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# Treatment and Re-Use of Raw Blackwater by *Chlorella vulgaris*-Based System

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**Abstract:** In this study, we examined a *Chlorella vulgaris*-based system as a potential solution to change liquid waste, such as blackwater, into valuable products for agriculture while protecting waters from pollution without technical demanding pre-treatment. To evaluate the possibility of nutrient removal and biomass production from raw blackwater, four blackwater dilutions were tested at lab-scale: 50%, 30%, 20%, and 10%. The results showed that even the less diluted raw blackwater was a suitable growth medium for microalgae *C. vulgaris*. As expected, the optimum conditions were observed in 10% blackwater with the highest growth rate (0.265 $d^{-1}$) and a nutrient removal efficiency of 99.6% for ammonium and 33.7% for phosphate. However, the highest biomass productivity (5.581 mg chlorophyll-a $L^{-1}$ $d^{-1}$) and total biomass (332.82 mg dry weight $L^{-1}$) were achieved in 50% blackwater together with the highest chemical oxygen demand removal (81%) as a result of the highest nutrient content and thus prolonged growth phase. The results suggested that the dilution factor of 0.5 followed by microalgae cultivation with a hydraulic retention time of 14 days could offer the highest biomass production for the potential use in agriculture and, in parallel, a way to treat raw blackwater from source-separation sanitation systems.

**Keywords:** feces; urine; wastewater treatment; nutrients; biomass; agriculture

## 1. Introduction

Municipal wastewater (WW) usually arrives as a mixture from toilets, kitchens, and bathrooms at a central WW treatment plant without closing material flows [1]. Nutrients in treated municipal water are, in most cases, lost and squandered through the discharge into water bodies, which can lead to eutrophication with detrimental effects to aquatic environments [2]. The two key nutrients in municipal WW are nitrogen (N) and phosphorous (P), which, if reused, could diminish not only eutrophication and potential pollution but their recovery could act as a sustainable fertilizer source [3]. In conventional WW treatment systems, enormous volumes of freshwater are required to transport the small volume of human excreta from the toilet to the WW treatment plant. Furthermore, nutrients from the toilet are diluted by rainwater, groundwater intrusion, and industrial WW. As a result, typical WW consists of more than 95% of water and only 5% of pollutants [4]. Therefore, the current challenge is to rethink the present WW treatment system and provide the technology to not only remove organic and inorganic compounds but also recover them in a sustainable way.

Population growth has increased the demand for synthetic fertilizers while concurrently depleting non-renewable phosphate rock. Therefore, the exploitation of alternative resources is required to produce new fertilizers [5]. The recovery of P from WW is a viable solution and may close the human-phosphorus cycle [6]. However, according to Mihelcic et al. [7], only approximately 22% of current P fertilizer demand could be satisfied if all the P from human feces and urine was recovered and returned to agriculture land. Employing source-separation sanitation (SSS) systems for blackwater (BW) treatment offers a promising option for efficient nutrient recovery from human feces and urine [8]. Nevertheless, technical solutions for nutrient removal from BW are still in the research phase and include compost filters, evaporation tanks, anaerobic digestion, and membrane reactors, with limitations such as inadequate removal of fecal indicators (with the exception of membrane reactors), filter clogging [9], and little to no nutrient removal [10].

Microalgae represent a sustainable and chemical-free option for WW treatment due to their ability to uptake nutrients, reduce biochemical oxygen demand (BOD), heavy metals, and pathogens [11], and due to their ability to tolerate high load WW [12]. They also represent an environmentally friendly alternative to the energy-intensive chemical and physical removal processes for P [13]. Furthermore, following WW remediation with microalgae using WW as a low-cost nutrient source for microalgae cultivation, harvesting the microalgae may prove to be a source for the production of different valuable byproducts, such as lipids, fertilizers, and biofuels [11]. Despite many studies that have shown the promise of microalgae for enhanced nutrient uptake and biomass production [11], a full-scale application of a microalgae-based systems for WW treatment is problematic due to several obstacles, such as limited light availability for algae growth, high pollution load, long hydraulic retention times (HRT) (up to 20 days), and a large surface area needed for algae ponds (20 g total suspended solids (TSS) $m^{-2}$ $d^{-1}$) [14]. According to Lavrinovičs and Juhna [13], the major limitations for microalgae-based WW treatment represents a lack of rapid and inexpensive biomass harvest method, a limitation to finite geographical locations, and WW with everchanging contamination with unknown influence on algal growth. Therefore, more research is needed in order to apply microalgae-based systems in real-life applications.

Several promising studies exist in the treatment of WW for different origins by microalgae-based systems, i.e., municipal [15], industrial [16], and agricultural systems [12]. However, research on source-separated BW treatment with microalgae-based systems is very limited and has focused solely on anaerobically pre-treated BW [8,17]. There is an increasing interest in the use of anaerobic digestion for treatment of source-separated BW [18] and, therefore, research on microalgae-based systems in relation to BW is focused on treating nutrient rich effluents from anaerobic digestion [8]. However, the production of biogas from source-separated BW can be economically questionable. According to Huihui He et al. [19], the annual economic viability of anaerobic reactors (e.g., upflow anaerobic sludge blanket) depends on inlet chemical oxygen demand (COD) concentration with an energy equilibrium at influent COD of 11,195 mg $L^{-1}$ and economic surplus at influent COD of 15,000 mg $L^{-1}$, which is up to 15-fold higher from COD values usually reported for the BW [20]. Since source-separated BW is rich in nutrients, it could present a suitable low-cost nutrient source for microalgae cultivation in its raw form, without technically demanding pre-treatment units, which could be applicable especially at a source where the quantities of water are small and more manageable in comparison to a common WW treatment plant. However, the question this paper strives to answer is whether source-separated BW is able to support microalgae growth also in its raw form due to unfavorable conditions for algae growth, such as a very high nutrient load, dark color, and changing N:P ratios.

This study aims to (a) evaluate efficiency of *Chlorella vulgaris*-based systems for raw source-separated BW treatment as a primary treatment step to avoid technical demanding pre-treatment; (b) determine the lowest dilution of raw BW that can still be treated in microalgae-based systems; and (c) determine the highest nutrient removal and biomass production. *C. vulgaris* was selected as a test organism because of its easy cultivation, its common presence in the environment, and its rapid growth and short generation time, as well as its good performance in different types of WW [21].

## 2. Materials and Methods

The source of BW (i.e., feces, urine, toilet paper, and flush water) was a low-flush (0.8 L water per flush) vacuum toilet (Jets™ Norway), a part of a source-separation sanitation (SSS) system located at a biogas plant in Ljubljana, Slovenia, which was used by male employees. In the SSS system, there was also a dry urinal, but the collected urine was not included in this study. A more detailed description of the SSS system employed for the BW collection is given by Žitnik et al. [22].

