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Cultivation of *Arthrospira platensis* in Brewery Wastewater

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Abstract: Cultivation of photosynthetic microorganisms in wastewater is a potential cost-effective method of treating wastewater and simultaneously providing the essential nutrients for high-value biomass production. This study investigates the cultivation of the cyanobacterium *Arthrospira platensis* in non-diluted and non-pretreated brewery wastewater under non-sterile and alkaline growth conditions. The system's performance in terms of biomass productivity, pollutant consumption, pigment production and biomass composition was evaluated under different media formulations (i.e., addition of sodium chloride and/or bicarbonate) and different irradiation conditions (i.e., continuous illumination and 16:8 light:dark photoperiod). It was observed that the combination of sodium bicarbonate with sodium chloride resulted in maximum pigment production recorded at the end of the experiments, and the use of the photoperiod led to increased pollutant removal (up to 90% of initial concentrations) and biomass concentration (950 mg/L). The composition of the microbial communities established during the experiments was also determined. It was observed that heterotrophic bacteria dominated by the phyla of Pseudomonadota, Bacillota, and Bacteroidota prevailed, while the cyanobacteria population showcased a dynamic behavior throughout the experiments, as it increased towards the end of cultivation (relative abundance of 10% and 30% under continuous illumination and photoperiod application, respectively). Overall, *Arthrospira platensis*-based cultivation proved to be an effective method of brewery wastewater treatment, although the large numbers of heterotrophic bacteria limit the usage of the produced biomass to applications such as biofuel and biofertilizer production.

Keywords: *Spirulina platensis*; brewery wastewater; biotreatment; prokaryotic community composition; biomass characterization

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1. Introduction

The photosynthetic cyanobacterium *Arthrospira platensis*, known as *Spirulina*, is widely recognized as a producer of active biological compounds, such as proteins, carbohydrates, polyunsaturated fatty acids, vitamins, and pigments (phycocyanin, chlorophyll-a and carotenoids), for food, feed, cosmetic, medical and agricultural applications [1–3]. However, the concentration and composition of its biomass is strongly regulated by the composition of the medium used for its cultivation. For example, a medium supplemented with excess nitrogen concentration can lead to high biomass protein content of up to 70% on a dry weight basis [4], while *Arthrospira* accumulates more carbohydrates (up to 60%) when

cultivated under phosphorus-limited conditions [5]. *Arthrospira* is currently cultivated at commercial scale and synthetic media are usually used for its growth. Additionally, selective bioprocess conditions (e.g., alkaline conditions, high salt concentrations, high pH value) are applied to favor *Arthrospira* growth and minimize bacterial contaminations [1]. Nevertheless, heterotrophic bacteria belonging to the phyla of Pseudomonadota, Bacteroidota, Bacillota, Actinomycetota, and Verrucomicrobia are usually detected in *Arthrospira* cultures [6,7] given that maintaining a monoculture in large-scale heterotrophic or mixotrophic cultivation systems when non-sterile conditions prevail constitutes a difficult endeavor.

Given that *Arthrospira* needs nutrient sources to grow, wastewaters containing adequate amounts of essential nutrients can be exploited as a sustainable feedstock for reduced-cost biomass production according to circular economy principles [1,4]. *A. platensis* has been previously investigated to bioremediate various types of effluents such as olive mill [8], fish farming [9], piggery [10], industrial (obtained by anaerobic digestion) [11,12], winery [13,14], and dairy [15] wastewater studying simultaneously the production of various active biological compounds. In the above studies, synthetic growth media were partly replaced with wastewater and/or wastewater was additionally treated prior cyanobacterial cultivation to reduce either its strong color or the toxic effects of high pollutant concentrations on *A. platensis* growth [8,10]. Although *A. platensis* has been successfully applied to remove nutrients from different wastewater types, limited information is currently available on the interactions of *A. platensis* with bacteria during wastewater treatment given the difficulty in maintaining the monoculture of the strain in the aforementioned processes due to high concentration of organic substrates [16].

In the present study brewery wastewater was used as a complete growth medium for *A. platensis* cultivation without dilution or pretreatment. Previous studies have confirmed that brewery wastewater can be efficiently utilized for cyanobacteria-based microbial consortia [16–18] and microalgae cultivation [19–21] due to the sufficient organic compounds (2000–6000 mg/L of chemical oxygen demand, COD), total nitrogen and phosphorous concentrations (25–80 and 10–15 mg/L, respectively) [16,17]. To the best of the authors' knowledge, this is the first study conducted on *A. platensis* cultivation using brewery wastewater as a sole growth medium. The aim of this study was to investigate the effects of brewery wastewater on biomass production and the biochemical composition of *A. platensis* under non-sterile and alkaline growth conditions. The addition of sodium chloride and/or sodium bicarbonate in combination with a high pH value was evaluated to enhance *A. platensis* growth, and based on the optimal results the effect of the photoperiod was also examined. Furthermore, the composition of the prokaryotic community was determined in the optimum conditions of biomass production.

2. Materials and Methods

2.1. Brewery Wastewater

Brewery wastewater was collected from the inlet of a local brewery's wastewater treatment plant [16]. The wastewater was filtered through 0.45 μm pore membrane filters to remove suspended solids and the filtrate was used as the growth medium for the microbial culture. After filtration, the wastewater was stored at −20 °C until use. The main physicochemical characteristics of the wastewater are presented in Table 1.

