Algal Research 70 (2023) 103013

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Cultivation of the PHB-producing cyanobacterium *Synechococcus leopoliensis* in a pilot-scale open system using nitrogen from waste streams

Marina Mariotto [a], Sophia Egloff [a], Ines Fritz [b], Dominik Refardt [a,*]

[a] Zurich University of Applied Sciences, Institute of Natural Resource Sciences, Grüentalstrasse 14, 8820 Wädenswil, Switzerland
[b] Universität für Bodenkultur Wien, Institut für Umweltbiotechnologie, Konrad-Lorenz-Strasse 20, 3430 Tulln an der Donau, Austria.

ARTICLE INFO

Original content: Cultivation of the PHB-producing cyanobacterium Synechococcus leopoliensis in a pilot-scale open system using nitrogen from waste streams (Original data)

Keywords:
Wastewater treatment
Liquid digestate
Aquaculture water
Bioplastic
Biopolymer
Nitrogen mass balance

ABSTRACT

PHB-producing cyanobacteria may provide the raw material for bio-based and biodegradable plastics. To commercialize photoautotrophic PHB production, their cultivation needs to be scaled up in open systems and to reduce costs and increase sustainability, nutrients must be obtained from waste streams. Here, the feasibility of these steps was verified. Different PHB-producing cyanobacteria were compared in laboratory-scale cultivations using either water from recirculating aquaculture systems or pre-processed liquid digestate as nutrient sources. Then, *Synechococcus leopoliensis* was cultivated in an open thin-layer photobioreactor (18 m^2, 200 L), were growth in mineral *Z*-medium was again compared to said waste streams. All cultivations 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 waste stream-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. Relative parameters such as nitrogen and energy conversion ratios indicate that cultivation with aquaculture water suffered from insufficient nitrogen supply to the culture, whereas the use pre-processed liquid digestate resulted in a substantial loss of nitrogen due to volatilization. All cultivations in the open system were continued in the laboratory, where cultures were starved for ten days under nutrient-depleted conditions (without nitrogen, phosphorus, or both). While PHB accumulation occurred, concentrations were comparatively low ($< 1\%_{dw}$). The comparison of the results suggests 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 considered for the optimization of the complete process.

1. Introduction

The worldwide consumption of plastics is expected to double by 2050 [1,2]. 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 [3,4]. In order to achieve political goals such as the European Green Deal [5] or the Sustainable Development Goals [6], which, among other things, pursue CO_2 neutrality and the protection of ecosystems, alternatives to conventional plastics must be found and commercialized.

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 [7]. PHB is synthesized by various bacterial strains including many cyanobacteria and is used by them as carbon storage [8]. The cultivation of PHB-producing organisms can be heterotrophic or photoautotrophic [9].

Heterotrophic cultivation generally results in higher PHB contents of 40 to over 90 % in dry weight [10,11]. 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 [12,13]. To produce PHB phototrophically, two cultivation phases are needed. In the first phase, cyanobacteria are cultivated under non-limiting conditions to rapidly build up high biomass densities. In the second phase, the culture

* Corresponding author.
E-mail address: dominik.refardt@zhaw.ch (D. Refardt).

https://doi.org/10.1016/j.algal.2023.103013
Received 27 July 2022; Received in revised form 10 January 2023; Accepted 10 February 2023
Available online 16 February 2023

is deprived of nutrients such as nitrogen or phosphorus, which triggers PHB accumulation [14].

Phototrophic PHB production with cyanobacteria usually results in lower PHB contents of below 10 % and up to 25 % in dry weight [15–18]. 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 production of feed and food as no organic carbon or arable land is needed, which makes the cultivation more sustainable [15].

Appropriate supply of nutrients, in particular nitrogen and phosphorus, is central for a productive cultivation of cyanobacteria [19]. The source of those nutrients, however, influences sustainability and resource efficiency of the cultivation. Especially in large-scale production, nutrient recycling using wastewater can improve resource efficiency and sustainability [20]. Many wastewaters are suitable for cultivation of cyanobacteria and microalgae [21]. Among them are liquid digestate and water from recirculating aquaculture systems [22,23].

Liquid digestate contains a high proportion of mineralized nutrients as these are not converted into gases during fermentation [24]. 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 before its application in cyanobacterial cultivation [25]. Part of this treatment is usually a separation of the solid and liquid fraction, during which phosphorus accumulates mainly in the solid fraction, organic carbon is largely removed, and nitrogen compounds remain in the liquid phase [24]. The cultivation of cyanobacteria and microalgae using liquid digestate as a nutrient source has been proven feasible. However, because ammonium is the main form of nitrogen in liquid digestate and its assimilation by microalgae releases protons, pH control may be necessary [23,26].

Water from aquaculture systems contains nitrogen compounds and other nutrients that are favorable for the cultivation of cyanobacteria and microalgae [22,27]. Nutrients originate from fish feed and are released into the water via excretion of the fish. In a recirculating aquaculture system, several treatment steps (e.g., solids removal, nitrification, and disinfection) are implemented to maintain a good water quality for the fish. This results in both 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 as well as in a favorable nutrient composition for cyanobacteria [28]. Nitrogen occurs mainly as nitrate and, while nutrient concentrations in aquaculture water are usually relatively low, high biomass yields can still be achieved if the wastewater is continuously supplied to a culture to balance water loss from evaporation [22].

To realize a commercial production system for photoautotrophic PHB, the upscaling of the cultivation is necessary [29]. Large-scale production is either performed in closed or open cultivation systems. Both come with their advantages and disadvantages: In closed systems, it is usually easier to control contaminations and operational parameters, but they call for higher initial investment costs. Open systems allow interactions with the environment, which allows easy access to natural resources (e.g., atmospheric CO_2). However, open systems cannot be fully controlled (e.g., temperature, pH) and contaminations by bacteria, protozoa, and different algae species are more likely [30]. In both types of systems, sunlight can be used as energy source and, thus, lowers the energy demand. So far, most literature documents the cultivation of PHB-producing cyanobacteria on a laboratory scale [31]. To our knowledge, only three cultivations have been reported on a larger scale, all in non-sterile tubular photobioreactors: *Synechocystis* sp. CCALA192 was cultivated in a 200 L-volume [32], a randomly mutated strain of *Synechocystis* sp. PCC6714 was cultivated in a 40 L-volume [33], and a wild consortium of cyanobacteria was cultivated in a 11.7 m^3-volume [34]. These cultivations confirm that an upscaling of the cultivation in closed or semi-closed systems under non-sterile conditions is possible. However, considering the commercialization of phototrophic PHB production, it is of interest to address the cultivation of single strains in open systems, as this would allow to approach the production cost of fossil plastics [35].

The aim of this study is to cultivate PHB-producing cyanobacteria at large scale in an open system (18-m^2 open thin-layer photobioreactor [36] with 200 L cultivation volume, see supplementary material S6) using selected wastewaters to supply nitrogen. 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 physicochemical parameters [21]. To find suitable species, an initial screening at laboratory scale was performed, upon which a promising species was cultivated at large scale using standard mineral medium as well as water from a recirculating aquaculture system and pre-processed liquid digestate. Finally, cultures were exposed to different nutrient-depleted 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 aquatilis* CCALA 190 was obtained from the Culture Collection of Autotrophic Organism of the Institute for Botany in Třeboň (CCALA). PCR analysis was performed to confirm their identity before and after the experiments (see Section 2.3).

2.2. Media

A mineral medium, *Z*-medium [37], and modifications thereof were used in all experiments. Unmodified mineral medium served as a control, while different types of wastewaters were used to replace its nitrogen source ($NaNO_3$) 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, which was treated with ultrafiltration and reverse osmosis at the plant, and water from two aquaculture systems at the Zurich University of Applied Sciences in Wädenswil, Switzerland (Table 1). Aquaculture water A was sampled from a recirculating aquaculture system stocked with burbot (*Lota lota*) and water withdrawal was after the solids removal unit. Aquaculture water B was sampled from a recirculating aquaponic system stocked with perch (*Perca fluviatilis*), vanilla (*Vanilla* sp.), and banana (*Musa* sp.), water withdrawal was after the solids removal unit, and the water was stored in an industrial bulk container at 4 °C for four months prior to use.

