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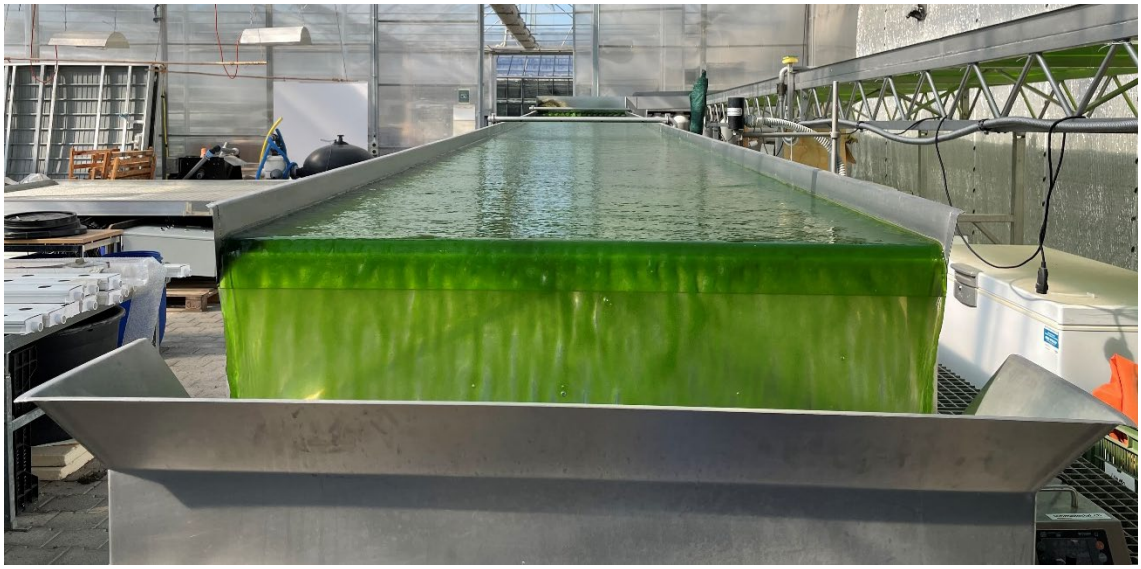

Cultivation of PHB-producing cyanobacteria in wastewater on laboratory and pilot-scale

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Cover image	Cultivation of *Synechococcus leopoliensis* on the open thin-layer PBR, photographed by Marina Mariotto

Abstract

The cultivation of PHB-producing cyanobacteria is a promising process to optain the raw material for bio-based and biodegradable plastics. To commercialise photoautotrophic PHB production, the cultivation needs to be scaled up in open cultivation systems. Further, the use of wastewater as nutrient source improves the resource efficiency of the process, making it cheaper and more sustainable. Here, the feasibility of these steps was tested. First, the suitability of different PHB-producing cyanobacteria was compared in laboratory scale cultivations. Then, the species *Synechococcus leopoliensis* was cultivated in an open thin-layer photobioreactor (18 m^2, 200 L), using a mineral medium, water from recirculating aquaculture systems and pre-processed liquid digestate as nutrient sources. Cultivations in all three media were successful. Cultivation in mineral medium resulted in both the highest final biomass yield (6 g L^{-1}) and productivity (0.7 g L^{-1} d^{-1}). Both wastewater-based media showed lower biomass yields and productivities (2 g L^{-1} and 0.25–0.3 g L^{-1} d^{-1}). However, due to differences in the cultivation conditions (e.g. temperature, nutrient supply), final biomass yield and productivity do not represent the performance of the cultivations adequately. Therefore, relative parameters such as nitrogen and energy conversion ratios were applied. All cultivations in the open system were continued in the laboratory, where cultures were starved for 10 days under nutrient-depleted conditions (without nitrogen, phosphorus and both). This yielded comparatively low amounts of PHB (< 1 $\%_{dw}$). The comparison of the results suggest that PHB yields were influenced more by the initial cultivation condition than by the specific type of nutrient depletion. Thus, while the cultivation with waste streams in an open system is feasible, environmental parameters seem to influence PHB yields considerably and must be taken into account for the optimisation of the complete process.

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1 Introduction

The worldwide consumption of plastics is expected to double by 2050 (Geyer et al., 2017; Lebreton & Andrady, 2019). Conventional plastics are mostly of fossil origin and non-biodegradable. Their use and production cause CO_2 emissions as fossil oil reserves are exploited and the mismanagement of the non-biodegradable plastics damages terrestrial and aquatic ecosystems (Chae & An, 2018; Worm et al., 2017). In order to achieve political goals such as the European Green Deal or the Sustainable Development Goals, which, among other things, pursue CO_2 neutrality and the protection of ecosystems, alternatives to conventional plastics must be found and commercialised (European Commission, 2019; United Nations Organisation, 2016).

Polyhydroxybutyrate (PHB) has promising properties to serve as an alternative to fossil plastics. It is chemically and physically similar to polypropylene (PP) and polyethylene (PE), yet it is biodegradable (McAdam et al., 2020). PHB is synthesised by various bacterial strains including many cyanobacteria and is used by them as carbon storage (Müller-Santos et al., 2021). The cultivation of PHB-producing organisms can be heterotrophic or photoautotrophic (Fritz et al., 2020). Heterotrophic cultivation generally results in higher PHB contents of 40 % to over 90 % in dry weight (Handrick et al., 2004; Trakunjae et al., 2021). However, for heterotrophic growth, organic carbon is needed. Traditionally, the organic carbon is derived from primary sugars (e.g. sugar cane or sugar beet), which directly compete with food and feed production (Shahzad et al., 2020; Sirohi et al., 2020). To produce PHB phototrophically, two cultivation phases are needed. In the first phase, the cyanobacteria are cultivated under non-limiting conditions, to reach high biomass values. In the second phase, the culture is deprived of nutrients such as nitrogen or phosphorus, which enhances the PHB accumulation (Singh et al., 2017). Phototrophic PHB production, i.e. with cyanobacteria, usually results in lower PHB contents of below 10 % and up to 25 % in dry weight (Drosg et al., 2015; Kaewbai-ngam et al., 2016; Panda et al., 2005; Panda & Mallick, 2007). Despite the lower PHB yield, there are several advantages to photoautotrophic production of PHB. Easily available natural resources such as sunlight as an energy source and CO_2 as an inorganic carbon source can be used for the production. Hence, there is no competition to the feed and food production as no organic carbon or arable land is needed, making the cultivation more sustainable (Drosg et al., 2015).

The supply of the right nutrients, such as nitrogen and phosphorus, is key for the growth of cyanobacteria (Klausmeier et al., 2004). The source of nutrients, however, influences the sustainability and resource efficiency of the cultivation. Especially in large-scale production, nutrient recycling through the use of wastewaters can improve resource efficiency and sustainability (Acién et al., 2012). Many

wastewaters are suitable for cyanobacteria and microalgae cultivation (Arias et al., 2020). Among them are liquid digestate and water from recirculating aquaculture systems.

Liquid digestate contains a high proportion of mineralised nutrients as these are not converted into gases during fermentation (Kaltschmitt et al., 2016). The untreated digestate contains many particulate substances and has a high chemical oxygen demand (COD). As this can lead to low translucency and high contamination risk, digestate needs to be treated for the application in cyanobacteria cultivation (Zhou et al., 2019). Part of this treatment is usually the separation of the solid and liquid fraction. In this process, phosphorus accumulates mainly in the solid matter, while nitrogen compounds remain in the liquid phase and are mainly present as ammonium (Kaltschmitt et al., 2016). The cultivation of cyanobacteria and microalgae using liquid digestate as a nutrient source has already proven to be feasible, although pH control may be necessary (Meixner et al., 2016; Pulgarin et al., 2021).

Water from aquaculture systems contains nitrogen compounds and other nutrients that are favourable for the cultivation of cyanobacteria and microalgae (Egloff et al., 2018; Guo et al., 2013). The nutrients are released into the water via the excretion of the fish. In a recirculating aquaculture system, the aim is to recycle the water. Therefore, several treatment steps (e.g. solids removal, nitrification and disinfection) are implemented to maintain a good water quality for the fish. These treatment steps result on one hand in low concentrations of suspended solids and COD, which lowers the risk of emergence of biological contaminants such as bacteria, green algae or protozoa in the cultures and on the other hand in favourable nutrient composition for cyanobacteria (Ebeling & Timmons, 2012). Nutrient concentrations in aquaculture water are usually relatively low. However, when the wastewater is used to balance water loss from evaporation, high biomass yields can still be achieved (Egloff et al., 2018). Nutrient supply by wastewaters has the potential to not only achieve high biomass yields but also increase resource efficiency and with that lowering the costs of large-scale cyanobacteria cultivation (Acién et al., 2012).

