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Microalgal Systems, a Green Solution for Wastewater Conventional Pollutants Removal, Disinfection, and Reduction of Antibiotic Resistance Genes Prevalence?

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Abstract: The low-efficiency rate of urban wastewater (UWW) treatment generates tons of discharged water with a high concentration of pollutants, pathogens and antibiotic-resistance genes (ARGs). Microalgal systems may be a green alternative to be implemented as a UWW polishing treatment. This study assessed the ability of *Chlorella vulgaris* and UWW autochthonous microalgal species (AMS) to simultaneously remove PO_4–P, and reduce the proliferation of coliforms and ARGs. AMS seems to be more promising due to: (i) the higher specific growth rate, μ_{max} (0.687 ± 0.065 d^{-1}); (ii) efficient PO_4–P removal (92.62 ± 0.10%); (iii) faster reduction of coliforms proliferation achieving concentrations below the limits of quantification (6 d); (iv) the reduction of *intl*1 and the ARGs *sul*1 and bla_{TEM} abundance in ca. of 70.4%, 69.2%, and 75.7%, respectively (9 d); and (v) the additional reduction of these genes in ca. of 97.1%, 94.2%, and 99.9%, respectively, after 5 d storage in the dark and at room temperature. Results also revealed that the high pH values in both microalgal systems (due to microalgal growth) were highly correlated with a reduction in the proliferation of coliforms, including *Escherichia coli*. In conclusion, using AMS as a final polishing treatment of UWW seems to be very promising.

Keywords: wastewater treatment; microalgae technology; reduction of pathogens proliferation; antibiotic resistance

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1. Introduction

Massive anthropogenic activities generate 359.4×10^9 m^3 of wastewater annually, and only 52% is treated before its discharge into natural water bodies [1]. The existing treatment methods in urban wastewater treatment plants (UWWTPs) are not effective either because [2–6]: (i) the conventional secondary treatments are ineffective in the reduction of contaminants of emerging concern, such as heavy metals, pharmaceuticals, and antibiotic-resistant bacteria and their genetic determinants (ARB&Gs); (ii) the implementation of tertiary treatments require high energy demands and thus are cost-effective; or (iii) some of the existent disinfection processes (such as chlorination or ozonation) imply the use of additional chemicals or the generation of other harmful by-products, some of them classified as carcinogenic compounds [7–9]. Hence, the continuous disposal of wastewater containing high concentrations of chemical pollutants, pathogens, and ARGs, can severely put freshwater quality at risk and jeopardize public health [1,10–13]. Reutilizing treated wastewater is seen as an alternative to reducing water demand, particularly in world regions that are affected by water scarcity. Treated UWW reutilization almost always implies a storage period for transportation or temporal need. Reports show that treated water storage may induce the regrowth of bacteria, namely ARB&Gs [14].

Therefore, the great challenge has been the development of reliable, effective, low-cost, and environmental-friendly strategies for pollutant removal and reduction of ARB&Gs

proliferation in UWWTP effluents that allows a safe reutilization of treated water. Biological final treatments, namely with microalgae technology, appear as a more environmentally friendly alternative, particularly due to their high metabolic flexibility and the non-generation of toxic by-products. Additionally, the biomass obtained at the end of the process can be converted to commercially valued products, such as biofuels or biofertilizers, paying off the photobioreactor implementation's costs [15,16]. Moreover, the operational conditions inherent to microalgal production (such as pH, dissolved oxygen and carbon dioxide concentrations, light exposure, and hydraulic retention time), were described to create hostile conditions for some potentially harmful bacteria, such as faecal coliforms [4,17–20]. Particularly, light exposure above 115 µmol m^{-2} s^{-1} has a crucial role in *E. coli* removal, in combination with DO values between 1.2–8.18 mg L^{-1} and alkaline pH (above 8.5) [21,22]. Besides, some physiological characteristics of microalgae may have a role in the reduction of pathogens' proliferation, including [18,20,23]: (i) the production of toxins and antimicrobial compounds; (ii) the content in extracellular polymeric substances (EPS), from which humic substances are part of the composition, and (iii) the capacity of co-sedimentation with bacteria.

The possibility of forming a consortium between microalgae and bacteria has several reported benefits related to cooperative interactions in nutrient uptake, microalgal growth, environmental resistance, and pathogens reduction. Pieces of evidence show that *Chlorella vulgaris* in consortium with bacteria from activated sludge improved the reduction of the abundance of *Pseudomonas aeruginosa*, *Escherichia coli*, and *Enterococcus faecalis* in 59, 56, and 55% when compared to the single-use of activated sludge. Moreover, a recent study highlighted the potential of *C. vulgaris* and a *C. vulgaris -Bacillus licheniformis* consortium to remove exogenous plasmids carrying ARGs, such as *sul*1, while keeping an efficient uptake of phosphate-phosphorus (PO_4–P) [24].