2.2. Microorganism and Culture Conditions of the Inoculum

The strain of *A. platensis* SAG 21.99 used in these experiments was obtained from the collection of the University of Göttingen (Sammlung von Algenkulturen der Universität Göttingen, Germany). The inoculum was produced by culturing *A. platensis* in diluted brewery wastewater (dissolved chemical oxygen demand: 180.14 ± 10.21 mg/L; nitrate: 12.04 ± 0.51 mg/L; ammonium: 5.08 ± 0.22 mg/L; orthophosphate: 6.32 ± 0.33 mg/L) supplemented with Zarrouk medium (containing (in g/L) $NaHCO_3$, 16.8; $NaNO_3$, 2.5; K_2SO_4, 1; NaCl, 1; K_2HPO_4, 0.5; $CaCl_2$, 0.04; Na_2EDTA, 0.08; $MgSO_4 \cdot 7H_2O$, 0.2; and $FeSO_4 \cdot 7H_2O$, 0.01) and 1.0 mL of trace elements (containing (in g/L): H_3BO_3, 2.86; $(NH_4)_6Mo_7O_{24}$, 0.02;

$MnCl_2 \cdot 4H_2O$, 1.8; Cu_2SO_4, 0.08; and $ZnSO_4 \cdot 7H_2O$, 0.22) [22]. Note that diluted brewery wastewater was used only in the inoculum. All experiments were conducted using non-diluted and non-pretreated wastewater. The inoculum cultures were incubated at 26 ± 1 °C under alkaline conditions (pH = 10 ± 0.5) and continuous magnetic stirring, without mechanical air supply and with continuous illumination from two cool white lamps with an average light intensity of 3000 lux (or 41 μmol/m^2/s).

Table 1. Physicochemical characteristics of the brewery wastewater used in the experiments. Data are presented as mean ± SD values from three separate measurements.

Physicochemical Characteristics	Value
d-COD (mg/L)	1670 ± 30.16
NO_3^--N (mg/L)	31.19 ± 1.25
NO_2^--N (mg/L)	ND [1]
NH_4^+-N (mg/L)	6.15 ± 0.23
PO_4^{3-}-P (mg/L)	9.46 ± 0.35
TKN (mg/L)	60.00 ± 3.85
pH	8.10 ± 0.20

[1] ND: not detected.

2.3. Experimental Design

The first sets of experiments were performed to examine *A. platensis* cultivation in four different brewery wastewater-based media: (1) untreated brewery wastewater, (2) brewery wastewater with 1 g/L NaCl, (3) brewery wastewater with 5 g/L $NaHCO_3$, and (4) brewery wastewater with 1 g/L NaCl and 5 g/L $NaHCO_3$. The cultures were incubated under the same conditions used for the inoculum cultures (3000 lux, continuous magnetic stirring, and without mechanical air supply) and the optimum medium was identified. The second set of experiments was performed based on the optimum brewery wastewater-based medium with which the highest pollutants removal, biomass, and pigment concentrations were achieved. In this set of experiments, the effect of the photoperiod (16:8 h light:dark) was studied to investigate the influence of different luminance conditions (i.e., continuous illumination and photoperiod) on pollutant removal in relation to biomass production.

In all experiments, the brewery wastewater-based media were subjected to a high-temperature short-time pasteurization procedure at 72 °C for 15 s to reduce the wastewater's indigenous bacterial population following Papadopoulos et al. [16] as *A. platensis* did not grow when the brewery wastewater was not pasteurized. All cultures included a working volume of 1 L and were inoculated with the inoculum of 20% v/v corresponding to the initial biomass concentration of 80.16 ± 2.12 mg/L. The initial pH value of the experiments was adjusted to 10 ± 0.2 using 5N NaOH solution and was kept constant during cultivation by using 5N NaOH or 5N H_2SO_4 solutions, while temperature was maintained at 26 ± 1 °C. All experiments were conducted in duplicate under non-aseptic conditions, continuous magnetic stirring, and without mechanical air supply.

2.4. DNA Extraction and Next-Generation Sequencing

The composition of the microbial communities established during the experiments was determined using rRNA sequencing. DNA extraction was conducted using 0.25 g of samples withdrawn from: (a) inoculum, (b) brewery wastewater before pasteurization, (c) brewery wastewater following pasteurization, (d) mid-exponential growth phase in experiments that included the highest pigment concentrations (11th day), and (e) the stationary phase in experiments performing the highest pigment contents (21st day). Biomedium samples were centrifuged for 15 min at 3500 rpm and the supernatant solution was removed. The cell pellet was subject to total genomic DNA extraction performed using a DNeasy PowerSoil Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions and the genome was sequenced by DNASense Apps Company (Aalborg, Denmark). Samples were processed using 16S rRNA gene amplicon sequencing targeting the bacterial

and archaeal variable region V4. DNA extraction and sequencing were successful for all samples yielding between 9636–104,081 reads following QC and bioinformatic processing.

2.5. Analytical Methods

The filtered brewery wastewater was analyzed for concentrations of dissolved chemical oxygen demand (d-COD), nitrate (NO_3^--N), nitrite (NO_2^--N), ammonium (NH_4^+-N), orthophosphate (PO_4^{3-}-P), total Kjeldahl nitrogen (TKN), and pH, according to Standard Methods [23]. The pH values were measured using a HANNA HI 5521 multiparameter instrument.

Total biomass concentrations (as dry weight), carbohydrate and lipid contents were determined as previously described by Papadopoulos et al. [16]. Protein concentration was determined following Markou et al. [22]. Briefly, 0.2 mg of dry biomass was suspended in 1.5 mL of 0.5 N NaOH and incubated in an agitated heating plate for 1 h. The resulting solution was centrifuged and the protein concentration was determined according to the Lowry assay [24] using bovine serum as a standard [22].

Chlorophyll was extracted from the wet biomass as previously described in Papadopoulos et al. [16]. Briefly, 5 mL of culture was centrifuged and subsequently washed twice with cold distilled water. The chlorophyll was extracted using 5 mL of 80% (*v*/*v*) acetone. The same procedure was also followed to determine the carotenoid content. Chlorophyll-a and carotenoid concentrations were calculated spectrophotometrically using the equations of Lichtenthaler and Buschmann [25]. Phycocyanin content was estimated as follows: 5 mL of fresh culture was centrifuged and then washed twice with cold distilled water. The concentrated biomass was re-suspended in 4 mL HCl 12 N [26] and the mixture was allowed to stand in a dark place for 24 h. The phycocyanin concentration and yield were calculated by measuring the optical densities at 652 and 620 nm using the equations proposed by Moraes et al. [26].

