Zurich University of Applied Sciences
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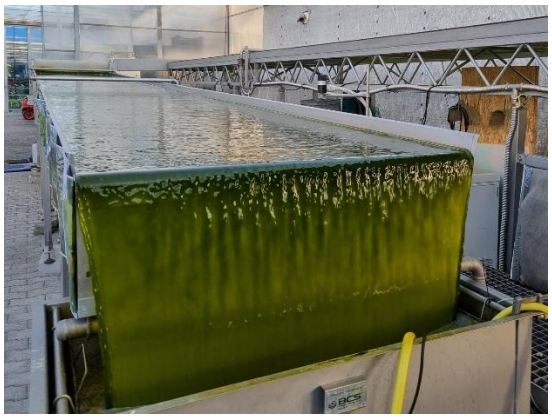

Cultivation of the carotenoid-producing microalgae *Chromochloris zofingiensis* at laboratory and pilot scale

Master's Thesis

by

Vozelj Pia

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Supervisors:

Dr. Dominik Refardt, Zurich University of Applied Sciences, Institute of Natural Resource Sciences

Sophia Egloff, Zurich University of Applied Sciences, Institute of Natural Resource Sciences

Dr. Tjaša Griessler Bulc, University of Ljubljana, Facility of Health Sciences

Abstract

The cultivation of microalgae is a promising way of obtaining natural carotenoids. However, their production is not yet cost-effectively sufficient to compete with current synthetic methods. To overcome crucial techno-economic challenges, studies are being conducted for selection of the ideal species with superior characteristics. One of the leading candidates is *Chromochloris zofingiensis*. Due to fast growth rate, robustness and tolerance to adverse culture conditions, it has the potential for commercial production. To make that happen, the cultivation needs to be scaled up in an open system. With this study, the feasibility of outdoor cultivation of *Chromochloris zofingiensis* was tested. Different media, nitrogen sources and media concentrations were investigated on a laboratory scale. Threefold BBM+3N achieved highest growth and was used for cultivation on an open thin-layer photobioreactor. Cultivation was proven to be successful, achieving high biomass density (11.75 g L^{-1}) and biomass productivity (1.02 g L^{-1} d^{-1}). Microalgal culture showed promising results, all while withstanding fluctuating outdoor conditions. The final amount of accumulated astaxanthin was 3.63 mg/g. With a laboratory experiment astaxanthin production was compared in BBM+3N and stressful conditions (high salinity and reduced nitrogen availability) after achieving high biomass. The combination of high salinity and nitrogen reduction (4.48 mg/g) enabled microalgae to produce more astaxanthin than the control (3.56 mg/g).

Acknowledgements

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1 INTRODUCTION

Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) is a valuable keta-carotenoid belonging to the xanthophyll family of red-orange pigments (Higuera-Ciapara et al., 2006). It has numerous applications in pharmaceutics, nutraceutics, cosmetics, aquaculture feed and functional food industries (Tang et al., 2020). One of the most abundant sources of natural astaxanthin are microalgae (Ambati et al., 2014). They are a diverse group of photosynthetic unicellular microorganisms that are biotechnologically interesting due to their ability to produce high-value components, including carotenoids, lipids, proteins and polysaccharides (Koren et al., 2021; Rahimi & Jazini, 2021).

There are beneficial health effects associated with astaxanthin consumption (Brendler & Williamson, 2019). Similarly like in microalgae and plants, the pigments play a protective role in the human body (Ambati et al., 2014; Gong & Bassi, 2016). Their antioxidant activity gives them the ability to protect the organism against a wide range of ailments, including cardiovascular problems, cancer and immunological problems (Higuera-Ciapara et al., 2006). Currently, the global astaxanthin market is dominated by synthetic derivates of petrochemical origin, which raises concerns related to food safety, potential toxicity, environmental pollution and sustainability (Hamed, 2016). Meanwhile natural astaxanthin has been approved for human consumption and has a superior antioxidant capacity in comparison to the synthetic derivates (Brendler & Williamson, 2019; EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2014). The increasing concerns for consumer safety and regulatory issues regarding the introduction of synthetic chemicals into the human food chain, further enhance interest in astaxanthin deriving from natural sources. With recognition of astaxanthin's value and bigger inclination towards natural astaxanthin, this promotes large-scale cultivation of microalgae that produce it (EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2014; Guedes et al., 2011).

Chromochloris zofingiensis, formerly known as *Chlorella zofingiensis* (Fučíková & Lewis, 2012), is one of the most promising carotenogenic chlorophytes (Liu et al., 2014). Besides its ability to produce astaxanthin, it can grow to high biomass densities and can survive under adverse environmental conditions (Liu et al., 2014). *Haematococcus pluvialis* has been known as the leading microalgal producer of astaxanthin (Kim et al., 2018), however, in recent years, *C. zofingiensis* has become recognized as a potential alternative (Liu et al., 2014). Albeit *H. pluvialis* accumulates the highest level of astaxanthin in nature (1.9-7% of dry weight) (Koopmann et al., 2022; Torzillo et al., 2003) and has more intracellular astaxanthin than *C. zofingiensis* (0.2-0.7 %) (Liu et al., 2014; Minyuk et al., 2020; Nishshanka et al., 2021), the

disadvantage may be compensated with mass cultivation. *C. zofingiensis* has a better tolerance to adverse culture conditions (including fluctuating temperatures, changeable weather conditions, biological contaminations, high salinity, high light irradiation and shear stresses), a higher growth rate, robustness for high cell densities, higher lipid productivity and better extractability of the target compounds due to a more fragile cell wall (Chen et al., 2017; Huo et al., 2018; Mao et al., 2018; Minyuk et al., 2020). These biotechnological traits provide *C. zofingiensis* with high potential to be a more suitable and commercially promising candidate for mass astaxanthin production than *H. pluvialis* (Liu et al., 2014; Sun et al., 2019).

C. zofingiensis is capable of meeting demands for multiple bioproducts (lipids, carbohydrates), yet the astaxanthin productivity does not meet the requirements necessary for commercial production (Chen et al., 2020). Currently, a viable production of natural pigments at a low-cost processing technology is unavailable (Rodrigues et al., 2014). Before this route can be exploited in an economical and environmentally sustainable manner, more research and optimization needs to happen (Osundeko et al., 2019). Outdoor cultivation utilizing natural sunlight for cultivation is an effective and sustainable way to reduce the overall cost of microalgal production and to allow mass cultivation (Borowitzka & Moheimani, 2013). While some sunlight is converted into chemical energy through photosynthesis, the majority of it is transformed into heat, thus increasing the temperature of the cultivation (Bhosale, 2004). *C. zofingiensis* can grow in the range of 20 °C to 30 °C, while the optimal temperature for the high astaxanthin yield is 25 °C to 30 °C (Ip, 2017; Liu et al., 2014). Furthermore, temperatures above 20 °C can help microalgae achieve high biomass productivity (Feng et al., 2011). Open cultivations in summer can experience temperature fluctuations in the range of 15 °C to 35 °C (Egloff et al., 2018; Mariotto, 2022; Pulgarin et al., 2021). There are few studies on cultivation of *C. zofingiensis* in open systems (Feng et al., 2011; Huo et al., 2018; Yuan et al., 2013). To our knowledge, there are only two studies where *C. zofingiensis* was cultivated outdoors in high temperature conditions, where temperatures reached up to 34-37 °C (Huo et al., 2018; Yuan et al., 2013). Since higher temperatures result in higher biomass, all while having access to natural resources, further studies on cultivation of *C. zofingiensis* in open systems are in the interest of commercializing its production (Gorgich et al., 2021). However, there is more risk associated with outdoor cultivations due to microalgae having to adapt to fluctuations of the surrounding environmental conditions. Apart from this, microalgae are more prone to contamination with protozoa, bacteria and fungi in open systems. Yet studies report *C. zofingiensis* is robust enough to withstand biological contamination, as well as adapt to outdoor environment (Huo et al., 2018).

A concern related to the cultivation of *C. zofingiensis* is the controlled accumulation of astaxanthin. The amount of carotenoid accumulation is related to the environment and

cultivation conditions (Ren et al., 2021). Carotenoids in microalgae are responsible for protecting algal cells against oxidative damage imposed by environmental conditions (Gorgich et al., 2021). With adequate culture conditions (pH, light, temperature) and nutrient availability, less carotenoids are being produced (Ren et al., 2021). Meanwhile adverse stresses, such as high salinity, high light and nutrient deficiency are key factors that trigger defence mechanisms of microalgae, which start accumulating secondary metabolites to survive (Bar et al., 1995; Li et al., 2009; Ren et al., 2021). Studies show that after a successful cultivation, microalgae can be exposed to stressful conditions to stimuli higher production of astaxanthin (Kou et al., 2020; Mao et al., 2018; Mulders et al., 2015).

The aim of the thesis was to investigate microalgal growth and astaxanthin production of *C. zofingiensis* on an open thin-layer photobioreactor using mineral medium. To find the optimal medium, growth of *C. zofingiensis* was observed in different treatments on a laboratory scale. Nitrate, ammonium and urea were used as nitrogen sources for cultivation. Simultaneously, two different media were compared: Bold's Basal Medium with triple nitrogen (CCAP, 2020) and another mineral medium by Doucha & Lívanský (2014). Furthermore, microalgal growth was observed in different concentrations of BBM+3N. Threefold BBM+3N was used in the outdoor experiment on an open thin-layer photobioreactor. Additionally, a laboratory experiment was conducted to observe astaxanthin production in microalgae in high salinity and reduced nitrogen availability conditions.

2 MATERIAL AND METHODS

2.1 Algal strain and preculture growth conditions

Chromochloris zofingiensis SAG 211-14 was obtained from the Culture Collection of Algae at Göttingen University (SAG).