Even though several studies report the effective use of *C. vulgaris* for conventional pollutants removals, such as nitrogen and phosphorus [25,26], few are related to disinfection and ARGs reduction of abundance [4,20,24]. To the authors' knowledge, none has evaluated the removal of conventional pollutants and the reduction of pathogens proliferation and ARGs abundance in the same system. Therefore, this study innovates by exploring the applicability and the dynamics of using microalgal systems for urban wastewater (UWW) polishing treatment to simultaneously remove conventional pollutants, reduce the proliferation of coliforms and reduce the abundance of ARGs.

2. Materials and Methods

2.1. Urban Wastewater Physicochemical Characterization

Experiments were performed using UWW freshly collected from the secondary clarifier from a conventional UWWTP located in Northern Portugal. This UWWTP has three treatment steps: (i) preliminary treatment (screening and grit removal); (ii) primary treatment (decantation); and (iii) biological treatment (activated sludge). It serves a population of less than 100,000 inhabitants and has a maximum daily average flow of approximately 2800 m^3 d^{-1} and 6300 m^3 d^{-1} of domestic and industrial wastewater, respectively. All samples were collected in sterile glass bottles, immediately transported to the laboratory, and processed.

UWW was characterized in terms of the following physicochemical parameters (Table 1): (i) pH, using a Consort's C6010 electrochemical analyzer (Brussels, Belgium); (ii) chemical oxygen demand (COD); following the 5220-D Standard Methods for the Examination of Water and Wastewater [27]; (iii) PO_4–P concentration, quantified according to the ascorbic acid colorimetric method described by Lee et al. [28]; (iv) nitrogen concentration based on nitrate–nitrogen levels (NO_3–N), according to the United States Environmental Protection Agency (USEPA)-approved Brucine Colorimetric Method [29], and (v) humic acids (HA) concentration, indirectly determined by UV spectroscopy at 254 nm (PG Instruments Ltd., T80 UV/VIS Spectrometer) [30]. COD, PO_4–P, NO_3–N and HA concentrations were determined, in triplicate, through calibration curves, after a dilution series with potas-

sium hydrogen phthalate (Merck, Germany), potassium dihydrogen phosphate (Aldrich, Germany), and humic acid sodium salt standard (Aldrich, Germany), respectively. Results are expressed in mg O_2 L^{-1} (COD) and mg L^{-1} (NO_3–N, PO_4–P and HA).

Table 1. Physicochemical characterization of the urban effluent collected after the secondary clarifier and used in this study.

Parameters	Values	Units
pH	8.6	-
Chemical oxygen demand (COD)	85.4 ± 4.7	mg O_2 L^{-1}
Humic Acids (HA)	10.7 ± 0.3	mg L^{-1}
Nitrate–nitrogen (NO_3–N)	<LOD [1]	mg L^{-1}
Phosphate–phosphorus (PO_4–P)	2.2 ± 0.1	mg L^{-1}

[1] LOD = 0.2 mg L^{-1}.

2.2. Photobioreactors Setup

C. vulgaris CCAP 211/11B obtained from the Culture Collection of Algae and Protozoa (CCAP, Oban, UK) was kept and grown in Organization for Economic Co-operation and Development (OECD) culture medium, under a light intensity of 250 µmol m^{-2} s^{-1} with a photoperiod of 24:0 light: dark cycle (LDC).

The experiment was carried out in 1-L photobioreactors (PBRs), using a working volume of 800 mL of UWW, and operated in batch mode, as depicted in Figure 1. The UWW treatment with *C. vulgaris* (CV) was tested in duplicate, and positive (C+, *C. vulgaris* + autoclaved UWW) and negative (C-, raw UWW) controls were used to validate the results. For the inoculation of the CV and C+ reactors, a pre-inoculum of *C. vulgaris* grown in OECD was cultured for 5 d to guarantee that cells were in the exponential phase. PBRs were inoculated for an initial microalgal cell concentration (MCC) of 4×10^4 cells mL^{-1}. All reactors were operated under a white light-emitting diode (LED, 4000 K), with a light intensity of 250 µmol m^{-2} s^{-1} and a photoperiod of 24:0 LDC, atmospheric air bubbling at 0.3 VVM (Sicce Airlight 3300, Pozzoleone, Italy), and at room temperature (20.0 ± 2.7 °C) for 9 d. Evaporation effects were restored daily by adding deionized sterilized water. CV and C+ PBRs suffered dilution effects of around 15%, due to the addition of pre-inoculum.