To prompt the accumulation of PHB in the cells, three different nutrient-depleted modifications of the mineral medium (without

Table 1
Concentrations (mg L^{-1}) of nitrogen and phosphorous in the different wastewaters used in this study.

	Pre-processed liquid digestate	Aquaculture water A	Aquaculture water B
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

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

Growth of cyanobacteria 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 was determined by light microscopy (phase contrast, 400-fold magnification) with a hemocytometer with Thoma ruling. Dry weight (dw) was either measured with a moisture analyzer (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 deionized 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 to 10^{-5}) of the culture samples was prepared and 5 μL of each dilution were spotted onto 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 hemocytometer with Fuchs-Rosenthal ruling.

PHB content was measured according to a modified method of Karr et al. [38]. The cyanobacterial 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. 1 mL of sulphuric acid (18.4 M) was added to the dried pellet. Samples were heated at 105 °C for 60 min to dissolve the biomass. Samples were cooled down to room temperature, 9 mL of sulphuric acid (7 mM) were added, and samples were filtered (0.45 μm). HPLC analysis was carried out with a Nexera system (Shimadzu, Kyoto, Japan) and a Repro-Gel H+ column (Dr. Maisch, Ammerbuch-Entringen, 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}. Measurements lasted 32 min. Results were standardized to percentage in dry weight ($\%_{dw}$) by dividing PHB concentration (mg L^{-1}) by 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, Loveland, CO).

The CHN content of dried algal biomass samples (100 mg) was determined by thermal conductivity and infrared spectroscopy (TruSpec Micro CHN, Leco, St. Joseph, MI). 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 obtain 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 with a commercial kit (NucleoSpin Tissue, Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. 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)) [39], 12.5 μL of DNA-polymerase (KAPA2G Robust HotStart ReadyMix, Roche, Rotkreuz, 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 s, annealing at 58 °C for 15 s, and extension at 72 °C for 15 s, and a final extension at 72 °C for 1 min. Amplified DNA was cleaned with a commercial kit (Gel and PCR Clean-up NucleoSpin, Macherey-Nagel) following the manufacturer's instructions. DNA samples were sent to a commercial sequencing service (Microsynth, Balgach, Switzerland) for Sanger sequencing and results were checked against the NCBI database using the BLAST algorithm.

pH was measured manually in the laboratory experiments (HQ40d multiprobe equipped with an INTELLICAL PHC108 sensor, Hach) and every minute with built-in systems in the open system (InPro 3253i SG/120, Mettler Toledo). Electrical conductivity was measured manually (HQ40d multiprobe equipped with a CDC401 sensor, Hach). Culture temperature was measured every minute with built-in systems (InPro 3253i SG/120, Mettler Toledo) in the open system.

Photosynthetically active photon flux density (PPFD, μmol s^{-1} m^{-2}) in the greenhouse was measured every minute with two PAR sensors (SKL2620, Skye Instruments, Llandrindod Wells, United Kingdom) that were placed above and below the glass cultivation platform of the open system. 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 $2.19 \cdot 10^{-4}$ [40]. To calculate the amount of absorbed solar energy (kWh), power was multiplied with the time difference from one measurement to the next (see supplementary material S4). To control for seasonal variation 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 A. clathrata, S. leopoliensis and S. aquatilis

Growth and PHB production of different species of cyanobacteria in different wastewater-based media was assessed in replicated laboratory-scale batch cultivations and repeated in two independent experiments to obtain reliable results. Three cyanobacterial species (*A. clathrata*, *S. leopoliensis*, and *S. aquatilis*) and a negative control without inoculum were cultivated in four different media (mineral medium, nitrogen replacement with pre-processed liquid digestate, nitrogen replacement with aquaculture water A (sterile-filtered (0.22 μm) and unfiltered); see Section 2.2). All combinations were replicated three-fold in the first and four-fold in the second experiment, yielding 48 and 64 cultivations, respectively. Both experiments lasted 21 days and were divided into a growth phase (14 days) and a starvation phase (seven days).

The growth phase was initiated by inoculating 10^5 cells mL^{-1} in a volume of 60 mL in 100-mL shake flasks followed by incubation at 25 °C, 1 % CO_2, 100 rpm agitation, and approximately 4′800 lx (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 biomass 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.

The starvation phase was initiated by transferring the cyanobacterial cultures to a nitrogen-depleted medium. To this end, 40 mL of the cultures were centrifuged (5 min, 7′200 *g*) and resuspended in 60 mL of different media as explained above. 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 S. leopoliensis in an open system

S. leopoliensis was chosen for upscaling of the cultivation because this

species grew reliably and produced the highest amount of PHB in the laboratory-scale cultivations. Three subsequent cultivations were carried out in mineral medium and two different modifications of it as described in Section 2.2 (unmodified mineral medium, nitrogen replacement with pre-processed liquid digestate and nitrogen replacement with aquaculture water B). Cultivations lasted 14 to 16 days and cultures were covered with a shade cloth during the first five to six days to prevent bleaching of the cultures. Where possible, operational parameters were kept the same in all cultivations. During the day, partial pressure of CO_2 in the medium was maintained at 5 to 10 mbar and during the night, CO_2 supply was stopped to save CO_2. Thickness of the suspension on the cultivation platform was set to 6 mm. In the medium that contained 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 [23]. Water loss from the system by evaporation was balanced by an automated supply of either desalinated tap water (cultivation with mineral medium and pre-processed liquid digestate) or aquaculture water (cultivation with aquaculture water). The addition of nutrients was handled differently in the three cultivations. When unmodified mineral medium was used, all 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 biomass dry weight approached the expected biomass yield (see supplementary material S7). The same strategy was used for the cultivations with aquaculture water and pre-processed liquid digestate, except for the supply of nitrogen, which was provided via the respective wastewater. Supply of nitrogen via aquaculture water depended on the corresponding water loss by evaporation. Supply of nitrogen via pre-processed liquid digestate occurred continuously with a peristaltic pump (on average, 34.6 mL h^{-1} of pre-processed liquid digestate (222 mg h^{-1} of nitrogen) were added to the system).

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. 40-mL samples were taken regularly to measure growth (dry weight, optical density, cell count), 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 electrical 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. The identity and presence of *S. leopoliensis* was confirmed at the beginning and the end of all three cultivations by sequencing.

At the end of every cultivation, culture samples were transferred into nutrient-depleted mineral media to accumulate PHB. Different nutrient-depleted media were tested (see Section 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 in the open system 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 lx. Each medium was replicated three-fold. Optical density, dry weight from a 1-mL sample, pH, PHB content, and number of protozoa were measured at days 0, 3, 7, and 10.

2.6. Data analysis

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

Data on growth and PHB accumulation from the laboratory cultivations were tested for effects of the different media and cyanobacterial species. To this end, a full factorial ANOVA (three species, four media) was fitted to optical density measured at the last day of the growth phase (day 14) and individual comparisons were carried with Tukey's HSD test. PHB content measured at the last day of the starvation phase (day 21) was analyzed with a Kruskal-Wallis test as assumptions for a parametric test (homoscedasticity, normally distributed residuals) were not fulfilled.

Cultivations in the open system were not replicated and, thus, no statistical analyses were carried out at this level. However, some relative values were used to compare the cultivations (see Section 2.3). The results of the subsequent PHB accumulation experiments were analyzed for effects of time and different nutrient-depleted media on changes in PHB content. Assuming that the different media, which were used during the growth experiments, influenced the PHB accumulation, a linear mixed-effects model (function lme of library nlme) was fitted to the data with sampling day (continuous) and nutrient-depleted medium (five levels) as independent predictors and the medium of the preceding cultivation in the open system as random effect. To achieve homoscedasticity and normally distributed residuals, data were square root-transformed.