Precultures were maintained in Bold's Basal Medium with 3-fold nitrogen (BBM+3N) containing: $NaNO_3$ (750 mg/L), $CaCl_2 \cdot 2H_20$ (25 mg/L), $MgSO_4 \cdot 7H_2O$ (75 mg/L), K_2HPO_4 (75 mg/L), KH_2PO_4 (175 mg/L), NaCl (25 mg/L), Na_2EDTA (4.5 mg/L), $FeCl_3 \cdot 6H_20$ (0.582 mg/L), $MnCl_2 \cdot 4H_2O$ (0.246 mg/L), $ZnCl_2$ (0.03 mg/L), $CoCl_2 \cdot 6H_2O$ (0.012 mg/L) and $Na_2MoO_4 \cdot 2H_2O$ (0.024 mg/L). Precultures were incubated at 20 °C, 2.0 % CO_2, continuous orbital shaking at 120 rpm and illumination of 121 µmol m^{-2} s^{-1} (7544 lux) (Multitron, Infors HT, Bottmingen, Switzerland). pH of every medium was adjusted 7.2 ± 0.1 with 1 M NaOH and media were autoclaved at 121 °C for 2 h prior to use.

2.2 Cultivation of *Chromochloris zofingiensis* on a laboratory scale using different media, nitrogen sources and media concentrations

Two laboratory experiments were carried out to determine the optimal mineral medium and its concentration.

In the first experiment, *Chromochloris zofingiensis* was cultivated in two different media: BBM+3N and another mineral medium by Doucha & Lívanský (2014). Due to the medium not having a name, we will refer to it as Doucha's Medium (DM for abbreviation). Treatments, which also differed by the source of nitrogen, were the following: i) BBM+3N with sodium nitrate (BBM+3N), ii) BBM+3N modification with ammonium chloride ($BBM+3N_{NH4Cl}$), iii) DM with potassium nitrate (DM_{KNO3}) and iv) DM with urea (DM_{UREA}) (see supplementary material S 1). To yield 1 g L^{-1} microalgal biomass, approximately 84.9 mg L^{-1} of nitrogen is needed (Doucha & Lívanský, 2006). All media were adjusted to contain an equimolar amount of nitrogen, which allowed microalgal growth up to 1.44 g L^{-1}.

In the second experiment, microalgae were cultivated in differently concentrated BBM+3N. Besides BBM+3N, which served as a control, two modifications were used that were twofold and threefold concentrated.

All cultivations were carried out in 100-mL Erlenmeyer flasks filled up to 60 mL with the respective mineral medium and each treatment was replicated three times. The initial concentration of cultures was 10^5 microalgal cells per mL. The experiments lasted 21 days.

Nutrients were added only at the beginning and the concentration of total nitrogen in the medium was followed weekly with photometric test kits (Hach-Lange, Rheineck, Switzerland). Sampling was done three times per week (Monday, Wednesday, Friday), at the same time the pH was measured and adjusted to 7.2 ± 0.2 with NaOH (0.5 M and 0.1 M). Incubator settings were the same as mentioned above.

2.3 The effect of high-salinity and reduced nitrogen availability on accumulation of astaxanthin in *Chromochloris zofingiensis*

Growth of *Chromochloris zofingiensis* and its astaxanthin content were observed under both high salinity and reduced nitrogen availability in a fully factorial experiment: i) control (BBM+3N), ii) high salinity (BBM+3N, 0.2 M NaCl), iii) reduced nitrogen availability (BBM), and iv) reduced nitrogen availability and high salinity (BBM, 0.2 M NaCl).

Before the experiment, microalgae were grown in BBM+3N, until they reached cell density of approximately $3 \cdot 10^8$ cells mL^{-1}. Cultures were then pooled, centrifuged for 5 minutes at 7197 rfc and resuspended in equal volumes of fresh media (i.-iv.). Each treatment was replicated three times.

Cell count and optical density were measured three times per week (Monday, Wednesday, Friday). pH was measured in the beginning and at the end of the experiment. Samples for astaxanthin analysis were taken only at the end of the experiment and samples from each treatment were pooled to obtain sufficient biomass for the analysis.

2.4 Cultivation of *Chromochloris zofingiensis* on an open thin-layer photobioreactor

The cultivation was carried out in an open thin-layer photobioreactor (18 m^2, 200 L, Fig. S 1), located in a greenhouse on Grüental campus of the Zürich University of Applied Sciences in Wädenswil, Switzerland. The photobioreactor has an inclined glass surface supported by a steel frame, on which the microalgal suspension circulates. The culture is captured in a tank and then pumped up with a centrifugal pump.

Based on the results of the laboratory experiments, threefold BBM+3N was chosen for cultivation of *Chromochloris zofingiensis*. The medium was prepared by filling the tank with deionised water (200 L) and adding dry nutrients of the mineral medium. Mineral medium was added in multiple batches (day 0, 14 and 21, respectively). This allowed a theoretical

microalgal growth up to 17.28 g/L. The nutrients were added based on the reached microalgal biomass and availability of nutrients in the medium.

A preculture was grown in the incubator with BBM+3N and then inoculated (18 L of preculture with OD_{750} of 0.63) on the open thin-layer photobioreactor. Pumping speed was chosen to obtain a liquid layer of 6 mm on the photobioreactor. CO_2 was not supplied in the first five days due to a technical issue. After, the partial pressure of CO_2 in the medium was set to 5 mbar during the day and turned off during the night. From day 10 on, the partial pressure was set to 10 mbar non-stop. At the start of the cultivation the pH was 6.90. In the first 12 days, the pH was not adjusted. Later, it was automatically adjusted with a pH controller to 7.3 ± 0.1 (pH Controller, Bluelab, Tauranga, New Zealand), by adding 1 M HCl. The water volume of the culture was kept constant with automatically supplied desalted water.

The cultivation took place from May 20th to June 24th 2022. Throughout the cultivation, the following metrics were determined every sampling day (Monday, Wednesday, Friday): optical density, cell count, Fv/Fm value and number of protozoa. Once the dry weight reached 1 g/L, it was also measured every sampling day. Concentration of nitrogen compounds and number of aerobic mesophilic bacteria were analysed once a week. Samples for CHN and astaxanthin analysis were taken at the end of the experiment.

2.5 Analytical methods

Growth of the microalgal suspension was monitored with three methods. Cell count was determined by light microscopy (phase contrast, 400-fold magnification) with a haemocytometer with Thoma ruling. Optical density was measured at 750 nm in a 96-well microtiter plate in a volume of 200 µL with four technical replicates with an automated plate reader (Infinite 200 Pro Tecan, Männedorf, Switzerland). Prior to dry weight analysis, the sample was centrifuged for 5 minutes at 7197 rfc. Dry weight was determined with moisture analyser (HB43-S-Halogen Moisture Analyzer, Mettler Toledo, Greifensee, Switzerland) from a 40 mL sample. To describe microalgal growth, average productivity and specific growth rate were calculated for dry weight and cell density (see supplementary material S 3).

Photosynthetic activity was followed with Pulse-Amplitude-Modulation Fluorometry (Mini-PAM-II, Photosynthesis Yield Analyzer, Walz, Effeltrich, Germany). The sample (400 µL) was kept in the dark until analysis.

Concentrations of the nitrogen compounds (total nitrogen, nitrate, nitrite and ammonium) in the water samples were measured with photometric test kits (Hach-Lange, Rheineck,

Switzerland). Prior to analysis, the samples were centrifuged for 5 minutes at 7197 rfc, then supernatant was filtered (0.45 µm).

The CHN content was determined with thermal conductivity and infrared spectroscopy (TruSpec Micro CHN, Leco Instruments Ltd., Mississauga, Canada). Prior to the analysis, the sample was washed and dried.

The results of the CHN analysis, dry weight, measured concentration of total nitrogen in the medium and the known nitrogen supply, allowed calculation of nitrogen mass balance. The nitrogen mass balance was calculated using the following equation:

$$N_{lost}\ (mg/L) = N_{supplied} - (Dry\ Weight\ (mg/L) \cdot N_{CHN\ analysis}\ (\%)) - N_{medium}\ (mg/L)$$

Astaxanthin content was determined with UV-VIS spectrometry, measured at a wavelength of 477 nm. A sample of 1-mL was obtained and centrifuged at 11'100 rcf. The dried algal biomass samples (200-250 mg) were then analysed.

Presence of contaminants (bacteria, protozoa, or other green microalgae) was quantified. Aerobic mesophilic bacteria (CFU/mL) were counted by plating a 180-µL sample and dilutions thereof on plate count agar (PCA) plates. The plates were incubated in the dark for two days at room temperature. Protozoa were identified using light microscopy (phase contrast, 200-fold magnification) with a haemocytometer with Fuchs-Rosenthal ruling and other green algae with haemocytometer with Thoma ruling.

To confirm the presence of *Chromochloris zofingiensis* on laboratory- and pilot-scale cultivations, DNA sequencing was performed. Samples (30-100 µL) were frozen in liquid nitrogen and homogenized with a 5 mm steel bead for 30 s at 30 Hz in a bead beating mill (Tissuelyser II, Qiagen, Germany). This was repeated twice for every sample. Genomic DNA was extracted with a commercial kit (NucleoSpin® Plant II, Macherey-Nagel, Düren, Germany), by following the instructions of the manufacturer. Polymerase chain reaction (PCR) was carried in 25-µL reaction volumes using the KAPA2G Robust PCR kit (Roche Sequencing), 1 µL of sample and 0.5 µM forward and reverse primer (Tab. S 5). Amplifications included an initial denaturation at 95 °C for 3 min and 35 cycles of denaturation at 95 °C for 15 s, annealing 58 °C for 15 s and extension at 72 °C for 45 s. Amplifications were visualized by gel electrophoresis using gel red. Selected samples were purified with a commercial kit (Gel and PCR Clean-up NucleoSpin®, Macherey-Nagel). The prepared DNA samples were sent to a sequencing service (Microsynth AG, Balgach, Switzerland) for Sanger sequencing. Results were checked against the NCBI database with the BLAST algorithm.

Temperature, pH and turbidity on the open thin-layer photobioreactor were measured with built-in sensors (InPro 3253i SG/120, Mettler Toledo). Photosynthetically active photon flux density (PPFD, µmol s^{-1} m^{-1}) was measured with two PAR sensors (SKL2620, Skye Instruments Ltd., Powys, United Kingdom), placed above and below the cultivation platform. The number of absorbed photons was calculated as the difference between upper and lower PAR sensor. Calculation for absorbed solar energy and energy conversion ratio were calculated with equations made by Mariotto (Mariotto, 2022). In laboratory experiments, pH was measured manually (HQ40d Hach).