### *2.1. Blackwater Collection and Characterization*

The BW was sampled from a 100 L stainless steel BW storage tank after 30 days of the SSS system operation in which, altogether, a total of 31 flushes were registered and ca. 43 L of the BW were collected. One part of the sample was used for the experiment, while the second part was used for the chemical analysis of pH, TSS, $BOD_5$, COD, ammonium nitrogen ($NH_4$-N), total nitrogen (TN), total Kjeldahl nitrogen (TKN), total phosphorus (TP), nitrate-nitrogen ($NO_3$-N), and nitrite-nitrogen ($NO_2$-N) performed according to standard methods [23]. The mechanical stirrers of the BW tank were activated for 5 min prior sampling to homogenize the collected BW.

### *2.2. Microalgae Inoculum Preparation*

Green microalgae *C. vulgaris* (strain 211/11S) was purchased as a live culture (20 mL) from the algae bank of the Culture Collection of Algae and Protozoa (CCAP, Scotland) and was used as a test organism. *C. vulgaris* was cultivated in a 2 L cultivation tank with a total volume of 1.5 L in a nutrient medium prepared from Plantfert U (8-8-7) (Chimro, Romania) diluted with distilled water ($dH_2O$) in a ratio of Plantfert U: $dH_2O$ 1:300 and sterilized until concentration has reached $10^6$ cells $mL^{-1}$. Afterwards, the culture of *C. vulgaris* was diluted with fresh nutrient medium prepared as described above and cultivated in bigger volumes until concentration $10^6$ cells $mL^{-1}$ was reached. The culture was maintained in an exponential growth phase. *C. vulgaris* was cultivated at an ambient temperature of 25 °C, which was in the optimal temperature range for its growth [24], using tubular fluorescent lamps (FLUORA L36W/77, OSRAM, Germany). Light irradiance was measured with a light meter (MS-1300, Voltracft, Italy) and was set to 5000 lux (150 μmol $m^{-2}$ $s^{-1}$) with a 16 h/8 h light/dark cycle [25]. Pure carbon dioxide ($CO_2$) (Messer, Slovenia) was introduced into the cultivation tank every 24 h for pH regulation. Continuous mixing was provided by magnetic stirrers (IKA, Germany, 250 rpm). Chlorophyll-a was analyzed daily in the cultivation tank and the relationship between cell counting and the measured chlorophyll-a concentrations of the cell suspension in the nutrient medium was determined following the method described in Section 2.4.1. The *C. vulgaris* in a nutrient medium with biomass of 13.3 mg chlorophyll-a $L^{-1}$, which was equivalent to $2.66 \times 10^7$ cells $mL^{-1}$, was an inoculum used for the experiment.

### *2.3. Experimental Design*

The experiment consisted of four different tests conducted in triplicates with a different dilution of BW, i.e., 50% BW, 30% BW, 20% BW, 10% BW, and a control (Table 1). The tested dilutions were chosen based on the COD value of the raw BW used for the experiments (6568 mg $L^{-1}$, Table 2) and the COD values of the WWs used as a growth media for microalgae reported in the literature [26]. In real-scale applications, BW can be diluted by mixing BW stream and greywater stream originating from sinks, shower, bath, kitchen, and laundry activities. According to Hernández Leal et al. [27], greywater represents about 70% of the domestic WW or more in the case of low-flush toilets.

**Table 1.** Composition of the experimental mixtures in different blackwater (BW) dilutions.

| | Unit | 50% BW | 30% BW | 20% BW | 10% BW | Control |
|---|---|---|---|---|---|---|
| Dilution factor | - | 0.5 | 0.3 | 0.2 | 0.1 | - |
| BW volume | mL | 640 | 384 | 256 | 128 | - |
| Distilled water | mL | 640 | 896 | 1024 | 1152 | - |
| *C. vulgaris* inoculum * | mL | 320 | 320 | 320 | 320 | 320 |
| Nutrient medium | mL | - | - | - | - | 1280 |

* cell density of $2.66 \times 10^7$ cells $mL^{-1}$.

**Table 2.** Raw blackwater composition at the source-separation unit used for the experiment.

| Parameter | Unit | Value |
|---|---|---|
| pH | - | 7.2 |
| Total suspended solids (TSS) | mg $L^{-1}$ | 2600 |
| Biochemical oxygen demand ($BOD_5$) | mg $L^{-1}$ | 3000 |
| Chemical oxygen demand (COD) | mg $L^{-1}$ | 6568 |
| Ammonium-nitrogen ($NH_4$-N) | mg $L^{-1}$ | 420 |
| Total nitrogen (TN) | mg $L^{-1}$ | 715 |
| Total Kjeldahl nitrogen (TKN) | mg $L^{-1}$ | 713 |
| Total phosphorous (TP) | mg $L^{-1}$ | 100 |
| Nitrate-nitrogen ($NO_3$-N) | mg $L^{-1}$ | 0.51 |
| Nitrite-nitrogen ($NO_2$-N) | mg $L^{-1}$ | <0.3 |

For each test, a 2 L photobioreactor (PBR) in batch-mode was used. Continuous stirring was provided by magnetic stirrers (IKA, Germany, 250 rpm). The tops of the PBRs were sealed with towel paper to avoid water evaporation and enable gas exchange. The cultivation conditions regarding temperature and illumination are described in Section 2.2. To maintain a pH close to value 7, which is an optimum for *C. vulgaris* growth [28], as well as to avoid inorganic carbon limitation [29] and inhibition due to high ammonia loads [30] (thus reducing stress and negative impact on growth and nutrient uptake) pure $CO_2$ (Messer, Slovenia) was introduced in PBRs on daily basis similar as in the cultivation tank. A control was used to evaluate growth of *C. vulgaris* in the nutrient medium without addition of raw BW.

### *2.4. Monitoring of the Microalgae-Based System*

During the experiment, once per day at the same time, two samples were taken from each PBR: Sample A (6 mL) for the determination of *C. vulgaris* growth and Sample B (50 mL) for chemical analysis. On days 3 and 7, sampling was not performed due to technical reasons.