3. Results and Discussion

3.1. Optimization of A. platensis Cultivation Using Brewery Wastewater

Brewery wastewater characterized by high organic load and essential nutrients (NO_3^--N and PO_4^{3-}-P) in sufficient amounts to support *A. platensis* growth, was applied as an alternative substrate to reduce the cost of cyanobacterial biomass production. Since all experiments were conducted under non-aseptic conditions, several parameters that favor *A. platensis* growth were examined such as high pH value and the addition of $NaHCO_3$ and/or NaCl. In general, alkaline pH values of 9.5–10.0 are considered optimum for *A. platensis* growth and ideal for pilot-scale cultivation [27,28] as they result in reduced bacterial contaminations [29]. Under high pH conditions, alkaliphilic cyanobacteria such as *A. platensis* can photosynthesize more efficiently due to elevated levels of dissolved inorganic carbon (DIC) whereas bicarbonate is the preferable carbon source, which is actively transported into the cells to be converted to CO_2 [30]. Furthermore, the addition of $NaHCO_3$ to the culture medium is recommended to ensure the growth of photosynthetic microorganisms [31]. Zarrouk medium contains 16.8 g/L of $NaHCO_3$; however, it was demonstrated that the addition of lower quantities (e.g., 5 g/L) in a medium containing high organic load positively influences the growth of *A. platensis* [8]. Zarrouk medium also contains 1 g/L of NaCl that affects the osmotic pressure of the medium, while lower salinity can impose an inhibitory effect on *A. platensis* growth [12]. In the present study, 1 g/L NaCl and/or 5 g/L $NaHCO_3$ was added into the brewery wastewater. Moreover, the initial pH value was adjusted to 10 ± 0.2 and ranged from 9.7 ± 0.2 to 10.3 ± 0.2 during the twenty-one-day *A. platensis* cultivation period.

Figure 1a,b show the consumption of pollutants (d-COD, NO_3^--N and PO_4^{3-}-P) as well as biomass growth (Figure 1c) for the four different brewery wastewater-based media examined (untreated brewery wastewater, brewery wastewater with NaCl addition, brewery wastewater with $NaHCO_3$ addition and brewery wastewater with NaCl and $NaHCO_3$ addition). According to Figure 1a,b similar pollutants consumption was observed during cultivation, and the consumption followed a similar pattern in all cases. Statistically

significant differences between data were evaluated using the t-student confidence interval, for 95% probability, for all pollutants and wastewater-based media examined. Wastewater-based media was found to have no significant effects on pollutants removal for all the paired data. The removal percentages of d-COD and NO_3^--N were over 90% and 70.5% for initial concentrations of 1660 ± 28.28 mg/L and 33.67 ± 1.91 mg/L, respectively. These results are slightly lower compared with those reported by Papadopoulos et al. [16] (over 95% and 80%, respectively) using a cyanobacterial-bacterial consortium dominated by the cyanobacterium *Leptolyngbya* sp., for the treatment in batch operation of brewery wastewater containing 2270 mg/L of initial d-COD and 30 mg/L of initial NO_3^--N concentrations. An efficient removal of initial COD and NO_3^--N concentrations ranging between 2200 and 11,000 mg/L and 1.85–9.25 mg/L, with values up to 40 and 50%, respectively, was also demonstrated in mixotrophic cultures of *A. platensis* in olive mill wastewater diluted and pre-treated with sodium hypochlorite [8]. Additionally, Spennati et al. [13] reported up to 90% COD reduction for an initial concentration of about 23,000 mg/L after co-cultivation of *A. platensis* with Chlorella vulgaris in diluted winery wastewater. Concerning the consumption of PO_4^{3-}-P, the removal efficiencies were low and ranged from 19.7 to 28.3% (Figure 1b), given that elevated pH values (up to 9) cause phosphorous precipitation reducing its availability to the microbial consortium [32]. PO_4^{3-}-P concentration increased slightly after day 14 probably due to enzymatic hydrolysis of organic forms of phosphorus to PO_4^{3-}-P [33]. The removal of NH_4^+-N (over 55% of the initial 5.17 ± 0.25 mg/L in all cases) can also be attributed to the high pH values as they can affect the ammonia stripping mechanism in wastewaters [32]. TKN removal was over 74% (of the initial value of 55.00 ± 4.31 mg/L) and the final concentrations of NO_2^--N ranged from 0 to 0.10 mg/L and did not exceed 1.30 ± 0.06 mg/L during the experiments (data not shown).