2.6 Data Analysis

Statistical analysis and data visualisation was performed with R (version 4.0.5) in Rstudio (version 2021.09.1).

Growth data from laboratory experiments were compared in different media and media concentrations, respectively. Growth results of optical density and cell count were analysed. The data is presented with mean and standard error of the mean. The significance level was set at $p < 0.05$. If assumptions for homogeneity of variance and normal distribution of the residuals were met, One-Way ANOVA was performed. If the required assumptions for an ANOVA weren't met, the Kruskal-Wallis test was performed.

The results of the experiment on the open thin-layer photobioreactor weren't statistically analysed since there was only one trial with no replicates. Same applies to laboratory experiment with high salinity and reduced nitrogen availability conditions, where few samples for astaxanthin analysis were available.

3 RESULTS

3.1 Cultivation of *Chromochloris zofingiensis* on a laboratory scale using different media and nitrogen sources

Cultivation of *Chromochloris zofingiensis* was successful in BBM+3N, DM_{KNO3} and DM_{UREA} (Fig. 1). Results indicate that both BBM and DM are suitable media, especially when using nitrates as a source of nitrogen. There were notable differences in growth in different treatments reported by cell count ($F = 2.67$, $p < 0.05$), in contrast to the observations made with optical density ($\chi^2 = 3.32$, $df = 3$, $p = 0.34$). The highest average productivity and maximum cell density was obtained with BBM+3N, followed by DM_{KNO3} and DM_{UREA} (Tab. 1). pH differed between the treatments ($F = 23.97$, $p < 0.001$). Initially, pH dropped in all treatments, making frequent adjustment necessary (Fig. S 3). Growth media also differed in terms of pH stability during the course of the experiment. BBM+3N required less addition of base (1-2 drops of 0.5 M NaOH, approx. 50-100 µL) than DM treatments (5-15 drops of 0.1 M NaOH, approx. 250-750 µL) on days 3, 5 and 7, respectively. BBM+3N experienced the least fluctuations in reaching the desired value (7.2 ± 0.2). This can be attributed to the potassium-phosphate buffer system that BBM+3N contains. $BBM+3N_{NH4Cl}$ was excluded as a possible nutrient source due to discoloration of the culture and unmaintainable pH.

Table 1: Growth of microalgae in different media and nitrogen sources

Medium	Nitrogen source	Average productivity (cells mL^{-1})		Maximum cell density (cells mL^{-1})		Highest specific growth rate (cells mL^{-1} d^{-1})	
		M	SD	M	SD	M	SD
BBM+3N	$NaNO_3$	$11.98 \cdot 10^6$	$0.91 \cdot 10^6$	$2.52 \cdot 10^8$	$1.92 \cdot 10^7$	0.99	0.03
	NH_4Cl	$6.90 \cdot 10^6$	$1.56 \cdot 10^6$	$1.45 \cdot 10^8$	$3.27 \cdot 10^7$	0.97	0.07
DM	KNO_3	$10.31 \cdot 10^6$	$1.26 \cdot 10^6$	$2.44 \cdot 10^8$	$4.41 \cdot 10^7$	0.97	0.08
	Urea	$6.60 \cdot 10^6$	$1.24 \cdot 10^6$	$1.40 \cdot 10^8$	$2.61 \cdot 10^7$	0.88	0.08

Microalgal cultures differed in colour (Fig. S 4). Ammonium chloride experimental treatment had an orange-brownish colour, while others had a murky green colour that slightly differed in the shade.

To summarize, the most successful treatment was BBM+3N. Due to successful cultivations and maintainable pH, it was chosen as the medium for cultivation on the photobioreactor and for laboratory experiment where different nutrient concentrations were compared.

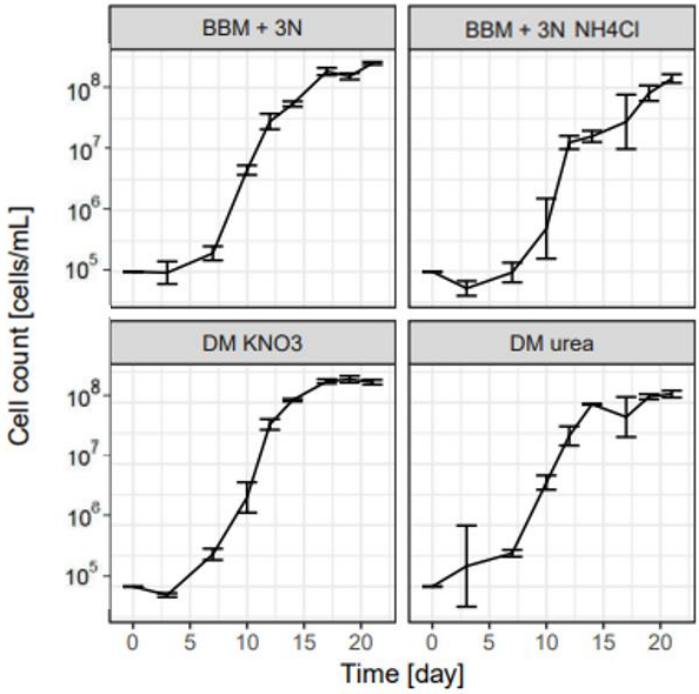

Figure 1: Microalgal growth in different media and nitrogen sources on a laboratory scale observed with cell count. Microalgal growth in different treatments is visualized with mean values of the replicates and standard error of the mean.

3.2 Cultivation of *Chromochloris zofingiensis* on a laboratory scale using different BBM+3N concentrations

Microalgae were successfully cultivated in all concentrations of BBM+3N (Fig. S 5, Fig. S 6). As expected, increasing the concentration of the medium resulted in higher cell density of microalgae. However, no significant differences between the growth of microalgae could be attributed to the concentration of the medium, neither with cell count (χ^2 = 2.00, df = 2, p = 0.37), nor with optical density (χ^2 = 0.70, df = 2, p = 0.71). The highest average productivity and maximum achieved cell density can be attributed to the threefold medium, followed by twofold and onefold medium (Tab. 2). Highest specific growth rate was in threefold treatments, followed by onefold and twofold. While the ranking of highest specific growth rate doesn't correlate to the supplied nutrient concentration, we don't find significant differences between the treatments. The pH value didn't differ between the media (F = 2.40, p < 0.10). The pH is lowest on the second day in all experimental treatments and by 7th day, it stabilizes (Fig. S 7).

Table 2: Growth of microalgae in different BBM+3N concentrations

BBM+3N	Average productivity (cells mL^{-1})		Maximum achieved cell density (cells mL^{-1})		Highest specific growth rate (cells mL^{-1} d^{-1})	
	M	SD	M	SD	M	SD
1-fold	$1.48 \cdot 10^7$	$1.58 \cdot 10^6$	$3.12 \cdot 10^8$	$3.32 \cdot 10^7$	0.78	0.09
2-fold	$2.06 \cdot 10^7$	$2.73 \cdot 10^6$	$4.33 \cdot 10^8$	$5.73 \cdot 10^7$	0.69	0.18
3-fold	$2.78 \cdot 10^7$	$3.18 \cdot 10^6$	$5.83 \cdot 10^8$	$6.68 \cdot 10^7$	0.91	0.14

Differences were visible in the colour of the cultures, suggesting that the physiology of the microalgal cells was influenced by the concentration of nutrients. Onefold BBM+3N cultivation had a murky green colour, while others with higher concentrations had a deep green colour (Fig. S 8).

Cultivation of *Chromochloris zofingiensis* in different concentrations of BBM+3N showed little effects on growth, yet the treatments contained similar amount of nitrogen at the end. The initial media concentrations varied according to the amount of supplied nitrogen (143 mg/L, 268 mg/L and 376 mg/L). While the ranking is still according to the initial concentrations, the differences between final concentrations were little (1.43 ± 0.12 mg/L, 2.52 ± 0.14 mg/L and 2.89 ± 0.23 mg/L). The initial concentration differed by approximately onefold between the treatments (SD = 95.21 mg/L), while the final concentrations had less deviation (SD = 0.64 mg/L).

3.3 The effect of high-salinity and reduced nitrogen on accumulation of astaxanthin in *Chromochloris zofingiensis*

Different treatments resulted in differences in the average astaxanthin content. Cultures that were exposed to two stress factors simultaneously (high salinity and reduced nitrogen availability), showed highest astaxanthin accumulation (4.48 mg/g), followed by high salinity, reduced nitrogen and BBM+3N (4.31 mg/g, 4.02 mg/g and 3.56 mg/g). Results show that the more stress the microalgae undergo, the higher the accumulation of astaxanthin.

The starting pH was comparable (7.16 ± 0.03), however through the experiment it changed depending on nitrogen availability. At the end of the experiment, pH increased to 7.68 in nitrogen treatments and decreased to 6.56 in reduced nitrogen availability treatments. Both pH values still present adequate environments for microalgae.

Colour differed between the treatments (Fig. 2). Control had a murky green colour, while high salinity treatment had more of a brown undertone. Both reduced nitrogen conditions had an orange colour.

Stress-inducing stimuli had an effect on the microalgal growth. Initially, growth continued for 4 days in all treatments (Fig. S 9). Following that, the number of microalgae became stationary in the control, while in stress conditions it decreased rapidly and then became stationary as well.

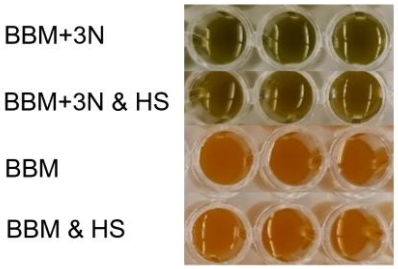

Figure 2: Appearance of treatments of the laboratory experiment investigating astaxanthin accumulation. The control and its replicates (BBM+3N) are in the first row, high salinity treatment (BBM+3N & HS) in second, nitrogen reduction treatment (BBM) in third and nitrogen reduction and high salinity (BBM & HS) in fourth.

