applied sciences

MDPI

Article

Examination of Photo-, Mixo-, and Heterotrophic Cultivation Conditions on *Haematococcus pluvialis* Cyst Cell Germination

Andreas Bauer * and Mirjana Minceva

Biothermodynamics, TUM School of Life Sciences, Technical University of Munich, Maximus-von-Imhof-Forum 2, 85354 Freising, Germany; mirjana.minceva@tum.de
* Correspondence: msc.andreas.bauer@tum.de; Tel.: +49-8161-71-6166

Abstract: The microalgae *Haematococcus pluvialis* is used for the biotechnological production of astaxanthin. The red carotenoid accumulates in the cytoplasm under unfavorable conditions. Astaxanthin synthesis is associated with the transformation of motile vegetative cells into non-motile cyst cells. In the industrial process, after harvesting, the cyst cells are mechanically disrupted, dried, and finally, astaxanthin is extracted with supercritical CO_2. The germination of the cyst cells represents an interesting alternative, replacing the mechanical cyst cell wall disruption. When cyst cells are exposed to favorable growth conditions, germination of the cyst cells occurs and zoospores are released after a certain time. These zoospores show a much weaker cell matrix compared to cyst cells. In this study, germination under phototrophic, mixotrophic, and heterotrophic conditions was examined. Glucose was used as the carbon source for mixotrophic and heterotrophic germination. Applying heterotrophic conditions, up to 80% of the cells were in the zoospore stage 49 h after the start of germination, and extraction yields of up to 50% were achieved using the solvent ethyl acetate for the extraction of astaxanthin from the algal broth containing zoospores. An extraction yield of up to 64% could be achieved by doubling the nitrate concentration and combining mixotrophic and heterotrophic cultivation.

Keywords: *Haematococcus pluvialis*; germination; cultivation conditions; extraction; astaxanthin; downstream processing

Citation: Bauer, A.; Minceva, M. Examination of Photo-, Mixo-, and Heterotrophic Cultivation Conditions on *Haematococcus pluvialis* Cyst Cell Germination. *Appl. Sci.* **2021**, *11*, 7201. https://doi.org/10.3390/app11167201

Academic Editor: You-Kwan Oh

Received: 28 June 2021
Accepted: 30 July 2021
Published: 4 August 2021

1. Introduction

The microalgae *Haematococcus pluvialis* (*H. pluvialis*) is the organism used for the biotechnological production of the red carotenoid astaxanthin [1]. Astaxanthin shows strong antioxidant and health-promoting properties, causing a huge increase in demand for biotechnologically produced astaxanthin [2]. Natural astaxanthin is mainly used as a food supplement and in the cosmetics industry [3]. *H. pluvialis* belongs to the Volvocales, which represent an order of flagellated green algae [4]. In nature, it is mainly found in temperate regions of the world, temporary water points, and natural or artificial freshwater pools [5]. The complex life cycle of *H. pluvialis* can be divided into a mobile and immobile phase, in which the cells exist as mobile vegetative cells and immobile aplanospores [6]. Under favorable growth conditions, the microalga live mainly as a green, flagellated vegetative cell (Figure 1a). The vegetative cells consist of a cell membrane and an extracellular gelatinous matrix characteristic of volvocalean motile cells [7]. Under unfavorable stress conditions, the species undergoes asexual reproduction producing aplanospores (Figure 1b) which, when mature accumulate astaxanthin (Figure 1c) and become cyst cells (Figure 1d) and may also produce zoospores, also accumulating astaxanthin (Figure 1e).

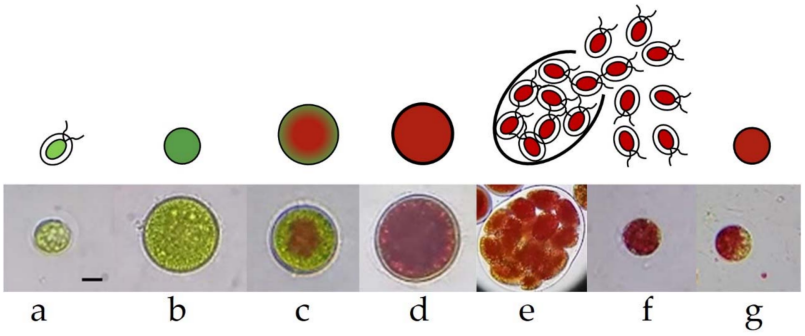

Figure 1. The cell cycle of *H. pluvialis*: (**a**) motile, flagellated cell, (**b**) aplanospore, (**c**) astaxanthin-accumulating aplanospore, (**d**) red cyst cell, (**e**) dividing cells (sporangium), (**f**) astaxanthin rich zoospores released from the sporangium, (**g**) aplanospore derived from astaxanthin containing zoospore. Scale bar: 10 µm.

In the final stage of cyst formation, *H. pluvialis* develops a rigid and resistant cell wall that consists of a trilaminar sheath, secondary wall, and the tertiary wall (Figure 1d) [7]. In principle, *H. pluvialis* can grow under phototrophic, heterotrophic, or mixotrophic conditions. When growing phototrophically, sunlight is used as energy and CO_2 as a carbon source [8]. With heterotrophic growth, microalgae take up organic molecules such as glucose or acetate [9,10] as their main source of nutrition, and no light is used during cultivation [11,12]. Glucose was used in several studies for the cultivation of *H. pluvialis* [13–15]. Mixotrophic cultivation is a variant of heterotrophic growth, where the organic carbon and CO_2 are assimilated simultaneously and respiratory and photosynthetic metabolism operate concurrently [16]. In literature, mixotrophic cultivation of vegetative *H. pluvialis* cells was done, using the sugars ribose, mannose, fructose, xylose, glucose, sodium acetate, glycerol, lactose, or sucrose at a concentration of 1.33 g L^{-1} (corresponds to 7.38 mM glucose) [17]. On an industrial scale, the upstreaming of *H. pluvialis* is done phototrophically in the form of a two-step process using closed photobioreactors. Open ponds are rarely used for the green phase of biomass production and are usually just used for the second step of cell stressing. In the first process step (Figure 1a,b), the algal biomass is cultivated phototrophically to high cell concentrations under optimal growth conditions, with a sufficient supply of nutrients such as nitrate and phosphate [18], gassing with CO_2 [19], and artificial lighting, especially by the red fraction of the light [20,21]. On an industrial scale, natural sunlight is usually used for cultivation [1]. Under so-called "stress conditions", nitrate and phosphate deficiency and light stress, and especially the blue light spectrum [21,22], the astaxanthin synthesis is initiated and further enhanced by CO_2 gassing [19] in the second process step (Figure 1c,d). Astaxanthin accumulation is accompanied by the formation of a resistant cyst cell wall, which impedes direct and efficient astaxanthin extraction. Thus, a complex downstream process is required, including centrifugation, mechanical cell wall disruption, and spray drying; finally, astaxanthin is extracted with supercritical CO_2 [23]. As a result, energy-intensive mechanical processes such as bead mills or high-pressure homogenization are required for the industrial cell wall disruption of the cyst cells. Up to three cycle repetitions are needed to achieve sufficient cell wall disruption efficiency using high-pressure homogenization [24]. Germination of *H. pluvialis* cysts represents an interesting alternative to mechanical cell wall disruption. When the cysts are exposed to growth conditions and sufficient nutrients are added, the mother cell begins to divide, forms a sporangium (Figure 1e), and releases zoospores (Figure 1f) containing astaxanthin. After a certain time, these also lose their flagella, round off, and form astaxanthin-containing non-motile aplanospores (Figure 1g) [25]. As shown in our previous study, it is possible to extract astaxanthin directly from these zoospores into a solvent. Thus, mechanical cell wall disruption, drying, and extraction with supercritical CO_2 could be replaced by a liquid–liquid extraction step, using a liquid–liquid chromatographic unit [26,27]. Nevertheless, the cultivation conditions to convert cyst cells

into zoospores, keeping the astaxanthin content high within the zoospores after release, are not yet fully investigated [26,28]. In previous studies, it could be determined that the nitrate concentration [25], the light intensity [29] and composition [30], and the age of the cyst cells [31] influence the germination, i.e., the proportion of released zoospores compared to non-germinated cysts and the astaxanthin content in the zoospores. In these studies, germination was performed at phototrophic conditions. It could be shown that at higher light intensities in comparison to lower ones, more zoospores were usually released from the cyst cells [25,29]. However, this was also accompanied by a degradation of astaxanthin at higher compared to lower light intensities. In addition, a stronger decrease of astaxanthin in the zoospores released was observed at higher compared to lower nitrate concentrations in the medium at the same light intensity [25]. In contrast, after the initiation of germination, an increase in the astaxanthin concentration of 20% at 12 h and a decrease of 30% at 24 h has also been reported [28]. In addition, compared to red or white light, it has been shown that germination with blue light has a positive effect on the number of zoospores released and their astaxanthin content [30].

The aim of this work was to develop a germination method that initiates rapid germination of as many cysts as possible while maintaining a high astaxanthin content in the released zoospores for the subsequent downstream process. Therefore, the influence of the CO_2 content on photo- and mixotrophic germination was examined. In addition, the influence of an external glucose source in comparison to cultivation without glucose was studied for two heterotrophic germinated cultures. The influence of light on germination was discussed using mixotrophic and heterotrophic cultivation.

2. Materials and Methods

2.1. Chemicals and Solvents

The solvents used for astaxanthin extraction from the red cyst cells and zoospores, ethyl acetate (≥99.5%), and dichloromethane (DCM) (99.8%), were purchased from Merck KGaA (Gernsheim, Germany). Deionized water was supplied in-house. For HPLC analysis Millipore 18 Mohm water, methanol (liquid chromatography grade) from VWR Chemicals (Darmstadt, Germany), and methyl-*tert*-butyl ether (MTBE) (>99.5%) from Honeywell (Israel) were used. K_2CO_3 (99%) and $KHCO_3$ (99%) for the preparation of the aqueous CO_2 buffer for *H. pluvialis* cultivation and germination were ordered from VWR Chemicals (Germany). Sulphuric acid (96%) from VWR Chemicals (Germany) and phenol (99%) from Alfa Aesar (Karlsruhe, Germany) was used for colourimetric detection of glucose, sugars, and extracellular polymeric substances (EPS) in the supernatant. Sodium nitrate (>99%) was purchased from VWR Chemicals (Germany). Anhydrous D(+)-glucose from Merck KGaA (Germany) was used as an organic carbon source. Bold Modified Basal Freshwater Nutrient Solution (BBM) was used as a culture medium. Medium preparation was done by diluting 20 mL BBM concentrate from Sigma-Aldrich (Darmstadt, Germany) with 980 mL deionized water to obtain the following composition (per liter): 11.42 mg H_3BO_3, 25.0 mg $CaCl_2{\cdot}2H_2O$, 0.49 mg $Co(NO_3)_2{\cdot}6H_2O$, 1.57 mg $CuSO_4{\cdot}5H_2O$, 50.0 mg EDTA (free acid), 4.98 mg $FeSO_4{\cdot}7H_2O$, 75 mg $mgSO_4{\cdot}7H_2O$, 1.44 mg $MnCl_2{\cdot}4H_2O$, 0.71 mg MoO_3, 0.003 mg $NiCl_2{\cdot}6H_2O$, 31.0 mg KOH, 0.003 mg KI, 175.0 mg KH_2PO_4, 75 mg K_2HPO_4, 25 mg NaCl, 250.0 mg $NaNO_3$, 0.002 mg Na_2SeO_3, 0.001 mg $SnCl_4$, 0.0022 mg $VOSO_4{\cdot}3H_2O$, and 8.82 mg $ZnSO_4{\cdot}7H_2O$. The pH of the culture medium was adjusted to 6.8 with sodium hydroxide (99%) from VWR Chemicals (Germany).

