0.900	0.046
Myristoleic acid 544-64-9	C14:1	0.103	0.016	0.268	0.156	0.791	0.370	0.836	0.515	0.000	0.000	0.025	0.047	0.824	0.131	0.551	0.121	0.270	0.163
Pentadecanoic acid 1002-84-2	C15:0	0.096	0.027	0.085	0.068	0.151	0.062	0.209	0.010	0.000	0.000	0.221	0.196	0.082	0.010	0.099	0.010	0.130	0.005
Pentadecenoic acid 84743-29-3	C15:1	0.016	0.011	0.190	0.391	0.362	0.151	0.465	0.162	0.040	0.046	0.172	0.141	0.764	0.026	0.671	0.038	0.565	0.224
Palmitic acid 57-10-3	C16:0	11.170	0.655	15.698	1.571	15.600	0.947	18.901	1.444	0.993	0.128	2.263	1.514	14.198	0.341	18.400	0.817	23.531	0.335
Palmitoleatic acid 373-49-9	C16:1	0.355	0.433	3.296	1.881	2.894	1.325	2.469	1.434	0.000	0.000	0.000	0.000	1.942	0.546	3.652	0.789	5.475	0.742
Heptadecanoic acid 506-12-7	C17:0	0.096	0.025	0.144	0.105	0.149	0.098	0.233	0.086	0.035	0.078	0.000	0.000	0.146	0.007	0.258	0.022	0.455	0.026
Heptadecenoic acid 29743-97-3	C17:1	1.820	0.471	2.825	0.225	2.758	0.397	3.013	0.713	0.000	0.000	0.013	0.023	2.766	0.086	2.535	0.285	2.211	0.218
Stearic acid 57-11-14	C18:0	0.247	0.107	0.486	0.324	0.502	0.306	0.668	0.266	0.274	0.005	0.264	0.027	0.436	0.018	2.339	0.420	6.387	0.225
Elaidic acid 1937-62-8	C18:1 trans	0.252	0.259	0.376	0.995	0.489	1.243	0.192	0.238	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Oleic acid 112-80-1	C18:1 cis	3.287	0.232	5.966	0.429	6.827	0.702	5.507	0.532	0.000	0.000	0.000	0.000	8.544	0.235	15.151	1.023	21.814	0.334
Linolelaidic acid 506-21-8	C18:2	0.082	0.083	0.015	0.039	0.015	0.042	0.120	0.186	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.215	0.429

Linoleic acid 60-33-3	**C18:2 (n-6)**	13.843	0.845	18.564	2.949	18.708	1.512	21.391	2.034	2.128	0.483	2.945	0.649	14.463	0.991	17.653	0.604	23.253	0.332
γ-Linolenic acid 506-26-3	**C18:3 (n-6)**	0.089	0.151	0.250	0.253	0.203	0.165	0.185	0.197	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
α-Linolenic acid 463-40-1	**C18:3 (n-3)**	4.678	1.432	9.127	2.076	8.776	2.125	9.935	3.163	0.000	0.000	0.000	0.000	8.526	0.515	8.346	0.875	8.146	0.414
Arachidic acid 506-30-9	**C20:0**	0.041	0.010	0.061	0.005	0.058	0.008	0.092	0.007	0.000	0.000	0.000	0.000	0.059	0.008	0.207	0.034	0.322	0.053
Gondoic acid 5561-99-9	**C20:1**	0.015	0.008	0.020	0.014	0.026	0.008	0.120	0.098	0.000	0.000	0.000	0.000	0.012	0.023	0.085	0.009	0.132	0.035
Eicosadienoic acid 5598-38-9	**C20:2**	0.026	0.015	0.028	0.013	0.026	0.013	0.092	0.189	0.000	0.000	0.000	0.000	0.047	0.019	0.073	0.011	0.066	0.045
Heneicosanionic acid 2363-71-5	**C21:0**	0.009	0.010	0.003	0.007	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.001
DGLA 1783-84-2	**C20:3 (n-6)**	0.000	0.000	0.000	0.000	0.000	0.000	0.004	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Arachidonic acid 506-32-1	**C20:4 (n-6)**	0.315	0.099	0.000	0.000	0.002	0.006	0.014	0.031	0.000	0.000	0.000	0.000	0.000	0.000	0.006	0.012	0.000	0.000
Eicosatrienoic acid 17046-5-2	**C20:3 (n-3)**	0.000	0.000	0.005	0.014	0.000	0.000	0.006	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.003
EPA 10417-94-4	**C20:5 (n-3)**	0.093	0.029	0.016	0.028	0.039	0.033	0.056	0.011	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Behenic acid 112-85-6	**C22:0**	0.063	0.004	0.068	0.008	0.070	0.017	0.081	0.007	0.000	0.000	0.000	0.000	0.045	0.014	0.111	0.014	0.224	0.020
Erucic acid 112-86-7	**C22:1**	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.002	0.009	0.008	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
13.16-Z-Docosadienoic acid	**C23:2**	0.000	0.000	0.000	0.000	0.003	0.009	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Tricosanoic acid 2433-96-7	**C:23**	0.018	0.012	0.019	0.013	0.021	0.015	0.039	0.005	0.000	0.000	0.000	0.000	0.000	0.000	0.004	0.008	0.032	0.028
Lignoceric acid 557-59-5	**C24:0**	0.144	0.029	0.127	0.012	0.129	0.019	0.136	0.006	0.000	0.000	0.000	0.000	0.248	0.016	0.299	0.030	0.450	0.015
DHA 6217-54-5	**C22:6 (n-3)**	0.640	0.288	0.000	0.000	0.000	0.000	0.004	0.012	0.000	0.000	0.000	0.000						
Nervonic acid 506-37-6	**C24:1**	0.285	0.098	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000						