In order to realise a commercial production system for photoautotrophic PHB, the upscaling of the cultivation is necessary (Chen, 2009). Large-scale production is either performed in closed or open photobioreactors (PBR). Both come with their advantages and disadvantages: In closed systems, it is usually easier to control contaminations and system parameters, but they call for higher initial investment costs. Due to their complexity, these systems have increased requirements in controlling all parameters. Open systems allow interactions with the environment. This enables easy accessibility of natural resources (e.g. atmospheric CO_2). However, with open systems, there is usually less control over system parameters (e.g. temperature, pH) and contaminations by bacteria, protozoa and different algal species are more likely (Zuccaro et al., 2020). Depending on where the open or closed systems are

placed, sunlight can be used as energy source and thus lowering the total energy demand. So far, most literature documents the cultivation of PHB-producing cyanobacteria on a laboratory scale (Yashavanth et al., 2021). To our knowledge, only two cultivations have been carried out on a pilot-scale, both in non-sterile tubular PBRs: *Synechocystis* sp. CCALA192 was cultivated in a 200 L volume (Troschl et al., 2018) and a randomly mutated strain of *Synechocystis* sp. PCC6714 was cultivated in a 40 L volume (Kamravamanesh et al., 2019). These cultivations confirm that an upscaling of the cultivation in closed systems under non-sterile conditions is possible. However, considering the commercialisation of phototrophic PHB production, it is of interest to address the cultivation in open systems, as this would allow to approach the production cost of fossil plastics (Panuschka et al., 2019).

The aim of this study is to attempt a cultivation of PHB-producing cyanobacteria in a large open system (open thin-layer PBR, see supplementary material S6) using wastewater for the nutrient supply. Combining the use of wastewater as nutrient source with the cultivation in an open system requires cyanobacterial species that perform robustly in the face of contaminations, different types of wastewater and fluctuating physico-chemical parameters (Arias et al., 2020). To find suitable species, an initial screening at laboratory scale was performed, upon which a promising species was cultivated in an open thin-layer PBR (200 L, 18 m^2, see supplementary material S6) using standard mineral medium as well as water from recirculating aquaculture system and pre-processed liquid digestate (Doucha & Lívanský, 2014). Finally, cultures are exposed to different nutrient-deficiency scenarios to investigate their role in triggering PHB accumulation.

2 Material and methods

2.1 Cyanobacteria

Experiments were carried out with three different strains of cyanobacteria. *Aphanothece clathrata* SAG 23.99 and *Synechococcus leopoliensis* SAG 1402-1 were obtained from the Culture Collection of Algae at Göttingen University (SAG). *Synechocystis aqutilis* CCALA 190 was obtained from the Culture Collection of Autotrophic Organism of the Institute for Botany in Třeboň (CCALA). Hereafter, these cyanobacteria strains are referred to as *Aphanothece, Synechococcus* and *Synechocystis.* PCR analysis was performed to confirm their identity before and after the study (see 2.3).

2.2 Media

A mineral medium (Z-medium, SAG, 2008) and modifications thereof were used in all experiments. Unmodified mineral medium served as a control, while different types of wastewaters were used to replace nitrogen ($NaNO_3$) equimolarly in modified versions of the mineral medium. The wastewaters used were a pre-processed liquid digestate from the Swiss Farmer Power biogas plant in Inwil, Switzerland and water from different aquaculture systems of the Institute of Natural Resource Sciences of the Zurich University of Applied Sciences in Wädenswil, Switzerland (Table 1).

Table 1: Specifications of the different wastewaters used in this study. Nutrient concentrations are given in mg L^{-1}

Used wastewater	*Pre-processed liquid digestate*	*Aquaculture water A*	*Aquaculture water B*
Origin	Biogas plant Swiss Farmer Power (SFPI, 2021)	Recirculating aquaculture system	Recirculating aquaponics system
Specifications	*Treatment before use:* Ultrafiltration and reverse osmosis	*Fish species:* burbot (*Lota lota*) *Water withdrawal:* After drum filter *Treatment before use:* Filtered (0.22 µm) and unfiltered	*Fish species:* perch (*Perca fluviatilis*) *Plants:* vanilla (*Vanilla* sp.) and banana (*Musa* sp.) *Water withdrawal:* After settling tank *Treatment before use:* Unfiltered and stored in intermediate bulk containers (IBC) at 4 °C
N_{tot}	6’419	32.1	35.3
NH_4-N	5’907	0.237	< 0.015
NO_2-N	7	0.484	< 0.015
NO_3-N	59	24.5	30.3
P_{tot}	-	1.43	0.95
PO_4-P	-	1.4	1.07

To prompt the accumulation of PHB in the cells, three different nutrient-depleted modifications of the mineral medium (without nitrogen and/or phosphorus) were prepared. $NaNO_3$ was omitted and

$Ca(NO_3)_2 \cdot 4\ H_2O$ was replaced with an equimolar amount of $CaCl_2$. K_2HPO_4 was replaced with an equimolar amount of KCl.

2.3 Analytical methods

The growth of the algae was determined with three different methods. Optical density at 750 nm (OD_{750}) was measured in 96-well plates and a volume of 200 µl with an automated plate reader (Infinite 200 Pro, Tecan, Männedorf, Switzerland). Cell density of the cyanobacteria was determined by light microscopy (phase contrast, 400-fold magnification) with a haemocytometer with Thoma ruling. Dry weight (dw) was either measured with a moisture analyser (HB43-S, Mettler Toledo, Greifensee, Switzerland) from a 40-ml sample or with a standard gravimetric method from a 1-ml sample. In both cases samples were centrifuged (5 min, 7'971 *g* and 10'000 *g*, respectively), decanted, washed with deionised water and dried at 105 °C.

Contaminants such as bacteria, protozoa and green algae were quantified as follows. Aerobic mesophilic bacteria (CFU ml^{-1}) were determined with plate count agar (PCA) plates. A dilution series (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) of the culture samples was prepared and 5 µl of each dilution were pipetted onto the PCA plates. Plates were incubated in the dark for two days at room temperature (ca. 23 °C). Concentrations of protozoa and green algae were quantified by light microscopy (phase contrast, 200-fold magnification) with a haemocytometer with Fuchs-Rosenthal ruling.

PHB content was measured according to a modified method of Karr et al. (1983). The cyanobacteria suspension (1 ml at an OD_{750} of 10 and 10 ml at an OD_{750} of 1) was centrifuged (8 min, 450 *g*), decanted, and dried overnight at 105 °C. Then 1 ml of sulphuric acid (18.4 M) was added to the dried pellet. The samples were heated at 105 °C for 60 min to dissolve the biomass. After the samples cooled down to room temperature, 9 ml of sulphuric acid (7 mM) were added, the samples were filtered (0.45 µm) and prepared for HPLC measurement. HPLC analysis was carried out with a Nexera system (Shimadzu, Reinach, Switzerland) and a Repro-Gel H+ column (Dr. Maisch, Ammerbuch, Germany) heated at 40 °C. Peaks were detected with a diode array detector (SPD-M30A, Shimadzu). The flow rate of the mobile phase (0.009 M H_2SO_4) was 0.8 ml min^{-1}. The measurement lasted 32 minutes. The results from the HPLC were standardised to percentage in dry weight ($\%_{dw}$) by dividing the PHB concentration (mg L^{-1}) by the dry weight (mg L^{-1}) and multiplying the result by 100 (see supplementary material S1).

The concentration of various nutrients and other compounds in the media (total nitrogen, nitrate, ammonium, nitrite, total phosphorus, phosphate and phenols) was measured with photometric test kits (Hach-Lange, Rheineck, Switzerland).

The CHN content of dried algal biomass samples (100 µg) was determined by thermal conductivity and infrared spectroscopy (TruSpec Micro CHN, Leco Instruments Ltd., Mississauga, Canada). The results of the CHN analysis in combination with the dry weight, the measured concentration of nitrogen compounds and the known nitrogen supply allow the calculation of nitrogen mass balances (see supplementary material S2). To gain more comparability between cultivations the nitrogen conversion ratio (mg g_{dw}^{-1}) was calculated by dividing the total supplied nitrogen (mg L^{-1}) of each cultivation by the dry weight increase (g L^{-1}) between the first and last day of the corresponding cultivation (see supplementary material S3).

DNA was extracted, amplified and sequenced. The DNA was extracted with a commercial kit (NucleoSpin®Tissue, Macherey-Nagel, Dueren, Germany) following the instructions of the manufacturer. The 16S rRNA gene was amplified with PCR using 1 µL of DNA sample, 1.25 µL of each primer (10 µM) (CYA106F, CYA781R(a) and CYA781R(b), Nübel et al., 1997), 12.5 µL of DNA-polymerase (KAPA2G Robust HotStart ReadyMix, Roche, Basel, Switzerland) and 7.75 µL of PCR grade H_2O. The PCR included an initial denaturation at 95 °C for 3 min, a series of 35 cycles of denaturation at 95 °C for 15 sec, annealing at 58 °C for 15 sec and extension at 72 °C for 15 sec, and ended with a final extension at 72 °C for 1 min. The DNA was purified with a commercial kit (Gel and PCR Clean-up NucleoSpin®, Macherey-Nagel) following the instructions of the manufacturer. The prepared DNA samples were sent to a commercial sequencing service (Microsynth, Belgach, Switzerland) for Sanger sequencing and the results were checked against the NCBI database using the BLAST algorithm.