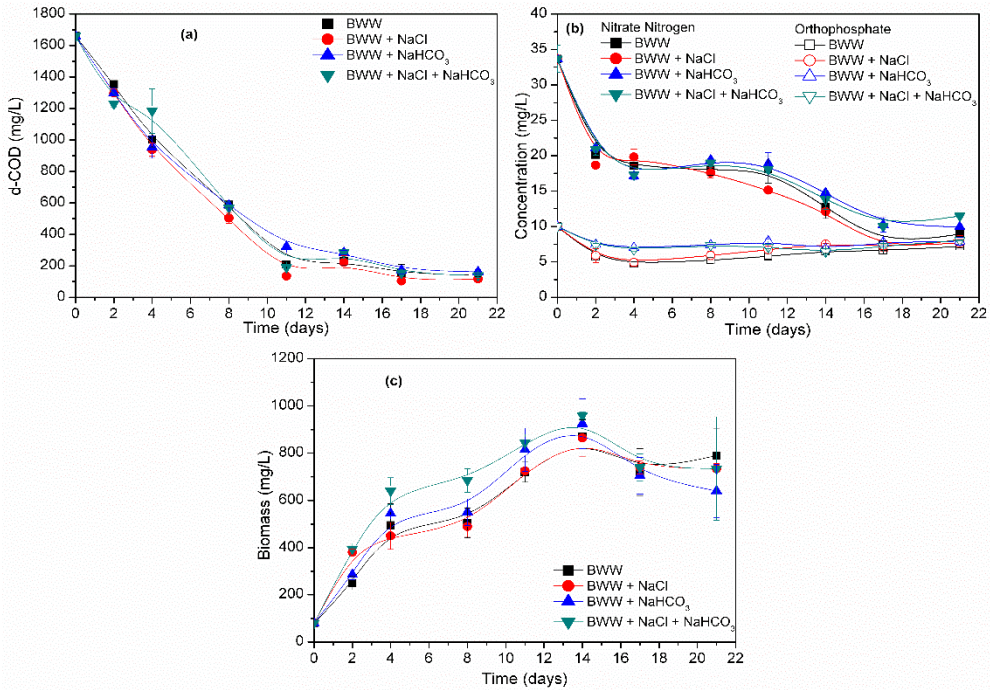

Figure 1. (**a**) d-COD consumption, (**b**) NO_3^--N and PO_4^{3-}-P consumption, and (**c**) growth of total biomass during *A. platensis* cultivation under continuous illumination in four different brewery wastewater-based media. Data present mean values of duplicate experiments ± standard deviations.

Total biomass concentration of *A. platensis* and bacterial populations (see also Section 3.3) reached its maximum on day 14 (Figure 1c) and was then observed to decrease, probably due to the decreasing numbers of bacteria as d-COD was depleted. Similar observations regarding bacterial dependence on d-COD concentration have also been made in previous studies. For example, Papadopoulos et al. [16] reported that bacterial populations reduced

in size after d-COD consumption in brewery wastewater, whereas cyanobacteria dominated until the end of the cultivation period achieving a maximum total biomass concentration of about 1000 mg/L. Lee et al. [34] also found that the ratio of bacteria to algae was highest at the early stage of cultivation in municipal wastewater when the concentration of soluble COD was high. Another potential reason for total biomass reduction after day 14 can be the consumption of pollutants as their concentrations were almost depleted at the specific time point. As seen in Figure 1c, the final biomass concentration ranged between 640 and 790 mg/L for all experiments performed. These concentrations were lower as compared with the contents observed for *A. platensis* cultivation in Zarrouk medium or wastewater supplemented with Zarrouk medium, where they usually exceed 1.5 g/L [15,35]. It is well-known that nitrogen concentration constitutes one of the most important factors for biomass production [36] and Zarrouk medium contains approximately 5-fold higher NO_3^--N concentration than the present study. However, the biomass concentrations observed here were similar to the contents reported for *A. platensis* cultivation in open ponds (0.5–1.0 g/L) [28]. As shown in Figure 1c, the addition of 5 g/L $NaHCO_3$ into the brewery wastewater appeared to positively influence biomass production during cultivation. Similar results were also obtained by Markou et al. [8] via addition of 5 g/L $NaHCO_3$ into olive mill wastewater achieving biomass concentration of approximately 300 mg/L.

The biomass pigment content was also examined in these experiments as it correlates positively with cyanobacterial biomass concentration [12]. Figure 2 shows the time course of chlorophyll-a, carotenoid and phycocyanin contents in the mixotrophic cultivation of *A. platensis* in different brewery wastewater-based media. The initial concentrations of chlorophyll-a, carotenoids, and phycocyanin were 6.19 ± 1.02, 1.07 ± 0.06, and 10.15 ± 1.13 mg/g dry weight (DW), respectively. As seen in Figure 2, these concentrations decreased over time, which can be attributed to the dominance of the bacterial population within the culture. After d-COD was completely consumed on day 14, the *A. platensis* population began to increase, and the concentration of pigments also increased at the end of the experiments. An exception to this trend, however, was recorded in the experiment with NaCl addition, where phycocyanin content remained almost constant throughout the experiment (Figure 2b). This wastewater-based medium affected positively the *A. platensis* population during cultivation (however the content of pigments was not the highest at the end of experiments, see below) or the level of sodium ions in this medium did not cause alterations in phycocyanin concentration [37]. The decreasing trend of phycocyanin content recorded during the experiments can be also due to the low nitrogen content of brewery wastewater as phycocyanin is utilized as a nitrogen source by *A. platensis* under nitrogen starvation conditions to sustain biomass growth [10,38]. Nitrogen concentration is also known to affect chlorophyll-a content which decreases in conditions of limitation [12]. Another possible reason for the decreasing trend of pigments content can be *A. platensis* adjusting to the different culture conditions.

Biomass pigment contents varied according to the brewery wastewater-based media composition. The addition of $NaHCO_3$ favored pigment content (Figure 2c,d), while the combination of $NaHCO_3$ with NaCl resulted in maximum pigment production recorded at the end of the experiment (Figure 2d). Specifically, in the medium containing $NaHCO_3$ with NaCl (Figure 2d), chlorophyll-a content was higher after 17 days of cultivation, thus indicating the growth of photosynthetic microorganisms [27]. The phycocyanin content was almost 2-fold higher (22.35 ± 1.34 mg/g DW) compared with that recorded in experiments presented in Figure 2a,b (without $NaHCO_3$ addition) and was higher than the wastewater-based medium containing only $NaHCO_3$ (Figure 2c). However, it was lower than the values recorded in phototrophic cultivations as mixotrophic cultivation limits the light requirements since photosynthetic cells use organic material as a source of carbon and energy [39]. In previous studies, phycocyanin concentrations recorded in *A. platensis* growing in Zarrouk medium using CO_2 as an additional inorganic carbon source ranged between 100 and 140 mg/g DW under light intensities that varied from

100 to 1300 μmol/m^2/s [40], and reached 170 mg/g DW when a combination of compressed air and CO_2 was applied [41]. The chlorophyll-a contents observed in this study were also lower (up to 4.79 ± 0.65 mg/g DW) compared with those synthesized in the *A. platensis* biomass that was cultivated in urea under similar irradiance levels (3500 lux) and ranged between 12 and 13 mg/g DW [42]. Carotenoids remained at low levels (Figure 2) thus indicating that light conditions did not cause oxidative stress [38]. Nevertheless, the results of the present study were similar to the data reported in mixotrophic *A. platensis* growth experiments [12,43]. In any case, apart from nitrogen concentrations in the culture medium, operational mode, temperature, light color and intensity, and harvesting time can also influence the pigment content in biomass [44,45].