#### 2.4.1. Microalgal Growth

In Sample A, optical density at 680 nm and chlorophyll-a were analyzed using a Nanocolor VIS spectrophotometer (MACHEREY-NAGEL, Germany) as indicators of *C. vulgaris* growth. The optical density was used as a means of monitoring dry weight with the coloration of the BW considered by measuring the matrix of the culture as a blank sample to calibrate the spectrophotometer before measuring the optical density of Sample A. As the matrix, the supernatant filtered through 0.45 µm cellulose acetate filter from centrifuged Sample A was used (see the procedure for Chlorophyll-a determination). The relationship between measured chlorophyll-a concentrations and cell count was determined as described below. Chlorophyll-a was analyzed according to a Vollenweider [31]. A 5 mL sample was centrifuged at 8000 rpm for 10 min with a UNIVERSAL 320 centrifuge (Hettich Zentrifugen, Germany). The supernatant was discarded and the sample was re-suspended in 8 mL of methanol (Sigma-Aldrich, USA). The suspension was kept in a water bath at 50 °C for 1 h and centrifuged

at 4000 rpm for 10 min. Chlorophyll-a concentration was determined spectrophotometrically and calculated using the following equation:

$$\text{Chlorophyll a}\left[\frac{\mu g}{mL}\right] = \frac{13.9 \times (E_{665} - E_{750}) \times 8}{V_{sample} \times l} \quad (1)$$

$E_{665}$ and $E_{750}$ are the absorbance of the chlorophyll-a suspension in methanol at 665 nm and 750 nm, respectively, and l is the width of the cuvette used.

The relationship between dry weight and measured optical density at 680 nm of the cell suspension in the nutrient medium was determined as follows. In 200 mL of nutrient medium, 40 mL of *C. vulgaris* inoculum was added and cultivated for 7 days in the conditions described in Section 2.3. Afterwards, the dilution with $dH_20$ in ranges 80%, 60%, 40%, 20%, and 0% was performed. A linear relationship ($R^2$ = 0.9718) between dry weight and measured optical density was obtained (data not shown). For each optical density measurement, three readings were obtained and an average value was used. Similarly, the relationship between cell counts and measured chlorophyll-a concentrations of the cell suspension in the nutrient medium was determined. Cell counting was performed according to a protocol of Moheimani et al. [32] by using a light microscope (CX31RBSF, Olympus, Japan) and a Neubauer chamber (Celeromics, France) with a 0.1 mm depth and a 0.0025 $mm^2$ total counting surface. Cell counts were correlated to measured chlorophyll-a concentrations using the same protocol as in correlating dry weight and measured optical density. A linear relationship ($R^2$ = 0.924) between cell counts and measured chlorophyll-a concentrations was obtained (data not shown), and the resulting empirical equation was the number of cells = $2 \times 10^7 \times$ [Chla]. For each chlorophyll-a measurement, three readings were obtained and an average value was used.

Specific growth rate (μ) and algal biomass productivity were calculated in the exponential phase of growth, according to Converti et al. [33] and Moheimani et al. [32], respectively.

#### 2.4.2. Chemical Analyses

Samples B were centrifuged at 11,000 rpm for 15 min (UNIVERSAL 320, Hettich Zentrifugen, Germany) and the supernatant was analyzed for COD, TN, $NH_4$-N, and orthophosphate ($PO_4$-P) using the Nanocolor VIS spectrophotometer (MACHEREY-NAGEL, Germany). For chemical analyses, the following standard protocols were used: ISO 6060:1989 for COD, EN 25663:1993 for TN, ISO 5664:1984 for $NH_4$-N, and ISO 6878:2004 for $PO_4$-P. TN and $PO_4$-P were analysed in all samples, while after day 2, COD and $NH_4$-N were analyzed every second day to minimize the reduction of the experimental mixture's volume. Before sampling, pH was measured directly in PBRs using a bench-top pH meter (HI-208, Hanna Instruments, Italy).

### *2.5. Statistical Analyses*

One-way analysis of variance (ANOVA) with Tukey's post hoc test and a linear correlation were used for statistical analyses. Normality of data was checked using Lilliefors test; all datasets were distributed normally. The analyses were conducted using the SPSS statistical package.

## 3. Results and Discussion

The characteristics of a medium used for algae cultivation are crucial for successful algae growth. Therefore, as a first step of the presented research, the properties of raw BW used for the experiment (Table 2) were analyzed and compared to literature data to evaluate the conditions for microalgae growth and biomass production as a base for system performance assessment. The pH of the BW was 7.2, which is appropriate for *C. vulgaris* growth [28] and similar to the pH of WW used for algae growth in other studies [12]. The COD concentrations were comparable with other studies on BW [34], but were up to 10-fold higher in comparison to municipal WW [35]. The $NH_4$-N concentrations were more than 3-fold lower as reported for raw BW [34], with the differences likely due to lower content of

urine (urine stream was not included in the experiment), while the TP concentrations were similar to the literature data [30].

### *3.1. The Microalgae Growth and Biomass Productivity on Blackwater*

The rate at which nutrients can be removed from WW is directly associated to algae growth rate and nutrient demands [17]. Thus, to gain more insight into the effect of *C. vulgaris* on carbon and nutrient removal, algal growth was measured. Raw BW supported the growth of *C. vulgaris* well in all tested dilutions (Figure 1). Several authors suggested that high $NH_4$-N concentrations, as in the current study, inhibit algae growth due to ammonia toxicity [30]. However, when the pH is kept neutral, ammonia inhibition is prevented, and the growth of algae on the nutrient-rich stream is possible [30]. In the present study, pH was regulated daily to near neutral levels and thus no toxic effects of high $NH_4$-N concentrations were noticed even in less diluted BW. *C. vulgaris* grew more gradually with a longer lag phase in less diluted BW, i.e., three days lag phase was observed for 50%, two days for 30%, one day for 20%, and no lag phase was observed for 10% BW. The reason for the prolonged lag phase in 50% and 30% BW was probably due to the extreme initial conditions, such as a darker color of the medium and consequent reduced light penetration (TSS of the un-diluted raw BW 2600 mg $L^{-1}$, Table 2). Although algae growth in 30% and 50% BW was more gradual in comparison to more diluted BW, the growth lasted longer, and thus the stationary phase was reached at days 9 and 11, respectively. Similarly, Fernandes et al. [17] observed the stationary phase at nearly 10 days when using *C. sorokiniana* to treat effluent from anaerobic digestion of BW. The growth curve of 10% BW was similar to the ones observed in studies with municipal WW as a growth medium [36] with the lag phase lasting less than a day. Further, in 20% BW the stationary phase was reached at day 6 and in 10% BW at day 5 due to reduced nutrients compared to 50% and 30% BW (Figure 2). Considering the high removal efficiency of $NH_4$-N (Figure 3), the slowing of the growth speeds might be the result of its rapid consumption.