To consider seasonal fluctuations between the cultivations in the data analysis, additional parameters were measured. In the laboratory, a multiprobe (HQ40d, Hach) was used to measure pH (INTELLICAL PHC108, Hach) and electric conductivity (CDC401, Hach). On the open thin-layer PBR, pH and temperature (InPro 3253i SG/120, Mettler Toledo) were measured with built-in systems. Photosynthetically active photon flux density (PPFD, µmol s^{-1} m^{-2}) was measured with two PAR sensors (SKL2620, Skye Instruments Ltd., Powys, United Kingdom) that were placed above and below the glass cultivation platform. The number of absorbed photons was calculated as the difference between the measurement of the upper and the lower sensor. The number of absorbed photons was converted into power (kW) by multiplying the number of absorbed photons with the factor 219 (Sager & McFarlane, 1997). Power was multiplied with the time difference from one measurement to the next, to calculate the amount of absorbed solar energy (kWh) (see supplementary material S4). This conversion from absorbed photons (µmol s^{-1} m^{-2}) to absorbed solar energy (kWh) allows the comparison of the energy uptake between cultures in different seasons. To gain more comparability between cultivations the energy conversion ratio (kWh g_{dw}^{-1}) was calculated by dividing the absorbed energy (kWh) of each

cultivation with the dry weight increase (g L^{-1}) between the first and last day of the corresponding cultivation (see supplementary material S5).

2.4 Laboratory experiments with *Aphanothece clathrata*, *Synechococcus leopoliensis* and *Synechocystis aquatilis*

Two similar laboratory experiments were carried out with *Aphanothece*, *Synechococcus* and *Synechocystis* in mineral medium and different modifications of it as described in 2.2 (nitrogen replacement with pre-processed liquid digestate and nitrogen replacement with aquaculture water A (filtered and unfiltered)). Experiments lasted 21 days and were divided into a growth phase (14 days) and a starvation phase (7 days).

Each cyanobacterial species and a negative control (without inoculation) was cultured in each medium, with each medium-species combination replicated three-fold in the first and four-fold in the second experiment. For each cultivation, 100'000 cells ml^{-1} were inoculated in a volume of 60 ml in 100-ml shake flasks and incubated at 25 °C, 1 % CO_2, 100 rpm agitation and approximately 4'800 lux (HT Multitron Pro, Infors, Bottmingen, Switzerland). During the growth phase of 14 days, the following metrics were determined: OD_{750} on days 0, 2, 4, 7, 9, 11, and 14, pH on days 0, 7, and 14, number of protozoa on days 2, 9, and 14, number of aerobic mesophilic bacteria in the media and pre-cultures on day 0, number of aerobic mesophilic bacteria in the cultures on days 2 and 14, and the dry weight on day 14. In the second experiment, the pH of the cultivations with medium containing pre-processed liquid digestate was adjusted to 7.5 on days 0, 2, 4, 7, 9, 11, and 14.

In the beginning of the starvation phase of seven days, the cyanobacteria cultures were transferred to a nitrogen-depleted medium. For transfer to the new medium, 40 ml of the cultures were centrifuged (5 min, 7'200 *g*) and the biomass was resuspended in 60 ml of the new medium. The cultures were incubated for another week under the same conditions as described above. During the starvation phase, the following metrics were determined: OD_{750} on days 14, 16, 18, and 21, pH on days 14 and 21, number of protozoa on days 16 and 21, number of aerobic mesophilic bacteria, dry weight and PHB content at the end of the starvation phase on day 21.

2.5 Cultivation of *Synechococcus leopoliensis* in an open thin-layer PBR

After the laboratory experiments, *Synechococcus* was chosen for the cultivation on the open thin-layer PBR (18 m^2, 200 L, see supplementary material S6). Reasons for this were that *Synechococcus* showed the most reliable growth and the highest PHB content in the preliminary experiments. The strain was cultivated in mineral medium and two different modifications of it as described in 2.2 (unmodified

mineral medium, nitrogen replacement with pre-processed liquid digestate and nitrogen replacement with aquaculture water B). The cultivations lasted 14 to 16 days and the cultures were shaded with a shade cloth (F50, Hortima, Hausen, Switzerland) during the first 5 to 6 days to prevent bleaching of the cultures. Most system parameters were set the same in all cultivations. During the day, partial pressure of CO_2 in the medium was kept at 5–10 mbar, while CO_2 supply was stopped overnight. The thickness of the cultures on the cultivation platform was set to 6 mm. In the cultivation with medium containing pre-processed liquid digestate pH was controlled with 1 M NaOH (pH Controller, Bluelab, Tauranga, New Zealand), as ammonium uptake causes a decrease in pH (Zheng et al., 2013). The addition of nutrients was handled differently depending on the nutrient source. For the cultivation with unmodified mineral medium, nutrients were added batchwise and always before they became limiting. For this purpose, it was assumed that the biomass yield depends on the nutrient supply. Nutrients were added once the dry weight of the cultivation was about to reach the assumed biomass yield (see supplementary material S7). The aquaculture water was fed to the system via a pump that was controlled by the amount of water that evaporated from the system. The pre-processed liquid digestate was added continuously with a peristaltic pump. On average, 34.6 ml h^{-1} of pre-processed liquid digestate and 222 mg h^{-1} of nitrogen were added to the system. In the cultivations with wastewater, nutrients other than $NaNO_3$ were added batchwise and always before they became limiting (see supplementary material S7).

The cultivation with mineral medium was carried out from the 09.08.2021 to the 23.08.2021, the cultivation with medium containing aquaculture water from the 23.08.2021 to the 06.09.2021 and the cultivation with medium containing pre-processed liquid digestate from the 18.09.2021 to the 04.10.2021. During the cultivations the following metrics were determined: dry weight from a 40-ml sample, OD_{750}, number of cyanobacteria cells, number of protozoa, number of aerobic mesophilic bacteria, concentration of relevant nitrogen compounds, percentage of CHN in the biomass, concentration of phenols (only for the cultivation with pre-processed liquid digestate) and electric conductivity (only for the cultivation with pre-processed liquid digestate). The cultivations with mineral medium and medium containing aquaculture water were sampled on days 0, 2, 4, 7, 9, 11, and 14. The cultivation with medium containing pre-processed liquid digestate was sampled on days 0, 2, 4, 6, 9, 11, 13, and 16. Additionally, PCR analysis was carried out in the beginning and at the end of the three cultivations.

After the cultivation on the open thin-layer PBR, cultures were transferred into nutrient-depleted mineral media to accumulate PHB. Different nutrient-depleted media were tested (see 2.2) as the nitrogen-depleted media used in the laboratory experiments did not result in high PHB contents. The cultures were centrifuged (5 min, 5'000 *g*) and resuspended in the nutrient-depleted media. The mineral medium and the exhausted medium from the cultivation on the open thin-layer PBR served as

controls. Cultivations had a volume of 150 ml in 250-ml shake flasks and were incubated for ten days at 25 °C, 2 % CO_2, 150 rpm agitation and approximately 12'600 lux. Each medium was replicated three-fold. OD_{750}, dry weight from a 1 ml sample, pH, PHB content and number of protozoa were measured on days 0, 3, 7, and 10.

2.6 Data analysis

The statistical analysis and data visualisation was performed with R (version 4.0.3) in RStudio (version 1.2.1335). The significance level was set at $p < 0.05$. The data is presented as the mean and standard error of the mean, wherever there were replicates in the experimental design.

The results of the OD_{750} measurement on the 14^{th} day as well as the PHB content on the 21^{th} of the laboratory experiments were analysed for differences between media and species. Additionally, the PHB content was analysed for an interaction of media and species. For the OD_{750} data, an ANOVA was calculated since the assumptions for this analysis (homogeneity of variance and normal distribution of the residuals) were given. Subsequently, post-hoc individual comparisons were carried out according to Tukey. The PHB data did not meet the required assumptions for an ANOVA. Hence, Kruskal Wallis tests were performed.

The results of the growth experiments on the open thin-layer PBR could not be analysed statistically, as each medium was only used once and there are thus no replicates of the cultivations. However, some relative values were used to compare the cultivations (see 2.3).

The results of the PHB accumulation experiments, which were carried out with the cultures from the growth experiments on the open thin-layer PBR, were analysed for differences between the nutrient-depleted media and changes in PHB content over time. Assuming that the different media, which were used during the growth experiments, influenced the PHB accumulation, a linear mixed effect model (function lme of library nlme) was calculated with the sampling day and nutrient-depleted media as independent predictors and the media of the growth experiment as random effect. The model was used to calculate an ANOVA. To meet the required assumptions for this analysis (homogeneity of variance and normal distribution of the residuals) a square-root transformation of the data was performed.