2.2. Cultivation of H. pluvialis Cyst Cells

The microalgae *H. pluvialis* (SAG number 192.80) was obtained from the University of Göttingen, Germany, immobilized on an agar. Parts of this culture were transferred to a 250 mL Erlenmeyer flask filled with 150 mL of Bold Modified Basal Freshwater Nutrient Solution (BBM). This pre-culture was placed on the shaker SHO-2D from Witeg Labortechnik GmbH (Wertheim, Germany) at a rotation of 100 rpm. Illumination was performed using a lamp containing 90 light-emitting diodes (LEDs). The light compositions

could be adjusted to "red light" with a maximum of the emitted light at 658 nm, and to "blue light" with a maximum at 451 nm. The pre-culture was continuously illuminated with "red light" at a light intensity (photon flux density) of 50 µmol m^{-2} s^{-1} for two weeks and consisted of a mixture of vegetative cells (Figure 1a) and aplanospores (Figure 1b). The light intensity was measured with the LightScout Quantum Meter from Spectrum Technologies, Inc. (Aurora, IL, USA). For the cultivation from the green flagellate stage to the red cyst cell, four photobioreactors, model HDC 1.100B from CellDEG GmbH (Berlin, Germany), were used (Figure 2). The reactors consist of a CO_2 buffer reservoir and a cultivation reactor, spatially separated by a hydrophobic membrane. An aqueous bicarbonate buffer solution is placed in the buffer reservoir, which releases CO_2 and allows bubble-free gassing of the microalgae culture through the membrane. The CO_2 is released through seven holes on the vessel's upper side, which is covered with a hydrophobic membrane. The actual cultivation reactor, which is placed above the buffer reservoir, has a spherical structure with a total volume of 250 mL and is made of transparent polystyrene. Four aliquots of the pre-culture were centrifuged at 5500 rpm with a centrifuge Sigma 3-16KL centrifuge from Sigma GmbH (Mannheim, Germany) for 5 min to cultivate the cysts for the germination experiments, and the supernatant was discarded. The cyst cells were suspended in distilled water and centrifuged, and the supernatant was removed. To each of the four samples, 200 mL of fresh BBM was added, whereby an optical density (OD) of 0.2 at 750 nm was set. This algal broth was transferred to the cultivation reactors. One hundred fifty milliliters of bicarbonate buffer containing 12.55 g K_2CO_3 and 36.04 g $KHCO_3$ were used for each reactor and placed in the buffer reservoir, which corresponds to the gassing of the microalgae culture with 3% v/v CO_2. The reactors were placed on the shaking plate and the rotation was set to 110 rpm. The cultivation was carried out at a light intensity of 100 µmol m^{-2} s^{-1} using the "red light". When all microalgae were present as immobile green aplanospores, the culture was stressed for seven days to accelerate the astaxanthin synthesis, using "blue light" at a light intensity of 200 µmol m^{-2} s^{-1}.

Figure 2. Photobioreactor HDC 1.100B filled with *H. pluvialis* cells at different cell stages.

2.3. Germinatin of H. pluvialis Cyst Cells

For germination, aliquots of the cyst cells were cultivated as described in Section 2.2 and centrifuged at 5500 rpm. The supernatant was removed and the biomass was washed with distilled water, centrifuged again, and the supernatant discarded. The cyst cells were germinated under a phototrophic, mixotrophic, or heterotrophic culture. The cyst cells were suspended in 200 mL BBM and 4 mM glucose was added to the mixotrophic or heterotrophic cultures. In a preliminary study, ribose, sodium acetate, and glucose were tested for mixotrophic cyst cell germination. More zoospores were released using glucose compared to ribose and sodium acetate (Figure S1) and enhanced germination of cyst cells were observed at these carbon concentrations compared to phototrophic germination. Additionally, in a preliminary study, glucose concentrations of 4, 8, 20, and 40 mM were used for mixo- and heterotrophic cyst cell germination, and the glucose concentration did not affect the germination efficiency (Figure S2). Thus, concerning resource consumption, the lowest investigated glucose concentration of 4 mM was used in the further course of experiments. Where germination was carried out with artificial light, "red light" was used, with light intensity as shown in Table 1.

Table 1. Applied germination conditions, each culture represents one biological sample.

Name	Germination Conditions
culture 1	phototrophic + CO_2 + 100 µmol m^{-2} s^{-1}
culture 2	phototrophic + ambient air + 100 µmol m^{-2} s^{-1}
culture 3	mixotrophic + 4 mM glucose + CO_2 + 100 µmol m^{-2} s^{-1}
culture 4	mixotrophic + 4 mM glucose + ambient air + 100 µmol m^{-2} s^{-1}
culture 5	phototrophic + CO_2 + 75 µmol m^{-2} s^{-1}
culture 6	phototrophic + ambient air + 75 µmol m^{-2} s^{-1}
culture 7	mixotrophic + 4 mM glucose + CO_2 + 75 µmol m^{-2} s^{-1}
culture 8	mixotrophic + 4 mM glucose + ambient air + 75 µmol m^{-2} s^{-1}
culture 9	heterotrophic + "no external carbon source" + ambient air
culture 10	heterotrophic + 4 mM glucose + ambient air
culture 11	phototrophic + ambient air + 75 µmol m^{-2} s^{-1}
culture 12	mixotrophic + 4 mM glucose + ambient air + 75 µmol m^{-2} s^{-1}
culture 13	heterotrophic + 4 mM glucose + ambient air
culture 14	heterotrophic + 4 mM glucose + 2xnitrate + ambient air
culture 15	mixo-/heterotrophic + 4 mM glucose + ambient air + (75 µmol m^{-2} s^{-1})
culture 16	mixo-/heterotrophic + 4 mM glucose + 2xnitrate + ambient air + (75 µmol m^{-2} s^{-1})

Four sets of experiments were carried out. In each set, four germination experiments were performed at different conditions using the same batch's cyst cell biomass. Thus, the "internal" state (nitrate, lipid, starch, astaxanthin content, etc.) of the cyst cells differs at the beginning of the germination for each set of experiments. Therefore, it is more reasonable to evaluate the tendencies within one set of an experiment than to compare absolute values between different sets of experiments. In the first set of experiments, the influence of gassing with 3% v/v CO_2 compared to cultivation at ambient air was examined using phototrophic culture 1 and 2 and mixotrophic culture 3 and 4 at 100 µmol m^{-2} s^{-1}. Germination conditions of cultures 1 to 4 were repeated using a light intensity of 75 µmol m^{-2} s^{-1} and are presented in Table 1 as culture 5 to culture 8. In the third set of experiments, cultures 9 to 12, the conditions of cultures 6 and 8 at 75 µmol m^{-2} s^{-1} were repeated (cultures 11 and 12). In addition, these two experiments were carried out in darkness by covering the reactors with aluminum foil for heterotrophic germination. Glucose was added to culture 10, while no further external carbon source was added to culture 9.

In the fourth and last series of experiments (cultures 13 to 16), all the cultures were germinated in the presence of 4 mM glucose. In cultures 14 and 16 additional sodium nitrate was added, resulting in a nitrate concentration two times higher than the one in the BBM medium. Cultures 13 and 14 were germinated in darkness. Cultures 15 and 16 were first germinated in the presence of red light, at a light intensity of 75 µmol m^{-2} s^{-1}. After the nitrate consumption (25 h after the start of the germination) the germination was continued in complete darkness.

2.4. Astaxanthin Extractability Analysis

Astaxanthin can be extracted directly from the zoospores into ethyl acetate without prior mechanical cell disruption or drying [26]. Several solvents, including ethyl acetate, methyl-*tert*-butyl ether, n-heptane, butan-1-ol, and dichloromethane, have been evaluated for the extraction of astaxanthin from germinated zoospores in our previous work [26]. Ethyl acetate showed the largest extraction yield; it is classified as a green solvent and was therefore chosen as an extraction solvent [32,33]. Consequently, the algal broth's extractability can be used as an indicator for the germination of cyst cells or the number of released zoospores. Therefore, liquid–liquid extraction was carried out as a single determination at a different time point after germination, to determine the amount of extractable astaxanthin into ethyl acetate. Two milliliters of algal culture were transferred into a 15 mL falcon tube and mixed with 2 mL ethyl acetate for 60 min using a Multi Bio RS-24 shaker from Biosan (Rīga, Latvia). The samples were then centrifuged at 5500 rpm

with a Sigma 3-16KL centrifuge from Sigma GmbH (Germany) for 15 min. The absorbance of the extracted carotenoids in the solvent was measured once with the SPECORD 50 Plus spectrophotometer from Analytik Jena (Thuringia, Germany) at 478 nm, and the astaxanthin concentration was calculated using a calibration curve. The astaxanthin content of the biomass was determined in triplicate (n = 3) at the beginning of germination (t = 0 h), at the time of the largest zoospore release (t = 49 h), and at the end of the series (t = 141 h) for each biological sample: cultures 5–16. For cultures 5–16, a double determination of the astaxanthin content of the biomass was additionally performed for the remaining times (t = 17 h, 25 h, 41 h, and 68 h). The astaxanthin concentration in ethyl acetate after the liquid–liquid extraction was obtained by a single determination for all cultures 1–16. The yield Y corresponds to the quotient of the astaxanthin content in the solvent after, to that in the biomass before extraction, and was calculated for the points in time examined, as presented in the following equation.

$$Y = \frac{m_{\text{astaxanthin extracted into the solvent}}}{m_{\text{astaxanthin in biomass before extraction}}}$$

In addition to the extraction experiments, the cell stage of the microalgae cultures was identified for cultures 5–16 with a light microscope, equipped with a Thoma hemocytometer from the BRAND GMBH + CO KG (Wertheim, Germany). A distinction was made between five different cell stages of *H. pluvialis*, cysts, dividing cells, zoospores, aplanospores, and empty cysts, as shown in Figure 1. Cell counting was performed for each measuring point in the form of a single determination.

2.5. Determination of the Biomass Concentration

The dry weight (DW) biomass concentration was measured in triplicate at every point in time after the start of germination for each culture. Therefore, three times 2 mL algal broth was transferred into microtubes from Eppendorf AG (Hamburg, Germany) and centrifuged at 5500 rpm for 15 min. The supernatant was used for the determination of sugar and nitrate content in the medium, as described in Section 2.7. The biomass was washed with distilled water and centrifuged again at 5500 rpm for 15 min and the supernatant was discarded. The microtubes with the dewatered biomass were stored at –80 °C and subsequently lyophilised with an Alpha 3-4 LSC basic freeze dryer from Martin Christ Gefriertrocknungsanlagen GmbH (Osterode am Harz, Germany). The DW was calculated from the weight difference between the microtube after freeze-drying and the empty microtube, divided by the volume of the taken algal broth, i.e., 2 mL.