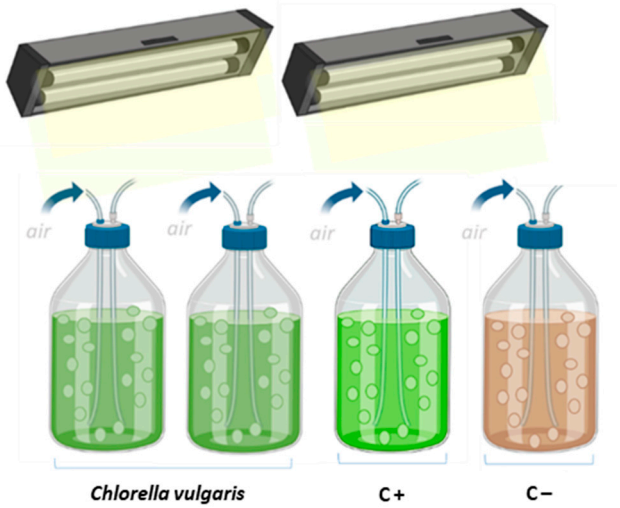

Figure 1. Experimental setup of the microalgal treatment, including the CV (*C. vulgaris* + UWW) PBR, positive (C+, *C. vulgaris* + autoclaved UWW), and negative (C−, UWW) PBRs controls.

2.3. Microalgal Biomass Quantification and Microalgae Identification

The microalgal biomass was monitored and quantified over time. For each reactor, samples of 100 µL were taken on days 0, 3, 6, and 9 in duplicate, and biomass was quantified

in duplicate by microscopic determination of the MCC using a Neubauer chamber (LEICA, DMLB, and Blau Brand, Steinheim, Germany).

In the different PBR conditions, the microalgal growth was evaluated and calculated, as before [26,31], in terms of: (i) maximum MCC (MCC_{max}), expressed in the number of cells per millilitre; (ii) specific growth rate (μ_{max}), determined by numerical regression of the experimental data at the exponential phase (between 3rd and 9th day) and expressed in d^{-1}; and (iii) average biomass productivities ($P_{X,avg}$), expressed in cells per volume per day. Autochthonous microalgal classes of C− PBR were identified by microscopic observation with an amplification of 40× (LEICA MC 120), according to their morphology.

2.4. Microalgal Pollutants Removal

Microalgal removal of pollutants from UWW was monitored over time at days 0, 1, 2, 3, 6, and 9 of the treatment assay. Samples, 7 mL, taken in duplicate for each PBR, were centrifuged at 1431× *g* (Eppendorf 5804 R centrifuge, Hamburg, Germany) for 10 min, and the supernatants were frozen at −20 °C until further analysis. Supernatants were characterized, in duplicate, in terms of pH (before frozen) and concentrations of COD, PO_4–P, according to the methods described in Section 2.1. Since the concentration of NO_3–N in the raw wastewater was below the limit of detection (<0.2 mg L^{-1}) it was not quantified during the experiment. Microalgal phosphorus removal was analyzed for all PBR conditions (CV, C+, and C−) in terms of mass removal (MR) and nutrient removal efficiencies (RE), as calculated before [25].

2.5. Enumeration of Culturable Microorganisms

Total heterotrophic and coliform bacteria (*E. coli* and other coliforms except for *E. coli*) were enumerated over time in each PBR. Samples of 1 mL were taken in duplicate at days 0, 3, 6, and 9, and were used to perform 10-fold dilution series. The enumeration of these culturable microorganisms was performed according to the membrane filtration method. For that, 1 mL of each serially 10-fold diluted sample was filtered through cellulose nitrate membrane filters (0.22 µm porosity; Whatman, Maidstone, UK) in duplicate. Membranes were incubated on Plate Count Agar (PCA, Merck, New York, NY, USA) at 30 °C for 48 h to enumerate total heterotrophs, and in Chromogenic Coliform Agar (CCA) at 36 °C for 24 h for *E. coli* (blue colony forming units) and other coliforms (pink colony forming units) enumeration.

2.6. DNA Extraction

A volume of 50 mL from each PBR was sampled on days 0 and 9. Samples were filtered through polycarbonate membranes (0.22 µm porosity; Whatman, UK), and total deoxyribonucleic acid (DNA) was extracted using the commercial kit Power Soil® DNA Isolation (MO BIO Laboratories, Inc., Carlsbad, CA, USA), according to the protocol provided by the manufacturer. Total DNA was quantified using the Qubit 3.0 Fluorometer (Invitrogen, Waltham, MA, USA) with Qubit® dsDNA HS assay kit (Invitrogen, USA), and DNA samples were stored at −20 °C before further analysis.