3 Results

3.1 Laboratory experiments with *Aphanothece clathrata, Synechococcus leopoliensis* and *Synechocystis aquatilis*

The cultivation of *Aphanothece*, *Synechococcus* and *Synechocystis* in mineral medium and its modifications, where wastewater provided nitrogen, was successful (Figure 1). Growth after 14 days, which was evaluated by OD_{750}, differed between species ($F_{2,78} = 11.12$, $p < 0.001$). *Synechococcus* and *Synechocystis* showed no differences in growth ($OD_{750} = 0.6–3.3$). *Aphanothece,* however, showed lower values ($OD_{750} = 0.6–2.4$). The growth of the cyanobacteria also differed between media ($F_{3,78} = 45.7$, $p < 0.001$). The mineral medium and medium containing filtered aquaculture water showed the best results ($OD_{750} = 1.2–3.6$ and $OD_{750} = 1.4–3.0$, respectively), while the cyanobacteria cultivated in the medium containing pre-processed liquid digestate ($OD_{750} = 0.5–1.5$) grew less. Cultivations in medium containing unfiltered aquaculture water reached high values ($OD_{750} = 0.5–2.8$) but must be interpreted with caution as they became contaminated with green algae (*Scenedesmus* sp.). The contamination with green algae was only found in the cultivations of *Aphanothece* and *Synechococcus*, where in some cultures cyanobacteria were displaced almost completely by green algae. No green algae cells were found in the *Synechocystis* cultures.

After the transfer of the cultures into a nitrogen-depleted medium, PHB accumulation over the course of seven days was observed in most cultures (see supplementary material S8). Performing the experiments twice revealed high variability in PHB accumulation and no effects could be attributed to the medium used during the growth phase ($\chi^2 = 2.74$, $p = 0.43$), the cultivated species ($\chi^2 = 1.94$, $p = 0.38$) or the interaction of the two ($\chi^2 = 18.59$, $p = 0.07$). The highest PHB contents were found in *Synechococcus* cultivated in medium containing unfiltered aquaculture water (2.4 % PHB_{dw}) and *Aphanothece* cultivated with medium containing pre-processed liquid digestate (2 % PHB_{dw}).

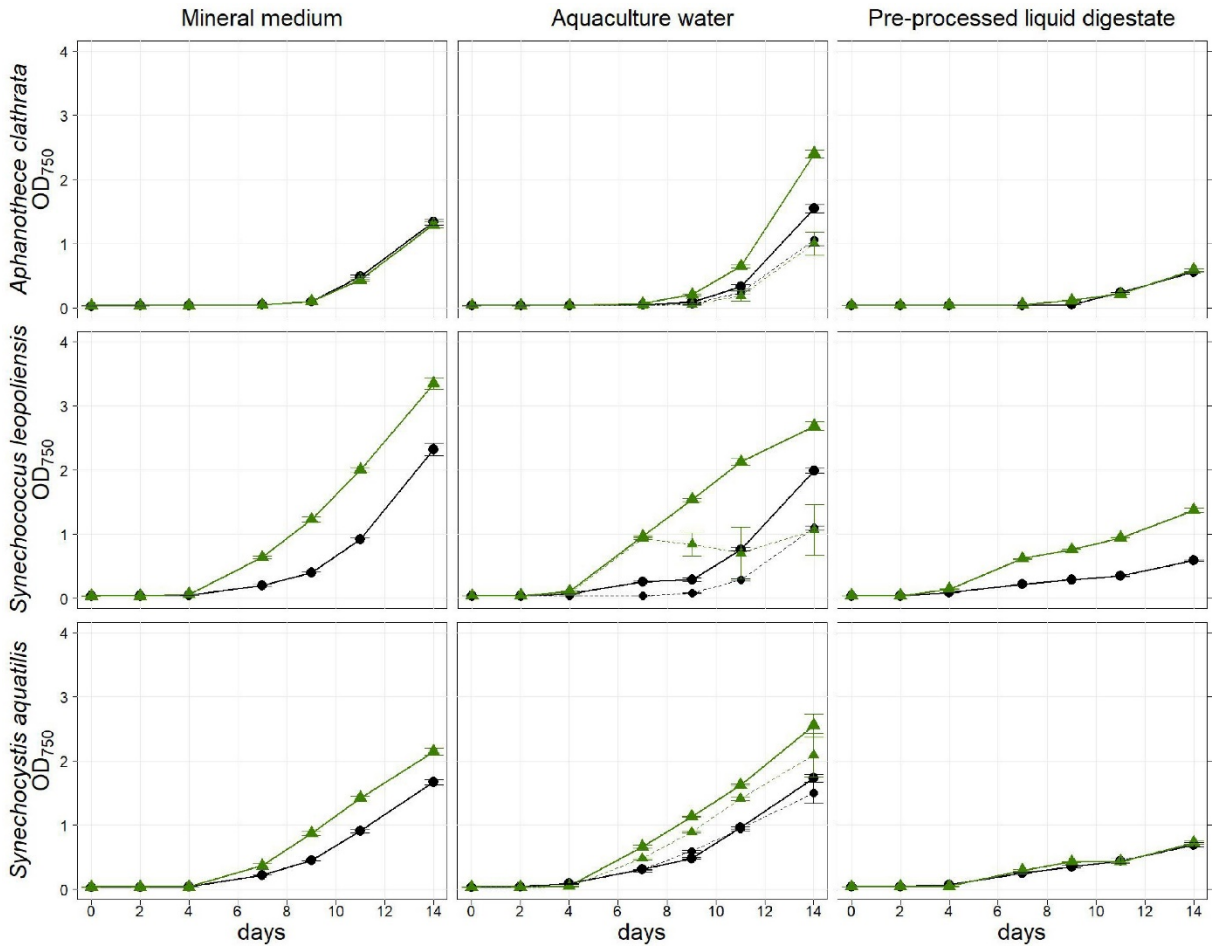

Figure 1: Cyanobacterial growth in two laboratory experiments (1st: black, circle, 2nd: green, triangle) measured via optical density. The achieved OD_{750} of the different cultivations in the different media «Mineral medium», «Aquaculture water» (solid: filtered, dashed: unfiltered) and «Pre-processed liquid digestate» are visualised with the mean values of the replicates and the standard error of the mean. Note that some of the cultures of *Aphanothece* and *Synechococcus* in unfiltered aquaculture water also contained green algae, which cannot be differentiated here

3.2 Cultivation of *Synechococcus leopoliensis* in an open thin-layer PBR

The cultivations of *Synechococcus* on the open thin-layer PBR in mineral medium as well as in its modifications, where wastewater provided nitrogen, were successful (Figure 2). *Synechococcus* cultivated in mineral medium showed the highest productivity with a maximum growth rate of 0.7 g L^{-1} d^{-1}, followed by the cultivation in medium containing pre-processed liquid digestate with 0.3 g L^{-1} d^{-1} and medium containing aquaculture water with 0.25 g L^{-1} d^{-1}. The final biomass was also highest in mineral medium (6 g L^{-1}) compared to the modified media containing wastewater (both 2 g L^{-1}). However, productivity and final biomass yield do not reflect the performance of *Synechococcus* in the different media adequately, as solar energy and nutrient inputs differed between the cultivations. To better compare cultivations, the produced biomass was expressed relative to solar irradiation and the nitrogen supply (see 2.3).

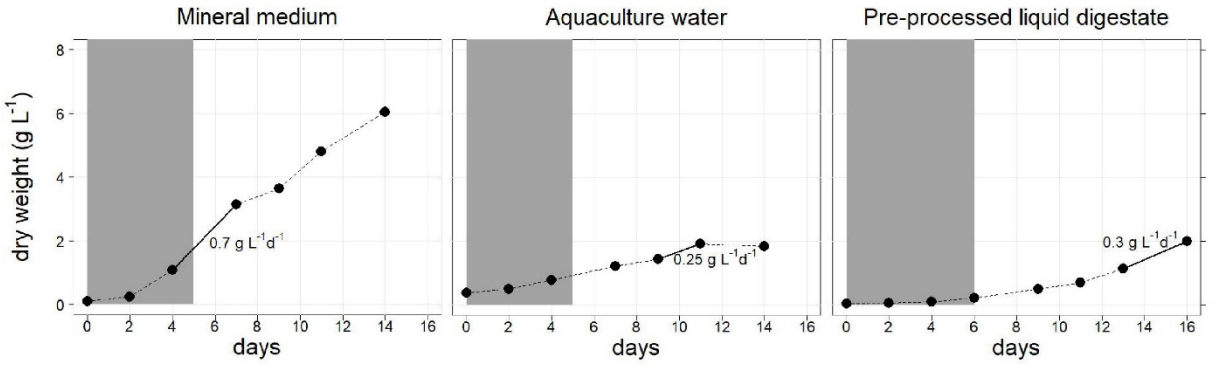

Figure 2: Dry weight of the three cultivations on the open thin-layer PBR. The days, on which the cultures were shaded are illustrated with a grey background. The most productive time is marked with the solid line and the value in the plots (g L^{-1} d^{-1})

The cultivation with mineral medium experienced the highest average solar energy input of 1 kWh m^{-2} d^{-1}, followed by the cultivations with medium containing aquaculture water (0.87 kWh m^{-2} d^{-1}) and pre-processed liquid digestate (0.55 kWh m^{-2} d^{-1}). Set in relation to the produced biomass, this results in an average energy conversion ratio of 0.22 kWh g_{dw}^{-1} for the cultivation in mineral medium, 0.6 kWh g_{dw}^{-1} for the cultivation in medium containing aquaculture water and 0.4 kWh g_{dw}^{-1} for the cultivation in medium containing pre-processed liquid digestate. Thus, when only looking at the final biomass yield and productivity, cultivations of *Synechococcus* in the media containing wastewater show similar performances. However, when differences in solar irradiation are considered, *Synechococcus* cultivated in medium containing pre-processed liquid digestate transforms the solar energy more efficiently into biomass than in medium containing aquaculture water. Also, when *Synechococcus* cultivated in medium containing pre-processed liquid digestate is compared to the cultivation in mineral medium, the uncorrected final biomass yield is three times higher, yet when the energy conversion ratio is considered, there is only a two-fold difference.