3. Results

3.1. Laboratory experiments with A. clathrata, S. leopoliensis and S. aquatilis

Cultivations of *A. clathrata*, *S. leopoliensis*, and *S. aquatilis* in mineral medium and its modifications, where wastewater provided nitrogen, were successful (Fig. 1). Growth (measured as optical density at day 14) differed between species ($F_{2,78} = 11.12$, $p < 0.001$), with *S. leopoliensis* and *S. aquatilis* achieving higher values (OD_{750} = 0.6–3.3) than *A. clathrata* (OD_{750} = 0.6–2.4). Growth of cyanobacteria also differed between media ($F_{3,78} = 45.7$, $p < 0.001$), with mineral medium and medium containing filtered aquaculture water yielding better results (OD_{750} = 1.2–3.6 and OD_{750} = 1.4–3.0, respectively) than medium containing pre-processed liquid digestate (OD_{750} = 0.5–1.5). While cultivations in medium containing unfiltered aquaculture water reached high values (OD_{750} = 0.5–2.8), these results must be interpreted with caution as cultures were contaminated with green algae (likely *Scenedesmus* sp.), which contributed to optical density. Contaminations only occurred in the cultivations of *A. clathrata* and *S. leopoliensis*, where cyanobacteria were displaced almost completely by green algae in some replicates. No contaminations with green algae were observed in cultures with *S. aquatilis*.

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 *S. leopoliensis* cultivated in medium containing unfiltered aquaculture water (2.4 % PHB_{dw}) and in *A. clathrata* cultivated in medium containing pre-processed liquid digestate (2 % PHB_{dw}).

3.2. Cultivation of S. leopoliensis in an open system

The cultivations of *S. leopoliensis* in the open system in mineral medium and its modifications, where nitrogen was provided via wastewater, were successful (Fig. 2). The highest productivity was achieved in mineral medium with a maximum growth rate of 0.7 g L^{-1} d^{-1}, followed by medium containing pre-processed liquid digestate (0.3 g L^{-1} d^{-1}) and aquaculture water (0.25 g L^{-1} d^{-1}). The final biomass yield was also

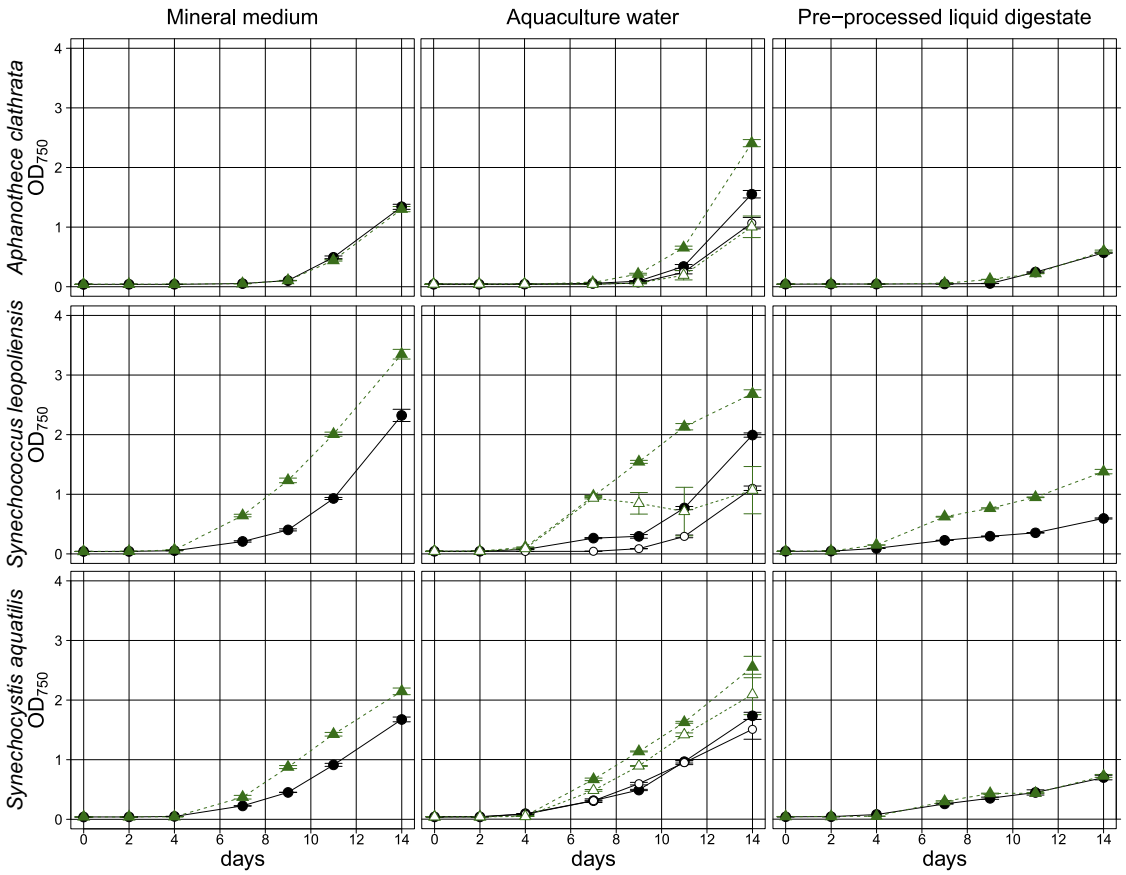

Fig. 1. Growth (measured via optical density at 750 nm) of three cyanobacterial species (rows) in three different media (columns) each. Two consecutive laboratory experiments (1st: black solid lines with circles, $n = 3$; 2nd: green dashed lines with triangles, $n = 4$) were carried out and data are shown as means ± standard error. Cultivations with aquaculture water were carried out both in filtered (open symbols) and unfiltered (closed symbols) medium. Note that growth of green algae in some cultures of *A. clathrata* and *S. leopoliensis* in unfiltered aquaculture water contributed to measurements of optical density. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

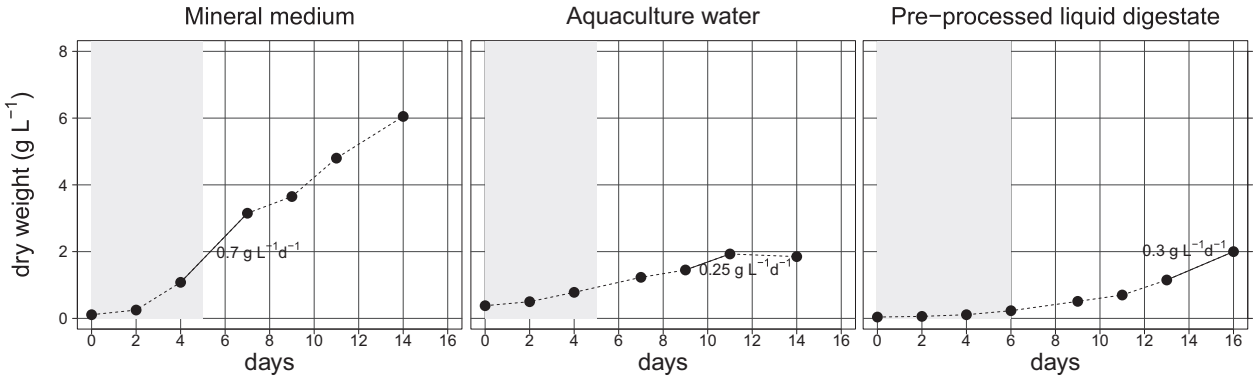

Fig. 2. Growth of *S. leopoliensis* in different media in an open system. Grey backgrounds indicate artificial shading of the cultures to prevent sunlight from damaging the cultures. Solid lines indicate the most productive period with the corresponding volumetric productivity.