2.6. Astaxanthin Quantification

To determine the biomass astaxanthin content, 5 mg freeze-dried biomass (Section 2.5) was broken up with mortar and pestle and astaxanthin was extracted, adding 10 mL of dichloromethane. This was repeated three times until only colourless debris was left. The dichloromethane was evaporated using a Heidolph Hei-VAP (Germany) rotary evaporator. The astaxanthin extract was saponified for 3 h at room temperature in the dark with acetone (2.25 mL), methanol (0.25 mL), and 0.05 M NaOH in methanol (0.5 mL). This extract was transferred to three 15 mL falcon tubes and mixed with 3 mL of petroleum ether. Additionally, 5 mL of 10 wt.% aqueous NaCl solution was added, mixed, and centrifuged at 5500 rpm for 5 min. The upper phase was transferred into a fresh NaCl solution. The washing step with NaCl solution was performed three times in total. Finally, the petroleum-rich phase was evaporated, and the astaxanthin extract was dissolved in solvent B (methanol, MTBE, water, 8:89:3, v/v) used for HPLC analysis and filtered with a 0.22 µm disposable nylon syringe filter before HPLC analysis. Quantification of the de-esterified astaxanthin was carried out on an HPLC unit (LC-20AB, Shimadzu, Japan) equipped with a diode array detector (SPD-M20A, Shimadzu, Japan) and a YMC carotenoid column (C30, 3 µm, 150 × 4.6 mm, YMC Co., Kyoto, Japan) according to our previous study [26]. As the mobile phase, solvent A (methanol, MTBE, water, 81:15:4, v/v) and

solvent B (methanol, MTBE, water, 8:89:3, v/v) were used. The gradient of the solvent A and B was as followed: 2% solvent B for 11 min, a linear gradient from 2% solvent B to 40% solvent B for 7 min, 40% solvent B for 6.5 min followed by a linear gradient to 100% solvent B for 2.5 min, 100% solvent B for 3 min, a linear gradient to 2% solvent B for 3 min, held for 3 min. The injection volume was 10 µL and the flow rate of the mobile phase was 1 mL min^{-1}. DIN 32645 was used for the calculation of the limit of detection (LOD) and limit of quantification (LOQ), using the calibration curve [34]. The calculated LOD and LOQ values were 1.85 and 5.51 mg L^{-1}, respectively.

2.7. Nitrate and Sugar Quantification

Nitrate and sugar content in the medium were determined using the supernatant of the biomass concentration samples from Section 2.5. The nitrate concentration was measured with UV/VIS spectroscopy, subtracting twice the absorbance at 275 nm from 220 nm [35]. The content of simple glucose, sugars, and oligo- and polysaccharides were determined colourimetrically, as described in [36]. Therefore, 400 µL supernatant and 40 µL 80% phenol solution was mixed with 1 mL sulphuric acid (96%) in a 2 mL microtube. The absorbance of the brown-yellow colour was measured spectroscopically at a wavelength of 488 nm. The calibration curve was determined with glucose in distilled water. The determination of glucose content was performed in triplicate at each measured time point. The nitrate content was determined by a single determination.

2.8. Statistical Analysis

To evaluate the biological reproducibility of the germination process, fixed germination conditions (mixo-/heterotrophic + 4 mM glucose + ambient air + (75 µmol m^{-2} s^{-1})) were repeated in triplicate. Therefore, the same cyst cell biomass was transferred into three photobioreactors, and 49 h after the start of germination, astaxanthin was extracted with ethyl acetate as described in Section 2.4. The astaxanthin content in the solvent was determined (Figure S3 in Supplementary Material). Since only a small relative standard deviation of ±5.2% was measured for astaxanthin in the ethyl acetate extracts, one experiment was carried out for each set of germination conditions to determine the influence of several parameters. Higher standard deviations were measured for the determination of the astaxanthin content in the biomass. Therefore, the astaxanthin content in the biomass was measured in triplicate. The differences in the astaxanthin content of the biomass at the beginning of the germination compared to different points in time after the start of germination were analysed using a two-tailed Student's t-test. Significant differences were found at $p < 0.05$. Measurement replicates are expressed as mean averages ± standard deviations.

3. Results and Discussion

The results and discussion of the germination experiments are presented, comparing the influence of CO_2 and ambient air on the phototrophic and mixotrophic germination at 100 µmol m^{-2} s^{-1} (culture 1 to culture 4) and 75 µmol m^{-2} s^{-1} (culture 5 to culture 8). In the third set of experiments (culture 9 to culture 12), the germination was performed at heterotrophic conditions, once with the addition of an external glucose source to the BBM and once without external glucose, and compared to a mixotrophic and phototrophic culture. In the last set of experiments (culture 13 to culture 16), the influence of nitrate on heterotrophic cultivation was investigated and a combination of mixo- and heterotrophic cultivation conditions was done.

3.1. Evaluation of Phototrophic, Mixotrophic, and Heterotrophic Cultivation Conditions on the Germination of H. pluvialis Cyst Cells

In the first set of experiments, the influence of CO_2 on the germination of phototrophic and mixotrophic cultures was examined. Here, culture 1 (phototrophic + CO_2 + 100 µmol m^{-2} s^{-1}), culture 2 (phototrophic + ambient air + 100 µmol m^{-2} s^{-1}), culture 3 (mixotrophic + 4 mM glucose + CO_2 + 100 µmol m^{-2} s^{-1}), and culture 4 (mixotrophic

+ 4 mM glucose + ambient air + 100 µmol m^{-2} s^{-1}) were germinated. As described in Section 2.4, astaxanthin was extracted with ethyl acetate from the algal broth in regular time intervals to evaluate the germination efficiency. As shown in Figure 3, CO_2 had a negative effect on germination in both, phototrophic and mixotrophic cultures, in comparison to ambient air. Forty-one hours after the start of germination under phototrophic conditions (culture 2), which was germinated at ambient air, 7.2 mg L^{-1} astaxanthin could be extracted into the solvent, whereas only 2.8 mg L^{-1} could be extracted under phototrophic conditions (culture 1), where additional CO_2 was gassed. As presented in Figure 3, a similar tendency could be seen under mixotrophic conditions, where 4.5 and 8.9 mg L^{-1} were extracted into ethyl acetate when germinated with additional CO_2 (culture 3) and at ambient air (culture 4), respectively. In addition, glucose showed a positive effect on the germination, as more astaxanthin was extracted in mixotrophic culture 3 (4.5 mg L^{-1}) compared to phototrophic culture 1 (2.8 mg L^{-1}), and mixotrophic culture 4 (8.9 mg L^{-1}) compared to phototrophic culture 2 (7.2 mg L^{-1}).

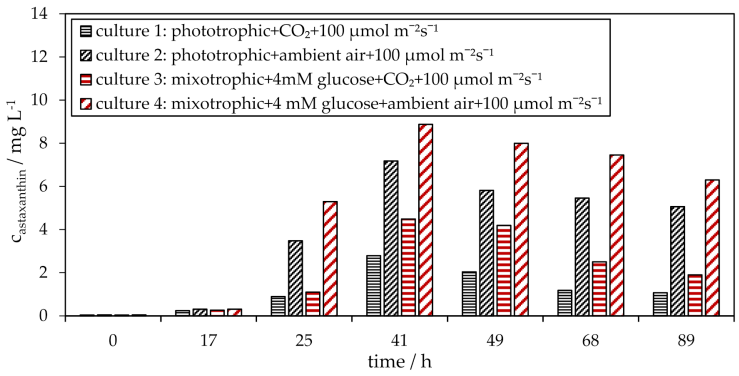

Figure 3. Astaxanthin concentration in the ethyl acetate extract of the phototrophic culture 1 (phototrophic + CO_2 + 100 µmol m^{-2} s^{-1}) and culture 2 (phototrophic + ambient air + 100 µmol m^{-2} s^{-1}), and the mixotrophic culture 3 (mixotrophic + 4 mM glucose + CO_2 + 100 µmol m^{-2} s^{-1}) and culture 4 (mixotrophic + 4 mM glucose + ambient air + 100 µmol m^{-2} s^{-1}) at different time points after the start of germination; data obtained by single determination.

In previous studies, it was shown that more zoospores are released from the cyst cells at higher light intensities in comparison to lower ones. However, the degradation of astaxanthin within the cells was also higher at higher light intensities compared to lower light intensities [25,29]. To counteract a possible degradation of intracellular astaxanthin, identical germination conditions as for culture 1 to culture 4 were applied in the next set of experiments (culture 5 to culture 8), but at a lower light intensity of 75 µmol m^{-2} s^{-1} compared to 100 µmol m^{-2} s^{-1}. In addition to the extraction with ethyl acetate, the cells were counted, and the biomass concentration and the astaxanthin content of the biomass were determined.

CO_2 and ambient air had a similar effect on the phototrophic germination of culture 5 (phototrophic + CO_2 + 75 µmol m^{-2} s^{-1}) and culture 6 (phototrophic + ambient air + 75 µmol m^{-2} s^{-1}) as in the first set of experiments. As presented in 4, 7.0 mg L^{-1} astaxanthin could be extracted into ethyl acetate at the phototrophic conditions at ambient air (culture 6) compared to 2.8 mg L^{-1} with additional CO_2 (culture 5), 49 h after the start of germination. The cell counting revealed that more zoospores were released from culture 6 compared to culture 5, which is in good accordance with the extracted amount of astaxanthin. As can be seen in Figures 4 and 5, 49 h after the start of germination, in culture 6, germinated at ambient air, 45% of the existing cells were in the zoospores stage, while in culture 5, germinated with additional CO_2, only 25% were found in that stage.

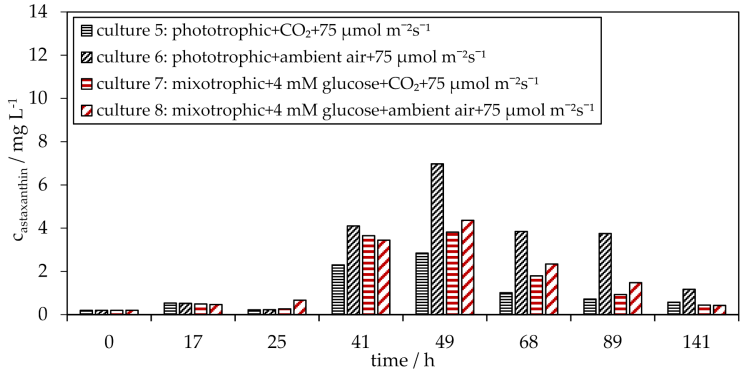

Figure 4. Astaxanthin concentration in the ethyl acetate extract of the phototrophic culture 5 (phototrophic + CO_2 + 75 µmol m^{-2} s^{-1}) and culture 6 (phototrophic + ambient air + 75 µmol m^{-2} s^{-1}), and mixotrophic culture 7 (mixotrophic + 4 mM glucose + CO_2 + 75 µmol m^{-2} s^{-1}) and culture 8 (mixotrophic + 4 mM glucose + ambient air + 75 µmol m^{-2} s^{-1}) at different time points after the start of germination; data obtained by single determination.