3.4 Cultivation of *Chromochloris zofingiensis* on the open thin-layer photobioreactor using threefold BBM+3N

Cultivation of *Chromochloris zofingiensis* on the open thin-layer photobioreactor using threefold BBM+3N was successful. Cultivation lasted for 35 days and reached a high biomass density (11.75 g L^{-1}). Highest biomass productivity was 1.02 g L^{-1} d^{-1} and average productivity was 0.429 g L^{-1} d^{-1}. Highest achieved growth rate was 0.19 g L^{-1} d^{-1}. When growth patterns, noted by cell count, showed gradual transition into linear growth, the experiment was ended (Fig. S 10). Meanwhile optical density (Fig. 3) and dry weight (Fig. S 11) continued to rise, however more gradually than previously observed.

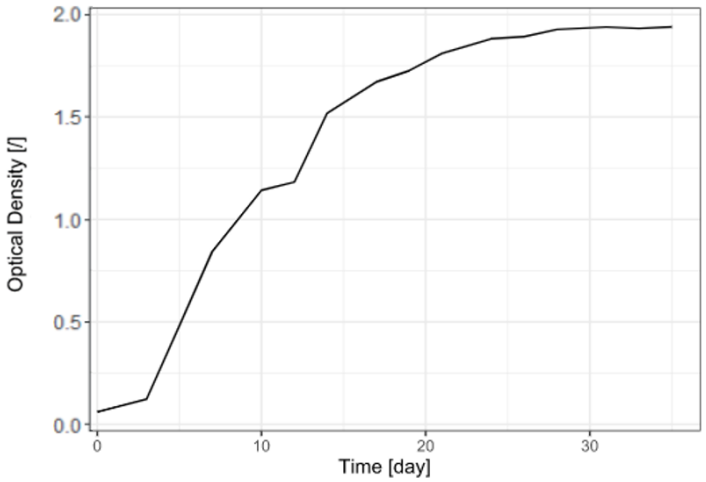

Figure 3: Microalgal growth on the open thin-layer photobioreactor observed with optical density at 750 nm

There was correlation between solar input, temperature, evaporation and microalgal biomass. Pearson's correlation coefficient reveals a distinct correlation between solar input and microalgal biomass (Pearson's r = 0.93), thus concluding higher solar input results in higher microalgal biomass. Temperature has a smaller but still notable correlation with biomass (Pearson's r = 0.74). In relation to the produced biomass, the average energy conversion ratio was 0.54 kWh g^{-1}. Throughout the cultivation, the average daily solar input was 1.51 kWh m^{-2}. Solar energy is partially transformed into heat, consequently increasing the temperature of the cultivation (Pearson's r = 0.79). With higher temperature of the cultivation, this results in more evaporation (Pearson's r = 0.83). Meanwhile Fv/Fm value didn't produce any meaningful results (Fig. S 12) and showed no correlation to the growth of the microalgae, available solar energy, daily evaporation and average daily temperature.

Growing microalgal culture and exposure to outdoor environment caused fluctuation of physico-chemical parameters. Temperature of the cultivation fluctuated from 10.8 °C to 37.8 °C, with the average temperature of 21.91 °C (Fig. S 13). Cultivation took place in a greenhouse in the summer, which caused the temperature to rise high during the day and cool off during the night. The state of the culture was also influenced by the pH, which fluctuated from 6.90 to 8.70 (Fig. S 14). As microalgae began growing, pH started to rise. Day and night fluctuations between day 5 and 10 can be explained by the setting of CO_2 supply. Once the CO_2 was supplied nonstop, the fluctuations of pH were smaller. When the pH was not controlled, its average value was 7.62 and it reached the peak value of 8.70. The rise in the pH coincided with microalgae starting to aggregate, sediment and discolour. Once the pH was maintained at an average of 7.42, microalgae were able to recover after the stress-inducing stimuli and continue to grow.

The nitrogen mass balance was calculated for the cultivation on the photobioreactor. This was conducted twice, at highest achieved growth (day 26) and at the end of the experiment (day 35). Microalgae have assimilated 61.03 % and 76.37 % of the supplied nitrogen, 22.47 % and 9.44 % remained in the medium, while 14.19 % and 16.50 % was lost into the atmosphere by day 26 and day 35, respectively. Out of the analysed nitrogen compounds, nitrate was the most abundant at all times. The amount of available nutrients influenced the colour of the cultivation. When being exposed to a harmful environment, microalgae had a slightly orange-brown undertone and once the stress-inducing stimuli was maintained, the colour turned to murky green. With more nutrients being added, the colour changed to deep green (Fig. 4).

Towards the end of the experiment, the amount of accumulated astaxanthin was observed. By then, microalgae have achieved high biomass density, still had a sufficient amount of nutrients available and weren't exposed to stressful conditions. By day 31 and 35, microalgae have produced 4.21 mg/g and 3.63 mg/g of astaxanthin, respectively.

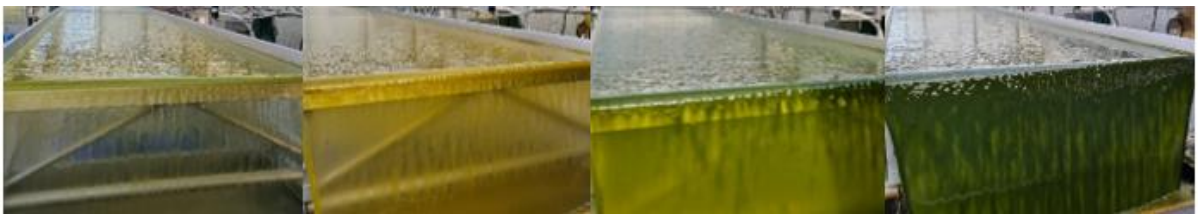

Figure 4: Change of appearance of the cultivation on the photobioreactor, recorded on day 5, 12, 17 and 35. In abundance of nutrients and with high cell density, the colour of the cultivation is deep green.

Enumeration of bacteria and protozoa indicated no deterioration of the culture throughout the cultivation. Protozoa and bacteria were present, meanwhile other green microalgae weren't. Number of bacteria increased throughout the experiment (Fig. S 16). Protozoa number fluctuated, either it was barely present or not detected at all (Fig. S 15). Despite the contaminants, the sequencing at the end of the cultivation on the photobioreactor detected *Chromochloris zofingiensis,* yet we cannot claim it was said microalgae exclusively. Since PCR sequencing is not quantitative, we can claim no *Chromochloris zofingiensis* was lost during the experiments. Furthermore, gel electrophoresis confirmed it to be present in all of the 5 targets and their respective amplicon lengths (see supplementary material S 10). Microscopy also found no evidence of other microalgae based on the cell shape and size. Same applies to all samples taken from laboratory cultivations.

4 DISCUSSION

With controlled cultivations in the laboratory, we were able to show that the choice of nitrogen source, medium and its concentration influences growth of *Chromochloris zofingiensis*. Successful growth was observed in BBM+3N, DM_{KNO3} and DM_{UREA}, meanwhile $BBM+3N_{NH4Cl}$ was excluded as a viable nutrient source. Different BBM+3N concentrations showed little differences. On a laboratory scale, both high salinity and reduced nitrogen availability as well as their combination promoted higher astaxanthin production than the control. Furthermore, the outdoor pilot-scale cultivation on the photobioreactor using threefold BBM+3N was successful.

Laboratory experiment using different media and nitrogen sources, showed that cultivation of *Chromochloris zofingiensis* was successful both in BBM+3N and DM, while among the nitrogen sources nitrates enabled highest growth. *Chromochloris zofingiensis* is mainly cultivated in BBM and BBM+3N (Azaman et al., 2017; Fernando et al., 2021; Mayers et al., 2020; Nishshanka et al., 2022; Rahimi & Jazini, 2021). To our knowledge, there are no other sources reporting usage of DM for cultivation of *Chromochloris zofingiensis*. By comparing BBM+3N and DM, we were able to show DM can also be used for the cultivation, however better results are achieved with BBM+3N. Yet all of the performance can't be attributed to the media, as the four treatments also differed by nitrogen source. Sodium nitrate-fed cultures had highest average productivity and microalgal growth rate, followed by potassium nitrate- and urea- fed cultures. Our results indicate that nitrate is the favourable nitrogen source under the investigated conditions, which is further confirmed by other studies that compared nitrogen sources (Arumugam et al., 2013; Li et al., 2008; Minyuk et al., 2020; Wu et al., 2013). This supports the conclusion that nitrates are the preferable nitrogen source for cultivation of *Chromochloris zofingiensis*. Growth rate was highest in urea-fed cultures in the first 10 days, then it was surpassed by nitrates and showed a considerable difference between the treatments. This finding coincides with another study where urea-fed cultures performed best in the first 2-3 days and were later outperformed by nitrates (Minyuk et al., 2020). However, contrary to our findings, there were little differences between nitrate- and urea-fed cultures (Minyuk et al., 2020). Therefore, our results didn't demonstrate the advantages of using urea as a nitrogen source. This could be attributed to the used medium. In our case, urea was the nitrogen source in DM, whereas in the previously mentioned study it was in BBM. This once again proves microalgae's slight inclination towards BBM+3N's combination of nutrients, rather than DM's. Even though the microalgal growth in urea- and ammonium- fed cultures didn't differ much, both studies excluded $BBM+3N_{NH4Cl}$ as a viable nutrient source due to visible discoloration and unmaintainable pH (Minyuk et al., 2019).

Controlling the pH value by adding NaOH was a key factor for successful growth. The importance of controlling the pH was demonstrated when microalgae were growing, thus causing acidification of the medium. Despite the addition of NaOH, pH still fluctuated in all treatments. In BBM+3N, the K_2HPO_4/KH_2PO_4 molar ratio corresponds to potassium-phosphate buffer, yet the buffer capacity of the medium is still insufficient to maintain the pH in the optimal range for the microalgae (Minyuk et al., 2019). The advantages of the buffer in BBM+3N were however demonstrated when adjusting the pH of the medium. While adjustment of pH in DM treatments was feasible, it showed less stability than BBM+3N treatment. Meanwhile, ammonium caused acidification of the culture to a larger extent and was unmaintainable. The acidification happens due to release of H^+ during the uptake of ammonium by microalgae and with the excess of the ammonium salt, this results in lower pH, thus creating an inhospitable environment for the microalgae to grow in (Minyuk et al., 2019; Morsy et al., 2016; Scherholz & Curtis, 2013).