The nitrogen supply over the course of the cultivation was highest in mineral medium (840 mg L^{-1}). The nitrogen supply with aquaculture water was 149 mg L^{-1} and with pre-processed liquid digestate 413 mg L^{-1} (see supplementary material S9). These values, set in relation to the final biomass yield, result in a nitrogen conversion ratio of 141 mg g_{dw}^{-1} in mineral medium, 101 mg g_{dw}^{-1} in medium containing aquaculture water and 206 mg g_{dw}^{-1} in medium containing pre-processed liquid digestate. The nitrogen conversion ratio in the cultivation with medium containing aquaculture water is lower compared to the mineral medium. This is explained by the limiting nutrient supply regime in the cultivation with medium containing aquaculture water, which was caused by the low nitrogen concentration of the aquaculture water and the dependency on evaporation for the provision of more wastewater. These factors resulted in an insufficient nitrogen supply. This also partially explains the low productivity in this medium. The insufficient nitrogen supply is also visible in the results of the CHN analysis, where the average proportion of nitrogen in the cells grown in medium containing aquaculture water (8.2 % N) is lower

($F_{1,12}$ = 22.14, p < 0.001) than in the cells grown in mineral medium (10.8 % N). The nitrogen conversion ratio in the cultivation with medium containing pre-processed liquid digestate is higher than with mineral medium, which indicates an inefficient use of nitrogen. This was caused by a nitrogen loss of over 50 % in the cultivation with medium containing pre-processed liquid digestate (Figure 3).

Depending on the nitrogen source, nitrogen was assimilated to varying degrees by the cyanobacteria (Figure 3 and supplementary material S9). In the cultivations with mineral medium and medium containing aquaculture water, nitrogen was mainly present as nitrate (NO_3^-). In these cultivations, 78.6 % and 72.3 % of the supplied nitrogen were assimilated by the cells. 4.8 % and 4.1 % of the supplied nitrogen remained in the media and 16.6 % and 23.6 % were lost to the atmosphere. In the cultivation with medium containing pre-processed liquid digestate, where nitrogen was mainly present as ammonium (NH_4^+), 46.2 % of nitrogen were assimilated by the cells, 2.3 % remained in the medium and 51.5 % were lost to the atmosphere.

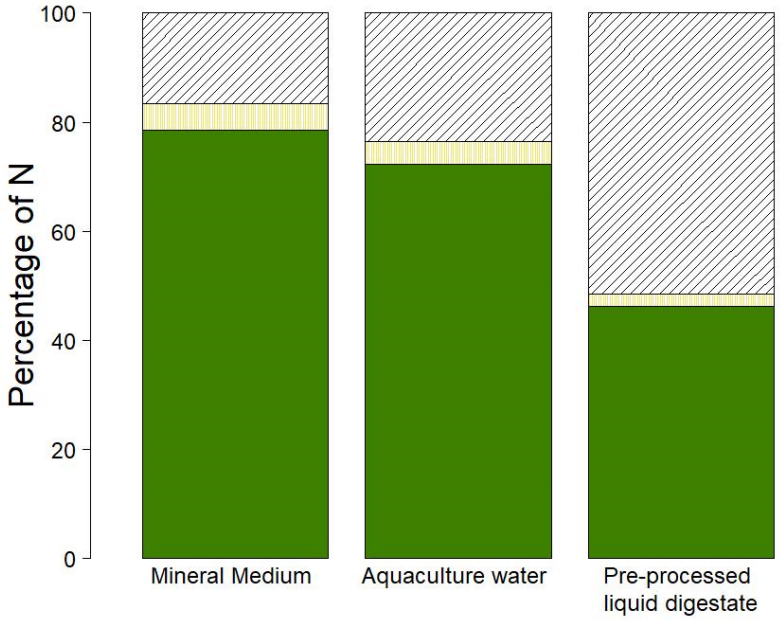

Figure 3: Nitrogen mass balance of the three cultivations on the open thin-layer reactor with nitrogen that was assimilated within the cells (green, solid), nitrogen that remained in the medium (yellow, stripped vertically) and nitrogen that was lost to the atmosphere (black, stripped diagonally)

In all three cultivations, bacteria and protozoa were present. The concentrations of protozoa in mineral medium and medium containing aquaculture water were relatively stable ($6.5 \cdot 10^3$ to $6.2 \cdot 10^5$ ml^{-1}) over the course of the cultivations, while in medium containing pre-processed liquid digestate the number of protozoa increased to 10^6 ml^{-1} until the 11th day and then decreased rapidly (Figure 4). The concentrations of aerobic mesophilic bacteria behaved differently in each cultivation (Figure 5). In mineral medium the number increased, in medium containing aquaculture water the number increased at first and decreased towards the end of the cultivation. In medium containing pre-processed liquid digestate the number remained relatively stable on a comparatively low level. Additionally, in the

cultivation with medium containing pre-processed liquid digestate, a contamination with green algae (*Scenedesmus* sp.) emerged. At the end of the experiment, green algae accounted for about 10 % of all cells. Despite the many contaminants, the sequencing of the 16S rRNA gene in the beginning and end of each cultivation confirmed that the cultivated strain was *Synechococcus leopoliensis* throughout the experiments.

The continuous addition of the pre-processed liquid digestate over the course of the cultivation led to an accumulation of phenols and an increase of the electric conductivity in the medium (see supplementary material S10 and S11). The concentration of phenols and the electric conductivity increased linearly from 0.27 to 1.9 mg L^{-1} and 0.7 to 4.6 mS cm^{-1}, respectively.

Some physico-chemical parameters such as temperature and pH fluctuated between day and night (see supplementary material S12 and S13). The open construction of the thin-layer PBR and its placement in a greenhouse caused temperatures to rise during the day and to drop overnight. Day and night fluctuations of the pH are explained by the setting of the CO_2 supply.

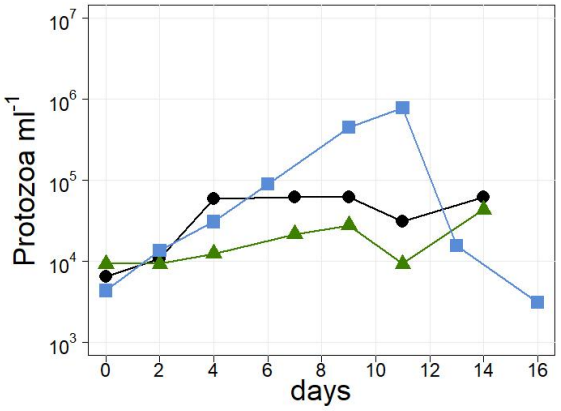

Figure 4: Concentration of protozoa in the cultivations with mineral medium (black, circle), medium containing aquaculture water (green, triangle) and medium containing pre-processed liquid digestate (blue, square)

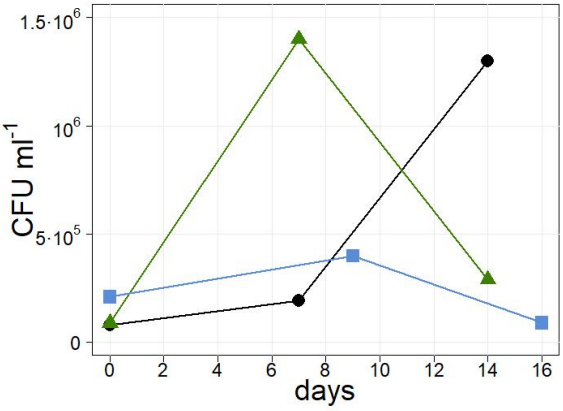

Figure 5: Concentration of aerobic mesophilic bacteria in the cultivation with mineral medium (black, circle), medium containing aquaculture water (green, triangle) and medium containing pre-processed liquid digestate (blue, square)

3.3 Accumulation of PHB in different nutrient-depleted media

After the transfer of the cultures from the open thin-layer PBR to the laboratory and into the nutrient-depleted media, PHB accumulation was observed over the course of ten days in most cultures (Figure 6). The results show that the PHB content increased over time ($F_{1,172} = 87.5$, $p < 0.001$). The comparison of the different media, however, does not show that the depletion of nutrients has a bigger effect on the PHB accumulation than the mineral medium or the exhausted medium from the cultivation on the open thin-layer PBR ($F_{4,172} = 1.77$, $p = 0.14$).