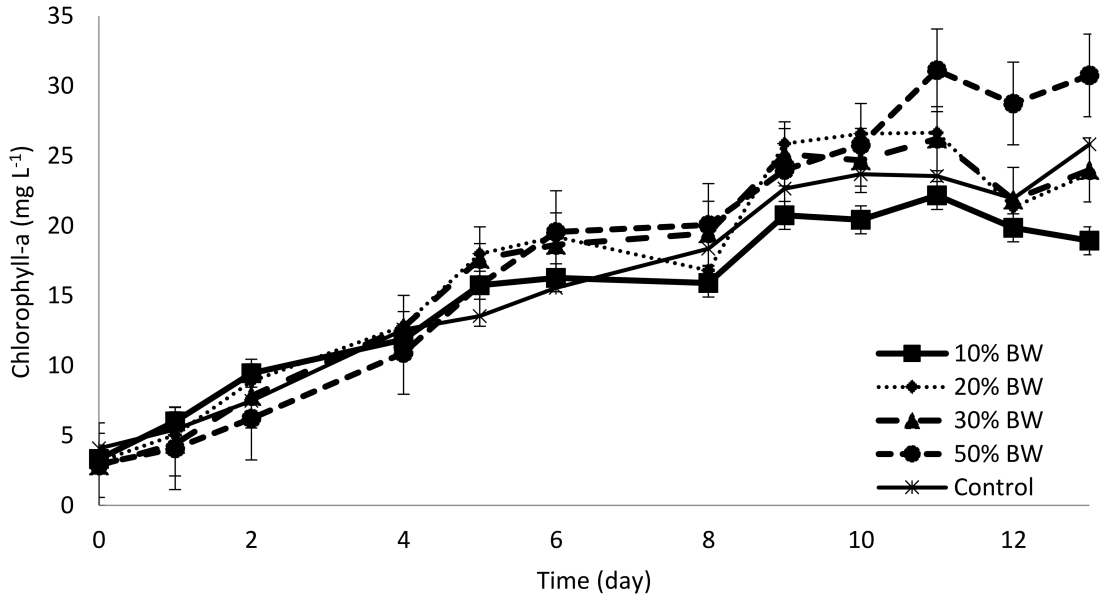


**Figure 1.** Concentrations of chlorophyll-a in different dilutions of blackwater (BW) ($n$ = 3).

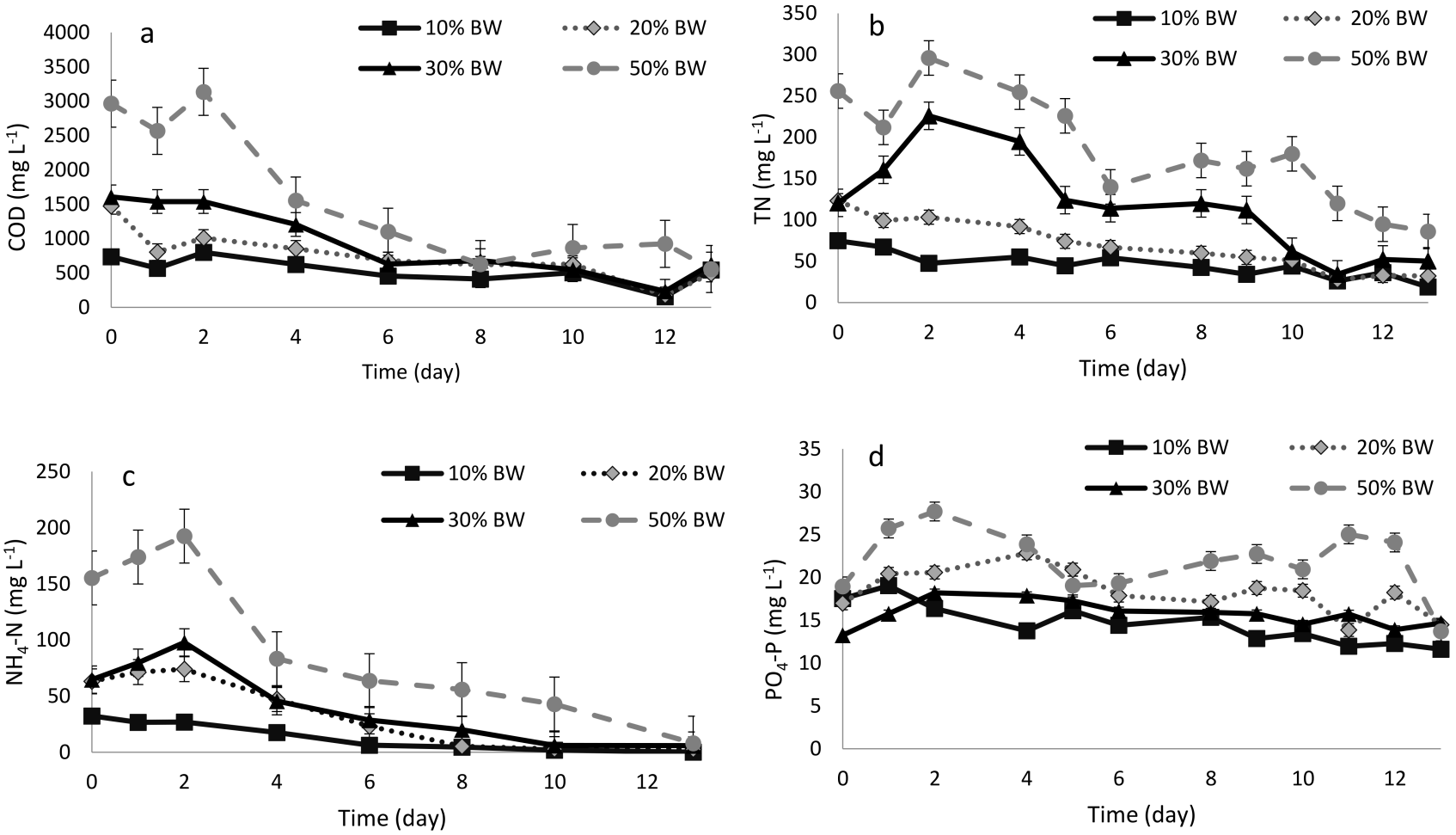


**Figure 2.** Concentration trends of chemical oxygen demand (COD) (**a**), total nitrogen (TN) (**b**), ammonium-nitrogen ($NH_4$-N) (**c**), and orthophosphate ($PO_4$-P) (**d**) in different dilutions of blackwater (BW) ($n$ = 3).