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 *S. leopoliensis* in the different media adequately, as solar energy and nutrient inputs differed between cultivations. To be able to compare cultivations, conversion ratios were calculated, which describe the amount of solar energy and nitrogen that were used to produce a unit of biomass (see Section 2.3).

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 *S. leopoliensis* in the media containing wastewater show similar performances. However, when differences in solar irradiation are considered, *S. leopoliensis* cultivated in medium containing pre-processed liquid digestate transforms the solar energy more

efficiently into biomass than in medium containing aquaculture water. Also, when *S. leopoliensis* 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 total amount of nitrogen supplied during the cultivation was highest in mineral medium (840 mg L^{-1}). The nitrogen supplied was lower with aquaculture water (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 211 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 strong nitrogen limitation in the cultivation with medium containing aquaculture water, which was caused both by the low nitrogen concentration in the aquaculture water and the dependency on evaporation for the supply of more wastewater. This is also reflected by the nitrogen content of the biomass, which is lower when cells were grown in medium containing aquaculture water (8.2 %) compared to mineral medium (10.8 %). The efficient use of a limited supply of nitrogen in the cultivation with aquaculture water contrasts with the high nitrogen conversion ratio in medium containing pre-processed liquid digestate, which indicates an inefficient use of nitrogen. This is likely explained by the large loss of nitrogen (>50 %) that occurred in this cultivation (Fig. 3).

The fraction of the supplied nitrogen that was assimilated by *S. leopoliensis* differed between cultivations (Fig. 3 and supplementary material S9). In the cultivations with mineral medium and medium containing aquaculture water, nitrogen was mainly present as nitrate (NO_3^-) and 78.6 % and 72.3 % were assimilated, 4.8 % and 4.1 % remained in the medium, and 16.6 % and 23.6 % were lost to the atmosphere. In the cultivation with medium containing pre-processed liquid digestate, nitrogen was mainly present as ammonium (NH_4^+) and 46.2 % were assimilated, 2.3 % remained in the medium, and 51.5 % were lost to the atmosphere.

In all three cultivations, the presence of bacteria and protozoa was observed. Numbers of protozoa in mineral medium and medium containing aquaculture water were comparatively stable (between $6.5{\cdot}10^3$ and $6.3{\cdot}10^4$ mL^{-1}) over the course of the cultivations, while in medium containing pre-processed liquid digestate numbers of protozoa increased to 10^6 mL^{-1} until day 11 and then decreased rapidly (Fig. 4). Numbers of aerobic mesophilic bacteria behaved differently in each cultivation (Fig. 4) and were between $8.1{\cdot}10^4$ and $1.4{\cdot}10^6$ mL^{-1}. Notably, numbers in medium containing pre-processed liquid and reached final values that were among the lowest observed.

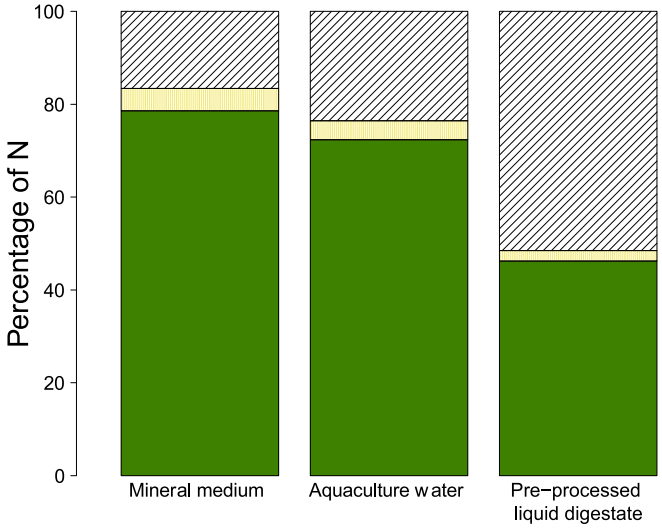

Fig. 3. Nitrogen mass balance of the three cultivations in the open system with nitrogen that was assimilated within the cells (green, solid), nitrogen that remained in the medium (yellow, striped vertically) and nitrogen that was lost to the atmosphere (black, striped diagonally). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In the cultivation with medium containing pre-processed liquid digestate, a contamination with green algae (likely *Scenedesmus* sp.) emerged, which amounted to approximately 10 % of all cells at the end of the cultivation. Despite this and other contaminations as described above, DNA sequencing at the beginning and end of each cultivation confirmed the presence of *S. leopoliensis*.

The continuous addition of 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). In correspondence with the continuous supply, concentration of phenols and electric conductivity increased linearly from 0.27 to 1.9 mg L^{-1} and 0.7 to 4.6 mS cm^{-1}, respectively.

Temperature fluctuated daily and strongly with shifts of up to 20 °C between 11 and 37 °C (see supplementary material S12). This reflects the placement of the open system in a greenhouse and emphasizes the robustness of *S. leopoliensis*. pH shifts occurred daily as well and were caused by the CO_2 supply, which was switched off during the night. pH remained between 7.2 and 9.5 throughout all cultivations.

3.3. Accumulation of PHB in different nutrient-depleted media

After the transfer of the cultures from the open system to the laboratory and into the nutrient-depleted media, PHB accumulation was observed over the course of ten days in most cultures (Fig. 5). While PHB content increased over time ($F_{1,172} = 87.5$, $p < 0.001$), there was no discernible effect of a specific type of nutrient depletion nor of nutrient depletion itself as it was also observed in samples that were transferred to fresh mineral medium ($F_{4,172} = 1.77$, $p = 0.14$). Rather, PHB accumulation appeared to depend on the medium and the environmental conditions during the cultivation in the open system before the starvation period. In all samples that were previously cultivated in mineral medium, PHB accumulation was most pronounced after day 7 of the starvation experiment. In these cultures, a maximum of 0.7 % PHB_{dw} was reached in the medium depleted for both nitrogen and phosphorus. Samples grown in medium containing aquaculture water prior to starvation appeared to accumulate PHB more gradually during the starvation period and reached comparatively low PHB contents (max. 0.2 % PHB_{dw}) with some cultures exhibiting a decline of PHB content between days 7 and 10. Cultures that were previously cultivated in medium containing pre-processed liquid digestate accumulated the highest amount of PHB. Accumulation was again more gradual and reached a maximum of 0.9 % PHB_{dw} in the medium that was phosphorus-depleted. To summarize, while PHB accumulation was observed, strong carry-over effects from the cultivation in the open system appear to influence it and interact with specific starvation regimes. Because the conditions of the growth phase where not replicated, specific inferences should be made with caution.

4. Discussion

In this study, it was tested on a laboratory scale whether several PHB-producing cyanobacterial species (*A. clathrata*, *S. leopoliensis* and *S. aquatilis*) can be cultivated in mineral medium and two media containing wastewater (aquaculture water and pre-processed liquid digestate). Results from cyanobacteria cultivations with aquaculture water can vary based on the composition of the water [41,42]. We therefore carried out the experiment twice. Despite between-experiment variation, *S. leopoliensis* exhibited a consistently good performance and was chosen for cultivation in mineral medium and media containing