The dynamic of the PHB accumulation seems to depend more on the medium in which the cyanobacteria were cultivated and the occuring cultivation conditions during the growth phase. In the cultures that were grown in mineral medium the PHB accumulation started after the 7th day of the starvation experiment and reached a maximum of 0.7 % PHB_{dw} in the medium that was nitrogen- and phosphorus-depleted. The cultures grown in medium containing aquaculture water showed the lowest PHB contents of the three cultures, after the starvation experiment. The PHB content increased after the 3rd day to a maximum of 0.5 % PHB_{dw} in the exhausted medium from the cultivation in the open thin-layer PBR. The PHB content decreased after the 7th day and on the 10th day a maximum of 0.2 % PHB_{dw} was reached in the medium that was nitrogen- and phosphorus-depleted. The cultures that were cultivated in medium containing pre-processed liquid digestate accumulated the most PHB. The accumulation started after the day of inoculation in the new media, kept increasing and reached a maximum of 0.9 % PHB_{dw} in the medium that was phosphorus-depleted. Even though the PHB accumulation seems to depend on the conditions of the growth phase, this cannot be confirmed statistically as the cultivations on the open thin-layer PBR were not repeated.

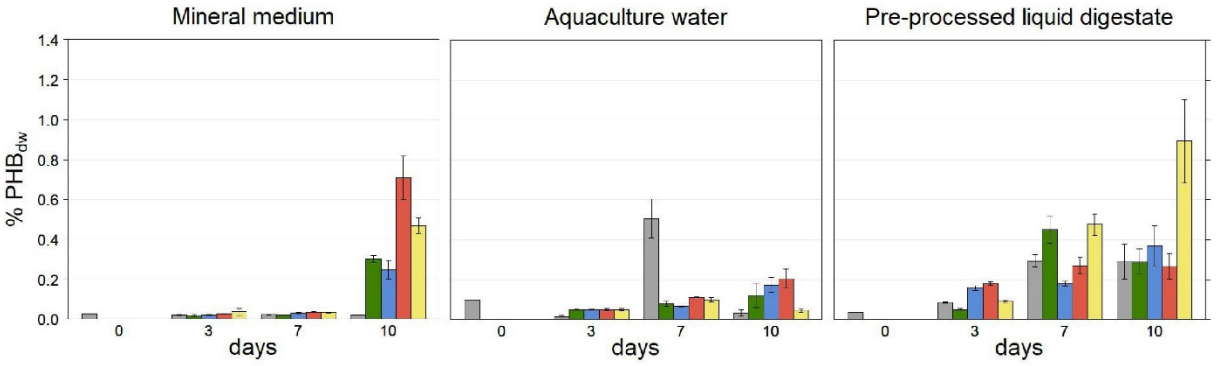

Figure 6: PHB accumulation in the different nutrient-depleted media (grey: exhausted medium from the cultivation on the open thin-layer PBR; green: mineral medium, blue: mineral medium –N, red: mineral medium –N–P, yellow: mineral medium –P) over time. The data is visualised with the mean values of the replicates and the standard error of the mean

4 Discussion

In this study, it was tested whether several PHB-producing cyanobacteria species (*Aphanothece*, *Synechococcus* and *Synechocystis*) could be cultivated in mineral medium and two media containing wastewater (aquaculture water and pre-processed liquid digestate) on laboratory scale. The most promising species *Synechococcus* was then cultivated with mineral medium and media containing wastewater on pilot-scale in an open thin-layer PBR. The cultivations in the three media on laboratory as well as pilot-scale were successful.

The scaled-up cultivation in the mineral medium, where growth was not nutrient-limited, showed the highest final biomass yield, the highest growth rate and the most efficient energy conversion ratio. Whether these values represent the best achievable performance of *Synechococcus* cannot be confirmed because the culture may have been limited, e.g. by solar irradiation. However, the results from this cultivation can be used as a reference to compare the wastewater-based cultivations to. In the cultivation of *Synechococcus* in the medium containing aquaculture water, a lower nitrogen conversion ratio (101 mg g_{dw}^{-1}) was observed than in the cultivation with mineral medium. This indicates that the cultivation was limited by the nitrogen supply. This assumption is also supported by the lower nitrogen content in the cells (8.2 %). Nitrogen limitation with aquaculture water as growth medium has already been observed by Egloff et al. (2018). When using aquaculture water as growth medium, the occurring nitrogen limitation indicates that cyanobacteria and microalgae cultivation can be used to fully treat this wastewater. The higher energy conversion ratio of the cultivation in the medium containing aquaculture water (0.6 kWh g_{dw}^{-1}) indicates that the cultivation was not limited by solar irradiation, as the cultivation in mineral medium showed that *Synechococcus* can grow with only 0.2 kWh g_{dw}^{-1}. The growth of *Synechococcus* in the medium containing pre-processed liquid digestate did not seem to have been limited by nutrient supply or solar irradiation as the nitrogen conversion ratio (206 mg g_{dw}^{-1}) and the energy conversion ratio (0.4 kWh g_{dw}^{-1}) were higher than in the cultivation with mineral medium. Other characteristics of the cultivation are more likely to have restrained the growth: 1) The cultivation was carried out in autumn. That led to slightly lower temperatures and therefore could have resulted in a slower growth pattern (Reynolds, 2006). 2) Ammonium as the nitrogen source was not ideal for *Synechococcus*. However, there are contradictory findings in the literature, whether ammonium or nitrate is the ideal nitrogen source for microalgae and cyanobacteria (Lin & Lin, 2011; Molloy & Syrett, 1988; Rossi et al., 2020). 3) Keeping the pH between 7 and 7.5 might have been too low as cyanobacteria usually prefer more alkaline conditions with pH from 7.5 to 10 (Nayak & Prasanna, 2007).

Overall, *Synechococcus* performed well in the mineral medium and the wastewater containing media under outdoor conditions and reached relatively high biomass concentrations. Other studies in which PHB-producing cyanobacteria were cultivated on pilot-scale, final biomass concentrations of 1.1 g L^{-1} and 3.2 g L^{-1} were achieved over the course of 16 to 20 and 14 days respectively (Troschl et al., 2018; Kamravamanesh et al., 2019). However, the results are not directly comparable as the cultivation systems, species and many other parameter differed considerably. A measure to improve comparability between cultivations was applied here with the calculation of relative parameters such as the nitrogen and energy conversion ratio.

The nitrogen mass balances in all three cultivations that were carried out in the open system revealed that there were losses of nitrogen in all cultivations of at least 16.6 %. In the cultivations with mineral medium and medium containing aquaculture water, nitrate was the main source of nitrogen, which is likely the reason that losses were moderate. In the cultivation with the medium containing pre-processed liquid digestate, ammonium was the main source of nitrogen, which is considerably more volatile and, hence, likely explains the higher nitrogen loss.

The loss of nitrate in the cultivations with mineral medium and medium containing aquaculture water may have been caused by denitrification. Denitrifying bacteria transform nitrate over nitrite into molecular nitrogen (N_2). This metabolism needs anaerobic conditions (Guieysse et al., 2013), which may occur in some parts of the open thin-layer PBR. Another contributing factor for the nitrate losses may be a metabolic pathway of cyanobacteria that results in nitrous oxide (N_2O) emissions (Weathers & Niedzielski, 1986; Guieysse et al., 2013; Plouviez et al., 2019; Plouviez & Guieysse, 2020; Plouviez et al., 2017). While emitting N_2 would be harmless to the environment, emitting the greenhouse gas N_2O (296 CO_2-eq over 100 years) would contribute to climate change (Ehhalt et al., 2001). The pathways of N_2O synthesis in microalgae and cyanobacteria are not fully understood yet, however, the N_2O emission factor is estimated to be 0.1–0.4 % of the nitrogen input (Plouviez et al., 2017). Thus, N_2O synthesis would only be a small contributor to the nitrogen losses.

The loss of ammonium in the cultivation with medium containing pre-processed liquid digestate may be explained through the transformation of ammonium into the volatile molecule ammonia. Ammonium and ammonia are in an equilibrium state depending on temperature and pH. The equilibrium leans towards ammonia the higher the pH and the warmer the temperature is (Emerson et al., 1975). Ammonia is toxic to humans and other animals and the deposition in the environment causes acidification and uncontrolled fertilisation. Therefore, the emissions should be minimised e.g. by keeping the pH below 7.5 (Behera et al., 2013; Bittman et al., 2014; Padappayil & Borger, 2021). In cultivations with *Chlorella vulgaris* in medium containing pre-processed liquid digestate, which were

carried out on the same system that was used in this study, comparable proportions of ammonium were lost to the atmosphere (Pulgarin et al., 2021), even when pH was kept below 7.3 (unpublished results). This indicates that controlling the pH value is not sufficient to minimise the emissions. Some processes that lead to nitrogen losses in cyanobacteria and algae cultivation are not sufficiently understood yet. Therefore, it can only be assumed whether nitrogen was emitted as N_2, N_2O or NH_3 during the cultivations of this study.