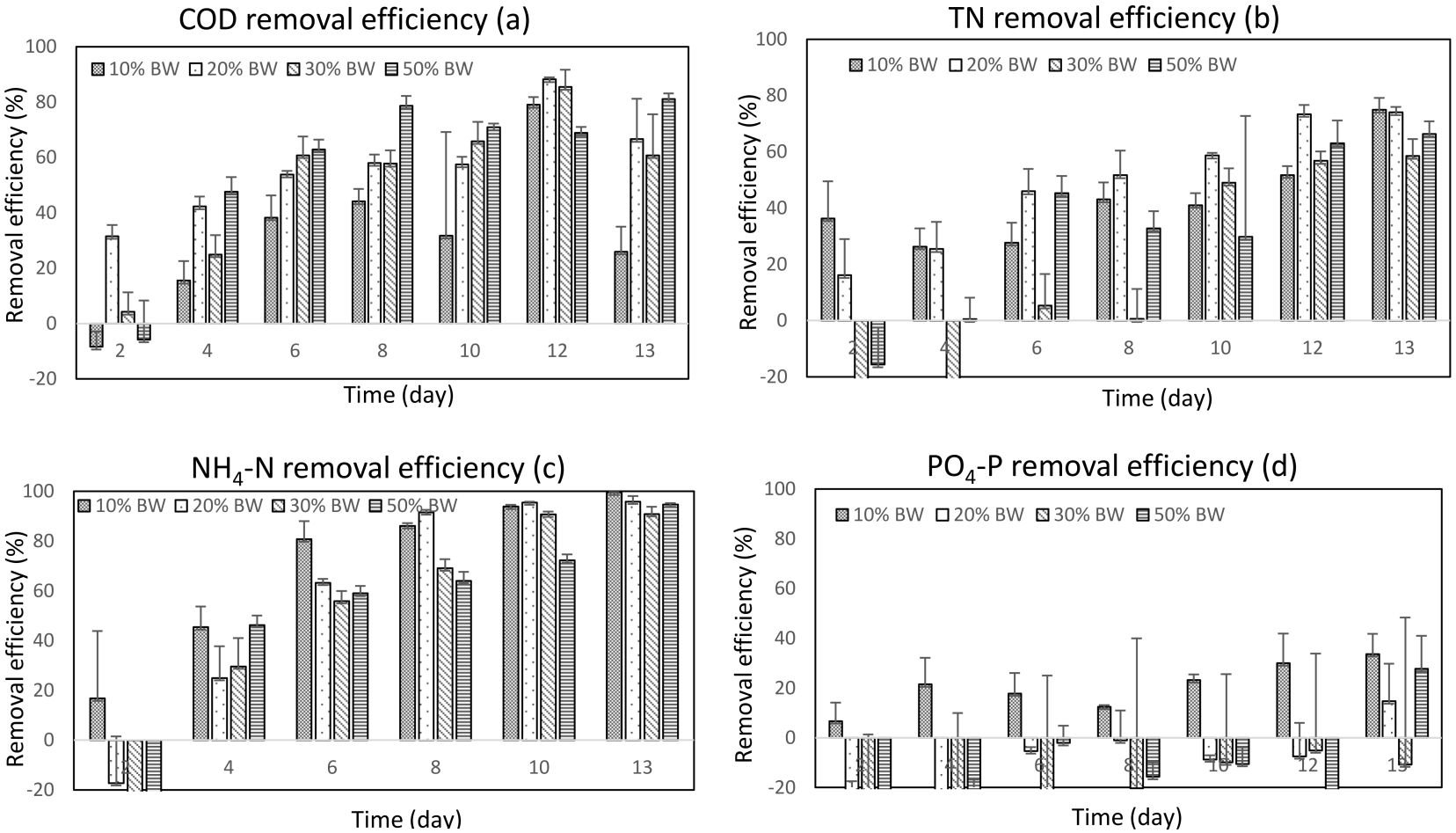


**Figure 3.** Removal efficiencies of chemical oxygen demand (COD) (**a**), total nitrogen (TN) (**b**), ammonium-nitrogen ($NH_4$-N) (**c**), and orthophosphate ($PO_4$-P) (**d**) in different dilutions of blackwater (BW) ($n$ = 3).

*C. vulgaris* grew faster in more dilute solutions with the highest average growth rate in 10% BW (Table 3). Several studies reported higher growth rates compared to the current study, most probably due to the more agreeable initial conditions employed in their studies. Wang et al. [37] reported up to 3.6-fold higher growth rate with *Chlorella* in different types of treated municipal WW due to a lighter medium color, up to 2-fold higher TP concentration, and 1.3-fold higher light intensity used than the one in the current study. Similarly, Ruiz-Martinez et al. [38] reported a 2.5-fold higher growth rate when employing a light intensity up to 209 μmol $m^{-2}$ $s^{-1}$ with a mixed algal culture in an effluent from an anaerobic membrane reactor. In the present study, mixotrophic growth of *C. vulgaris* was employed by exchanging light and dark cycles (16 h/8 h) since in real-scale applications algae ponds are kept under natural day/night conditions. During the dark cycle, *C. vulgaris* had a heterotrophic growth using organic carbon from BW as a carbon source. This was also evident from the reduction of COD in all tested dilutions with patterns similar to the $NH_4$-N reduction (Figure 3). The autotrophic growth of *C. vulgaris* is indicated by daily rises of pH in all BW dilutions (Figure 4) [39]. Although the average growth rate during the exponential phase was the highest in 10% BW, maximum biomass productivity was the highest in 50% BW due to an elongated growth period. Biomass productivity in 50% BW was higher compared to 30%, 20%, and 10% BW for 1.03, 1.28, and 1.35-fold, respectively (Table 3).