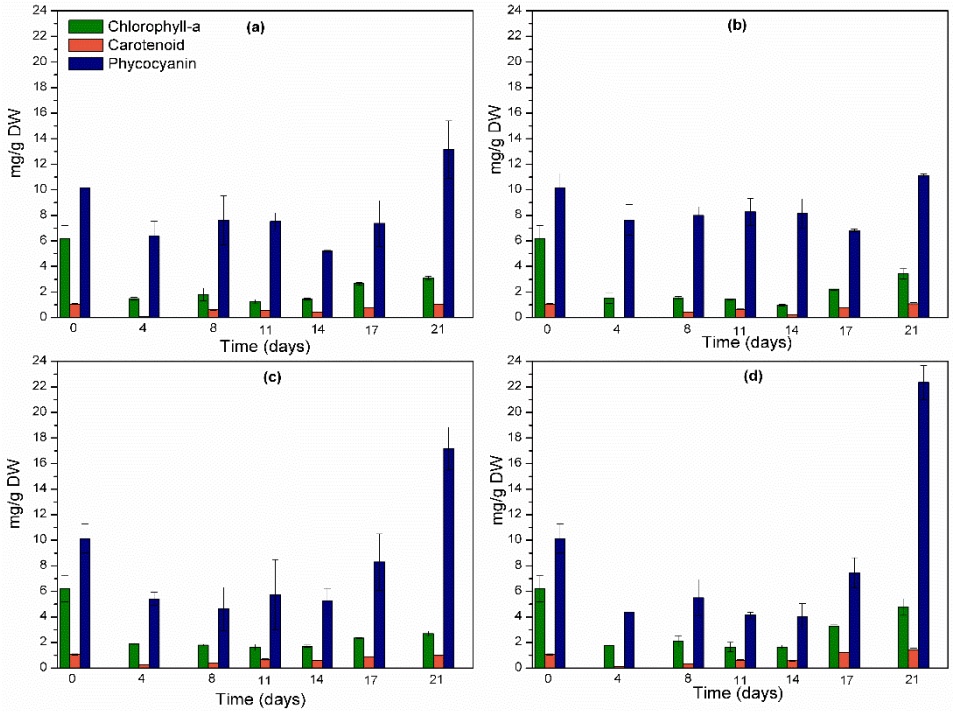

Figure 2. Pigments content into total biomass during *A. platensis* cultivation under continuous illumination in (**a**) untreated brewery wastewater, (**b**) brewery wastewater with 1 g/L NaCl, (**c**) brewery wastewater with 5 g/L $NaHCO_3$, and (**d**) brewery wastewater with 1 g/L NaCl and 5 g/L $NaHCO_3$. Data present mean values of duplicate experiments ± standard deviations.

The biochemical composition of the produced biomass was also studied to determine any effects of the different brewery wastewater-based media on *A. platensis* cultivation. The initial inoculum used contained 25.07 ± 0.84% carbohydrates, 45.13 ± 1.78% protein and 7.11 ± 0.62% lipids (Figure 3), which is in agreement with the typical chemical composition of A. platensis (15–25% carbohydrates, 55–70% proteins, and 4–7% lipids) [12,46]. The protein content remained relatively low (up to 19%) throughout the experiments, as the low nitrogen concentration in the culture media limited their synthesis [10,38]. In addition, nitrogen-limiting conditions result in increased carbohydrate or lipid contents, since cells tend to store energy in the form of these metabolites [44]. In the experiments where $NaHCO_3$ was not added to the wastewater (Figure 3a,b), carbohydrate content decreased until day 11 and an increase was observed after day 14 reaching the values of 14.83 ± 2.55% and 24.37 ± 2.23%, respectively, until the end of cultivation. On the contrary, their percentages remained constant in the experiments with $NaHCO_3$ addition (Figure 3c,d) and did not change significantly compared with the initial values obtained. Lipid contents remained low in all experiments (up to 1.74 ± 0.04%) (Figure 3a–d).