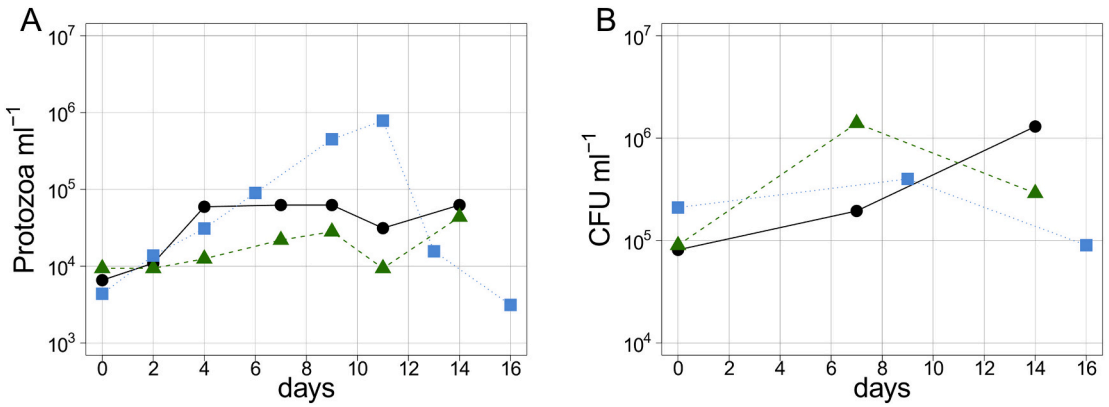

Fig. 4. Concentration of protozoa (A) and aerobic mesophilic bacteria (B) in the cultivations with mineral medium (black, circle, solid), medium containing aquaculture water (green, triangle, dashed) and medium containing pre-processed liquid digestate (blue, square, dotted). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

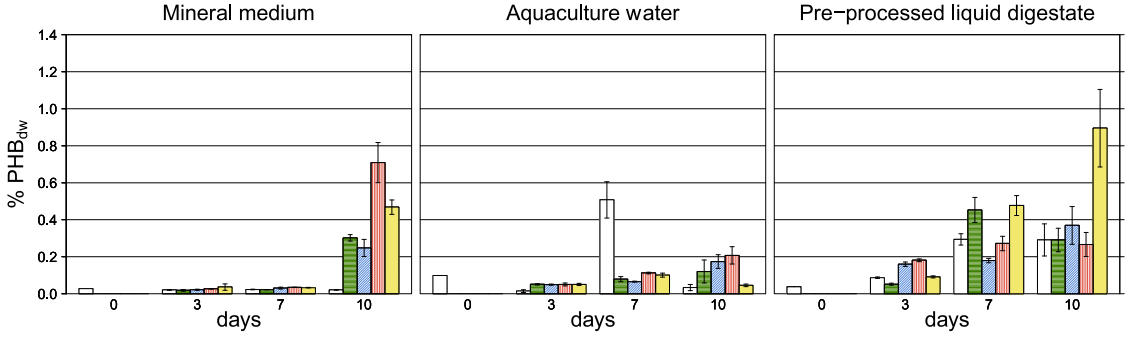

Fig. 5. PHB accumulation in *S. leopoliensis* during ten days in different starvation media (from left to right: exhausted medium from the cultivation in the open system (white, solid), mineral medium (green, striped horizontally), mineral medium —N (blue, striped diagonally), mineral medium —N —P (red, striped vertically), mineral medium —P (yellow, solid). Panels show starvation experiments derived from cultures previously cultivated in the open system in different media (title of panel). Data show mean values and the standard error of the mean ($n = 3$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

wastewater on a large scale in an open system. All cultivations in the three media on both laboratory and large 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 best energy conversion ratio. Some of obtained values can likely be improved because a substantial portion (approximately 50 %) of the sunlight does not enter the greenhouse [22]. Nevertheless, they can be used as a reference to compare the wastewater-based cultivations.

When *S. leopoliensis* was cultivated in medium where nitrogen was supplied via aquaculture water, conversion ratio of nitrogen into biomass was 28 % below that of the cultivation in mineral medium. This indicates that growth was limited by the nitrogen supply and is also supported by the nitrogen content in the biomass that was 24 % lower than in the biomass grown in mineral medium. Furthermore, the nitrogen limitation in the cultivation with aquaculture wastewater is also reflected in the increased energy conversion ratio, which indicates that light limitation was not an issue, as the cultivation in mineral medium required 2.7 times less solar energy to build the same amount of biomass. Nitrogen limitation in cultivations with aquaculture water was also observed in a previous study with *Chlorella vulgaris* and *Tetradesmus obliquus* [22] and suggests that cultivation of cyanobacteria or microalgae can be used to fully treat this wastewater.

Growth of *S. leopoliensis* in the medium containing pre-processed liquid digestate did not appear to be limited by nitrogen supply or solar irradiation as the nitrogen conversion ratio was 2.1 times higher and the energy conversion ratio 1.8 times higher than in the cultivation with mineral medium. Likely, other characteristics of the cultivation restrained growth: 1) The cultivation was carried out in autumn, with slightly lower temperatures and, therefore, slower growth [43]. 2) Ammonium as the nitrogen source was not ideal for *S. leopoliensis*. However, there are contradictory findings in the literature, whether ammonium or nitrate is the ideal nitrogen source for microalgae and cyanobacteria [44–46]. 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 [47].

Overall, *S. leopoliensis* performed well in mineral medium and wastewater-containing media under semi-outdoor conditions and reached relatively high biomass concentrations. In other studies in which PHB-producing cyanobacteria were cultivated on a larger 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 [32,33]. However, the results are not directly comparable as the cultivation systems, species and many other parameters 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 why losses were moderate. In the cultivation with the medium containing pre-processed liquid digestate, ammonium was the main source of nitrogen, which, due to its volatility

as ammonia, likely explains the higher nitrogen loss of >50 % [23,48]. Furthermore, supply of CO_2 was switched off during the night to save CO_2. This caused corresponding pH shifts towards more alkaline conditions and may have further promoted the loss of nitrogen. Indeed, lower losses (~30 %) were observed in a cultivation of *Chlorella vulgaris* with a pH continuously maintained close to 7 under similar conditions (unpublished results).

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 [49], which may occur in some parts of the open system, presumably in biofilms, where exchange of nutrients with the circulating medium is possible [50]. Another contributing factor for the nitrate losses may be a metabolic pathway of cyanobacteria that results in nitrous oxide (N_2O) emissions [49,51–54]. 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 [55]. 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 [52]. Thus, N_2O synthesis would only be a small contributor to the nitrogen losses. Finally, it is conceivable that herbivorous protozoa consumed *S. leopoliensis* and, thus, shunted a part of the assimilated nitrate back into the circulating medium as ammonium [56], which was then subsequently lost by volatilization.

The loss of ammonium in the cultivation with medium containing pre-processed liquid digestate may be explained through the transformation of ammonium into volatile 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 [57]. Ammonia is toxic to humans and other animals and deposition in the environment causes acidification and eutrophication. Therefore, emissions should be minimized, e.g., by keeping the pH below 7.5 [58–60]. In cultivations with *Chlorella vulgaris* in medium containing pre-processed liquid digestate, which were carried out in the same system that was used in this study, comparable proportions of ammonium were lost to the atmosphere [23], even when pH was kept below 7.3 (unpublished results). This indicates that controlling the pH value is not sufficient to minimize the emissions and that the causes of nitrogen loss in cyanobacteria and algae cultivation are not sufficiently understood yet.

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 [14,16], the data of this study did not reveal a clear dependency of the PHB accumulation on nutrient depletion. Possibly, cultivation conditions during the growth phase interfered with subsequent PHB accumulation. As outlined above, the three cultivation regimes differed in many aspects that caused variation in growth rates and nitrogen content of the biomass, and, likely, resulted in cultures that differed in their physiological states prior to their transfer to the laboratory for the starvation phase. It has been shown that temperature, pH, incubation time, availability of carbon, and the timing of the light-dark cycle have an effect on PHB accumulation [61–63]. The results of this study caution that the effect of environmental fluctuations, which are inevitable when upscaling is performed, must be better understood.

Biological contaminants, such as protozoa, other microalgae, bacteria, or fungi, as well as the reduced level of control are challenges that open systems usually entail [64]. They do, however, not pose unsurmountable problems as *S. leopoliensis* remained the dominant species throughout the cultivations in the open system. One reason that may have kept growth of heterotrophic microorganisms at a low level was the low availability of organic carbon in the wastewaters used in this study. Water from recirculating aquaculture systems passes solids filtration and a nitrification unit multiple times before being exchanged, which results in a low COD (27 mg O_2 L^{-1}) and a much lower BOD (<2 mg O_2 L^{-1}) [65], which, together, indicate a very low biodegradability of <10 %. Moderate bacterial growth was confirmed repeatedly [41,42]. Liquid digestate contains only organic carbon with a low degradability and, in our case, solids were removed by ultrafiltration. The final product has a C:N ratio < 1 [23]. Because we dosed liquid digestate according to the nitrogen demand of the culture, the supply of carbon was negligible.