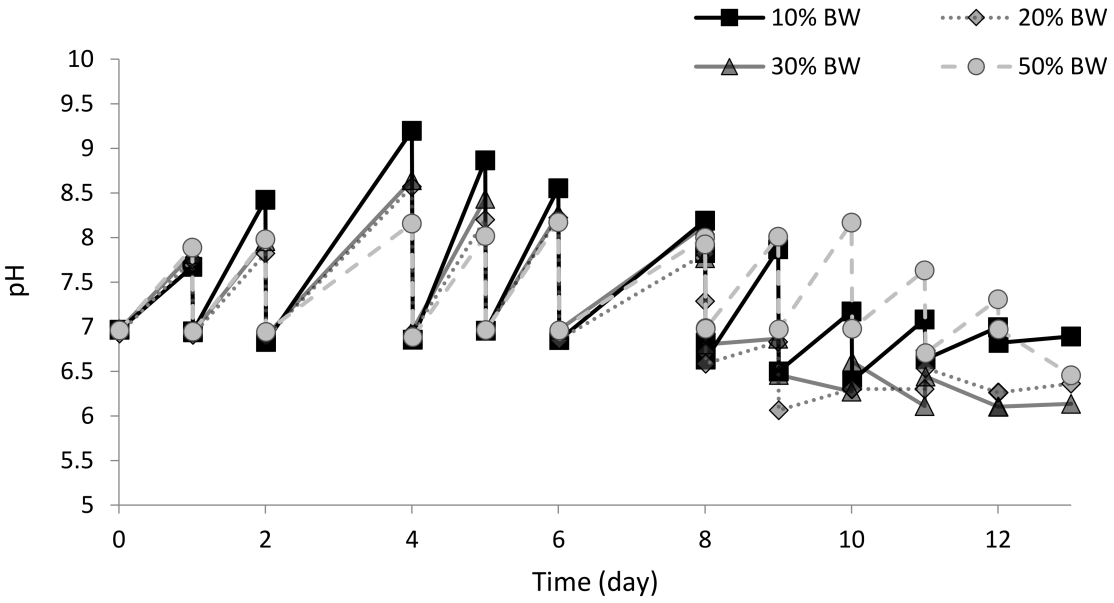


**Figure 4.** Fluctuations of pH in photobioreactors (PBRs) with different dilutions of blackwater (BW) and daily carbon dioxide ($CO_2$) insufflation ($n$ = 3).

The final biomass after 14 days of the experiment (Table 3) was the highest in 50% BW and the lowest in 10% BW for all measured parameters. However, no statistically significant variations of biomass parameters (Table 3) occurred between different BW dilutions (including control) meaning that 50% BW supported *C. vulgaris* growth well. In 50% BW, *C. vulgaris* reached a 10-fold higher biomass in terms of cell concentration and chlorophyll-a than the initial inoculum (compared to 6-fold increase in the control). Although in less diluted BW (50% and 30%) initial conditions were not favorable for microalgae growth, the overall growth at the end of the exponential phase in 50% BW reached up to 150% increase compared to more diluted BW and up to 120% increase compared to the control due to higher nutrients (Figure 2) and thus elongated growth period.

**Table 3.** Average growth rates, maximum biomass productivity during exponential phase and total biomass of *Chlorella vulgaris* with standard deviations in different blackwater (BW) dilutions ($n$ = 3). Chla: chlorophyll-a.

| Parameter | Unit | 50% BW | 30% BW | 20% BW | 10% BW | Control |
|---|---|---|---|---|---|---|
| Growth rate (μ) | $d^{-1}$ | 0.188 ± 0.01 | 0.215 ± 0.02 | 0.227 ± 0.03 | 0.265 ± 0.01 | 0.178 ± 0.01 |
| Biomass productivity | mg Chla $L^{-1}$ $d^{-1}$ | 5.581 ± 0.30 | 5.417 ± 0.48 | 4.349 ± 1.16 | 4.177 ± 0.14 | 3.758 ± 0.56 |
| Cell concentration | No. of cells $mL^{-1}$ | $5.96 \times 10^7 \pm 1.43 \times 10^6$ | $4.80 \times 10^7 \pm 2.20 \times 10^6$ | $4.75 \times 10^7 \pm 3.78 \times 10^6$ | $3.79 \times 10^7 \pm 9.34 \times 10^5$ | $5.16 \times 10^7 \pm 3.39 \times 10^6$ |
| Chlorophyll-a | mg $L^{-1}$ | 29.79 ± 0.72 | 23.99 ± 1.10 | 23.74 ± 1.89 | 18.93 ± 0.47 | 25.82 ± 0.87 |
| Dry weight | mg $L^{-1}$ | 332.82 ± 9.67 | 248.26 ± 12.40 | 229.83 ± 15.72 | 223.33 ± 5.89 | 279.67 ± 4.92 |

The algal biomass yields (Table 3) are comparable with the studies growing microalgae on municipal WW [40] or animal husbandry WW [41]. However, Wang et al. [12] and Fernandes et al. [17] reported higher biomass yields while cultivating microalgae at higher light intensities compared with the current research and using a UV-mutated strain of *C. vulgaris* [12]. According to Daliry et al. [25], a light intensity of 5000 lux (150 µmol $m^{-2}$ $s^{-1}$) is the optimum for *C. vulgaris* and was followed in the current experiment. The shadow effect in 50% and 30% BW could have occurred not only due to the increased turbidity but also due to fast-growing bacteria present in the BW [22].

### 3.2. Performance of the Microalgae-Based System

Overall performance of the system was assessed by calculating removal efficiencies and mass balance of monitored parameters, i.e., COD, TN, $NH_4$-N, and $PO_4$-P. The results of the mass balance analysis showed that 0.32 g, 0.14 g, 0.17 g, and 0.10 g of TN was retained by the system for 50%, 30%, 20%, and 10% BW, respectively. Furthermore, 0.02 g of $PO_4$-P was retained by the system for 50%, and 10% BW; 0.01 g of $PO_4$-P was retained by the system for 30% and 20% BW. The results of ANOVA analysis revealed statistically significant difference ($p < 0.05$) between parameter concentrations of different BW dilutions with the exception of 10% and 20% BW for COD, 20% and 30% BW for TN and $NH_4$-N, and 20% and 50% for $PO_4$-P. Nevertheless, no statistically significant variations of COD and nutrient removal occurred between different BW dilutions meaning that 50% BW enabled microalgae treatment. The exception is $PO_4$-P removal in 10% BW, which was statistically significantly different ($p < 0.05$) from less diluted BW. However, no statistically significant variations of $PO_4$-P removal occurred between 20%, 30%, and 50% BW.

From Figure 2, it is evident that COD and nutrients in BW dilutions were constantly reduced throughout the experiment with some exceptions for $PO_4$-P even after microalgae reached its stationary growth phase. This was probably caused by aerobic bacterial activity in the PBRs stimulated by the photosynthetic oxygenation of *C. vulgaris*; several authors have reported a symbiotic relationship between *C. vulgaris* and aerobic bacteria [35]. The trends of COD, TN, $NH_4$-N, and $PO_4$-P showed an initial increase in BW dilutions over the first two culture days followed by a decrease over the remaining portion of the experiment with a second increase noticed for COD in 30%, 20%, and 10% BW after culture Day 12 (Figure 2). The initial increase was mostly present in less diluted BW (50% and 30%) with the longest lag phases (Figure 1). The reason can be attributed to the algae cell death as a response to the initial extreme conditions and thus to the release of cellular nutrients into the medium [42]. The initial $NH_4$-N and $PO_4$-P increase may be explained by the degradation of complex organic compounds in the BW due to bacterial activity naturally present in BW, especially in the tests with high BW dilutions with no or very little starting COD and TN increase. After the second culture day, all nutrients decreased rapidly due to fast assimilation by algae, with the exception of $PO_4$-P. The second increase in COD for 30%, 20%, and 10% BW at the last culture day was most probably due to the depletion of $NH_4$-N resulting in the start of death phase in which cell density decreases (Figure 1) with the release of cellular nutrients into the medium.