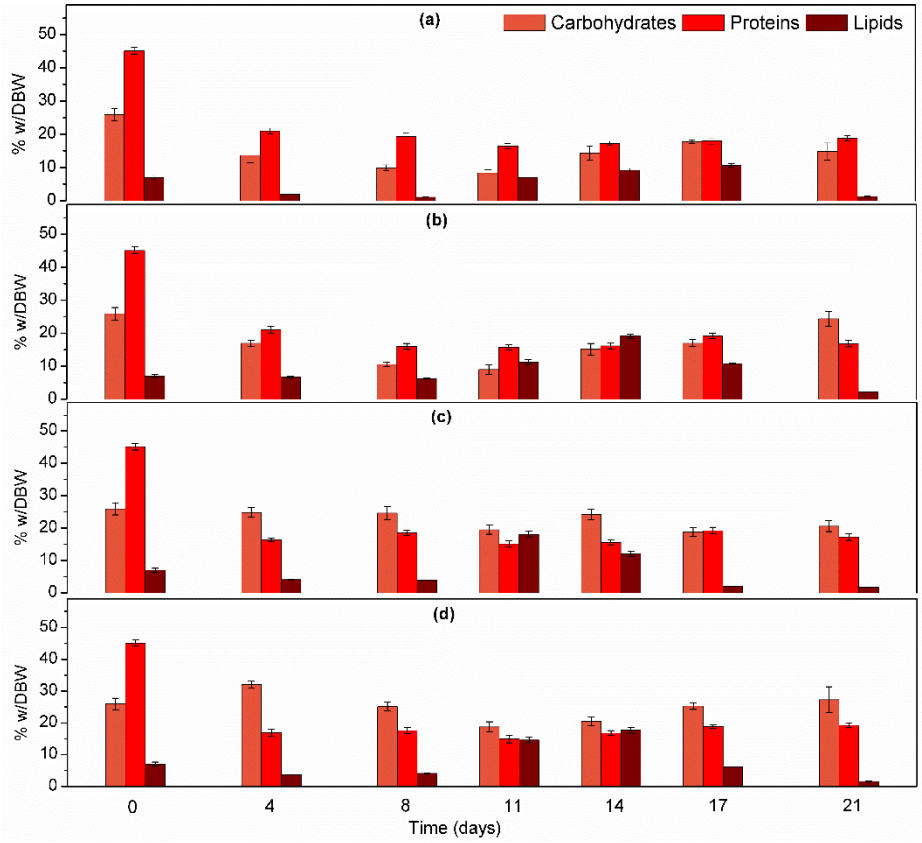

Figure 3. Carbohydrate, protein, and lipid contents in total biomass during *A. platensis* cultivation under continuous illumination in (**a**) untreated brewery wastewater, (**b**) brewery wastewater with 1 g/L NaCl, (**c**) brewery wastewater with 5 g/L $NaHCO_3$, and (**d**) brewery wastewater with 1 g/L NaCl and 5 g/L $NaHCO_3$. Data present mean values of duplicate experiments ± standard deviations.

3.2. Effect of Photoperiod on A. platensis Cultivation in Brewery Wastewater

The effect of the photoperiod (16:8 h light:dark) was evaluated to examine its influence on pollutant consumption, biomass growth, and biochemical composition of *A. platensis* in terms of proteins, carbohydrates, lipids, and pigments. Within the range of brewery wastewater-based media examined, the maximum pigment content was higher when sodium chloride and sodium bicarbonate were added (Figure 2d). Therefore, this wastewater-based medium was selected to study the effect of the photoperiod (16:8 h light:dark) on pollutants consumption and biomass production and composition. Based on the experimental data presented in Figure 4a,b, the consumption rate of pollutants in experiments applying the photoperiod was higher until day 14 compared with the consumption rate observed in continuous illumination conditions, while after this time the removal rates were almost the same for all experiments conducted. The photoperiod is considered crucial for stable, high cell density cultures of *A. platensis* [47]. This is supported by the results shown in Figure 4c, where the final biomass concentration of about 950 mg/L was higher than that recorded in the experiments conducted using the same brewery wastewater-based medium under continuous illumination.

Pigment concentrations also showed the same trend, i.e., a decrease in pigments occurred during cultivation and an increase was observed at the end of the cultivation period (Figure 5a), which can probably be attributed to the predominance of bacterial populations in the cultures (see also Section 3.3). However, the highest pigment concentration was achieved under continuous illumination conditions (Figure 2d).

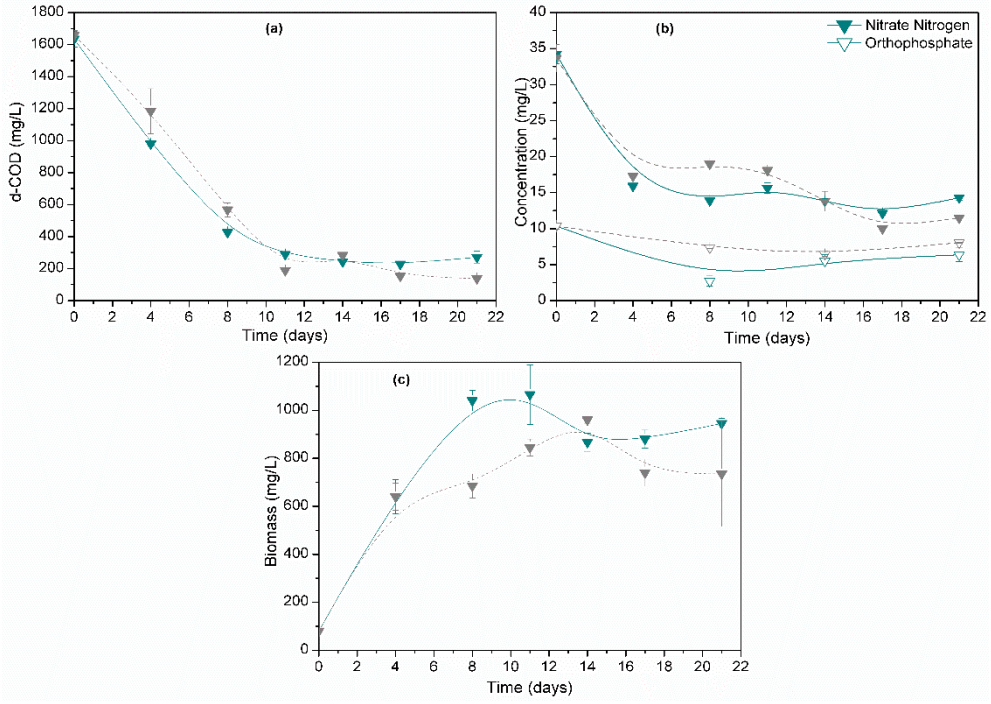

Figure 4. (**a**) d-COD consumption, (**b**) NO_3^--N and PO_4^{3-}-P consumption, and (**c**) growth of total biomass applying photoperiod (16:8 h light:dark) during *A. platensis* cultivation in brewery wastewater with 1 g/L NaCl and 5 g/L $NaHCO_3$. Data present mean values of duplicate experiments ± standard deviations. Grey symbols with dash lines represent the experiments conducted using the same brewery wastewater-based medium under continuous illumination.