The contamination by competing microalgae species, as has been observed here, has been reported previously. This issue could be addressed by adjusting cultivation conditions such that growth of the target species is favored [66]. Indeed, we observed fewer competing microalgae when cultures were moved to the large open system, possibly because conditions were more favorable for our target species. Another solution being proposed is the creation of conditions that specifically select for the desired product [67]. As PHB serves as an energy storage, the intermittent creation of famine periods can select for species that accumulate PHB [34].

In the cultivation with medium containing pre-processed liquid digestate, an emerging contamination of protozoa threatened to compromise the cyanobacteria culture as it reached levels that were previously found to be sufficiently high to destroy a culture of *C. vulgaris* [41]. However, the number of protozoa declined again without active measures being taken. Possibly, this was due to increasing concentrations of phenols and salts, caused by the continuous addition of pre-processed liquid digestate. Phenols are toxic to aquatic organisms and the reported toxicity values suggest that effects may already occur at the phenol concentrations that were reached here [68]. If this was the reason for the decline of protozoa in the culture, the use of certain wastewaters for cyanobacteria cultivation may not only complicate matters but also provide solutions regarding contamination control [69].

5. Conclusion

In this study, growth and PHB production of the cyanobacterial species *A. clathrata*, *S. leopoliensis*, and *S. aquatilis* was compared at laboratory scale when nitrogen was supplied from waste streams. Based on these results, *S. leopoliensis* was cultivated repeatedly in a large open system under semi-outdoor conditions in a greenhouse, again using wastewater to supply nitrogen. While this is a further step towards a sustainable phototrophic production of PHB [35], results also revealed challenges specific to the upscaling. First, nitrogen mass balances showed that, while the removal efficiency of nitrogen from the medium was above 95 % in all three cultivations, a substantial fraction was lost to the atmosphere. At a larger scale of cultivation, this will entail grave environmental consequences and may also become a cost factor. Second, while in principle possible, the triggering of PHB accumulation by nutrient starvation was greatly dependent on prior cultivation conditions and points to the need of a better understanding of the role of environmental variation in process control.

CRediT authorship contribution statement

Marina Mariotto: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Sophia Egloff:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision. **Ines Fritz:** Writing – review & editing, Supervision. **Dominik Refardt:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

No potential financial or other interests that could influence the outcomes of the research are perceived.

Data availability

Cultivation of the PHB-producing cyanobacterium Synechococcus leopoliensis in a pilot-scale open system using nitrogen from waste streams (Original data) (Dryad)

Acknowledgements

The authors would like to thank Simone Vögeli and Jonas Windisch for their assistance during sampling, all members of the aquaculture systems research group for the provision of aquaculture water, Nicola Rhyner for advice on the genetic analyses, Nicolas Pirolet for advice on sample preparation for HPLC analysis, Roger Fehér for HPLC analysis, and Rahel Wanner for CHN analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2023.103013.