The PHB content in all cultures was relatively low (< 2.4 $\%_{dw}$). Although most studies about phototrophic PHB production confirm an increased PHB accumulation with a depletion of nitrogen or phosphorus (Kaewbai-ngam et al., 2016; Singh et al., 2017), the data of this study did not reveal a clear dependency of the PHB accumulation on nutrient depletion. Possibly, the cultivation conditions during the growth phase affected the PHB accumulation negatively. The use of the three different media had consequences, such as different growth rates and final biomass yields or different states of nutrient deprivation in the cyanobacteria. Thus, the cultures that were transferred to the laboratory for the starvation phase were likely in different physiological states. Also, the fluctuation of certain parameters during the growth phase might have affected the PHB accumulation. It has been shown that temperature, pH, incubation time, availability of carbon as well as light and dark cycle have an effect on the PHB accumulation (Ansari & Fatma, 2016; Kamravamanesh et al., 2017; Monshupanee & Incharoensakdi, 2014). For a commercialisation of the PHB production, the accumulation phase also needs to be tested in larger cultivation volumes.

As stated before, *Synechococcus* performed well under outdoor conditions in the scaled-up system, even though contaminants were present in the cultivations and some physico-chemical parameters fluctuated in the open thin-layer PBR. The emergence of contaminants (e.g. protozoa or green algae) and the less controllable parameters are challenges that open systems usually entail (Ugwu et al., 2008). The cultivations in this study, however, were not drastically affected by these challenges. *Synechococcus* remained the dominant species throughout the cultivations in the open thin-layer PBR, despite the contaminating organisms. In the cultivation with medium containing pre-processed liquid digestate, an emerging contamination of protozoa threatened to compromise the cyanobacteria culture. However, the number of protozoa declined after the 11^{th} day without active measures being taken. Most certainly, the decline was due to the increasing concentration of phenols and salts, which was caused by the continuous addition of pre-processed liquid digestate (Pulgarin et al., 2021). Phenols are toxic compounds, which affect aquatic ecosystems negatively and directly impact the abundance of protozoa even in low concentrations (Saha et al., 1999). This cultivation in the medium containing pre-processed liquid digestate shows that the use of certain wastewaters in cyanobacteria cultivation do not solely bring challenges but also solutions regarding contamination control (Wang et al., 2013).

Overall, the results of this study show that a sustainable and commercially successful phototrophic PHB production in an open system with the application of wastewater for nutrient supply is possible. The achieved PHB yields confirm that there is an accumulation of PHB, but also suggest that there is still more research required to optimise and complete the scaled-up process of phototrophic PHB production.

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Supplementary material

S1 Calculation of PHB content in dry weight

$$\%_{\mathrm{dw}} = \frac{PHB\ [\mathrm{mg\ L^{-1}}]}{dw\ [\mathrm{mg\ L^{-1}}]} \cdot 100$$

S2 Calculation of nitrogen mass balance

$$N_{lost}\ [\mathrm{mg\ L^{-1}}] = N_{supplied}\ [\mathrm{mg\ L^{-1}}] - (dw\ [\mathrm{mg\ L^{-1}}] \cdot N_{cells}\ [\%]) - N_{medium}\ [\mathrm{mg\ L^{-1}}]$$

S3 Calculation of the nitrogen conversion ratio

$$biomass\ [\mathrm{g\ L^{-1}}] = dw_{end}\ [\mathrm{g\ L^{-1}}] - dw_{start}\ [\mathrm{g\ L^{-1}}]$$

$$nitrogen\ conversion\ ratio\ \left[\mathrm{mg\ g_{dw}^{-1}}\right] = \frac{N_{supplied}\ [\mathrm{mg\ L^{-1}}]}{biomass\ [\mathrm{g\ L^{-1}}]}$$

S4 Conversion of PPFD (µmol s^{-1} m^{-2}) into power (kW) and energy (kWh) per area (m^2)

The number of absorbed photons was measured with two PAR sensors that were placed above (PAR 1) and below (PAR 2) the glass cultivation platform. During the first 5–6 days, the reactor was shaded. To prevent the cultures from bleaching. To take this into account in the calculations, the measured value of PAR 1 was divided by the factor 2 (only 50 % of light passed through the shade cloth) during the shaded time period.

$$absorbed\ photons\ [\mathrm{\mu mol\ m^{-2}\ s^{-1}}] = PAR\ 1\ [\mathrm{\mu mol\ m^{-2}\ s^{-1}}] - PAR\ 2\ [\mathrm{\mu mol\ m^{-2}\ s^{-1}}]$$

$$power\ per\ area\ [\mathrm{kW\ m^{-2}}] = absorbed\ photons\ [\mathrm{\mu mol\ m^{-2}\ s^{-1}}] \cdot 219\ [\mathrm{kW\ \mu mol^{-1}}]$$

$$\Delta t\ [\mathrm{h}] = \frac{time_{i+1}\ [\mathrm{sec}] - time_i\ [\mathrm{sec}]}{3'600}$$

$$energy\ per\ area\ [\mathrm{kWh\ m^{-2}}] = power\ per\ area\ [\mathrm{kW\ m^{-2}}] \cdot \Delta t\ [\mathrm{h}]$$

S5 Calculation of the energy conversion ratio

$$area\ [\mathrm{m^2}] = 18$$

$$volume\ in\ reactor\ [\mathrm{L}] = 200$$

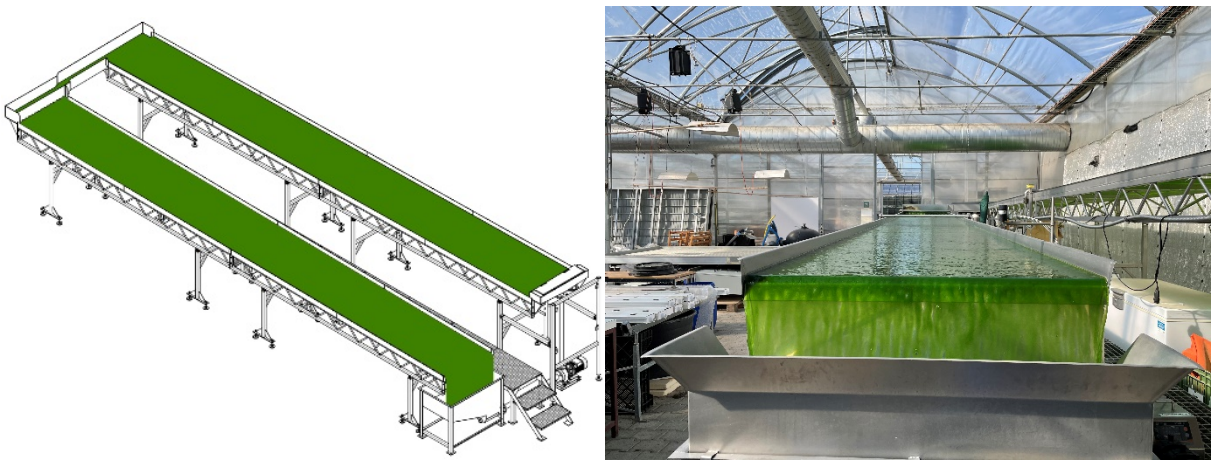
$$total\ energy\ [\mathrm{kWh}] = \sum energy\ per\ area\ [\mathrm{kWh\ m^{-2}}] \cdot area\ [\mathrm{m^2}]$$

$$total\ biomass\ [\mathrm{g_{cdw}}] = (cdw_{end} - cdw_{start}) \cdot volume\ of\ reactor\ [\mathrm{L}]$$

$$energy\ conversion\ ratio\ [\mathrm{kWh\ g_{dw}^{-1}}] = \frac{total\ energy\ [\mathrm{kWh}]}{total\ biomass\ [\mathrm{g_{dw}}]}$$

S6 Open thin-layer PBR

The open thin-layer photobioreactor (Doucha & Lívanský, 2014) has a volume of 200 L and a sun exposed surface of 18 m^{-2}. The reactor is placed in a greenhouse on the campus Grüental in Wädenswil, which belongs to the Institute of Natural Resource Sciences of the Zurich University of Applied Sciences.



S7 Dependency between biomass yield and nutrient supply

According to Doucha and Lívanský (2006), 84.9 mg L^{-1} nitrogen and 9 mg L^{-1} phosphorus are needed to yield 1 g L^{-1} algal biomass. The mineral medium used in this study (Z-medium, SAG, 2008) contains, when concentrated one-fold, 76.96 mg L^{-1} nitrogen and 5.51 mg L^{-1} phosphorus. Dividing the nutrient concentration of the Z-medium by the nutrients needed for 1 g L^{-1}, the biomass yield that can be achieved with a one-fold concentrated Z-medium can be calculated.