Based on the results, we have opted for BBM+3N. Evidence suggests BBM+3N with nitrate as a source of nitrogen, was the most promising out of the investigated treatments. Furthermore, the pH didn't fluctuate as much as in other treatments, making it easier to manage and causing less stress to microalgae while adjusting it.

The effect on the growth of microalgae in different concentrations of BBM+3N showed little differences in biomass densities. This coincides with a study stating that nitrate in concentrations from 100 mg/L to 300 mg/L doesn't show significant differences on biomass productivity (Toumi & Politaeva, 2021). The microalgae growth curves in different media had similar tendencies - the lag, exponential and stationary phase coincide between the media (Blair et al., 2014; Toumi & Politaeva, 2021). Higher concentration resulted in higher cell density, which is consistent with the finding that high nitrogen concentration leads to high biomass density (Lin & Lin, 2011). However, the observed differences are dismissible. This is further confirmed with nitrogen mass balance. While CHN analysis wasn't performed in this case, we can estimate the amount of nitrogen loss. Initial nitrogen concentrations differed, yet at the end nitrogen was nearly completely exhausted and equal in all treatments. Furthermore, if we take into account microalgae have a limit of nitrogen uptake and can absorb maximum 10 % of nitrogen, this leaves us nitrogen loss (29 %, 62 %, and 73 %, respectively). With this, we see the higher the concentration of the medium, the higher the loss of nitrogen.

Cultivation of *Chromochloris zofingiensis* on the photobioreactor using threefold BBM+3N was successful. To our knowledge, our cultivation achieved highest produced biomass of *Chromochloris zofingiensis* on an outdoor pilot-scale, which can also be attributed to the duration of the experiment (Corrêa et al., 2022; Feng et al., 2011; Yuan et al., 2013). A study

by Correa et al. (2022) was the most comparable considering temperature, medium and both cultivations taking place outdoor. In their study, *Chromochloris zofingiensis* was cultivated on a pilot-scale in outdoor tubular photobioreactors using BBM (Tab. S 4). The duration of their experiment was 96 days, however it was also done during autumn (September to December). The final biomass density was 0.92 g/L and biomass productivity 0.016 mg L^{-1} d^{-1} (Corrêa et al., 2022). This is considerably lower than our results. Temperature could be a contributing factor to this, as cultures exposed to higher temperatures (above 20 °C) reach higher biomass productivity (Tab. S 4). This was proven by a study conduced in outdoor flat plate photobioreactor using BG-11 (Feng et al., 2011). In the course of 10 days, highest biomass productivity was 0.58 g L^{-1} d^{-1}. In our case, a comparable biomass productivity happened by day 14 (0.51 g L^{-1} d^{-1}). Considering the span of time, Feng et al. (2011) achieved higher biomass productivity faster. By allowing our culture to continue growing longer, microalgae were able to achieve almost twofold higher biomass productivity.

Our results show that *Chromochloris zofingiensis* can withstand temperature fluctuations from 10.8 °C to 37.8 °C and can be successfully cultivated in these conditions. Similarly, a study on outdoor cultivation of *Chromochloris zofingiensis* during the high-temperature season, reported the cultivation was possible in temperatures reaching up to 37 °C (Huo et al., 2018). Another study reported growth of the *Chromochloris zofingiensis* outdoor in the temperature range of 21.5 °C to 34.5 °C (Yuan et al., 2013). There are few studies investigating growth of *Chromochloris zofingiensis* in outdoor high temperature conditions, however the aforementioned studies confirm a successful cultivation is possible.

Chromochloris zofingiensis tolerates pH from 5.5 to 8.5 (Liu et al., 2014) and once this range was exceeded, microalgae started to aggregate. Reason for this was most likely high pH. Production of exopolymeric substance (EPS), which enables adherence of microalgal cells is linked to environmental stressors. Once the conditions of the cultivation started endangering microalgae, aggregation into biofilms occurred via cellular encapsulation into EPS matrix (Schriber & Venable, 2019). pH being the cause behind this is further confirmed by all other environmental conditions remaining the same during the time of aggregation. The full recovery of the microalgae after the stress-inducing stimuli verified their robustness.

High solar input increases temperature of the cultivation and with that enables higher biomass production. Average daily solar input was 1.51 kWh m^{-2}, which was both sufficient for achieving high biomass and not harmful to the microalgae. A study on same photobioreactor cultivating *Chlorella vulgaris* using aquaculture water and pre-processed liquid digestate reported energy conversion ratio of 0.6 kWh m^{-2} and 0.4 kWh m^{-2}, respectively (Mariotto, 2022). While our

results (0.54 kWh m^{-2}) were that of *Chromochloris zofingiensis* cultivated in threefold BBM+3N, a similar efficiency of energy conversion ratio was found.

The main source of nitrogen for the microalgae was nitrate. While the majority was taken up by microalgae (76.37 %), some of it was lost into the atmosphere. Nitrogen mass balance revealed that 16.50 % of the supplied nitrogen was lost. A study conducted on the same photobioreactor with *Chlorella vulgaris* reported a similar loss at 16.60 % (Mariotto, 2022). Since nitrate was the most abundant nitrogen compound, it's likely the explanation for moderate losses in the cultivation. Some loss is expected due to denitrification, a process in which denitrifying bacteria convert inorganic nitrate into N_2 gas (Ramli et al., 2020). Since photobioreactors with greater air surface contact allow more gas exchange to the air, more nitrogen is being released into the atmosphere (Romero-Villegas et al., 2018). While release of N_2 isn't harmful, same can't be said for N_2O, which is a greenhouse gas that can be generated with nitrite reduction to N_2O by microalgae in outdoor cultivation systems (Plouviez et al., 2017). While this process isn't fully understood yet, the estimation of emission is 0.1-0.4 % of the nitrogen input load (Plouviez et al., 2017). A possible mitigation solution is reducing overall losses in nitrogen, which consequently extends to smaller N_2O losses.

Combination of reduced nitrogen availability and high salinity was the ideal condition for astaxanthin production, showing an additive effect between them. Salinity stress can inhibit cell growth and change the shape and structure of microalgal cells (Ermis & Altinbas, 2020), while lower concentration of nitrogen is found to significantly decrease cell count and photosynthetic activity (Dixit et al., 2020). Our results show stress-inducing conditions enabled microalgal growth to continue for 4 day and after, treatments experienced rapid decrease in cell count, followed by stationary phase. High salinity and nitrogen depletion also have a significant effect on biochemical composition of microalgae (Haris et al., 2022; Zarrinmehr et al., 2020). Stress results in excess photooxidation in microalgae, which accordingly stimulates accumulation of anti-oxidative secondary carotenoids to protect the microalgal cells from getting damaged (Kou et al., 2020; Mao et al., 2020; Mulders et al., 2015; Chen et al., 2017). Albeit we had few results available due to low biomass of microalgae in these treatments, our findings confirm both high salinity and nitrogen reduction promote astaxanthin accumulation. In other studies, accumulation of astaxanthin in 0.25 M NaCl high salinity conditions was 2.8 mg/g (Kou et al., 2020) and 3.1 mg/g in 0.2 M NaCl (Mao et al., 2018), while our results show higher accumulation (4.31 mg/g). Reduced nitrogen availability achieved 2.0-2.5 mg/g of astaxanthin in another study (Mulders et al., 2015), which was less than in our case (4.02 mg/g). While our control (3.56 mg/g) achieved less astaxanthin than other treatments, its astaxanthin content was still higher than those of previously mentioned studies on *Chromochloris zofingiensis* in stressful conditions (Kou et al., 2020; Mao et al., 2020; Mulders

et al., 2015). To our knowledge, there are no other studies comparing astaxanthin accumulation in *Chromochloris zofingiensis* in both high salinity and reduced nitrogen availability conditions.

Even without inducing stress on microalgae in the cultivation on photobioreactor, the astaxanthin content results were promising. Results of control in the laboratory experiment and those of at the end of the cultivation on the photobioreactor were comparable (3.56 mg/g and 3.63 mg/g, respectively). Interestingly, on day 31 of the photobioreactor cultivation the astaxanthin accumulation was higher (4.21 mg/g) and comparable to stress-inducing treatments on a laboratory scale. The reason behind this could be elevated pH on day 31 in comparison to lower pH on day 35, however we cannot claim this. While there are different kinds of stress that can trigger astaxanthin production, some are less resource consuming and easily controlled. Possibly, by introducing nitrogen starvation and high salinity conditions to microalgae that were successfully cultivated on the photobioreactor, astaxanthin accumulation could be even higher. This could be done by no longer supplying nutrients to the microalgae and adding NaCl to the cultivation. As previously mentioned, for laboratory experiment on astaxanthin accumulation, replicates of each treatment had to be pooled due to not enough biomass available for astaxanthin analysis. With upscaling on the photobioreactor, astaxanthin content could be analysed every sampling day, thus providing us with better understanding on astaxanthin production outdoors.