2.7. Quantification of 16S rRNA, intI1 and Antibiotic Resistance Genes

To determine the abundance of 16S rRNA, *intI*1, and the ARGs *sul*1 and bla_{TEM}, a quantitative polymerase chain reaction (qPCR, StepOneTM Real-Time PCR System, Life Technologies, Carlsbad, CA, USA) was performed in duplicate for each DNA extract [32]. Quantification was attained in duplicate through a standard curve method as described in a StepOne™ Real-Time PCR System (Life Technologies, Carlsbad, CA, USA) [33], and to avoid any probable qPCR inhibition, target genes were evaluated in a serially diluted sample [34]. The abundance values of each gene (GA) were expressed as the average of log gene copy number per 100 mL, and the *intI*1 and ARGs prevalence was determined according to normalization with the respective 16S rRNA abundance values (GA copy number per 100 mL/16S rRNA gene copy number per 100 mL). Moreover, the percentage

of reduction of resistance genes (RRG) was calculated as presented in Equation (1): where GA_{T0} corresponds to the GA at the beginning of the experiment (T0), and GA_{T9} to the GA on the last day (T9).

$$RRG = \frac{GA_{T0} - GA_{T9}}{GA_{T0}} \times 100 \quad (1)$$

2.8. Treated Water Storage

The potential of reusing the treated water was explored for the microalgal system that revealed the best UWW treatment results. Hence, at the end of the experiment, microalgae cells were harvested by centrifugation at 1431× *g* (Eppendorf 5804 R centrifuge, Hamburg, Germany) for 10 min, and water (supernatant) was stored for 5 d, in the dark, and at room temperature. Water stability in terms of 16S rRNA, *intl*1, and ARGs abundance and prevalence was performed as described in the previous section.

2.9. Statistical Analysis

Experimental data were analyzed using GraphPad Prism 8.0. A Shapiro-Wilk test of normality was performed before the analysis of variance (ANOVA) to confirm the normal distribution of the residuals. A two-way ANOVA with Tukey's multi-comparison test was used to find differences between growth parameters, P–PO_4 removal parameters, *E. coli*, other coliforms and total heterotrophs, and multiple t-student tests were used to compare ARG abundance, over time and between the PBRs. A Pearson correlation was also established among all physicochemical and microbiological parameters. All statistical analyses were performed at a significance level of 0.05.

3. Results and Discussion

3.1. Microalgal Growth

The microalgal growth profile along the UWW treatment is depicted in Figure 2, and the calculated growth parameters are presented in Table 2. In this experiment, light was provided by an artificial source, LED, with continuous illumination (24:0 LDC). Although it may not represent a real-life scenario, considering that sun is the main cost-effective light source, it has been proven that microalgal N and P uptake efficiencies are higher when a continuous light supply is applied, and the absence of a dark period reduces the proliferation of bacteria [35]. The possibility of using artificial light even in open-space photobioreactors (open raceways) was shown recently [36]. Hence, to improve microalgal efficiency, this experiment was designed considering microalgal systems operated in closed photobioreactors with light provided by a low-cost artificial light supply (LEDs).

Table 2. Growth parameters determined for each PBR: CV (*C. vulgaris* + UWW), C+ (*C. vulgaris* + autoclaved UWW), and C− (raw UWW). Values with a common superscript letter and within the same column are not statistically different ($p > 0.05$).

PBR	MCC_{max} (Cells mL^{-1})	μ_{max} (d^{-1})	$P_{X,avg}$ (Cells mL^{-1} d^{-1})
CV	$(6.3 \pm 0.4) \times 10^{5\,a}$	$0.087 \pm 0.002^{\,a}$	$(1.7 \pm 0.6) \times 10^{4\,a}$
C+	$(8.1 \pm 0.2) \times 10^{5\,b}$	$0.115 \pm 0.045^{\,a}$	$(5.1 \pm 0.2) \times 10^{4\,b}$
C−	$(5.5 \pm 0.1) \times 10^{5\,a}$	$0.687 \pm 0.065^{\,b}$	$(9.0 \pm 0.3) \times 10^{4\,c}$

μ_{max}: maximum specific growth rate; MCC_{max}: maximum microalgal cell concentration; $P_{X,avg}$: average biomass productivity.