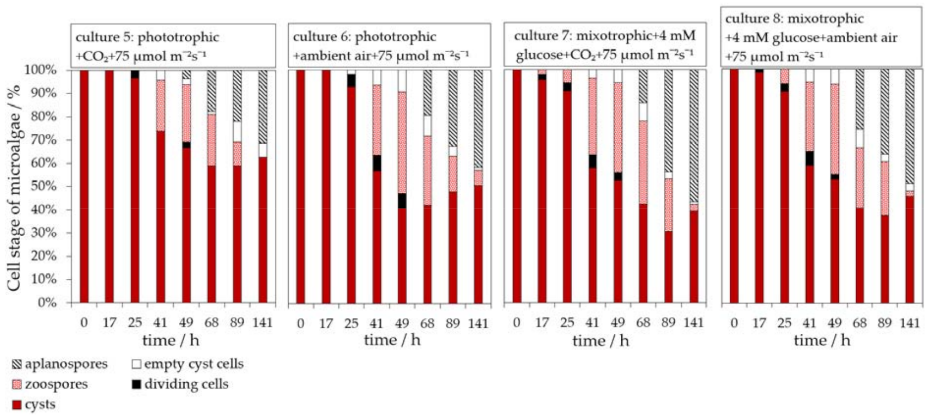

Figure 5. Relative distribution of the cell stages of *H. pluvialis* at different points in time after the start of germination for culture 5 (phototrophic + CO_2 + 75 µmol m^{-2} s^{-1}) and culture 6 (phototrophic + ambient air + 75 µmol m^{-2} s^{-1}), and culture 7 (mixotrophic + 4 mM glucose + CO_2 + 75 µmol m^{-2} s^{-1}) and culture 8 (mixotrophic + 4 mM glucose + ambient air + 75 µmol m^{-2} s^{-1}).

In addition, as presented in Figure 6, 49 h after the start of germination the measured astaxanthin content in the biomass was higher in the phototrophic culture 6 germinated at ambient air (1.9 wt.%) than in phototrophic culture 5 (1.3 wt.%) germinated with additional CO_2, which might be another reason for the higher concentration of astaxanthin extracted with ethyl acetate.

Forty-nine hours after the start of germination, only slightly more astaxanthin (4.4 mg L^{-1}) could be extracted with ethyl acetate under mixotrophic conditions at ambient air (culture 8) compared to 3.8 mg L^{-1} with additional CO_2 (culture 7). At this point in time, in cultures 7 and 8, approximately 38% of the cells were at the zoospore stage, which is slightly lower compared to phototrophic culture 6, which was approximately 45% at that stage. Thus, as shown in Figure 4, the concentration of astaxanthin extracted with ethyl acetate at this time point was lower for the mixotrophic cultures. Additional to the lower amount of released zoospores, this was also due to the lower astaxanthin content in the biomass at mixotrophic conditions with additional CO_2 (culture 7, 1.1 wt.%) and ambient air (culture 8, 1.27 wt.%) compared to phototrophic conditions at ambient air (culture 6, 1.88 wt.%), 49 h after the start of germination (Figure 6).

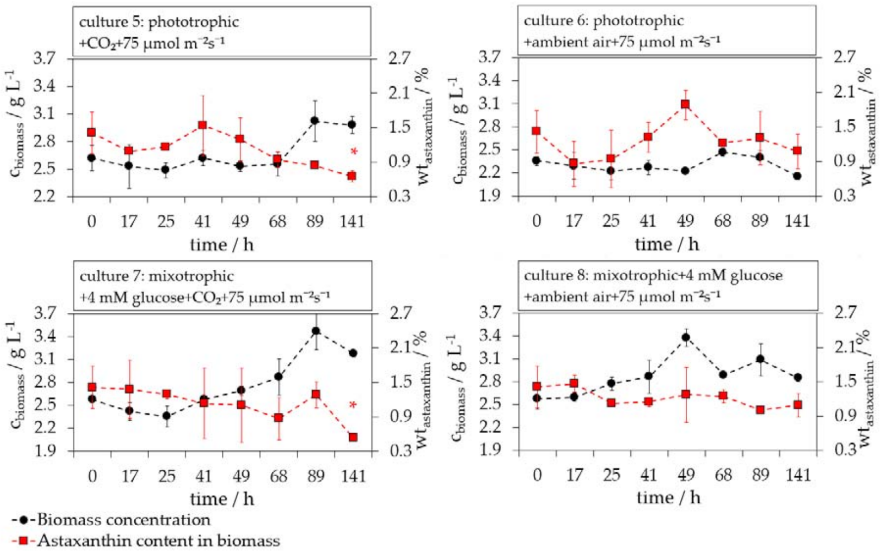

Figure 6. Time course of the biomass concentration and the astaxanthin content in the algal biomass of phototrophic culture 5 (phototrophic + CO2 + 75 µmol m^{-2} s^{-1}) and culture 6 (phototrophic + ambient air + 75 µmol m^{-2} s^{-1}), and mixotrophic culture 7 (mixotrophic + 4 mM glucose + CO2 + 75 µmol m^{-2} s^{-1}) and culture 8 (mixotrophic + 4 mM glucose + ambient air + 75 µmol m^{-2} s^{-1}). Biomass concentration: error bars show ±SD, n = 3. Astaxanthin content: error bars show ±SD, n = 3 at t = 0 h, t = 49 h, and t = 141 h and ±SD, n = 2 at t = 17 h, t = 25 h, t = 41 h, t = 68 h, and t = 89 h of one biological sample; asterisks indicate significant differences (*t*-test) of measured value vs. control (t = 0 h) at $p < 0.05$.

The initial astaxanthin content in the cyst cells of cultures 5–8 was 1.4 wt.%. In the phototrophic culture 5 and mixotrophic culture 7, the CO_2 gassing resulted in a significant reduction ($p < 0.05$) of the intracellular astaxanthin content, to 0.66 and 0.53 wt.%, compared to its starting value 141 h after the start of the germination. In contrast, in phototrophic culture 6, which was germinated at ambient air, 49 h after the start of germination an increase of the astaxanthin content in the biomass to 1.88 wt.% was measured and then declined slightly to 1.08 wt.% at 141 h after the start of germination (Figure 6). Additionally, in the mixotrophic culture 8, the astaxanthin content of the biomass remained relatively stable and was at 1.08 wt.% 149 h after the start of germination. The extraction yield for cultures 5–8 was calculated as the mass of astaxanthin that was extracted into ethyl acetate to the mass of astaxanthin in the biomass before the extraction with ethyl acetate (Section 2.4). The highest astaxanthin extraction yield was reached 49 h after the start of germination for the phototrophic culture 6 cultivated at ambient air with 16%, compared to 9% for the phototrophic culture 5 germinated with additional CO_2. An extraction yield of 11% was reached for the mixotrophic culture 8, germinated at ambient air, and a yield of 8% for the mixotrophic culture 7, in which additional CO_2 was supplied.

In the first set of experiments (culture 1–4), the greatest amount of astaxanthin could be extracted under mixotrophic conditions at ambient air (culture 4). In the second set of experiments (culture 4–8), most astaxanthin was extracted under phototrophic conditions at ambient air (culture 6). However, the tendencies of the extracted amount of astaxanthin are similar within the first and second set of experiments for the cultivation conditions applied, with exception of the results of mixotrophic conditions at ambient air. Only the values under mixotrophic conditions at ambient air and 100 µmol^{-2} s^{-1} (culture 4) and mixotrophic conditions at ambient air at 75 m^{-2} s^{-1} (culture 8) show a deviation when compared to the other three germination conditions. Thus, it was assumed that the change of the light intensity from 100 to 75 µmol m^{-2} s^{-1} did not influence the tendencies obtained within the first set and second set of experiments, but that there was a problem in

germination for mixotrophic cultivation at ambient air and 100 µmol m^{-2} s^{-1} (culture 8) compared to mixotrophic conditions at ambient air and 75 µmol m^{-2} s^{-1} (culture 4).

To evaluate whether the cyst cell germination is enhanced under phototrophic or mixotrophic conditions at ambient air, in the third set of experiments, phototrophic (culture 11) and mixotrophic (culture 12) germination at ambient air and a light intensity of 75 µmol m^{-2} s^{-1} was repeated. Since CO_2 had a negative effect on phototrophic and mixotrophic germination, it was assumed that it is not fixed by the cyst cell and that the overall photosynthetic activity of the cysts is reduced. To show that *H. pluvialis* cyst germination can be performed independently of photosynthetic activity, two heterotrophic conditions were applied for the germination of cultures 9 and 10. Both cultures were grown in dark conditions, without light, and at ambient air. Heterotrophic culture 9 was germinated without an external carbon source and depended on internal carbon reserves such as starch, fatty acids, or triacylglycerides (TAG) for energy production. Heterotrophic culture 10 was germinated with additional glucose in the BBM.

As shown in Figure 7, significantly more astaxanthin could be extracted into ethyl acetate in heterotrophic culture 9 (15.0 mg L^{-1}), germinated without an external carbon source, and heterotrophic culture 10 (24.8 mg L^{-1}), germinated with more glucose than in the phototrophic culture 11 (7.5 mg L^{-1}) and mixotrophic culture 12 (8.3 mg L^{-1}), 49 h after the start of germination. Similar to mixotrophic culture 4 (8.9 mg L^{-1}) and phototrophic culture 2 (7.2 mg L^{-1}), more astaxanthin could be extracted into ethyl acetate at mixotrophic conditions of culture 12 (8.3 mg L^{-1}) compared to the phototrophic conditions of culture 11 (7.5 mg L^{-1}). As shown in Figure 8, 49 h after the start of germination, 33% of the cells were in the zoospore stage in the mixotrophic culture 12 and 28% were in the zoospore stage in the phototrophic culture 11. In heterotrophic conditions with additional glucose (culture 10), 82% of the cells were in the zoospore stage, whereas without an external carbon source (culture 9) 62% were present at this cell stage, 49 h after the start of germination. According to the data presented in Figure 7 for heterotrophic culture 10, germinated at ambient air approx. 70% more astaxanthin could be extracted into ethyl acetate than in the heterotrophic culture 9, germinated without an external carbon source, 49 h after the start of germination.

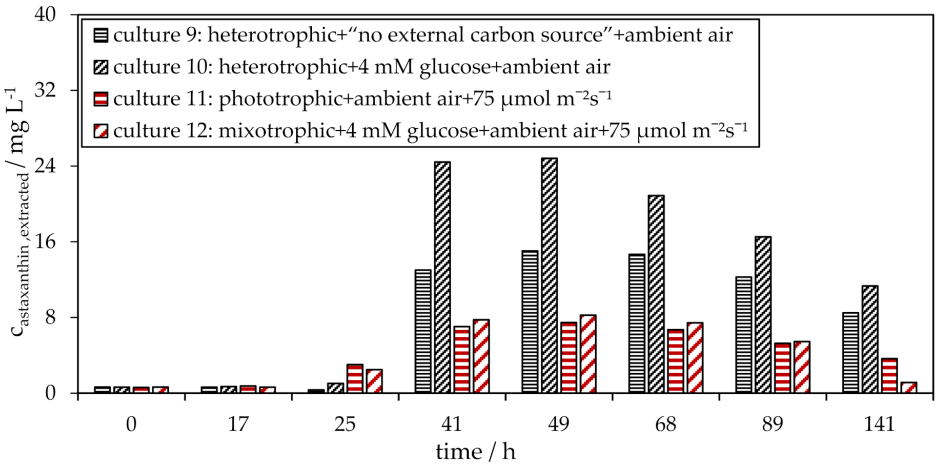

Figure 7. Astaxanthin concentration in the ethyl acetate extract of the heterotrophic culture 9 (heterotrophic + "no external carbon source" + ambient air) and culture 10 (heterotrophic + 4 mM glucose + ambient air), phototrophic culture 11 (phototrophic + ambient air + 75 µmol m^{-2} s^{-1}) and mixotrophic culture 12 (mixotrophic + 4 mM glucose + ambient air + 75 µmol m^{-2} s^{-1}) at different time points after the start of germination; data obtained by single determination.