A study compared growth and astaxanthin production in *Chromochloris zofingiensis* and *Haematococcus pluvialis* in BBM medium (Nishshanka et al., 2022). Final astaxanthin concentrations reached a significantly higher value with *Haematococcus pluvialis* (14.28 mg/g) than with *Chromochloris zofingiensis* (3.97 mg/g). However, final biomass concentration was higher in *Chromochloris zofingiensis* (1.06 g/L) and almost twofold of *Haematococcus pluvialis* (0.57 g/L) (Nishshanka et al., 2022). Similar results of final astaxanthin content (3.63 mg/g) were found by our study. Since *Chromochloris zofingiensis* can achieve approximately twofold higher biomass and with highest reported accumulated astaxanthin by other studies being 7 mg/g (Minyuk et al., 2020), it's comparable to *Haematococcus pluvialis*. Furthermore, on large-scale outdoor cultivation, *Chromochloris zofingiensis* shows better characteristics with its robustness and ability to achieve high biomass densities while undergoing adverse environmental conditions, making it less difficult to cultivate (Huo et al., 2018). Another important factor that limits outdoor cultivation is contamination with protozoa, fungi, bacteria and other microalgae. In our case, biological contaminants didn't show a negative effect on growth of microalgae, nor did the fluctuating environmental conditions obstruct microalgae in achieving high biomass density. Meanwhile, contaminants in outdoor cultivation of *Haematococcus pluvialis* easily affect the cultivation, sometimes even leading to failure of the

whole cultivation (Fei et al., 2022). *Haematococcus pluvialis* may have advantage in amount of accumulated astaxanthin, however *Chromochloris zofingiensis* has better biomass production and shows less limitations for large-scale outdoor cultivation.

Overall, results of this study show that *Chromochloris zofingiensis* can be cultivated in an open outdoor system using threefold BBM+3N. It can achieve high biomass density, withstand fluctuating outdoor conditions and even recover after aggregating. With more trials on the photobioreactor using BBM+3N, our findings on microalgal growth and astaxanthin production in an outdoor cultivation can be further confirmed. While the amount of astaxanthin showed promising results, laboratory experiment suggests more astaxanthin could be accumulated under stress conditions, thus more research on this in open systems could contribute to making a commercial production possible.

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SUPPLEMENTARY MATERIAL

S 1 Media recipes for cultivation of *Chromochloris zofingiensis*

Table S 1: Bold Basal Medium with 3-fold nitrogen & modification

	BBM+3N	**BBM+NH_4Cl**
Stocks	*g/L*	*g/L*
(1.1) $NaNO_3$	75	
(1.2) NH_4Cl		47.2
(2) $CaCl_2 \cdot 2H_20$	2.5	2.5
(3) $MgSO_4 \cdot 7H_2O$	7.5	7.5
(4) K_2HPO_4	5.7	5.7
(5) KH_2PO_4	17.5	17.5
(6) NaCl	2.5	2.5
(7) Trace elements		
Na_2EDTA	0.75	0.75
$FeCl_3.6H_20$	0.097	0.097
$MnCl_2.4H_2O$	0.041	0.041
$ZnCl_2$	0.005	0.005
$CoCl_2.6H_2O$	0.002	0.002
$Na_2MoO_4.2H_2O$	0.004	0.004
Medium	*mL/L*	*mL/L*
Stock solutions 1 - 6	10	10
Stock solution 7	6	6

Table S 2: Douchas Medium and modifications

	DM + KNO3	**DM + CH_4N_2O**
	g/L	*g/L*
KNO_3	0.89	
CH_4N_2O		0.26
KH_2PO_4	0.06	0.06
$MgSO_4 \cdot 7H_2O$	0.05	0.05
Na_2EDTA Fe	0.01	0.01
$CaCl_2 \cdot 2H_20$	0.03	0.03
	mL/L	*mL/L*
Microelements 1	0.48	0.48
Microelements 2	0.48	0.48

Table S 3: Microelements in Douchas Medium

Microelements 1	*g/L*
H_3BO_3	0.5160
$CuSO_4 \cdot 5H_2O$	0.5730
$MnCl_2 \cdot 4H_2O$	1.6470
$CoSO_4 \cdot 7H_2O$	0.3080
$ZnSO_4 \cdot 7H_2O$	1.3390
Microelements 2	*g/L*
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.0860
$(NH_4)VO_3$	0.0070

S 2 Open thin-layer photobioreactor

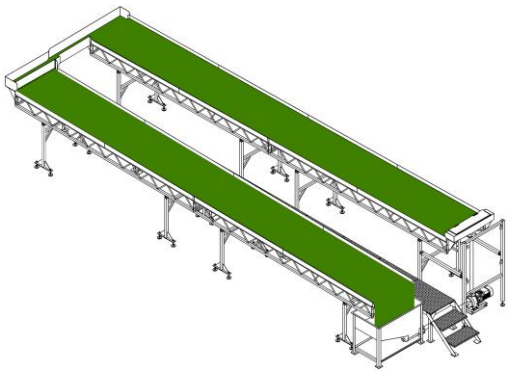


Figure S 1: Drawing of the open thin-layer photobioreactor used in this study

S 3 Calculation of average productivity and specific growth rate

Average productivity and specific growth rate were calculated and cell density and dry weight as follows:

$P_{CD} = (n_1 - n_0) / (t_1 - t_0)$

$P_{DW} = (DW_1 - DW_0) / (t_1 - t_0)$

$\mu = \ln (n_1 / n_0) / (t_1 - t_0)$*

$\mu = \ln (DW_1 / DW_0) / (t_1 - t_0)$*

P_{CD} – *average productivity considering cell density increase (cell mL^{-1} d^{-1})*

P_{DW} – *average productivity considering dry weight increase (g L^{-1} d^{-1})*

n_0 – *starting cell density (cell mL^{-1})*

n_1 – *final cell density (cell mL^{-1})*

$(t_1 - t_0)$ – *duration of cultivation (d)*

μ – *specific growth rate (d^{-1})*

DW_0 – *starting dry weight (g L^{-1})*

DW_1 – *final dry weight (g L^{-1})*

*Specific growth rate was calculated for the exponential phase, as noted by graphs with logarithmic scale.

S 4 Cultivation of *Chromochloris zofingiensis* on a laboratory scale using different media and nitrogen sources

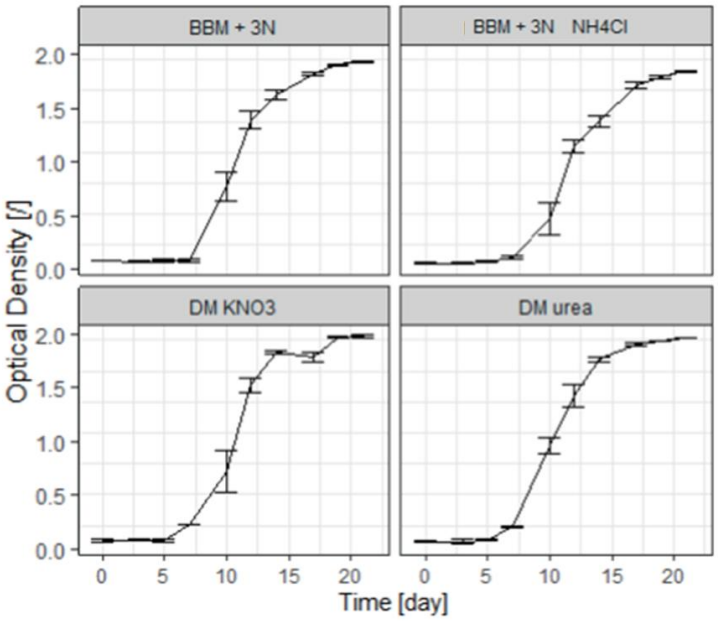


Figure S 2: Microalgal growth in different media and nitrogen sources observed with optical density at 750 nm. Microalgal growth in different treatments is visualized with mean values of the replicates and standard error of the mean.

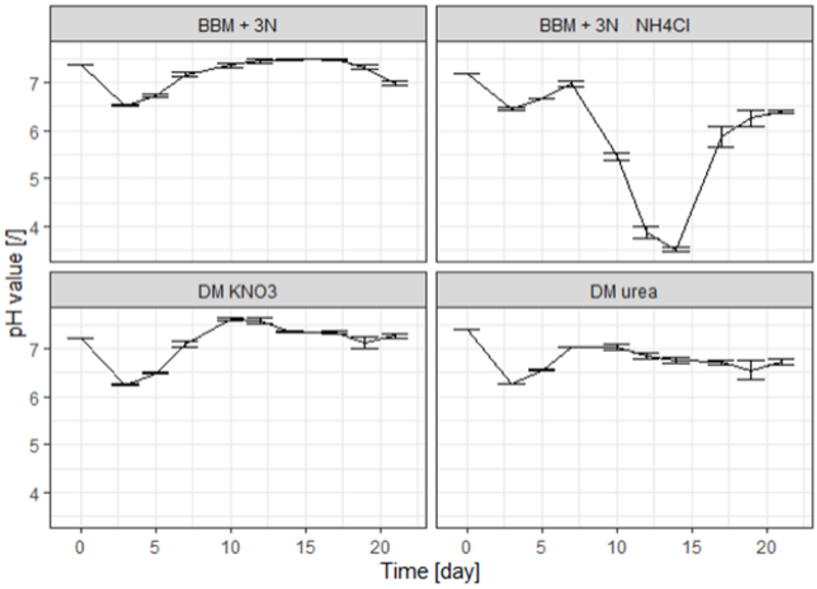


Figure S 3: pH variation throughout the laboratory cultivation using different media and nitrogen sources. pH in different treatments is visualized with mean values of the replicates and standard error of the mean.