PBRs inoculated with *C. vulgaris* (CV and C+) showed similar growth behavior until day 6, with no significant differences ($p > 0.05$) in the μ_{max} values, 0.087 ± 0.002 d^{-1} and 0.115 ± 0.045 d^{-1}, respectively. No lag phase was observed in any of these PBRs; however, in the CV PBR, a deceleration and a possible stationary phase started after day 6. On the other hand, microalgal growth in the C+ reactor was in a plain exponential phase at the end of the experiment (day 9). In terms of productivity, microalgae grown in the C+ PBR attained a 3-fold higher $P_{X,avg}$ than in the CV PBR. Moreover, *C. vulgaris* on C+ PBR

reached the highest X_{max}, (8.1 ± 0.2) × 10^5 cells mL^{-1} ($p < 0.05$), probably due to the greater availability of organic carbon from the UWW sterilization process.

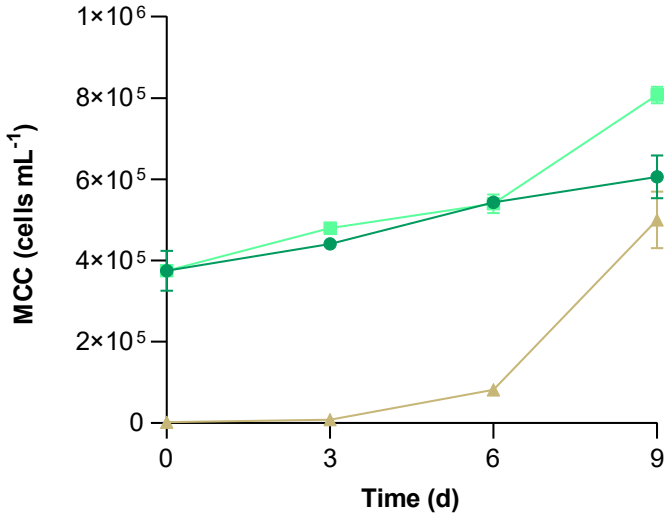

Figure 2. Microalgal cell concentration (MCC, average ± standard deviation) over time (d) for each PBR: ■ CV (*C. vulgaris* + UWW); ■ C+ (*C. vulgaris* + autoclaved UWW); and ■ C− (raw UWW).

After a lag phase of 3 d, autochthonous microalgal species (AMS) belonging to microalgal classes *Cyanophyceae*, *Chlorophyceae*, and *Bacillariophyceae* (Figure 3), started to proliferate exponentially in the C− reactor until the end of the experiment, attaining the highest μ_{max} (0.687 ± 0.065 d^{-1}) and $Px_{,avg}$ ((9.0 ± 0.3) × 10^4 cells mL^{-1} d^{-1}) ($p < 0.05$). The best results in terms of microalgal growth in C− can be associated, to some extent, with the cooperation effects between bacteria and microalgae and greater adaptation of AMS to the UWW than *C. vulgaris* [37].

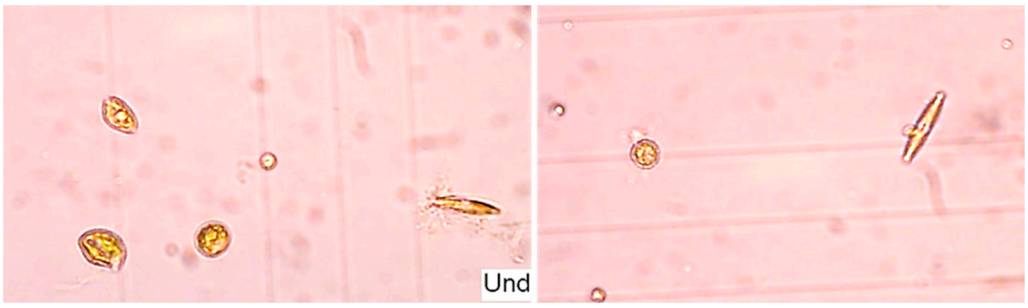

Figure 3. Microscopic observations (40×) of UWW autochthonous microalgal species detected in the C− PBR.

3.2. Physical-Chemical Characteristics of UWW and Conventional Pollutants Removal

The pH variations were plotted over time (Figure 4). In the CV and C+ PBRs, the pH values started to increase on day 2, while in the C− PBR, it only occurred on day 3, along with microalgal growth. In contrast with the present study, a pH decrease was reported in photobioreactors with other microalgal species described as hyperproducers of humic acids, such as *Euglena* sp. [38]. The co-production of other acid compounds such as itaconic acid by these microalgae was responsible for the pH decrease in such photobioreactors [39]. In the present study, the observed pH increase was probably due to the photosynthetic process. As microalgae consume CO_2, a cell internal conversion of HCO_3^- occurs to be further used as a carbon source for photosynthesis. This process leads to the accumulation of OH^- in the cells; to neutralize these ions, an increase in H^+ uptake from the culture medium occurs, leading to a cell-external increase in pH [39]. The correlation between the

microalgal growth and pH is supported by the exponential growth of microalgal cells and pH increase in both the C+ and C− PBRs, and the stationary phase of *C. vulgaris* between days 6 and 9.