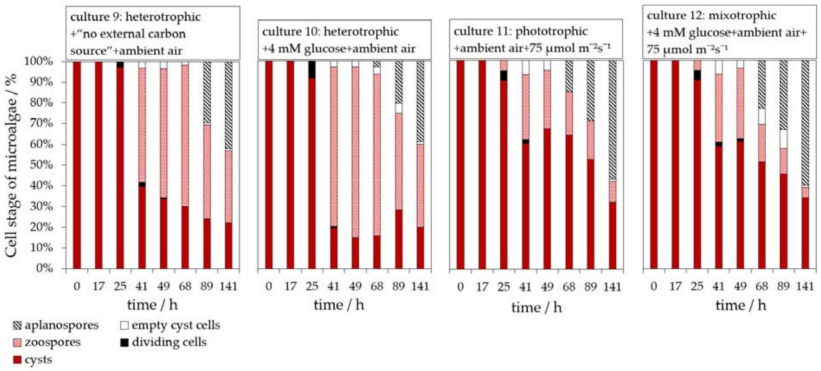

Figure 8. Relative distribution of the cell stages of *H. pluvialis* at different points in time after the start of germination for the heterotrophic culture 9 (heterotrophic + "no external carbon source" + ambient air) and culture 10 (heterotrophic + 4 mM glucose + ambient air), phototrophic culture 11 (phototrophic + ambient air + 75 µmol m^{-2} s^{-1}) and mixotrophic culture 12 (mixotrophic + 4 mM glucose + ambient air + 75 µmol m^{-2} s^{-1}).

As presented in Figure 9, the initial astaxanthin content in the cyst biomass of culture 9–12 was 1.51 wt.% and no decline of the astaxanthin content in the biomass was measured at heterotrophic conditions without an external carbon source (culture 9) and with glucose (culture 10). In culture 10, an increase of the astaxanthin content to 2.28 wt.% was measured 49 h after the start of germination, with a relative standard deviation of 36%. Similar to the previous set of experiments, the astaxanthin content in the biomass at mixotrophic conditions (culture 12) fell slightly in the course of germination ($p > 0.05$) to 1.11 wt.% 149 h after the start of germination, and the astaxanthin content of the phototrophic culture 11 decreased slightly to 1.42 wt.% ($p > 0.05$). A stronger decrease of the astaxanthin biomass content in *H. pluvialis* at higher light intensity compared to germination without light has already been described in the literature. There, the astaxanthin content decreased from 454 initially to 46 pg $cell^{-1}$ for phototrophic illuminated cells at 240 µmol m^{-2} s^{-1} and 116 pg $cell^{-1}$ for heterotrophic cultures (without external glucose) [29].

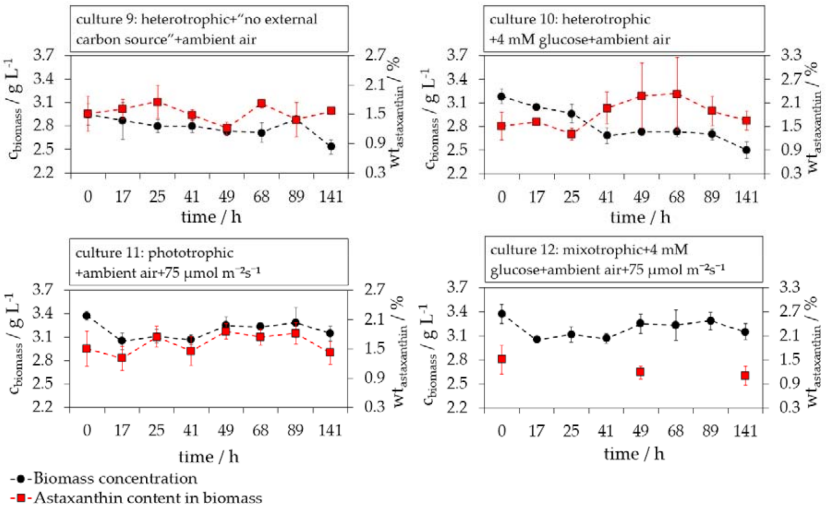

Figure 9. Time course of the biomass concentration and the astaxanthin content in the algal biomass of heterotrophic culture 9 (heterotrophic + "no external carbon source" + ambient air) and culture 10 (heterotrophic + 4 mM glucose + ambient air), phototrophic culture 11 (phototrophic + ambient air + 75 µmol m^{-2} s^{-1}) and mixotrophic culture 12 (mixotrophic + 4 mM glucose + ambient air + 75 µmol m^{-2} s^{-1}). Biomass concentration: error bars show ±SD, n = 3. Astaxanthin content: error bars show ±SD, n = 3 at t = 0 h, t = 49 h and t = 141 h and ±SD, n = 2 at t = 17 h, t = 25 h, t = 41 h, t = 68 h and t = 89 h of one biological sample; asterisks indicate significant differences (*t*-test) of measured value vs. control (t = 0 h) at $p < 0.05$.

The highest astaxanthin extraction yield of 50% was reached for germination at heterotrophic conditions and additional external glucose (culture 10), 49 h after the start of germination. The second highest extraction yield was 45% at heterotrophic conditions without an external glucose source (culture 9). Based on the large difference in the concentration of astaxanthin extracted with ethyl acetate, 24.8 mg L^{-1} compared to 15 mg L^{-1} (Figure 7), and the number of released zoospores of 82% to 62% (Figure 8) for heterotrophic culture with glucose in comparison without glucose, an even more significant difference of the yields would have been expected. However, due to the higher astaxanthin content measured in the biomass at heterotrophic conditions with external glucose (culture 10), 49 h after the start of germination, in comparison to without external glucose (culture 9), the yields almost converge. Significantly lower yields were reached with 22% and 16% for the mixotrophic (culture 12) and phototrophic (culture 11) germination conditions, respectively.

In the previous set of experiments, the highest yield, the highest concentration of astaxanthin extracted into ethyl acetate, and the number of released zoospores were reached at heterotrophic conditions with additional glucose (culture 10). These conditions were repeated in the following set of experiments as culture 13. Additionally, a heterotrophic culture with external glucose and twice the nitrate concentration was germinated (culture 14) to rule out a limitation of the germination due to nitrate deficiency. In addition, it was found in the previous set of experiments that zoospore formation was faster under phototrophic and mixotrophic conditions (culture 11 and 12) compared to heterotrophic conditions (culture 9 and 10). However, the maximum number of released zoospores and the extracted amount of astaxanthin in ethyl acetate was higher at heterotrophic conditions compared to mixotrophic or phototrophic conditions. For that reason, two cultures were germinated; mixotrophic and heterotrophic cultivation conditions were combined. These two cultures were cultivated at a light intensity of 75 µmol m^{-2} s^{-1} until nitrate was consumed to accelerate the zoospores formation, and further cultivated at heterotrophic conditions (without light) to reach a high total number of released zoospores. One of these cultures was germinated at double nitrate content of the medium (culture 16), while the other culture was germinated without additional nitrate (culture 15).

As shown in Figure 10, a doubling of the nitrate content in heterotrophic germination conditions hardly affected the concentration of astaxanthin extracted in ethyl acetate, as can be seen when comparing culture 13 (12.9 mg L^{-1}) and culture 14 (12.6 mg L^{-1}). When mixotrophic and heterotrophic germination conditions were combined, the doubling of the nitrate content showed an influence on the concentration of astaxanthin extracted into ethyl acetate. There, 49 h after the start of germination, the concentration of astaxanthin extracted with ethyl acetate was 17.1 mg L^{-1} for double nitrate concentration (culture 16) and 8.4 mg L^{-1} when no additional nitrate was added (culture 15). Why the combination of mixotrophic and heterotrophic germination conditions at a nitrate concentration of the BBM (culture 15) with 8.4 mg L^{-1} was worse compared to the double nitrate concentration (culture 16) with 17.1 mg L^{-1} and the heterotrophic culture 13 (12.9 mg L^{-1}) and culture 12 (12.6 mg L^{-1}) cannot be clarified in this context. The combination of mixotrophic and heterotrophic germination conditions represents a complex procedure in which the influence of nitrate and the light intensity must be further investigated.

As presented in Figure 11, 49 h after the start of germination at double nitrate concentration of the mixo-/heterotrophic conditions (culture 16), 84% of cells were at the zoospore stage, although only 40% were in that cell stage at mixo-/heterotrophic conditions with the nitrate of the BBM (culture 15). A comparison of the two heterotrophic cultivation conditions with the nitrate of the BBM (culture 13) and double nitrate (culture 14)) show that the nitrate concentration had little influence on the maximum number of released zoospores, which was approximately 70% for both cultivation conditions, 49 h after the start of germination. The amount of zoospores released shows a good correlation with the concentration of astaxanthin extracted with ethyl acetate for all examined conditions.

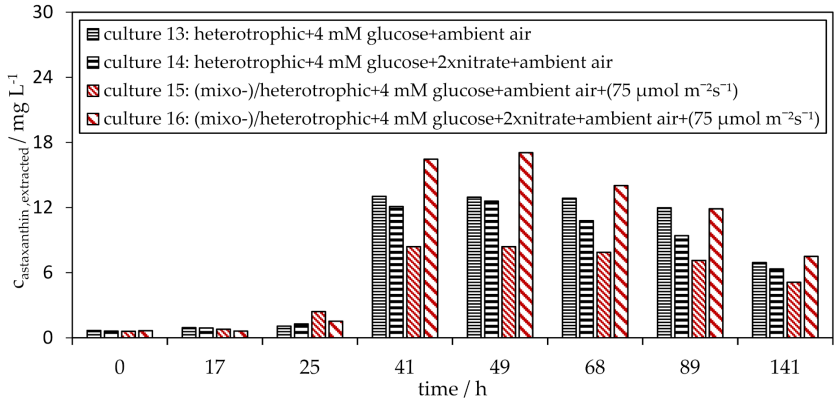

Figure 10. Astaxanthin concentration in the ethyl acetate extract of the heterotrophic culture 13 (heterotrophic + 4 mM glucose + ambient air) and culture 14 (heterotrophic + 4 mM glucose + 2xnitrate + ambient air), and culture 15 (mixo-/heterotrophic + 4 mM glucose + ambient air + (75 µmol m^{-2} s^{-1}) and culture 16 (mixo-/heterotrophic + 4 mM glucose + 2xnitrate + ambient air + (75 µmol m^{-2} s^{-1}); data obtained by single determination.