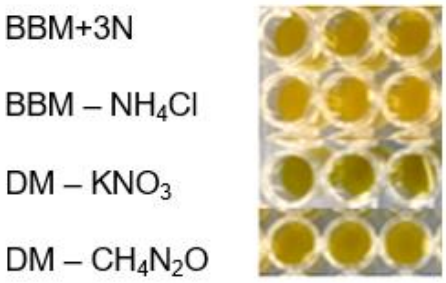


Figure S 4: Appearance of different cultivations using different media and nitrogen sources (Row 1: BBM+3N; Row 2: BBM+NH_4Cl; Row 3: Douchas medium with KNO_3; Row 4: Douchas medium with CH_4N_2O)

S 5 Cultivation of Chromochloris zofingiensis on a laboratory scale using different BBM+3N concentrations

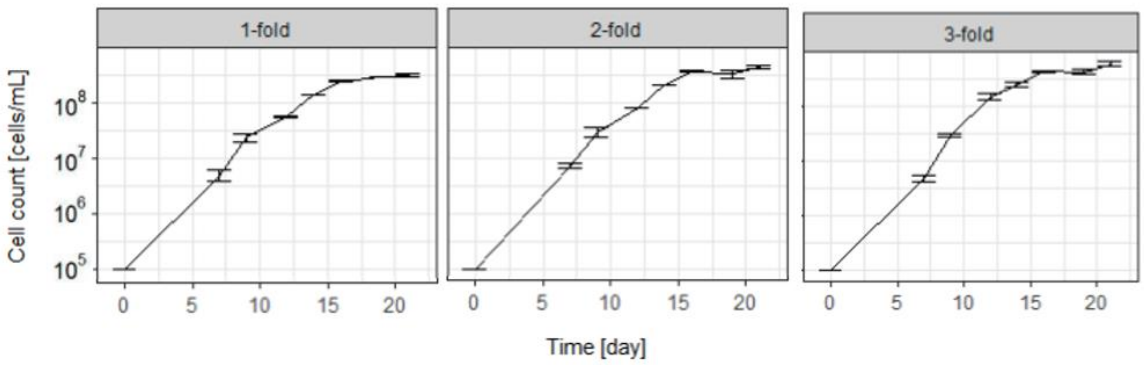


Figure S 5: Microalgal growth in different BBM+3N concentrations observed with cell count. Microalgal growth in different treatments is visualized with mean values of the replicates and standard error of the mean.

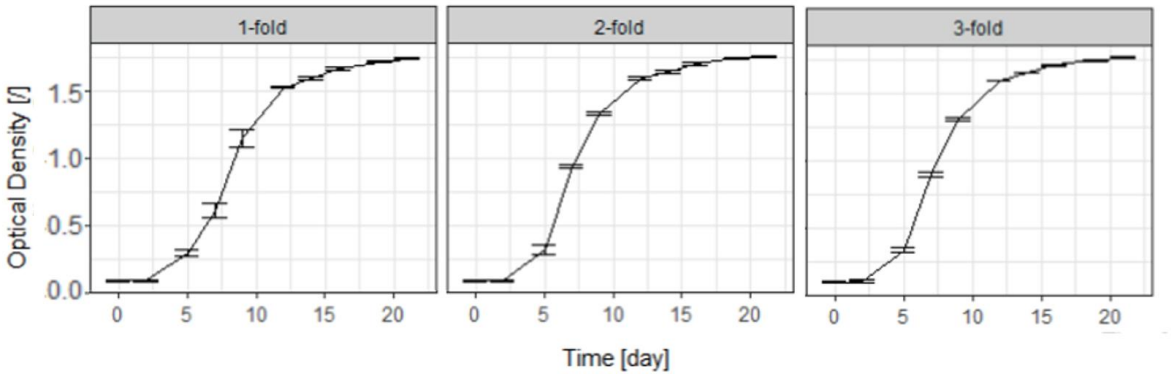


Figure S 6: Microalgal growth in different BBM+3N concentrations observed with optical density at 750 nm. Microalgal growth in different treatments is visualized with mean values of the replicates and standard error of the mean.

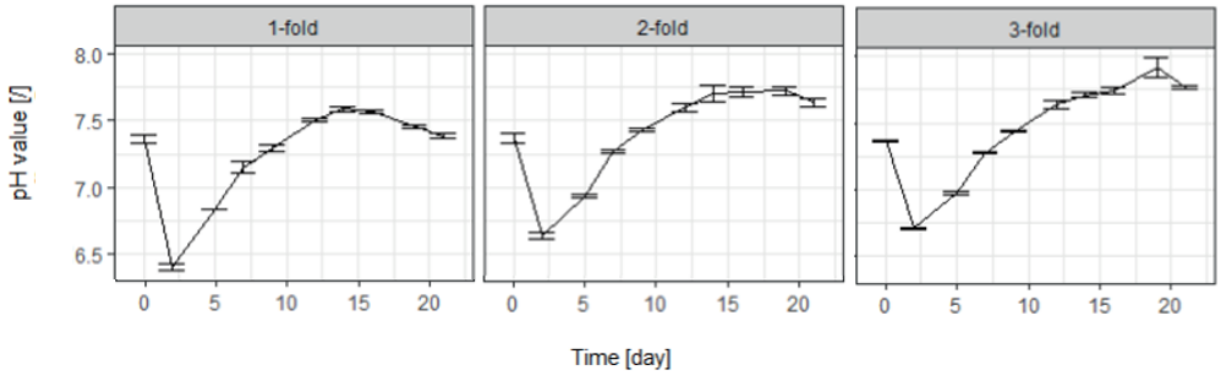


Figure S 7: pH variation throughout the laboratory cultivation using different BBM+3N concentrations. Microalgal growth in different treatments is visualized with mean values of the replicates and standard error of the mean.

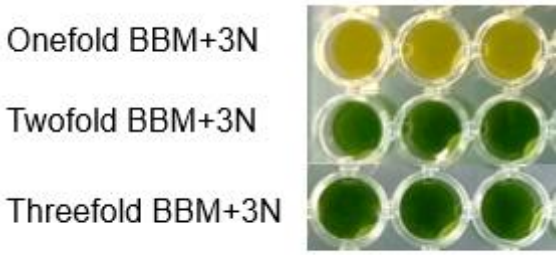


Figure S 8: Appearance of different cultivations using different BBM+3N concentrations and their replicates (Row 1: Onefold BBM+3N, Row 2: Twofold BBM+3N, Row 3: Threefold BBM+3N)

S 6 The effect of high-salinity and reduced nitrogen availability on accumulation of astaxanthin in *Chromochloris zofingiensis*

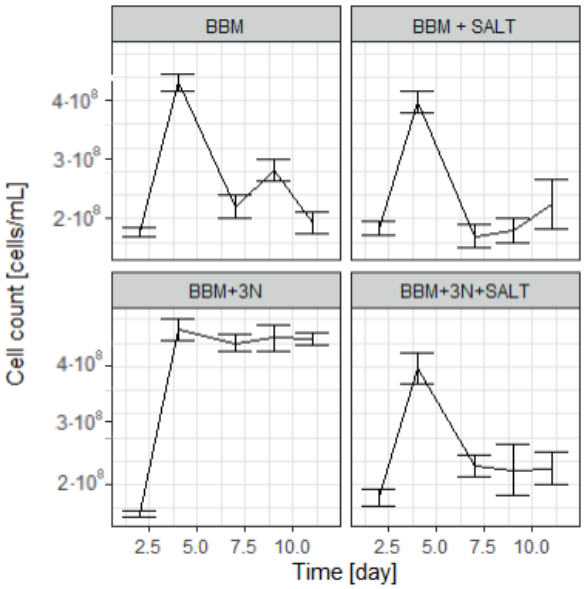


Figure S 9: Growth of *Chromochloris zofingiensis* in high-salinity and nitrogen reduced media observed with cell count. Microalgal growth in different treatments is visualized with mean values of the replicates and standard error of the mean.

S 7 Cultivation of *Chromochloris zofingiensis* on the open thin-layer photobioreactor using BBM+3N

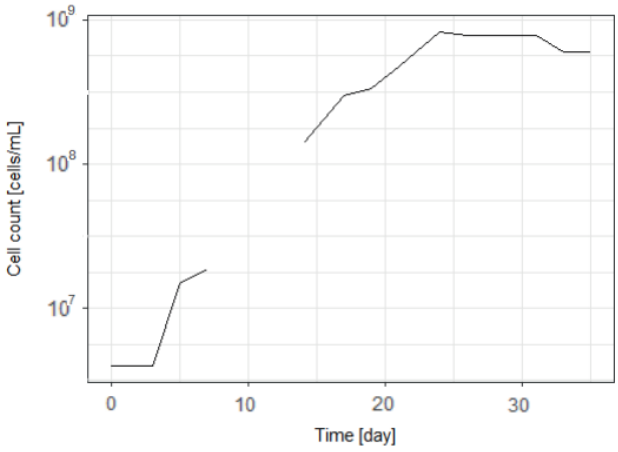


Figure S 10: Microalgal growth on the open thin-layer photobioreactor observed with cell count

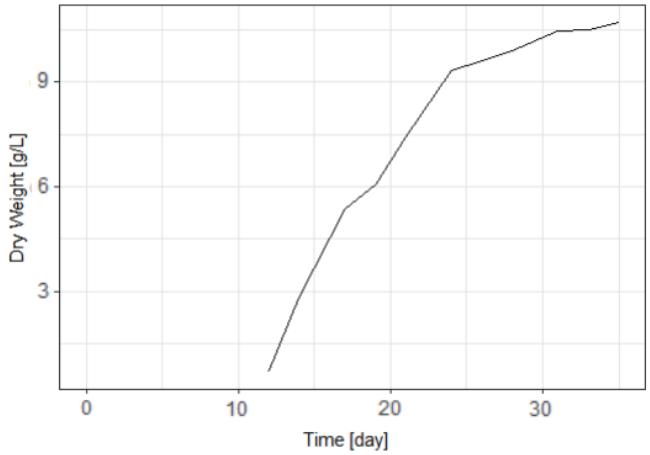


Figure S 11: Microalgal growth on the open thin-layer photobioreactor observed with dry weight

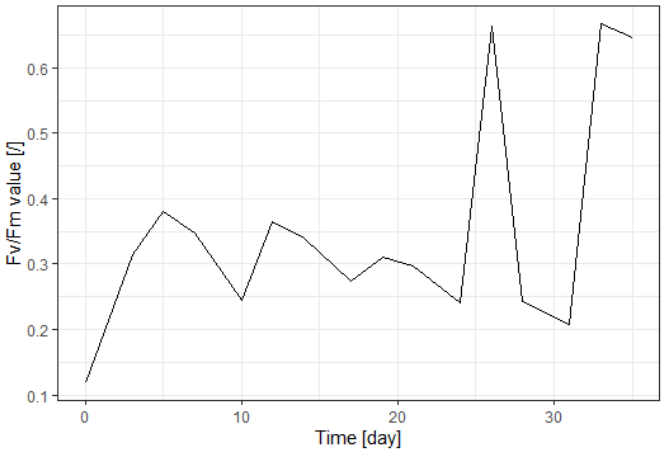


Figure S 12: Fv/Fm value throughout the cultivation on the open thin-layer photobioreactor