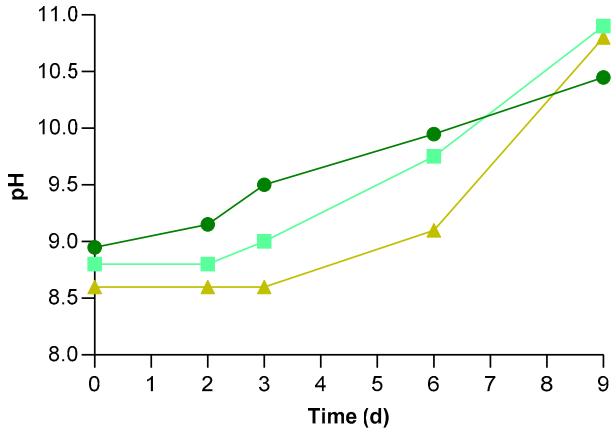

Figure 4. pH variation (average ± standard deviation) over time (d) for each PBR: ■ CV (*C. vulgaris* + UWW); ■ C+ (*C. vulgaris* + autoclaved UWW); and ■ C− (raw UWW).

As with any other living organism, microalgae are mainly composed of C, N, and P, typically with molar ratios $C_{106}H_{181}O_{45}N_{16}P$ [35]. Consequently, the assimilation mechanisms of these elements are linked [35]. Hence, the advantages of using microalgae-based treatments is the capacity of these organisms to remove conventional pollutants, such as phosphates, nitrates, and COD, frequently detected in the final effluents of conventional UWWTPs, at concentrations up to 40 mg L^{-1} of total phosphorus, 30 mg L^{-1} of NO_3–N and 160 mg O_2 L^{-1} of COD, respectively [7,26,40]. To confirm this ability, variations of PO_4–P concentration over time were registered (Figure 5), and the phosphorus MR and RE were calculated (Table 3) after 9 d of the microalgal treatment. Variations in the N–NO_3^- concentration were not determined because their content in the initial wastewater was already below the detection limit (0.2 mg L^{-1}).

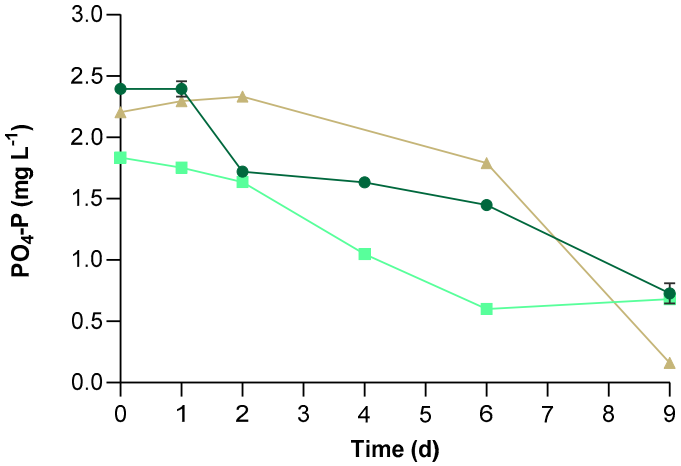

Figure 5. Variation of phosphate–phosphorus (PO_4–P) concentration (average ± standard deviation) over time (d), expressed in mg L^{-1}, for each PBR: ■ CV (*C. vulgaris* + UWW); ■ C+ (*C. vulgaris* + autoclaved UWW); and ■ C− (raw UWW).

The initial PO_4–P concentration of UWW was close to the EU legislation limit for UWWTPs with less than 100,000 population equivalent (2 mg L^{-1}) [41], and a slightly lower concentration was detected in the C+ PBR. Microalgae removed PO_4–P in all PBRs, revealing REs above 62% (Table 3). The profile of phosphorus removal over time followed

the microalgal growth, with the highest RE and MR attained by AMS in the C− PBR, 92.6 ± 0.1% and 2.04 ± 0.03 mg L^{-1}, respectively. These results are similar to the ones obtained using *Chlorella sorokiniana* and *C. vulgaris* by Fan et al. [42], Mujtaba & Lee [43], and Ferro et al. [44], and 5% higher than those described for *C. vulgaris* by Salgado et al. [25]. Although it was not possible to calculate NO_3–N removal efficiencies, due to the linkage between the macronutrients, it is expected that both *C. vulgaris* and AMS could remove this conventional pollutant as well as other species of nitrogen such as NH_4^+ [25,31,45].