Calculation for nitrogen:

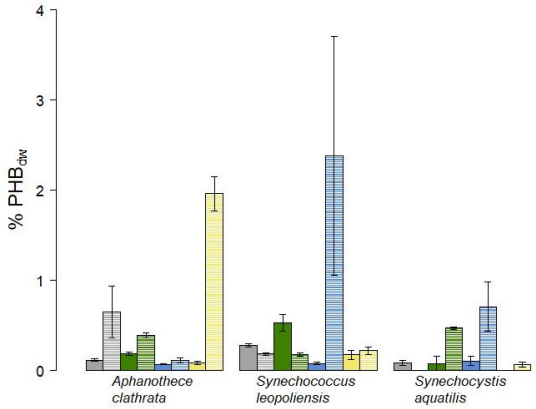
$$achievable\ biomass = \frac{76.96\ mg\ L^{-1}}{84.9\ mg\ L^{-1}} = 0.9\ g\ L^{-1}$$

Calculation for phosphorus:

$$achievable\ biomass = \frac{5.51\ mg\ L^{-1}}{9\ mg\ L^{-1}} = 0.6\ g\ L^{-1}$$

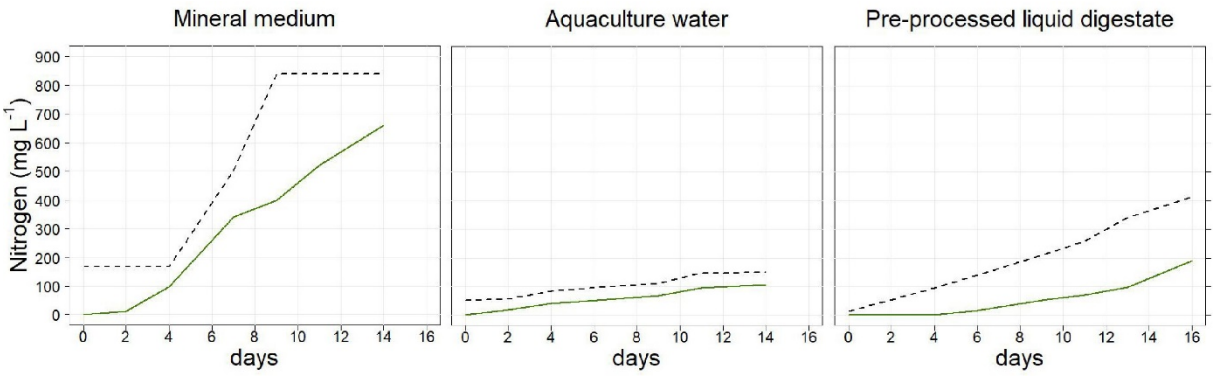
According to the calculations, phosphorus is the limiting nutrient and therefore a one-fold concentrated Z-medium can yield 0.6 g L^{-1} biomass.

S8 PHB contents in the cultures of the laboratory experiments



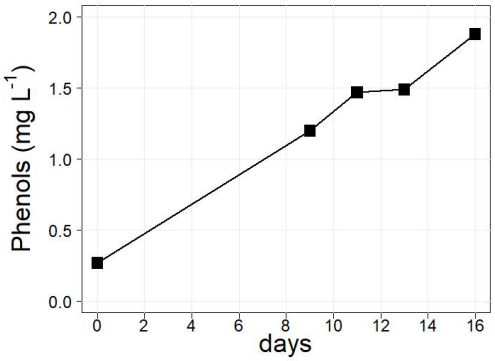
The figure shows the PHB content on the 7th day of the starvation phase (day 21 of the laboratory experiment). The values represent the mean values of the replicates with the standard error of the mean. The three species are visualised with each medium used during the growth phase (grey: mineral medium; green: medium containing filtered aquaculture water; blue: medium containing unfiltered aquaculture water; yellow: medium containing pre-processed liquid digestate) in the two experiments (1st: solid; 2nd: hatched horizontally).

S9 Nitrogen supply and assimilation in the cyanobacterial cells



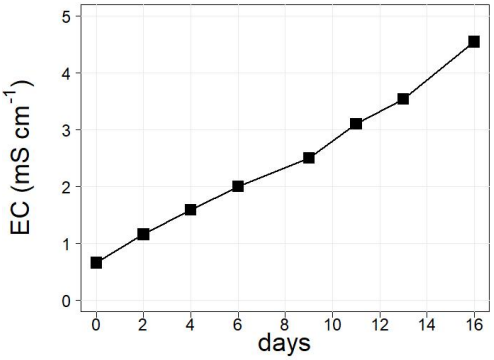
The figure shows the absolute values of nitrogen that was supplied to the cultures on the open thin-layer PBR (black, dashed) and nitrogen that was assimilated by the cyanobacteria over the course of the corresponding cultivation (green, solid).

S10 Concentration of phenols in the cultivation with medium containing pre-processed liquid digestate



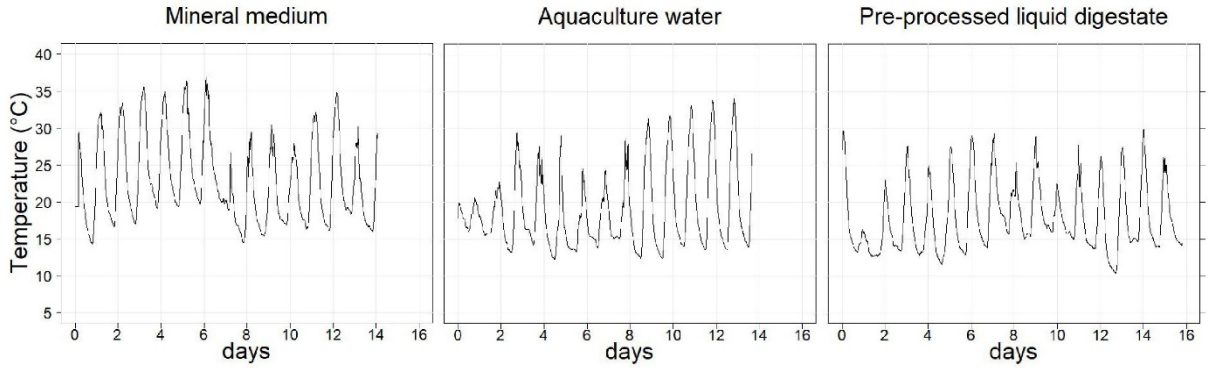
The figure shows the rise of the concentration of phenols over the course of the cultivation with medium containing pre-processed liquid digestate. The increase was caused by the continuous addition of pre-processed liquid digestate which is high in phenols.

S11 Development of the electric conductivity (EC) in the cultivation with medium containing pre-processed liquid digestate



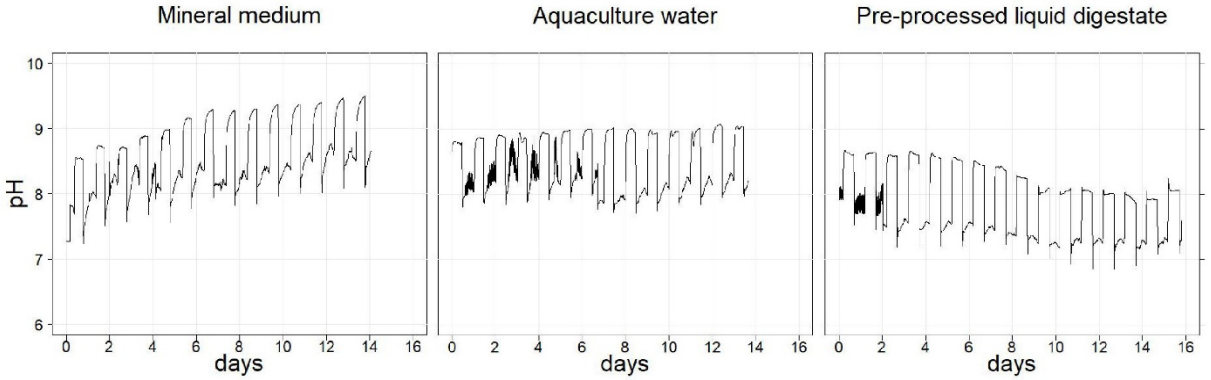
The figure shows the increase of the electric conductivity (EC) over the course of the cultivation with medium containing pre-processed liquid digestate. The increase was caused by the continuous addition of pre-processed liquid digestate which is high in salts.

S12 Temperature profiles of the three cultivations on the open thin-layer PBR



The figure shows the temperature profiles of the three cultivations on the open thin-layer PBR over the course of the cultivations. The temperature fluctuations are caused by natural temperature fluctuations between day and night. Temperatures increased over the day and decreased again over the night.

S13 pH values in the three cultivations on the open thin-layer PBR



The figure shows the pH values of the three cultivations on the open thin-layer PBR over the course of the cultivations. The pH value fluctuated between day and night as the CO_2 supply was stopped over the night.