Table S 4: Cultivations of *Chromochloris zofingiensis* using mineral medium on an outdoor pilot scale

Study	**Medium**	**Duration**	**Cultivation temperature**	**Final biomass density**	**Highest productivity**
This study	BBM+3N	35 days	10.8 °C to 37.8 °C	11.75 g L^{-1}	1.02 g L^{-1} d^{-1}
Corrêa et al., 2022	BBM	96 days	4.0 °C to 24.6 °C	0.92 g L^{-1}	0.016 mg L^{-1} d^{-1}
Feng et al., 2011	BG-11	10 days	10.0 °C to 22.0 °C	0.90 g L^{-1}	0.58 g L^{-1} d^{-1}

S 8 Physico-chemical parameters recorded during the cultivation on open thin-layer photobioreactor

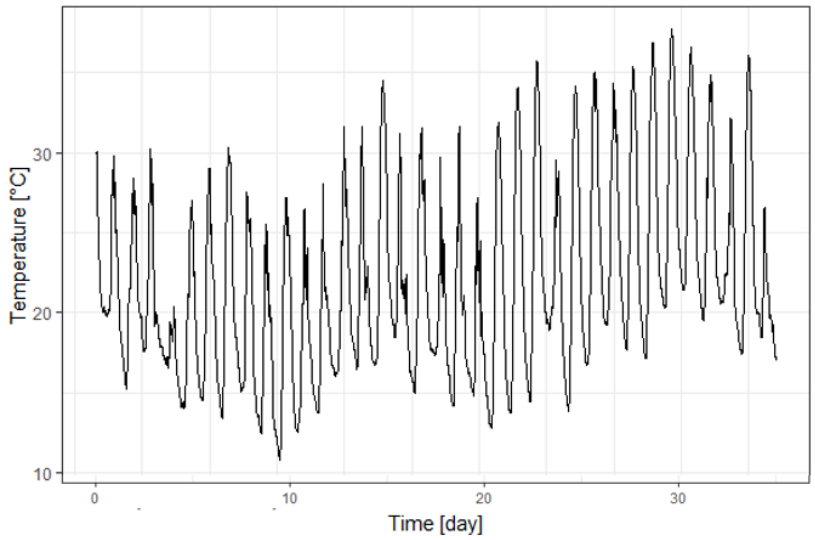


Figure S 13: Temperature throughout the cultivation on open thin-layer photobioreactor

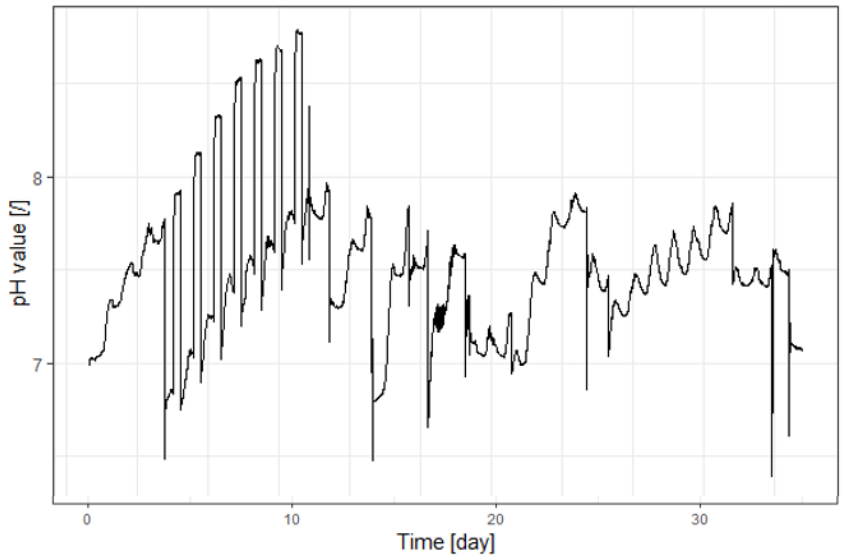


Figure S 14: pH throughout the cultivation on open thin-layer photobioreactor

S 9 Contamination with protozoa and bacteria during the cultivation on open thin-layer photobioreactor

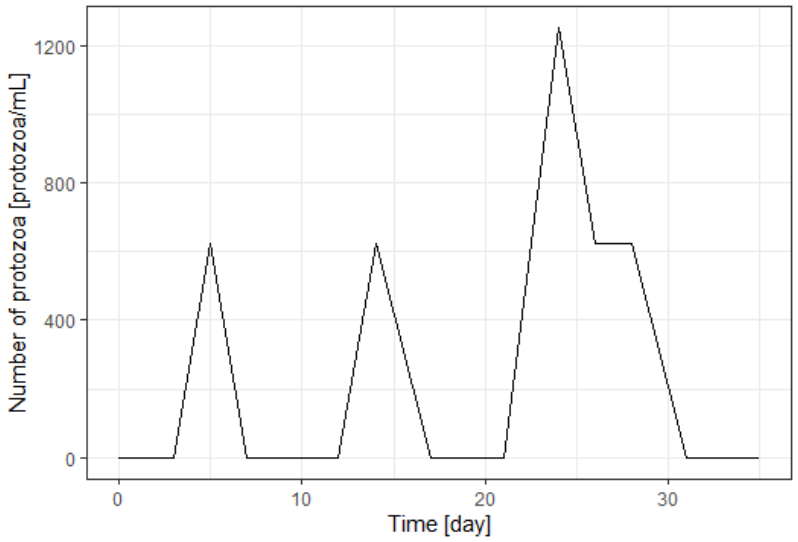


Figure S 15: Number of protozoa in the cultivation on open thin-layer photobioreactor

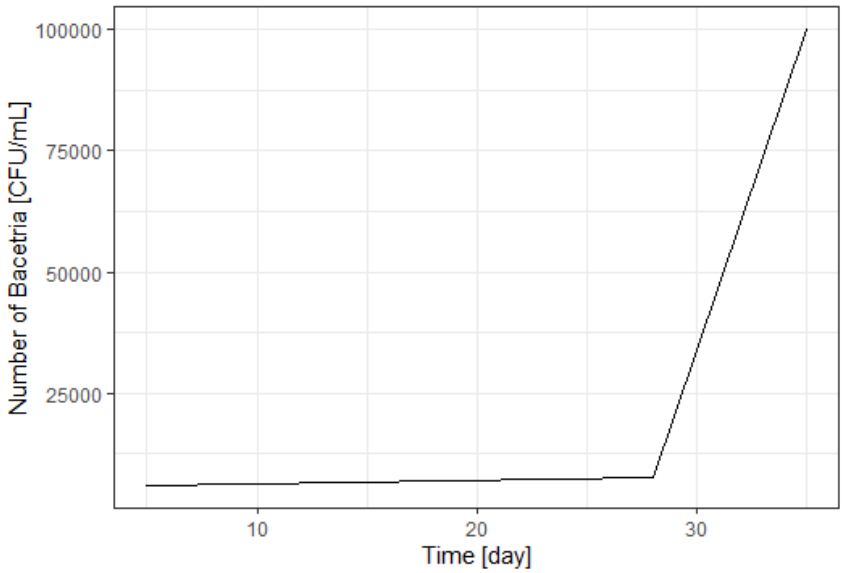
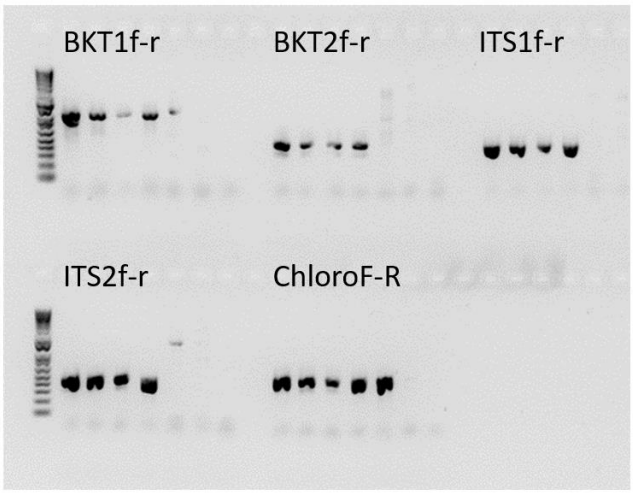


Figure S 16: Number of bacteria in the cultivation on open thin-layer photobioreactor

S 10 Gel electrophoresis results



Samples:

1. Open thin-layer photobioreactor cultivation from 24.6.
2. Open thin-layer photobioreactor cultivation from 27.6.
3. *Chromochloris zofingiensis* from laboratory cultivation
4. *Chlorella vulgaris*
5. *Arthrospira platensis*

Table S 5: Primers and their sequence, target and amplicon length

Name of the primer	Primer Sequence	Target	Amplicon Length
BKT1f	ATACTGCCGCATTCGTTGAC	β-Ketolase gene of *Chromochloris*	980 bp
BKT1r	GCACACATTGGGGCTTACAA		
BKT2f	TATGCGCATGTTGTAAGCCC	β-Ketolase gene of *Chromochloris*	414 bp
BKT2r	TTTCTCCCAGGGCATTGTCT		
ITS1f	TGCACTTCCCTGTTATCGGT	ITS1 of *Chromochloris*	328 bp
ITS1r	TGACCCAACTACCAAAGGCA		
ITS2f	ATCGGTTGGCAGGGATAGTG	ITS1 of *Chromochloris*	466 bp
ITS2r	ACCACAAGCAACAACTTCGT		
ChloroF	TGGCCTATCTTGTTGGTCTGT	18S rRNA gene	473 bp
ChloroR	GAATCAACCTGACAAGGCAAC		

Note: From “Moro CV, Crouzet O, Rasconi S, Thouvenot A, Coffe G, Batisson I, Bohatier J. New design strategy for development of specific primer sets for PCR-based detection of Chlorophyceae and Bacillariophyceae in environmental samples. Appl Environ Microbiol. 2009 Sep;75(17):5729-33”