Table 3. Initial PO_4–P concentration and removal parameters determined for each PBR: CV (*C. vulgaris* + UWW), C+ (*C. vulgaris* + autoclaved UWW), and C− (raw UWW). Values with a different letter within the same column are statistically different ($p < 0.05$).

PBR	P_0 (mg L^{-1})	MR (mg L^{-1})	RE (%)
CV	2.40 ± 0.02 ^{a}	1.67 ± 0.09 ^{a}	69.60 ± 3.44 ^{a}
C+	1.83 ± 0.02 ^{c}	1.15 ± 0.03 ^{b}	62.91 ± 0.61 ^{b}
C−	2.20 ± 0.01 ^{b}	2.04 ± 0.03 ^{c}	92.62 ± 0.10 ^{c}

MR: mass removal; P_0: initial phosphorus concentration; RE: removal efficiency.

Figure 6 depicts the variation of COD and HA concentrations over time in all PBRs. Initial UWW COD concentration values in all PBRs were below the maximum (125 mg O_2 L^{-1}) established by the Council Directive 1991/271/EEC [46]. In the C+ PBR, the sterilization process of UWW led to a 34% increase in the initial COD concentration (Figure 6A), since the organic matter degradation may have originated from polyphenolic, humin, and humic and fulvic acids [47]. On the other hand, the CV PBR had a significantly lower ($p < 0.05$) concentration of COD values due to a dilution effect of the *C. vulgaris* pre-inoculum.

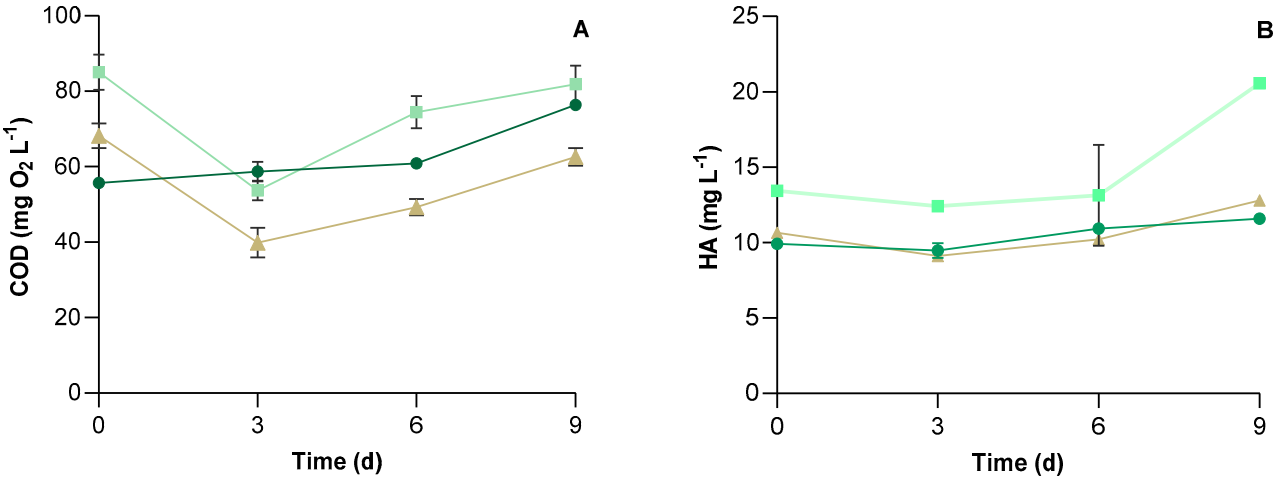

Figure 6. Variation of chemical oxygen demand (COD) (**A**) and humic acid (HA) (**B**) concentration (average ± standard deviation) over time (d), expressed in mg O_2 L^{-1} and mg L^{-1}, respectively, for each PBR: ■ CV (*C. vulgaris* + UWW); ■ C+ (*C. vulgaris* + autoclaved UWW); and ■ C− (raw UWW).