References

[1] R. Geyer, J.R. Jambeck, K.L. Law, Production, use, and fate of all plastics ever made, Sci. Adv. 3 (2017), e1700782, https://doi.org/10.1126/sciadv.1700782.
[2] L. Lebreton, A. Andrady, Future scenarios of global plastic waste generation and disposal, Palgrave Commun. 5 (2019) 6, https://doi.org/10.1057/s41599-018-0212-7.
[3] Y. Chae, Y.-J. An, Current research trends on plastic pollution and ecological impacts on the soil ecosystem: a review, Environ. Pollut. 240 (2018) 387–395, https://doi.org/10.1016/j.envpol.2018.05.008.
[4] B. Worm, I. Jubinville, C. Wilcox, J. Jambeck, Plastic as a persistent marine pollutant, Annu. Rev. Environ. Resour. 42 (2017) 1–26, https://doi.org/10.1146/annurev-environ-102016-060700.
[5] Publications Office of the European Union, The European Green Deal (Document 52019DC0640), European Commission, Brussels, Belgium, 2019. https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX:52019DC0640.
[6] United Nations, Transforming our world: The 2030 agenda for sustainable development (A/RES/70/1), United Nations. https://sdgs.un.org/publications/transforming-our-world-2030-agenda-sustainable-development-17981, 2015.
[7] B. McAdam, M. Brennan Fournet, P. McDonald, M. Mojicevic, Production of polyhydroxybutyrate (PHB) and factors impacting its chemical and mechanical characteristics, Polymers 12 (2020) 2908, https://doi.org/10.3390/polym12122908.
[8] M. Müller-Santos, J.J. Koskimäki, L.P.S. Alves, E.M. de Souza, D. Jendrossek, A. M. Pirttilä, The protective role of PHB and its degradation products against stress situations in bacteria, FEMS Microbiol. Rev. 45 (2021) 1–13, https://doi.org/10.1093/femsre/fuaa058.
[9] I. Fritz, K. Meixner, M. Neureiter, B. Drosg, Comparing heterotrophic with phototrophic PHA production: concurring or complementing strategies? in: Martin Koller (Ed.), The Handbook of Polyhydroxyalkanoates: Microbial Biosynthesis and Feedstocks, 1st ed., CRC Press, 2020, pp. 331–356, https://doi.org/10.1201/9780429296611.
[10] R. Handrick, S. Reinhardt, P. Kimmig, D. Jendrossek, The “intracellular” poly(3-hydroxybutyrate) (PHB) depolymerase of rhodospirillum rubrum is a periplasm-located protein with specificity for native PHB and with structural similarity to extracellular PHB depolymerases, J. Bacteriol. 186 (2004) 7243–7253, https://doi.org/10.1128/JB.186.21.7243-7253.2004.
[11] C. Trakunjae, A. Boondaeng, W. Apiwatanapiwat, A. Kosugi, T. Arai, K. Sudesh, P. Vaithanomsat, Enhanced polyhydroxybutyrate (PHB) production by newly isolated rare Actinomycetes rhodococcus sp. Strain BSRT1-1 using response surface methodology, Sci. Rep. 11 (2021) 1896, https://doi.org/10.1038/s41598-021-81386-2.
[12] K. Shahzad, I.M.I. Ismail, N. Ali, M.I. Rashid, A.S.A. Summan, M.R. Kabli, M. Narodoslawsky, M. Koller, LCA, sustainability and techno-economic studies for PHA production, in: Martin Koller (Ed.), The Handbook of Polyhydroxyalkanoates: Kinetics, Bioengineering, and Industrial Aspects, 1st ed., CRC Press, 2020, pp. 455–486.
[13] R. Sirohi, J. Prakash Pandey, V. Kumar Gaur, E. Gnansounou, R. Sindhu, Critical overview of biomass feedstocks as sustainable substrates for the production of polyhydroxybutyrate (PHB), Bioresour. Technol. 311 (2020), 123536, https://doi.org/10.1016/j.biortech.2020.123536.
[14] A.K. Singh, L. Sharma, N. Mallick, J. Mala, Progress and challenges in producing polyhydroxyalkanoate biopolymers from cyanobacteria, J. Appl. Phycol. 29 (2017) 1213–1232, https://doi.org/10.1007/s10811-016-1006-1.
[15] B. Drosg, I. Fritz, F. Gattermayr, L. Silvestrini, Photo-autotrophic production of poly(hydroxyalkanoates) in cyanobacteria, Chem. Biochem. Eng. Q. 29 (2015) 145–156, https://doi.org/10.15255/CABEQ.2014.2254.
[16] A. Kaewbai-ngam, A. Incharoensakdi, T. Monshupanee, Increased accumulation of polyhydroxybutyrate in divergent cyanobacteria under nutrient-deprived photoautotrophy: an efficient conversion of solar energy and carbon dioxide to polyhydroxybutyrate by Calothrix scytonemicola TISTR 8095, Bioresour. Technol. 212 (2016) 342–347, https://doi.org/10.1016/j.biortech.2016.04.035.
[17] B. Panda, N. Mallick, Enhanced poly-β-hydroxybutyrate accumulation in a unicellular cyanobacterium, Synechocystis sp. PCC 6803, Lett. Appl. Microbiol. 44 (2007) 194–198, https://doi.org/10.1111/j.1472-765X.2006.02048.x.
[18] B. Panda, L. Sharma, N. Mallick, Poly-β-hydroxybutyrate accumulation in Nostoc muscorum and Spirulina platensis under phosphate limitation, J. Plant Physiol. 162 (2005) 1376–1379, https://doi.org/10.1016/j.jplph.2005.05.002.
[19] C.A. Klausmeier, E. Litchman, T. Daufresne, S.A. Levin, Optimal nitrogen-to-phosphorus stoichiometry of phytoplankton, Nature 429 (2004) 171–174, https://doi.org/10.1038/nature02454.
[20] F.G. Acién, J.M. Fernández, J.J. Magán, E. Molina, Production cost of a real microalgae production plant and strategies to reduce it, Biotechnol. Adv. 30 (2012) 1344–1353, https://doi.org/10.1016/j.biotechadv.2012.02.005.
[21] D.M. Arias, J. García, E. Uggetti, Production of polymers by cyanobacteria grown in wastewater: current status, challenges and future perspectives, N.Biotechnol. 55 (2020) 46–57, https://doi.org/10.1016/j.nbt.2019.09.001.
[22] S. Egloff, F. Tschudi, Z. Schmautz, D. Refardt, High-density cultivation of microalgae continuously fed with unfiltered water from a recirculating aquaculture system, Algal Res. 34 (2018) 68–74, https://doi.org/10.1016/j.algal.2018.07.004.
[23] A. Pulgarin, A.G. Kapeller, M. Tarik, S. Egloff, M. Mariotto, C. Ludwig, D. Refardt, Cultivation of microalgae at high-density with pretreated liquid digestate as a nitrogen source: fate of nitrogen and improvements on growth limitations, J. Clean. Prod. 324 (2021), 129238, https://doi.org/10.1016/j.jclepro.2021.129238.
[24] M. Kaltschmitt, H. Hartmann, H. Hofbauer (Eds.), Energie aus Biomasse: Grundlagen, Techniken und Verfahren, 3rd ed., Springer, 2016.
[25] J. Zhou, Y. Wu, J. Pan, Y. Zhang, Z. Liu, H. Lu, N. Duan, Pretreatment of pig manure liquid digestate for microalgae cultivation via innovative flocculation-biological contact oxidation approach, Sci. Total Environ. 694 (2019), 133720, https://doi.org/10.1016/j.scitotenv.2019.133720.
[26] K. Meixner, I. Fritz, C. Daffert, K. Markl, W. Fuchs, B. Drosg, Processing recommendations for using low-solids digestate as nutrient solution for poly-ß-hydroxybutyrate production with Synechocystis Salina, J. Biotechnol. 240 (2016) 61–67, https://doi.org/10.1016/j.jbiotec.2016.10.023.
[27] Z. Guo, Y. Liu, H. Guo, S. Yan, J. Mu, Microalgae cultivation using an aquaculture wastewater as growth medium for biomass and biofuel production, J. Environ. Sci. 25 (2013) S85–S88, https://doi.org/10.1016/S1001-0742(14)60632-X.
[28] J.M. Ebeling, M.B. Timmons, Recirculating aquaculture systems, in: James H. Tidwell (Ed.), Aquaculture Production Systems, Wiley, 2012, pp. 245–277, https://doi.org/10.1002/9781118250105.ch11.
[29] G.-Q. Chen, A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry, Chem. Soc. Rev. 38 (2009) 2434–2446, https://doi.org/10.1039/B812677C.
[30] G. Zuccaro, A. Yousuf, A. Pollio, J.-P. Steyer, Microalgae cultivation systems, in: A. Yousuf (Ed.), Microalgae Cultivation for Biofuels Production, Academic Press, 2020, pp. 11–29, https://doi.org/10.1016/B978-0-12-817536-1.00002-3.
[31] P.R. Yashavanth, D. Meenakshi, K.M. Soumen, Recent progress and challenges in cyanobacterial autotrophic production of polyhydroxybutyrate (PHB), a bioplastic, J. Environ. Chem. Eng. 9 (2021), 105379, https://doi.org/10.1016/j.jece.2021.105379.
[32] C. Troschl, K. Meixner, I. Fritz, K. Leitner, A.P. Romero, A. Kovalcik, P. Sedlacek, B. Drosg, Pilot-scale production of poly-β-hydroxybutyrate with the cyanobacterium Synechocystis sp. CCALA192 in a non-sterile tubular photobioreactor, Algal Res. 34 (2018) 116–125, https://doi.org/10.1016/j.algal.2018.07.011.
[33] D. Kamravamanesh, D. Kiesenhofer, S. Fluch, M. Lackner, C. Herwig, Scale-up challenges and requirement of technology-transfer for cyanobacterial poly (3-hydroxybutyrate) production in industrial scale, Int. J. Biobased Plast. 1 (2019) 60–71, https://doi.org/10.1080/24759651.2019.1688604.
[34] E. Rueda, M.J. García-Galán, A. Ortiz, E. Uggetti, J. Carretero, J. García, R. Díez-Montero, Bioremediation of agricultural runoff and biopolymers production from cyanobacteria cultured in demonstrative full-scale photobioreactors, Process Saf. Environ. Prot. 139 (2020) 241–250, https://doi.org/10.1016/j.psep.2020.03.035.
[35] S. Panuschka, B. Drosg, M. Ellersdorfer, K. Meixner, I. Fritz, Photoautotrophic production of poly-hydroxybutyrate – first detailed cost estimations, Algal Res. 41 (2019), 101558, https://doi.org/10.1016/j.algal.2019.101558.
[36] J. Doucha, K. Lívanský, High density outdoor microalgal culture, in: R. Bajpai, A. Prokop, M. Zappi (Eds.), Algal Biorefineries, Springer, 2014, pp. 147–173, https://doi.org/10.1007/978-94-007-7494-0_6.
[37] R. Staub, Ernährungsphysiologisch-autökologische Untersuchungen an der planktischen Blaualge Oscillatoria rubescens DC, Schweiz. Z. Hydrol. 23 (1961) 82–198, https://doi.org/10.1007/BF02505618.
[38] D.B. Karr, J.K. Waters, D.W. Emerich, Analysis of poly-β-hydroxybutyrate in rhizobium japonicum bacteroids by ion-exclusion high-pressure liquid chromatography and UV detection, Appl. Environ. Microbiol. 46 (1983) 1339–1344, https://doi.org/10.1128/aem.46.6.1339-1344.1983.
[39] U. Nübel, F. Garcia-Pichel, G. Muyzer, PCR primers to amplify 16S rRNA genes from cyanobacteria, Appl. Environ. Microbiol. 63 (1997) 3327–3332.
[40] J.C. Sager, J.C. McFarlane, Radiation, in: R.W. Langhans, T.W. Tibbitts (Eds.), Plant Growth Chamber Handbook, Iowa State University, 1997, pp. 1–29.
[41] Y. Tejido-Nuñez, E. Aymerich, L. Sancho, D. Refardt, Treatment of aquaculture effluent with Chlorella vulgaris and Tetradesmus obliquus: the effect of pretreatment on microalgae growth and nutrient removal efficiency, Ecol. Eng. 136 (2019) 1–9, https://doi.org/10.1016/j.ecoleng.2019.05.021.