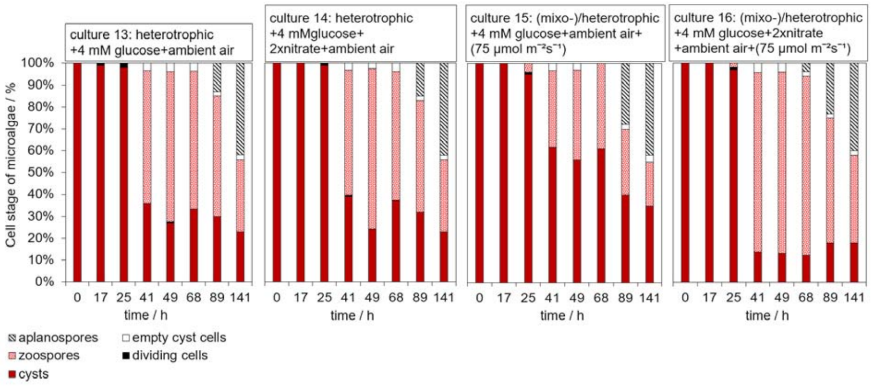

Figure 11. Relative distribution of the cell stages of *H. pluvialis* at different times after the start of germination for the heterotrophic culture 13 (heterotrophic + 4 mM glucose + ambient air) and culture 14 (heterotrophic + 4 mM glucose + 2xnitrate + ambient air), and culture 15 (mixo-/heterotrophic + 4 mM glucose + ambient air + (75 µmol m^{-2} s^{-1}) and culture 16 (mixo-/heterotrophic + 4 mM glucose + 2xnitrate + ambient air + (75 µmol m^{-2} s^{-1}); data obtained by single determination.

As shown in Figure 12, the astaxanthin content in the biomass remained relatively constant for the culture conditions examined ($p > 0.05$). Increased supply of glucose for glycolysis could result in an increased carbon flow into the citric acid cycle and might prevent the consumption of astaxanthin fatty acid esters for energy production.

The highest extraction yield of 64% was reached in this set of experiments for the combination of mixotrophic and heterotrophic germination conditions at double nitrate concentration (culture 16). The second highest yield of 56% was reached at heterotrophic conditions at double nitrate content (culture 14), followed by 45% at the nitrate content of the BBM (culture 13). Though slightly more astaxanthin was extracted into ethyl acetate at heterotrophic conditions at the nitrate content of the BBM (culture 13) compared to the heterotrophic conditions with double nitrate concentration (culture 14), the yield was higher at heterotrophic conditions of double nitrate concentration. This is due to the slightly lower astaxanthin content in the biomass, 49 h after the start of germination at heterotrophic conditions and double nitrate (culture 14), compared to the heterotrophic conditions with the nitrate concentration of the BBM (culture 13). The lowest extraction yield of 26% was reached in this set of experiments for the combination of the mixotrophic and heterotrophic cultivation conditions at the nitrate content of the BBM (culture 15)

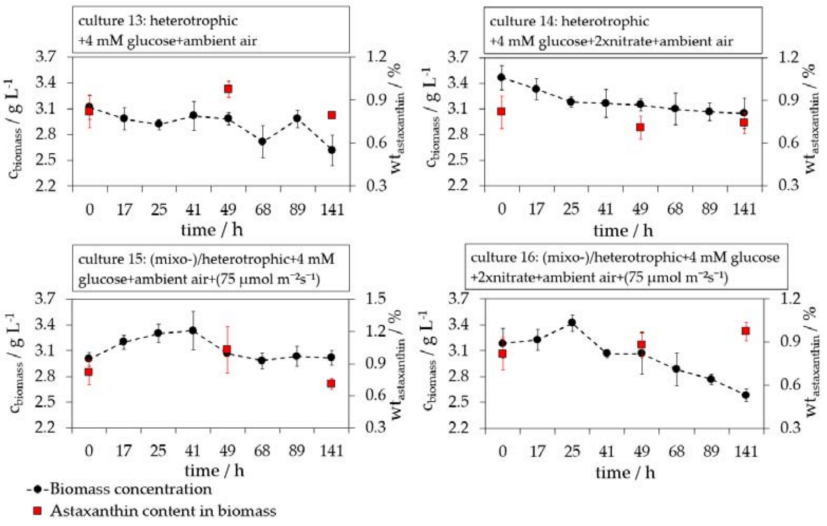

Figure 12. Time course of the biomass concentration and the astaxanthin content in the algal biomass of heterotrophic culture 13 (heterotrophic + 4 mM glucose + ambient air) and culture 14 (heterotrophic + 4 mM glucose + 2xnitrate + ambient air), and culture 15 (mixo-/heterotrophic + 4 mM glucose + ambient air + (75 µmol m^{-2} s^{-1})) and culture 16 (mixo-/heterotrophic + 4 mM glucose + 2xnitrate + ambient air + (75 µmol m^{-2} s^{-1})). Biomass concentration: error bars show ±SD, n = 3. Astaxanthin content: error bars show ±SD, n = 3 at t = 0 h, t = 49 h and t = 141 h and ±SD, n = 2 at t = 17 h, t = 25 h, t = 41 h, t = 68 h and t = 89 h of one biological sample; asterisks indicate significant differences (*t*-test) of measured value vs. control (t = 0 h) at $p < 0.05$.

3.2. Influence of CO_2 on Phototrophic and Mixotrophic H. pluvialis Cyst Cell Germination

A systematic study of the influence of the CO_2 content on the germination of *H. pluvialis* cells has not yet been carried out. In literature, phototrophic germination of *H. pluvialis* was done at different levels of CO_2, gassing at ambient air [30], every 10 min for 10 s with CO_2 supplemented air [29], but also continuously with flue gas with 3.5 +/− 0.5% *v*/*v* CO_2 [25], making it difficult to compare these data to the results obtained in our study for the influence of CO_2 on germination. In general, in *H. pluvialis*, CO_2 fixation takes place in both C3 and C4 photosynthetic pathways [37,38]. CO_2 is fixed by the enzyme ribulose-1,5-bisphosphate carboxylase during the Calvin cycle and glyceraldehyde-3-phosphate is formed, which is required for the further synthesis of carbohydrates and the basic structure of lipids, which are processed, among other components, into secondary metabolites such as astaxanthin [38,39]. As shown in the first two sets of experiments (culture 1–8), photo- and mixotrophic cyst cell germination was not enhanced by the addition of CO_2 compared to germination at ambient air. A constant influx of CO_2 and fixation via the Calvin Cycle might shift the equilibrium of degradation to formation of internally stored carbon sources. Thus, reduced energy for germination would be available, as storage compounds are synthesized and not used for germination, which might ultimately result in a reduced release of zoospores. In contrast, reduced photosynthetic activity and degradation of the chloroplast through *H. pluvialis* cyst formation has been reported and might explain that excess of CO_2 supplied cannot be used efficiently for growth [40]. To what extent the activity of the chloroplasts in the formed zoospores influences the number of germinated zoospores and their CO_2 uptake cannot be clarified in this context.

3.3. Influence of Glucose on the H. pluvialis Cyst Cell Germination

In microalgae, glucose is processed to generate energy for cell growth and maintenance via glycolysis within the cell [41]. During *H. pluvialis* cyst germination, glycolysis and fatty acid oxidation provide the primary energy and precursor for a variety of biosynthetic pathways. Starch, which is the most widely used storage polysaccharide in algae, is the carbohydrate source during germination and zoospore formation [42,43]. It has been

shown in phototrophic *H. pluvialis* cyst germination that the genes and enzymes involved in the ß-oxidation of fatty acids and glycolysis show upregulation and downregulation to different degrees during germination. Stored starch is degraded via the upregulated α-amylase during germination, providing dextrin and maltose for glucose metabolism [42]. It was shown that the germination was enhanced at mixotrophic in comparison to the phototrophic conditions, at otherwise identical parameters. The results were confirmed for CO_2 under mixotrophic conditions (culture 3 and 7) compared to phototrophic conditions (culture 1 and 5), and for ambient air under mixotrophic conditions (culture 4 and 12) compared to phototrophic conditions (culture 2 and 11). The mixotrophic conditions at ambient air (culture 8) compared to the phototrophic conditions at ambient air (culture 6) might present an exception, which might be due to the state of the cyst cells at the beginning of germination.

Additionally, the results suggest that the heterotrophic germination of *H. pluvialis* cysts is enhanced by adding external glucose into the medium (culture 10), compared to germination, which is based on the mobilization of intracellular stored carbon sources only (culture 9) (Figure 8).

The sugar analysis, described in Section 2.7, detects the reaction of simple sugars, oligosaccharides, polysaccharides, and their derivatives, including methyl esters with free-reducing groups with phenol and sulphuric acid, which react to give an orange-brown color [36]. Although the heterotrophic culture 9 was germinated without an external carbon source, a sugar concentration of approximately 150 mg L^{-1} could be measured in the medium at the start of germination, which is probably due to extra polymeric saccharides of the cyst cells (Figure 13).

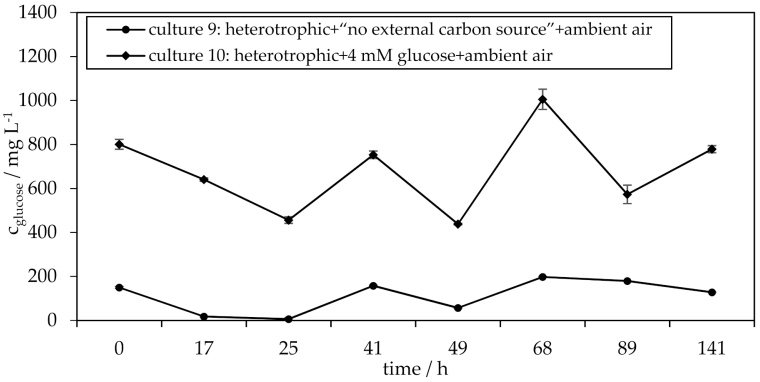

Figure 13. Time course of the glucose concentration in the medium of heterotrophic culture 9 (heterotrophic + "no external carbon source" + ambient air without external glucose) and culture 10 (heterotrophic + 4 mM glucose + ambient air with external glucose); error bars show ±SD, n = 3.

For both heterotrophic cultures, a decrease in glucose concentration was observed 25 h after the start of germination, which indicates active glucose consumption. The increase of the sugar concentration after 41 h in both cultures is most probably attributed to the presence of extra polymeric saccharides, parts of the mannose-containing cyst cell wall, and hexoses of the cell-matrix of the zoospores [7], which might be partly dissolved in the aqueous supernatant and detected using the analytical method for the sugar quantification.

In all heterotrophic cultures examined, the astaxanthin content in the biomass remained relatively constant ($p > 0.05$). A possible reason for the stable astaxanthin content in the biomass could be the increased supply of glucose for glycolysis that results in an increased carbon flow into the citric acid cycle. It is therefore possible that the need to break down astaxanthin fatty acid esters for energy production and provision of the carbon skeletons for the citric acid cycle is reduced by adding external glucose.

3.4. Influence of Light on the H. pluvialis Cyst Cell Germination

Light influences the speed of nitrate uptake, while nitrate plays an essential role in the growth and synthesis of astaxanthin [38] and cyst germination in *H. pluvialis* [25,28,29]. The time course of the nitrate concentrations of culture 5 to culture 8 is presented in Figure 14. As can be seen, all nitrate was nearly consumed 17 h after the start of germination in the cultures; an identical light intensity of 75 µmol m^{-2} s^{-1} was applied to all cultures.