The main mechanism of the N and P removal in algae-based systems is expected to be biomass uptake. According to Fernandes et al. [17], higher growth rates are generally associated with higher nutrient uptake rates, and thereby result in faster removal of nutrients from WW. This is in accordance with the results of the current study as 10% BW with the highest growth rate among tested BW dilutions (Table 3) is accompanied with the highest $NH_4$-N and $PO_4$-P removals (Figure 3). Removal of COD was the highest in 50% BW since other mechanisms than microalgal assimilation, such as aerobic bacterial activity, play important roles in COD reduction. The reduction of COD could only be attributed to the microalgae in the axenic culture conditions. Similarly, TN removal is not associated only with the microalgal assimilation of N. Other processes, such as stripping and bacteria uptake, may play significant roles in TN reduction. Because tubular fluorescent lamps were employed, which according to the producer information (OSRAM, Germany) emit significantly less ultraviolet light than sun-light

does (wavelength range 380 nm and higher), it can be hypothesized that the effect of photo-oxidation on BW components in the current experiment was minimal.

#### 3.2.1. COD Removal

In order to monitor removal dynamics throughout the experiment, removal efficiencies were calculated for every other experimental day (Days 2, 4, 6, 8, 10, 12, and 13 (final removal efficiency)) for monitored parameters. Figure 3 shows positive COD removal efficiency after culture day 2 for all tested BW dilutions with the highest removal efficiency achieved in 50% BW throughout the experiment. Final COD removal efficiency for 50% BW (HRT 14 days) was 81%; however, by culture day 4, almost half of COD was removed (48%) with 63% of COD being removed by day 6 and 79% by day 8. The lowest COD removal was observed for 10% BW throughout the experiment, with 79% removal reached by culture day 12. Removal efficiencies of COD for 30% and 20% BW were quite similar, with the removal efficiencies ranging between the less and the most diluted BW. After day 6, the COD levels dropped significantly in all BW dilutions (Figure 2) leaving only less reachable organic carbons in the batch media, causing nutrient deficiency for further heterotrophic algae growth. COD removal in the current study was in range [43] or up to 2.4-fold higher than reported by other studies [15]. The good growth and considerable COD reduction in diluted BW with the pattern similar to $NH_4$-N reduction (Figure 3) indicated that *C. vulgaris* used organic carbon as a source of energy and a substrate for cell growth in the dark cycle when transferring from autotrophic to heterotrophic metabolism. During the light cycle, the source of carbon for *C. vulgaris* was inorganic carbon from $CO_2$ introduced in PBRs on a daily basis and from bacterial respiratory activity. Part of the COD was also removed by bacteria naturally present in BW; however, this proportion is unknown.

#### 3.2.2. $NH_4$-N and TN Removal

The results of $NH_4$-N and TN removal presented in Figure 3 show positive removal efficiencies after culture day 2 for $NH_4$-N and after culture day 4 for TN in all BW dilutions. In the initial BW, the majority (60%) of the N was in the form of $NH_4$-N and the rest was organic N. The reason for the difference between $NH_4$-N and TN concentrations was the presence of a urinal at the collection facility for men employees, and the lower concentration of urine in the BW as a result. Negative $NH_4$-N and TN removals were observed in less diluted BW until culture days 2 and 4, respectively, due to the acclimatization of microalgal biomass to raw BW. The highest final TN removal of 75% was reached in 10% BW and the lowest final TN removal of 59% was observed in 30% BW (Figure 3). $NH_4$-N was the most effectively removed in 10% BW with a removal efficiency of 45% reached by Day 4; remarkably, 80% removal reached by day 6, and 99.6% removal reached by the end of the experiment. $NH_4$-N removal was also quite efficient for 50%, 30%, and 20% BW dilutions with final $NH_4$-N removal efficiencies of 94.8%, 90.9%, and 95.9%, respectively. $NH_4$-N removal efficiencies at the beginning of the cultivation varied between different BW dilutions; however, in the last few culture days, they became more similar due to $NH_4$-N depletion. From Figures 1 and 2, it is evident that $NH_4$-N exhaustion was reached by culture days 6, 8, 10, and 13 for 50%, 30%, 20%, and 10% BW, respectively. In the current study, TN [26] and $NH_4$-N removal efficiencies [14] were in the range with the data reported in the literature. The correlation of biomass and N removal is not found to be proportional in the current research, which is in range with studies on municipal WW treatment by *C. vulgaris* [44]. TN and $NH_4$-N could also be reduced as a result of ammonia gas ($NH_3$) stripping influenced by the increase in pH due to photosynthetic activity in the PBRs (Figure 4) causing the $NH_3$:$NH_4$ equilibrium to shift towards $NH_3$ production [45]. However, N gas ($N_2$) stripping due to denitrification is excluded because dissolved oxygen concentrations in PBRs at the beginning of the light cycle were above 7 mg $L^{-1}$ (data not shown). In the study performed on municipal WW by Delgadillo-Mirquez et al. [26], 50% of the TN was assimilated by the microorganisms and 33% of the TN was eliminated by stripping.

#### 3.2.3. $PO_4$-P Removal

The removal of $PO_4$-P in the current study was much lower than expected and was generally up to 3-fold lower in comparison to other studies [26]. $PO_4$-P was recovered only in 10% BW throughout the experiment, and 50% and 20% BW at culture day 13 (Figure 3). Maximum $PO_4$-P removal of 33.7% was achieved in 10% BW on day 13 with 21.5% removal achieved on day 4 and 17.9% removal achieved on day 6. In 50%, 30%, and 20% BW, $PO_4$-P was released instead of being recovered most of the time. This could be explained with the composition of the BW being mainly feces. According to Bai and Wang [46], a solid fraction of feces contains 84% to 93% organic matter, meaning that most of the P in fresh feces is in organic form. The BW in the current study was collected through the vacuum toilet in the BW storage tank with very little aeration; thus, it can be assumed that most of the P remained trapped in organic form until the start of the experiment. Because of good oxygenation of the experimental mixtures, it can be hypothesized that organic P was decomposed to inorganic form in the PBRs. Due to the high amount of organic matter in the BW used considerable amounts of $PO_4$-P were continuously released from the organic fraction of the BW during the experiment. In fact, in less diluted BW (50%, 30%, and 20%) more $PO_4$-P was released from the organic fraction of the feces then being assimilated by microalgae resulting in negative $PO_4$-P removal. Negative P removal was not observed by other authors studying microalgae-based treatment of BW [17] or other types of WW [26], most probably because the experiments were conducted on at least partly treated WW in contrast with the raw BW used in the current study.