[42] Y. Tejido-Nuñez, E. Aymerich, L. Sancho, D. Refardt, Co-cultivation of microalgae in aquaculture water: interactions, growth and nutrient removal efficiency at laboratory- and pilot-scale, Algal Res. 49 (2020), 101940, https://doi.org/10.1016/j.algal.2020.101940.
[43] C.S. Reynolds, The Ecology of Phytoplankton, Cambridge University Press, 2006.
[44] Q. Lin, J. Lin, Effects of nitrogen source and concentration on biomass and oil production of a Scenedesmus rubescens like microalga, Bioresour. Technol. 102 (2011) 1615–1621, https://doi.org/10.1016/j.biortech.2010.09.008.
[45] C.J. Molloy, P.J. Syrett, Interrelationships between uptake of urea and uptake of ammonium by microalgae, J. Exp. Mar. Biol. Ecol. 118 (1988) 85–95, https://doi.org/10.1016/0022-0981(88)90232-8.
[46] S. Rossi, R. Díez-Montero, E. Rueda, F. Castillo Cascino, K. Parati, J. García, E. Ficara, Free ammonia inhibition in microalgae and cyanobacteria grown in wastewaters: photo-respirometric evaluation and modelling, Bioresour. Technol. 305 (2020), 123046, https://doi.org/10.1016/j.biortech.2020.123046.
[47] S. Nayak, R. Prasanna, Soil pH and its role in cyanobacteria abundance in rice field soils, Appl. Ecol. Env. Res. 5 (2007) 103–113, https://doi.org/10.15666/aeer/0502_103113.
[48] J. García, R. Mujeriego, M. Hernández-Mariné, High rate algal pond operating strategies for urban wastewater nitrogen removal, J. Appl. Phycol. 12 (2000) 331–339, https://doi.org/10.1023/A:1008146421368.
[49] B. Guieysse, M. Plouviez, M. Coilhac, L. Cazali, Nitrous oxide (N2O) production in axenic Chlorella vulgaris microalgae cultures: evidence, putative pathways, and potential environmental impacts, Biogeosciences 10 (2013) 6737–6746, https://doi.org/10.5194/bg-10-6737-2013.
[50] M.P. Mezzari, M.L.B. da Silva, R.S. Nicoloso, A.M.G. Ibelli, M. Bortoli, A. Viancelli, H.M. Soares, Assessment of N2O emission from a photobioreactor treating ammonia-rich swine wastewater digestate, Bioresour. Technol. 149 (2013) 327–332, https://doi.org/10.1016/j.biortech.2013.09.065.
[51] P.J. Weathers, J.J. Niedzielski, Nitrous oxide production by cyanobacteria, Arch. Microbiol. 146 (1986) 204–206, https://doi.org/10.1007/BF00402352.
[52] M. Plouviez, A. Shilton, M.A. Packer, B. Guieysse, N2O emissions during microalgae outdoor cultivation in 50 L column photobioreactors, Algal Res. 26 (2017) 348–353, https://doi.org/10.1016/j.algal.2017.08.008.
[53] M. Plouviez, A. Shilton, M.A. Packer, B. Guieysse, Nitrous oxide emissions from microalgae: potential pathways and significance, J. Appl. Phycol. 31 (2019) 1–8, https://doi.org/10.1007/s10811-018-1531-1.
[54] M. Plouviez, B. Guieysse, Nitrous oxide emissions during microalgae-based wastewater treatment: current state of the art and implication for greenhouse gases budgeting, Water Sci. Technol. 82 (2020) 1025–1030, https://doi.org/10.2166/wst.2020.304.
[55] D. Ehhalt, M. Prather, F. Dentener, R. Derwent, E. Dlugokencky, E. Holland, I. Isaksen, J. Katima, V. Kirchhoff, P. Matson, P. Midgley, M. Wang, Atmospheric chemistry and greenhouse gases, in: Climate Change 2001: The Scientific Basis, Cambridge University Press, 2001, pp. 239–288.
[56] J.R. Dolan, Phosphorus and ammonia excretion by planktonic protists, Mar. Geol. 139 (1997) 109–122, https://doi.org/10.1016/S0025-3227(96)00106-5.
[57] K. Emerson, R.C. Russo, R.E. Lund, R.V. Thurston, Aqueous ammonia equilibrium calculations: effect of pH and temperature, J. Fish. Res. Bd. Can. 32 (1975) 2379–2383, https://doi.org/10.1139/f75-274.
[58] S.N. Behera, M. Sharma, V.P. Aneja, R. Balasubramanian, Ammonia in the atmosphere: a review on emission sources, atmospheric chemistry and deposition on terrestrial bodies, Environ. Sci. Pollut. Res. 20 (2013) 8092–8131, https://doi.org/10.1007/s11356-013-2051-9.
[59] S. Bittman, J.R. Brook, A. Bleeker, T.W. Bruulsema, Air quality, health effects and management of ammonia emissions from fertilizers, in: E. Taylor, A. McMillan (Eds.), Air Quality Management: Canadian Perspectives on a Global Issue, Springer, 2014, pp. 261–277, https://doi.org/10.1007/978-94-007-7557-2_12.
[60] R.P. Padappayil, J. Borger, Ammonia toxicity, in: StatPearls, StatPearls Publishing, 2021 (accessed December 14, 2021), http://www.ncbi.nlm.nih.gov/books/NBK546677/.
[61] S. Ansari, T. Fatma, Cyanobacterial polyhydroxybutyrate (PHB): screening, optimization and characterization, PLoS One. 11 (2016), e0158168, https://doi.org/10.1371/journal.pone.0158168.
[62] D. Kamravamanesh, S. Pflügl, W. Nischkauer, A. Limbeck, M. Lackner, C. Herwig, Photosynthetic poly-β-hydroxybutyrate accumulation in unicellular cyanobacterium Synechocystis sp. PCC 6714, AMB Express. 7 (2017) 143, https://doi.org/10.1186/s13568-017-0443-9.
[63] T. Monshupanee, A. Incharoensakdi, Enhanced accumulation of glycogen, lipids and polyhydroxybutyrate under optimal nutrients and light intensities in the cyanobacterium Synechocystis sp, J. Appl. Microbiol. 116 (2014) 830–838, https://doi.org/10.1111/jam.12409. PCC 6803.
[64] C.U. Ugwu, H. Aoyagi, H. Uchiyama, Photobioreactors for mass cultivation of algae, Bioresour. Technol. 99 (2008) 4021–4028, https://doi.org/10.1016/j.biortech.2007.01.046.
[65] S. Kamali, V.C.A. Ward, L. Ricardez-Sandoval, Dynamic modeling of recirculating aquaculture systems: effect of management strategies and water quality parameters on fish performance, Aquac. Eng. 99 (2022), 102294, https://doi.org/10.1016/j.aquaeng.2022.102294.
[66] S. Lan, L. Wu, D. Zhang, C. Hu, Effects of light and temperature on open cultivation of desert cyanobacterium Microcoleus vaginatus, Bioresour. Technol. 182 (2015) 144–150, https://doi.org/10.1016/j.biortech.2015.02.002.
[67] P.R. Mooij, G.R. Stouten, M.C. van Loosdrecht, R. Kleerebezem, Ecology-based selective environments as solution to contamination in microalgal cultivation, Curr. Opin. Biotechnol. 33 (2015) 46–51, https://doi.org/10.1016/j.copbio.2014.11.001.
[68] W. Duan, F. Meng, H. Cui, Y. Lin, G. Wang, J. Wu, Ecotoxicity of phenol and cresols to aquatic organisms: a review, Ecotoxicol. Environ. Saf. 157 (2018) 441–456, https://doi.org/10.1016/j.ecoenv.2018.03.089.
[69] H. Wang, W. Zhang, L. Chen, J. Wang, T. Liu, The contamination and control of biological pollutants in mass cultivation of microalgae, Bioresour. Technol. 128 (2013) 745–750, https://doi.org/10.1016/j.biortech.2012.10.158.