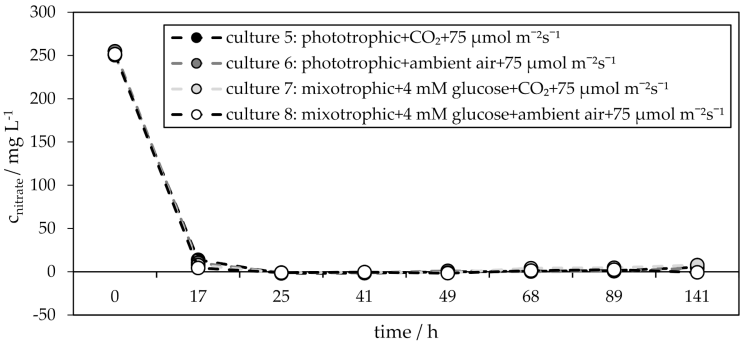

Figure 14. Time course of the nitrate concentration in the supernatant of the phototrophic culture 5 (phototrophic + CO_2 + 75 µmol m^{-2} s^{-1}) and culture 6 (phototrophic + ambient air + 75 µmol m^{-2} s^{-1}), and mixotrophic culture 7 (mixotrophic + 4 mM glucose + CO_2 + 75 µmol m^{-2} s^{-1}) and culture 8 (mixotrophic + 4 mM glucose + ambient air + 75 µmol m^{-2} s^{-1}).

The time course of the nitrate concentration for the third set of experiments (culture 9 to culture 12) is presented in Figure 15. In the illuminated phototrophic and mixotrophic (culture 11 and 12) nitrate is already consumed 17 h after the start of germination, while it is depleted after 25 h in heterotrophic conditions (culture 9 and 10). Similar tendencies were observed in the literature, where nitrate was consumed two days earlier in a *H. pluvialis* cyst culture illuminated at 300 µmol m^{-2} s^{-1} compared to 150 µmol m^{-2} s^{-1} [25]. The faster nitrate consumption was accompanied by a trend toward the faster release of zoospores for the illuminated cultures, whereby for all cultivation conditions the maximum zoospore release was reached 49 h after the start of germination (Figure 8).

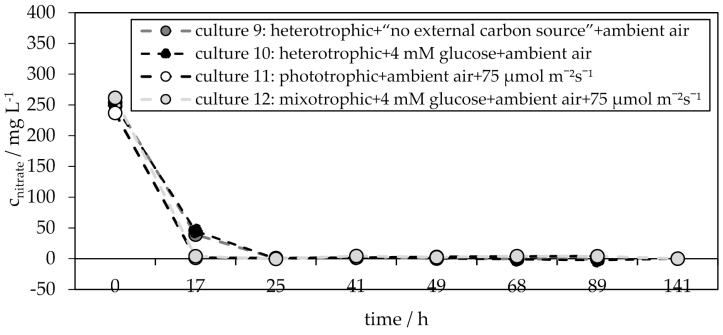

Figure 15. Time course of the nitrate concentration in the supernatant of the culture 9 (heterotrophic + "no external carbon source" + ambient air) and culture 10 (heterotrophic + 4 mM glucose + ambient air), phototrophic culture 11 (phototrophic + ambient air + 75 µmol m^{-2} s^{-1}) and mixotrophic culture 12 (mixotrophic + 4 mM glucose + ambient air + 75 µmol m^{-2} s^{-1}).

As shown in Figure 16, a similar trend was also measured for the heterotrophic conditions of cultures 13 and 14, when compared to the experiments where mixotrophic and heterotrophic conditions were combined (cultures 15 and 16). These were cultivated at a light intensity of 75 µmol m^{-2} s^{-1} until nitrate was consumed and further cultivated at heterotrophic conditions (without light).

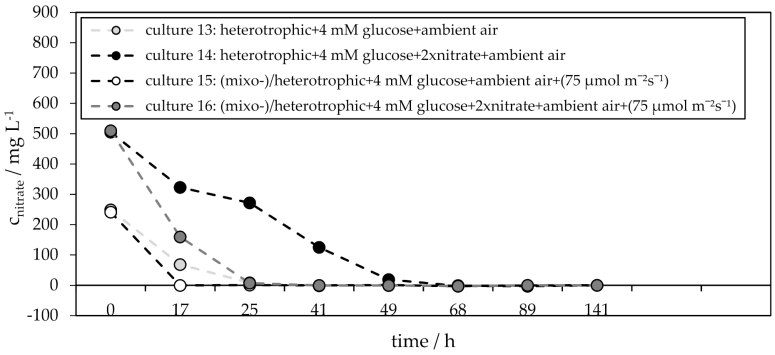

Figure 16. Time course of the nitrate concentration heterotrophic culture 13 (heterotrophic + 4 mM glucose + ambient air) and culture 14 (heterotrophic + 4 mM glucose + 2xnitrate + ambient air), and culture 15 (mixo-/heterotrophic + 4 mM glucose + ambient air + (75 µmol m^{-2} s^{-1}) and culture 16 (mixo-/heterotrophic + 4 mM glucose + 2xnitrate + ambient air + (75 µmol m^{-2} s^{-1}); data obtained by single determination.

In general, the rate of nitrate and nitrite uptake in microalgae depends on the nitrogen status of the cells, the presence of other nitrogen compounds, and light conditions [39,40]. Nitrate enters the cell via specific transport systems, followed by a reduction to nitrite in the cytosol. Nitrite is then transported to the chloroplast where it is reduced to ammonium. Finally, ammonium is incorporated into L-glutamate through the glutamine/glutamate cycle [40] and allows the formation of amino acids and cell growth. In the absence of nitrate, supplemented carbon in the form of CO_2 or glucose is stored as starch, TAGs, or fatty acids. In conclusion, the nitrate uptake and formation of zoospores were accelerated in the illuminated phototrophic and mixotrophic conditions compared to the heterotrophic conditions. However, the illumination itself did not enhance the number of released zoospores, as more zoospores were released at heterotrophic conditions (culture 9 and 10) compared to the phototrophic and mixotrophic conditions (culture 11 and 12) (Figure 8). Light stress as a reason for the overall lower number of released zoospores of illuminated cultures compared to heterotrophic cultures can be excluded, as the vegetative cell growth (Figure 1a) to receive the biomass for the germination experiments was also performed at the light conditions of 75 µmol m^{-2} s^{-1} and lower starting biomass concentrations compared to the germination experiments. Growth rates of approximately 0.4 d^{-1} were reached for the vegetative cell growth. Thus the exact influence of light on the germination, apart from the nitrate uptake, has to be examined further.

4. Conclusions

The germination of the cyst cell of *H. pluvialis* represents an interesting alternative to conventional mechanical cell wall disruption [26]. In the industrial process, phototrophic cultivation is used for the growth and accumulation of astaxanthin. It was shown that by applying phototrophic conditions, nearly all cyst cells can be germinated, though this is always accompanied by a significant loss of astaxanthin [25,29]. In this work, initially, the influence of the CO_2 content of the aeration on photo- and mixotrophic germination was investigated. It was found that more zoospores for both cultivation conditions and consequently more astaxanthin could be extracted into ethyl acetate when gassed with ambient air compared to 3% v/v CO_2. These results led to the assumption that the carbon supply during germination is not obtained by CO_2 fixation, but rather due to the metabolism of internal carbon sources, such as fatty acids, starch, and TAGs. Thus, two heterotrophic cultures were germinated, in which the number of released zoospores could be further increased by adding 4 mM glucose. In heterotrophic cultures, astaxanthin extraction yields of up to 50% could be achieved and the astaxanthin content remained relatively constant in the biomass within the entire period of germination. The illumination of mixotrophic cultures with 75 µmol m^{-2} s^{-1}, compared to heterotrophic cultivation, results in an accelerated release of zoospores due to faster nitrate uptake, while the maximal

number of released zoospores was higher in heterotrophic cultures. With twice the nitrate content and short-term illumination of the culture until nitrate depletion, the extraction yield could be increased up to 64%. Transferring the heterotrophic germination from the orbital shaker and photobioreactors used to a fermenter may improve the oxygen transfer, resulting in a further increase in the number of zoospores released. To increase the yield, i.e., increase the number of released zoospores, the metabolism during heterotrophic germination should be understood at a genetic level and different carbon sources need to be tested, as they enter the cell metabolism through different metabolic pathways [41].

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app11167201/s1. Figure S1. Absorption of ethyl acetate at 478 nm after liquid–liquid extraction of phototrophic and mixotrophic germinated *H. pluvialis* cyst cells at defined time intervals. Influence of CO_2 on the phototrophic and mixotrophic (sodium acetate, glucose, and ribose) cyst cell germination at 100 µmol m^{-2} s^{-1}, Figure S2. Absorption of ethyl acetate at 478 nm after liquid–liquid extraction of mixotrophic and heterotrophic germinated *H. pluvialis* cyst cells at 4, 8, 20, and 40 mM glucose at defined time intervals, Figure S3. Astaxanthin concentration in ethyl acetate extract, 49 h after the start of germination combining mixo- and heterotrophic germination conditions, illuminating the cultures for 25 h at 75 µmol m^{-2} s^{-1} followed by cultivation in the dark for three independently germinated cultures of the same cyst cell biomass (mixo-/heterotrophic + 4 mM glucose + ambient air + (75 µmol m^{-2} s^{-1})).