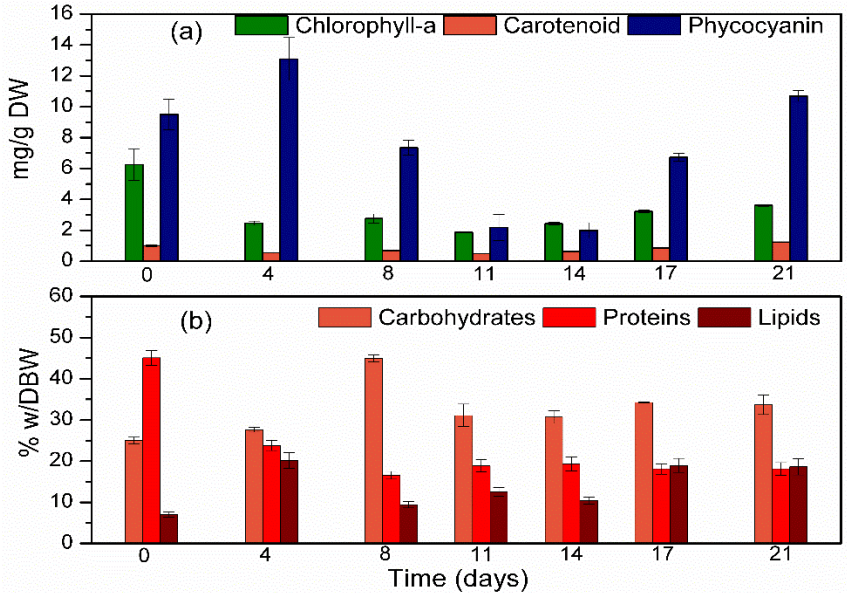

Figure 5. (**a**) Pigments content and (**b**) carbohydrate, protein and lipid contents into total biomass applying photoperiod (16:8 h light:dark) during *A. platensis* cultivation in brewery wastewater with 1 g/L NaCl and 5 g/L $NaHCO_3$. Data present mean values of duplicate experiments ± standard deviations.

In a study by Xue et al. [48], light harvesting efficiency also increased under continuous illumination and low light intensities (lower than 150 µmol/m^2/s), leading to a rise in pigments. Nevertheless, the low nitrogen concentration in the growth media inhibited higher pigment accumulation [45]. Regarding the biomass analysis, it was observed that percent-

ages of carbohydrates and lipids were higher (about 33.66 ± 2.36% and 18.64 ± 1.98%, respectively, at the end of cultivation) when the photoperiod was applied compared with continuous illumination (Figure 5b). Protein content remained low (18.14 ± 1.52% at the end of cultivation) due to the low level of nitrogen in the growth medium [12].

3.3. Prokaryotic Community Dynamics during the Process

Advanced understanding of the biological mechanisms dominant in the applied bioprocesses employed for the treatment and valorization of biowaste can be enabled via determination of the microbial profile via next generation sequencing [49]. In a natural environment, photosynthetic microorganisms grow symbiotically with bacterial species. The CO_2 generated by these bacteria is utilized as inorganic carbon source by microalgae/cyanobacteria, while oxygen, as well as extracellular polymeric substances (EPSs) released by microalgae/cyanobacteria, are used by bacteria for growth [50]. Although heterotrophic bacteria are usually isolated from *Arthrospira* cultures [51], limited information is currently available on the interactions of *Arthrospira* with bacteria during wastewater treatment.

Herein, the composition of the prokaryotic community was characterized in the inoculum, the brewery wastewater before and after pasteurization, as well as the mid- and end-stages of cultivation in experiments conducted using the highest pigment concentrations (i.e., with NaCl and $NaHCO_3$ addition under continuous illumination and photoperiod application). The bacterial community structures were determined at different taxonomic levels, including dominant phyla and classes (Figure 6). The most abundant phyla in the inoculum comprised cyanobacteria (56.2%), followed by Pseudomonadota (20%), Planctomycetes (15%), Bacteroidota (10%), and Kiritimatiellaeota (5%). Low percentages (up to 1%) of Verrucomicrobia, WPS-2, and Actinomycetota were also detected. The predominant phyla in the brewery wastewater before pasteurization were Pseudomonadota and Bacillota (Figure 6a). Classes of the dominant phyla cyanobacteria, Pseudomonadota, Bacillota, Bacteroidota, and Planctomycetes are presented in Figure 6b. The phylum cyanobacteria consisted of *Oxyphotobacteria*, while Pseudomonadota were dominated by *Gammaproteobacteria* and *Alphaproteobacteria*. In Bacillota, Bacteroidota and Planctomycetes, the classes *Bacilli*, *Bacteroidia,* and *Planctomycetacia* were dominant, respectively. It is worth noting that *A. platensis* did not grow when the brewery wastewater was not pasteurized (data not shown), thus a short pasteurization process was applied to reduce bacterial populations. As shown in Figure 6a, a 20% reduction in the population of Bacillota appeared to favor *A. platensis* growth. However, bacteria were dominant in all cultures due to the wastewater's high organic load [34] apparently affecting the overall productivity of *A. platensis*. More specifically, at the midpoint of experiments when COD levels were still high, the cyanobacterial population was low (~2% of the total number of OTUs), while Pseudomonadota, Bacillota, and Bacteroidota were the dominant phyla. By the end of the cultivation period, the relative abundance of cyanobacteria increased to 10% in the experiments that employed NaCl and $NaHCO_3$ addition under continuous illumination, while a further increase to 30% was monitored in the photoperiod experiments. These results were consistent with those of pigment concentration where the values obtained were higher at the end of cultivation.

Although the brewery wastewater used as substrate in this study included a high d-COD value and therefore high bacterial populations were expected during *A. platensis* cultivation, similar bacterial communities were additionally observed in experiments using synthetic culture media. A study by Yuan et al. [7] reported that the relative abundance of *Arthrospira* in raceway ponds was 50%, while Pseudomonadota, Bacteroidota and Archaea consisted the rest of the major phyla of prokaryotes. Vardaka et al. [6] investigated bacterial contamination in commercially available *Arthrospira* products originating from open ponds of different geographical origin demonstrating that heterotrophic bacteria belonging to the phyla Pseudomonadota, Bacillota, and Bacteroidota were detected in the end products. In another study, Pseudomonadota were the most common bacteria isolated from *Arthrospira* cultures in open ponds [51]. A population of gram-negative

heterotrophic *Bacilli* was also detected in *Arthrospira* cultures when glycerol was used as a carbon substrate [43]. Amongst prokaryotic microorganisms, pathogens similar to those found in wastewater streams [52] were also reported in commercially available *Arthrospira* products [6,53]. In general, high salinity in a *Arthrospira* growth medium can prevent the growth of pathogenic microorganisms [51]. Therefore, similar methods compared with those applied to prevent pathogenic bacteria from developing within *Arthrospira* cultures grown in a synthetic medium can be also applied when brewery wastewater is used, given that brewery wastewater contains negligible amounts of heavy metals [19]. Moreover, it should be noted that although *Arthrospira* cultivated in wastewaters cannot be used in food production, the biomass formed can be utilized in low-value applications such as the production of biofuels or biofertilizers [54].