Delgadillo-Mirquez et al. [26] observed 100% $PO_4$-P removal in the experiments with *C. vulgaris* on municipal WW. However, they reported that other processes than recovery by microalgae were the main role in $PO_4$-P removal, such as the adsorption of phosphate on the cell surface, and chemical precipitation due to high pH values. Abiotic P removal normally occurs at pH 9 to 11 [47]. The pH value in the current study slightly exceeded 9 only in 10% BW on day 4 (Figure 4); therefore, the $PO_4$-P precipitation might have only minor influence in the removal of $PO_4$-P. Regarding the correlation between algal biomass (Figure 1) and residual $PO_4$-P in the medium (Figure 2), only in 10% BW could correlation be observed, which is similar as reported by Choi and Lee [44]. Therefore, it can be assumed that the main mechanism of P removal in 10% BW was accumulation into algal biomass. Several studies with high P removal reported P as a limiting nutrient for microalgae growth in their experiments [17]. In parallel, several authors attributed low P removal to N limitation encountered by the cultures [48].

### *3.3. The Role of N:P Ratio*

WW N:P ratios depend on the origin of WW [17] and, in concentrated BW, such as raw BW in the present study, N:P ratio usually varies from 20 to 30 [30]. The N:P ratio of raw BW used in the present study was low (7) indicating N-limitation of microalgal culture [17]. The results show that *C. vulgaris* stopped growing when $NH_4$-N was depleted (Figures 1 and 2) demonstrating that $NH_4$-N was strongly linked with algal growth as a major limiting factor. According to Choi and Lee [44] the maximum TN removal treating municipal WW with *C. vulgaris* occurs at N:P ratio of 11–15; however, in the present study, very efficient N removal up to 99.6% was achieved at a lower N:P ratio of 7. In the research of Delalglio-Mirquez et al. [26], N:P ratio of the medium did not affect $PO_4$-P removal; however, as they reported, phenomena other than P uptake by microalgae could be responsible for P removal in their study. Low N:P ratio of the BW in the current study, with the initial $NH_4$-N concentrations in the range of treated BW [10], is the consequence of low content of urine as a result of sole male users of the experimental toilet together with separate collection of urine not being part of this experiment. According to Oarga-Mulec et al. [9] an increase in $NH_4$-N in BW is usually caused by hydrolysis of urea. Furthermore, during the BW collection in-situ, ammonia volatilization processes could have occurred since the BW storage tank was connected to an air filtration system. However, toilet facilities are usually used by both genders and, in such systems, due to higher urine content, higher N:P ratios are expected, and thus higher microalgae biomass yields and more efficient P removal can be achieved.

### *3.4. Microalgae-Based Systems for Blackwater Treatment in a Real-Scale*

Microalgae-based treatment of source-separated BW is feasible for small to medium settlements rather than for a single household [49] where it is more realistic to treat BW at the source with less quantities of water in comparison to a common WW treatment plant. The investment for an algae bioreactor as a resource-oriented solution in Slovenia is estimated to be 150 €/population equivalent (PE) and 24 €/year/PE for operation and management costs, respectively, for a settlement with 50–500 inhabitants with an area requirement of 3 $m^2$/PE [49]. Such a microalgae reactor due to usage of sun energy and free-carbon source which are BW itself and $CO_2$ from flue gas in the case that microalgae reactor will be located beside the source-separation sanitation at the biogas plant could lower the environmental foot-print of BW treatment in comparison with other technologies (e.g., sequencing batch reactor). Furthermore, if water and P scarcity will become more compelling in the future, WW treatment will need to harness all possible opportunities to increase efficiency and revenues, in order to remain sustainable. Opportunities include recovering resource content in WW and find synergy with other sectors, e.g., agriculture for byproducts. The most cost-effective reuse of microalgae biomass at a household level or at a level of small to medium settlements is as a fertilizer for lawns [50]. The results of this study showed that the amount of TN and TP in dry algae biomass (50% BW after the end of the experiment) is 36.2 g TN/kg and 11 g TP/kg, respectively, which resulted in N:P ratio of *C. vulgaris* biomass needed for fertilizing grass (3) [50].

## 4. Conclusions

The results of this study demonstrated that even less diluted raw blackwater (BW) is a suitable growth medium for microalgae since *C. vulgaris* could adapt well in 50% BW. The optimum conditions, as expected, were in 10% BW achieving the highest growth rate and a nutrient removal of 99.6% for ammonium ($NH_4$-N) and 33.7% for phosphate ($PO_4$-P). However, the $NH_4$-N limitation could not support the expected phosphorous removal in 50% BW and other less diluted BW. Usually, this would not be a problem due to toilets being used by both men and women. Biomass productivity and total biomass were the highest in 50% BW due to the highest nutrient content and thus prolonged growth phase. *C. vulgaris* performed well in 50% BW and $NH_4$-N consumption (94.8%); however, the needed hydraulic retention time (HRT) was 14 days. High HRTs are a major drawback of algae-based wastewater (WW) treatment systems and a compromise between affordable HRTs, appropriate treatment efficiencies, and biomass production is required regarding the selected purpose. Therefore, a dilution factor of raw BW up to 0.5 and a HRT of 14 days could be a promising solution for raw BW treatment if the microalgae-based WW treatment system is designed for byproducts (e.g., nutrients, bio-stimulants) to be reused in agriculture or for other applications (e.g., biofuels, feed, pharmacology). This means that nutrient-rich WW like BW represents a valuable feedstock to reduce the costs of microalgae cultivation, which will ultimately increase the cost-competitiveness of microalgae-based products. Greywater represents about 70% of domestic WW (the rest is BW), part of which (ca. 30%) could be used to dilute BW to achieve the proper conditions for microalgae cultivation, while 40% of greywater remains available for other household activities (irrigation, washing cars, etc.). When treating BW at the source, the volumes of BW are much lower in comparison to common treatment plants, meaning that surface area of algae ponds and the longer HRT should not be the limiting parameters. Based on the results that 50% BW was suitable as a growth medium for microalgae, future research should explore even less diluted BW and define the upper limits of BW concentrations still suitable for treatment with microalgae, and prove the results by scaling-up the system to pilot scale. Microalgae-based systems are a promising and sustainable solution to change liquid waste (BW) with the use of sun energy and cost-free carbon source (e.g., $CO_2$ from flue gas) into valuable products while protecting waters from pollution.

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