Author Contributions: Conceptualization, A.B. and M.M.; methodology, A.B. and M.M.; validation, A.B.; formal analysis, A.B.; investigation, A.B.; resources, A.B. and M.M.; data curation, A.B.; writing—original draft preparation, A.B.; writing—review and editing, A.B. and M.M.; visualization, A.B.; supervision, M.M.; project administration, M.M.; funding acquisition, M.M. Both authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by FEDERAL MINISTRY OF ECONOMIC AFFAIRS AND ENERGY (BMWi), grant number ZF4025031 SB8 and A. Bauer was funded from a fellowship granted by FOUNDATION OF GERMAN BUSINESS (sdw). This work was supported by the German Research Foundation (DFG) and the Technical University of Munich within the funding program Open Access Publishing.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Li, X.; Wang, X.; Duan, C.; Yi, S.; Gao, Z.; Xiao, C.; Agathos, S.N.; Wang, G.; Li, J. Biotechnological production of astaxanthin from the microalga *Haematococcus pluvialis*. *Biotechnol. Adv.* **2020**, *43*, 107602. [CrossRef]
2. Shah, M.R.; Liang, Y.; Cheng, J.; Daroch, M. Astaxanthin-Producing Green Microalga *Haematococcus pluvialis*: From Single Cell to High Value Commercial Products. *Front. Plant Sci.* **2016**, *7*, 531. [CrossRef]
3. Astaxanthin Market Size, Share & Trends Analysis Report By Source, by Product (Dried Algae Meal, Oil, Softgel), by Application (Nutraceutical, Cosmetics, Aquaculture and Animal Feed), and Segment Forecasts, 2020–2027. 2020. Available online: https://www.grandviewresearch.com/industry-analysis/global-astaxanthin-market (accessed on 17 February 2021).
4. Richmond, A. (Ed.) *Handbook of Microalgal Culture: Biotechnology and Applied Phycology*; Blackwell Publishing Ltd.: Hoboken, NJ, USA, 2004.
5. Czygan, F.C. Blood-rain and blood-snow: Nitrogen-deficient cells of *Haematococcus pluvialis* and *Chlamydomonas nivalis*. *Arch. Mikrobiol.* **1970**, *74*, 69–76. [CrossRef]
6. Zhang, C.; Liu, J.; Zhang, L. Cell cycles and proliferation patterns in *Haematococcus pluvialis*. *Chin. J. Oceanol. Limnol.* **2016**, *35*, 1205–1211. [CrossRef]
7. Hagen, C.; Siegmund, S.; Braune, W. Ultrastructural and chemical changes in the cell wall of *Haematococcus pluvialis* (Volvocales, Chlorophyta) during aplanospore formation. *Eur. J. Phycol.* **2002**, *37*, 217–226. [CrossRef]
8. Bux, F.; Chisti, Y. (Eds.) *Algae Biotechnology*; Springer Science + Business Media: Berlin, Germany, 2016.
9. Kobayashi, M.; Kakizono, T.; Yamaguchi, K.; Nishio, N.; Nagai, S. Growth and astaxanthin formation of *Haematococcus pluvialis* in Heterotrophic and Mixotrophic conditions. *J. Ferment. Bioeng.* **1992**, *74*, 17–20. [CrossRef]
10. Wan, M.; Zhang, Z.; Wang, J.; Huang, J.; Fan, J.; Yu, A.; Wang, W.; Li, Y. Sequential Heterotrophy–Dilution–Photoinduction Cultivation of *Haematococcus pluvialis* for efficient production of astaxanthin. *Bioresour. Technol.* **2015**, *198*, 557–563. [CrossRef]

11. Hu, J.; Nagarajan, D.; Zhang, Q.; Chang, J.-S.; Lee, D.-J. Heterotrophic cultivation of microalgae for pigment production: A review. *Biotechnol. Adv.* **2018**, *36*, 54–67. [CrossRef] [PubMed]
12. Boyle, N.R.; Morgan, A.J. Flux balance analysis of primary metabolism in *Chlamydomonas reinhardtii*. *BMC Syst. Biol.* **2009**, *3*, 4. [CrossRef]
13. Dong, Q.-L.; Zhao, X.-M. In situ carbon dioxide fixation in the process of natural astaxanthin production by a mixed culture of *Haematococcus pluvialis* and *Phaffia rhodozyma*. *Catal. Today* **2004**, *98*, 537–544. [CrossRef]
14. Gong, X.-D.; Chen, F. Rapid detection of heterotrophic growth of *Haematococcus pluvialisusing* indirect conductimetry. *Biotechnol. Tech.* **1997**, *11*, 841–844. [CrossRef]
15. Oncel, S.S.; Sukan, F.V.; Imamoglu, E.; Gunerken, E. Comparison of different cultivation modes and light intensities using mono-cultures and co-cultures of *Haematococcus pluvialis* and *Chlorella zofingiensis*. *J. Chem. Technol. Biotechnol.* **2010**, *86*, 414–420. [CrossRef]
16. Cheirsilp, B.; Torpee, S. Enhanced growth and lipid production of microalgae under mixotrophic culture condition: Effect of light intensity, glucose concentration and fed-batch cultivation. *Bioresour. Technol.* **2012**, *110*, 510–516. [CrossRef]
17. Pang, N.; Chen, S. Effects of C5 organic carbon and light on growth and cell activity of *Haematococcus pluvialis* under mixotrophic conditions. *Algal Res.* **2017**, *21*, 227–235. [CrossRef]
18. Nahidian, B.; Ghanati, F.; Shahbazi, M.; Soltani, N. Effect of nutrients on the growth and physiological features of newly isolated *Haematococcus pluvialis* TMU1. *Bioresour. Technol.* **2018**, *255*, 229–237. [CrossRef] [PubMed]
19. Chekanov, K.; Schastnaya, E.; Solovchenko, A.; Lobakova, E. Effects of CO_2 enrichment on primary photochemistry, growth and astaxanthin accumulation in the chlorophyte *Haematococcus pluvialis*. *J. Photochem. Photobiol. B Biol.* **2017**, *171*, 58–66. [CrossRef] [PubMed]
20. Katsuda, T.; Lababpour, A.; Shimahara, K.; Katoh, S. Astaxanthin production by *Haematococcus pluvialis* under illumination with LEDs. *Enzym. Microb. Technol.* **2004**, *35*, 81–86. [CrossRef]
21. Xi, T.; Kim, D.G.; Roh, S.W.; Choi, J.-S.; Choi, Y.-E. Enhancement of astaxanthin production using *Haematococcus pluvialis* with novel LED wavelength shift strategy. *Appl. Microbiol. Biotechnol.* **2016**, *100*, 6231–6238. [CrossRef] [PubMed]
22. Sun, H.; Kong, Q.; Geng, Z.; Duan, L.; Yang, M.; Guan, B. Enhancement of cell biomass and cell activity of astaxanthin-rich *Haematococcus pluvialis*. *Bioresour. Technol.* **2015**, *186*, 67–73. [CrossRef]
23. Panis, G.; Carreon, J.R. Commercial astaxanthin production derived by green alga *Haematococcus pluvialis*: A microalgae process model and a techno-economic assessment all through production line. *Algal Res.* **2016**, *18*, 175–190. [CrossRef]
24. Praveenkumar, R.; Lee, J.; Vijayan, D.; Lee, S.Y.; Lee, K.; Sim, S.J.; Hong, M.E.; Kim, Y.-E.; Oh, Y.-K. Morphological Change and Cell Disruption of *Haematococcus pluvialis* Cyst during High-Pressure Homogenization for Astaxanthin Recovery. *Appl. Sci.* **2020**, *10*, 513. [CrossRef]
25. Choi, Y.Y.; Hong, M.-E.; Sim, S.J. Enhanced astaxanthin extraction efficiency from *Haematococcus pluvialis* via the cyst germination in outdoor culture systems. *Process. Biochem.* **2015**, *50*, 2275–2280. [CrossRef]
26. Bauer, A.; Minceva, M. Direct extraction of astaxanthin from the microalgae *Haematococcus pluvialis* using liquid–liquid chromatography. *RSC Adv.* **2019**, *9*, 22779–22789. [CrossRef]
27. Minceva, M.; Bauer, A. Method of Extracting a Pigment from Microalgae. Patent WO2020089173, 7 May 2020.
28. Praveenkumar, R.; Lee, K.; Oh, Y.-K. Breaking dormancy: An energy-efficient means of recovering astaxanthin from microalgae. *Green Chem.* **2015**, *17*, 1226–1234. [CrossRef]
29. Fábregas, J.; Domínguez, A.; Maseda, A.; Otero, A. Interactions between irradiance and nutrient availability during astaxanthin accumulation and degradation in *Haematococcus pluvialis*. *Appl. Microbiol. Biotechnol.* **2003**, *61*, 545–551. [CrossRef]
30. Ma, R.; Thomas-Hall, S.R.; Chua, E.T.; Eltanahy, E.; Netzel, M.E.; Netzel, G.; Lu, Y.; Schenk, P.M. Blue light enhances astaxanthin biosynthesis metabolism and extraction efficiency in *Haematococcus pluvialis* by inducing haematocyst germination. *Algal Res.* **2018**, *35*, 215–222. [CrossRef]
31. Vidhyavathi, R.; Venkatachalam, L.; Kamath, B.S.; Sarada, R.; Ravishankar, G.A. Differential expression of carotenogenic genes and associated changes in pigment profile during regeneration of *Haematococcus pluvialis* cysts. *Appl. Microbiol. Biotechnol.* **2007**, *75*, 879–887. [CrossRef]
32. Alfonsi, K.; Colberg, J.; Dunn, P.J.; Fevig, T.; Jennings, S.; Johnson, T.A.; Kleine, H.P.; Knight, C.; Nagy, M.A.; Perry, D.A.; et al. Green chemistry tools to influence a medicinal chemistry and research chemistry based organisation. *Green Chem.* **2007**, *10*, 31–36. [CrossRef]
33. Green Chemistry at Pfizer. Available online: https://www.dbu.de/media/130508051211a525.pdf (accessed on 10 March 2021).
34. Berechnung der Verfahrensstandardabweichung und Nachweis-, Erfassungs- und Bestimmungsgrenze aus einer Kalibrierung gemäß DIN 32645. Available online: https://www.uni-due.de/imperia/md/content/iac/git_erw_1.pdf (accessed on 10 January 2021).
35. American Public Health Association. *APHA Method 4500-NO3: Standard Methods for the Examination of Water and Wastewater*; American Public Health Association: Washington, DC, USA; American Water Works Association: Denver, CO, USA; Water Environment Federation: Alexandria, VA, USA, 1992.
36. Dubois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.A.; Smith, F. Colorimetric Method for Determination of Sugars and Related Substances. *Anal. Chem.* **1956**, *28*, 350–356. [CrossRef]

37. Li, K.; Cheng, J.; Lu, H.; Yang, W.; Zhou, J.; Cen, K. Transcriptome-based analysis on carbon metabolism of *Haematococcus pluvialis* mutant under 15% CO_2. *Bioresour. Technol.* **2017**, *233*, 313–321. [CrossRef] [PubMed]
38. Zhao, Y.; Hou, Y.; Chai, W.; Liu, Z.; Wang, X.; He, C.; Hu, Z.; Chen, S.; Wang, W.; Chen, F. Transcriptome analysis of *Haematococcus pluvialis* of multiple defensive systems against nitrogen starvation. *Enzym. Microb. Technol.* **2020**, *134*, 109487. [CrossRef]
39. Tibocha-Bonilla, J.D.; Zuñiga, C.; Godoy-Silva, R.D.; Zengler, K. Advances in metabolic modeling of oleaginous microalgae. *Biotechnol. Biofuels* **2018**, *11*, 241. [CrossRef]
40. Pick, U.; Zarka, A.; Boussiba, S.; Davidi, L. A hypothesis about the origin of carotenoid lipid droplets in the green algae *Dunaliella* and *Haematococcus*. *Planta* **2018**, *249*, 31–47. [CrossRef] [PubMed]
41. Morales-Sánchez, D.; Martinez-Rodriguez, O.A.; Kyndt, J.; Martinez, A. Heterotrophic growth of microalgae: Metabolic aspects. *World J. Microbiol. Biotechnol.* **2014**, *31*, 1–9. [CrossRef]
42. Li, Q.; Zhang, L.; Liu, J. Examination of carbohydrate and lipid metabolic changes during *Haematococcus pluvialis* non-motile cell germination using transcriptome analysis. *Environ. Biol. Fishes* **2018**, *31*, 145–156. [CrossRef]
43. Tunçay, H.; Findinier, J.; Duchêne, T.; Cogez, V.; Cousin, C.; Peltier, G.; Ball, S.; Dauvillée, D. A Forward Genetic Approach in *Chlamydomonas reinhardtii* as a Strategy for Exploring Starch Catabolism. *PLoS ONE* **2013**, *8*, e74763. [CrossRef] [PubMed]