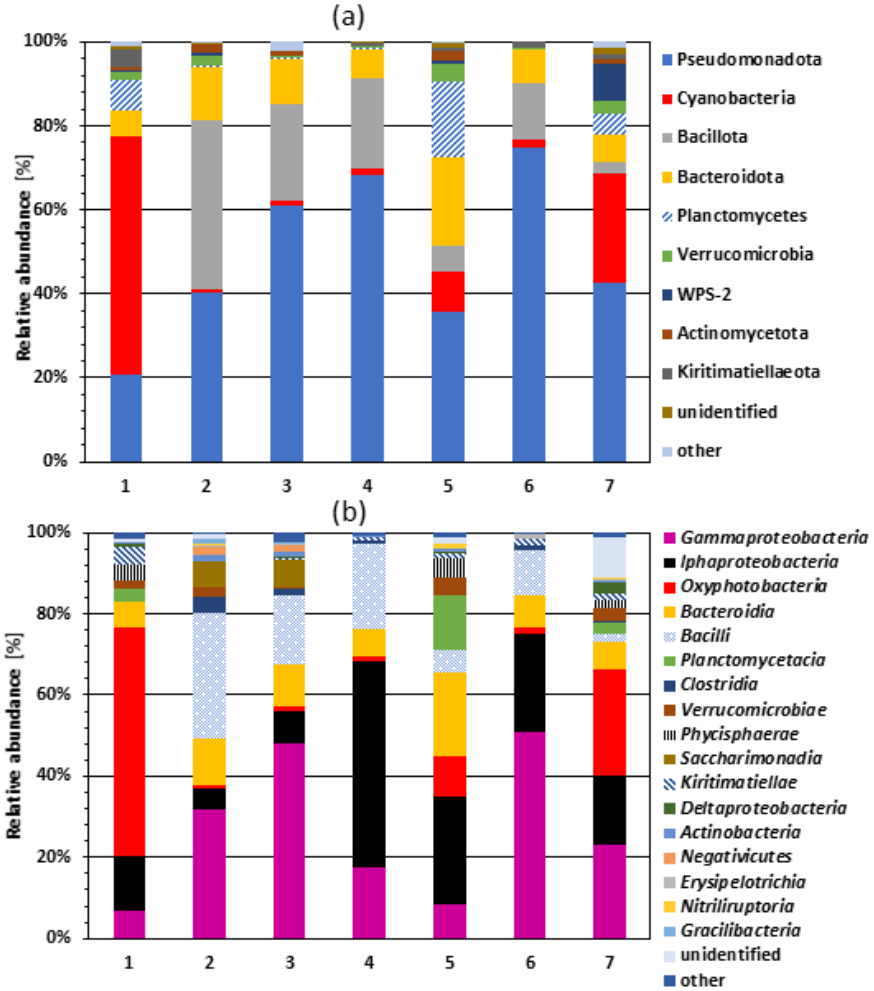

Figure 6. Relative abundance of major taxonomic groups at (**a**) phylum and (**b**) class level for bacteria and archaea. The most abundant operational taxonomic units (OTUs) at different levels for all experiments are listed (>1% in at least one sample), while all other OTUs were combined and shown as "other". The samples analyzed were denoted using numbers as follows: (1) inoculum, (2) brewery wastewater before pasteurization, (3) brewery wastewater following pasteurization, (4) mid-exponential growth phase (11th day) during *A. platensis* cultivation under continuous illumination in brewery wastewater with 1 g/L NaCl and 5 g/L $NaHCO_3$, (5) stationary phase (21st day) during *A. platensis* cultivation under continuous illumination in brewery wastewater with 1 g/L NaCl and 5 g/L $NaHCO_3$, (6) mid-exponential growth phase (11th day) applying photoperiod (16:8 h light:dark) during *A. platensis* cultivation in brewery wastewater with 1 g/L NaCl and 5 g/L $NaHCO_3$, and (7) stationary phase (21st day) applying photoperiod (16:8 h light:dark) during *A. platensis* cultivation in brewery wastewater with 1 g/L NaCl and 5 g/L $NaHCO_3$.

4. Conclusions

This study demonstrates the potential of *Arthrospira platensis* to treat non-diluted and non-pretreated brewery wastewater. The addition of $NaHCO_3$ and NaCl into the wastewater combined with high pH values favored *Arthrospira* growth. As all experiments were conducted under non-aseptic conditions, heterotrophic bacteria dominated by the phyla of Pseudomonadota, Bacillota, and Bacteroidota were also detected in the cultures. The abundance of heterotrophic bacteria was also highly associated with the high initial COD value of the brewery wastewater and negatively affected *Arthrospira* growth. Nevertheless, the symbiosis of *A. platensis* with bacteria achieved effective pollutants removal (over 90%, 70.5%, 28%, 55%, and 74% for d-COD, NO_3^--N, PO_4^{3-}-P, NH_4^+-N, and TKN, respectively) and biomass production. The maximum biomass concentration (about 950 mg/L) was achieved when the photoperiod was applied. The photoperiod also favored the accumulation of carbohydrates and lipids (33.66% and 18.64%, respectively), while protein and pigment concentrations remained low in all experiments due to the low nitrogen content of the wastewater. The biomass produced from the bioremediation of brewery wastewater can be used for biofuels or biofertilizer production. However, further research to optimize culture conditions in terms of *A. platensis* population is required for pilot and/or industrial application.

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