Nevertheless, the COD values lowered in the C+ in the first 3 d, as represented in Figure 6A, suggesting that *C. vulgaris* underwent mixotrophic metabolism. This type of metabolism allows cells to use organic carbon for respiration, besides inorganic carbon for photoautotrophy [48]. In the C− PBR, the COD decreased in the same period, most likely due to its consumption by the bacteria from the UWW. However, COD decrease by AMS should not be neglected, even though they were at lower concentrations at this first 3 d. After that, COD concentration increased over time, reaching slightly lower values in the C− PBR (62.5 ± 2.3 mg O_2 L^{-1}) than in the CV PBR (75.7 ± 2.7 mg O_2 L^{-1}), but still lower than legislation limits. In wastewater, COD can have many origins. However, in this present scenario, the increase in COD concentration over time probably occurred

due to the excretion of metabolic products, such as EPS, of which HA, a stable organic matter form, is a component [47]. Some microalgal species naturally secrete humic acids, while others secret humic acid-like substances along with EPS release in the presence of certain stressors [39,49]. Indeed, several studies related to wastewater treatment report the excretion of EPS by *Chlorella* sp., including HA, although to a lesser extent when compared to other HA hyperproducers such as *Euglena* sp. [38,50–52].

Hence, HA may have contributed to the COD increase. This occurrence is supported since the COD (Figure 6A) and HA content (Figure 6B) increased after day 3, to which a Pearson correlation was found to some extent (r 0.7–0.8). In addition, a high correlation between HA and MCC values (Pearson correlation, $r > 0.9$, $p < 0.050$) in the C− PBR was observed (Figure S1).

In summary, these results led to the conclusion that both *C. vulgaris* and AMS are efficient microalgal systems for PO_4–P removal and organic matter stabilization from UWW.

3.3. Cultivable Bacterial Abundance

Microbial abundance was determined for all PBRs in terms of *E. coli*, other coliforms except *E. coli*, and total heterotrophic bacteria (Figure 7). As expected, total coliforms were not detected in the C+ PBR. Both *C. vulgaris* and AMS had high similarity in their effects on these cultivable bacterial groups after 9 d, reducing coliforms to values lower than the limit of quantification (5 CFU mL^{-1}). *E. coli* reduction was faster (after 3 d) than other coliforms (after 6 d), and no statistical differences were observed in these parameters between the CV and C− PBRs ($p > 0.05$). According to the Pearson correlation for the CV PBR (Figure S1), aside from a strong negative correlation and significance between *C. vulgaris* MCC and *E. coli* concentration ($r > 0.9$ and $p < 0.05$), it was not possible to establish other clear and significant correlations (Figure S1). However, the pH increase seems to have a role, as observed in the study of Davies-Colley [53], in which a pH above 8.5 induced the inactivation of *E. coli*. While optimal pH conditions for faecal coliform growth are between 6.5 and 7.5, pH levels above 8.5 are very effective in their removal [22]. The pH has also been reported as playing an important role in reducing the proliferation of pathogens in wastewater stabilization ponds, in which microalgae had a crucial role in water alkalization as a result of photosynthesis [54]. Moreover, other studies report a reduction of four orders of magnitude in the concentration of total coliforms and *E. coli* after a pH increase from 8.4 to 10.5, and a total coliform reduction rate of 92% in high retention ponds for UWWT at a pH of 9.4 [55,56].

Indeed, the decrease of coliforms (except *E. coli*) abundance over time was highly negatively correlated with pH increase in the CV and C− PBRs ($r > 0.9$ and $p < 0.05$), as well as with microalgal growth and HA production in the CV ($r > 0.9$ and $p < 0.05$) (Figure S1). Moreover, HAs have adverse effects on pathogenic bacteria in the presence of light due to photo-oxidation, since HAs act as exogenous photosensitizers [18]. In photo-oxidation, the photosensitizers absorb light and transfer i energy to other molecules, leading to the development of reactive oxygen species that damage the cell membranes of microorganisms [18]. Therefore, the results from the present study suggest that the decrease in the abundance of coliforms is related to *C. vulgaris* growth, indicating that this is an efficient biological system to promote UWW disinfection.

Regarding total heterotrophic bacteria, the initial abundance in C+ PBR was lower than in CV and C− PBRs, due to the sterilization process of UWW, which also reduced total coliforms to non-detectable levels. Furthermore, the total heterotrophs content (6.9 logs CFU 100 mL^{-1}) in the C+ PBR confirmed that the *C. vulgaris* pre-inoculum was a microalgae-bacteria consortium. Additionally, the increase of the total heterotrophs in the C+ PBR over time, when compared to the CV PBR suggests that bacteria from the *C. vulgaris* consortium competed with bacteria from UWW in the CV PBR. In the C+ PBR, a decrease in the total heterotrophs content was observed from days 3 to 9, probably due to nutrient starvation caused by the rapid microalgae growth or the production of any antibacterial compound. More constant values of total heterotrophs were detected in the CV PBR,

suggesting that a microalgae-bacteria consortium was well established [57]. Microalgae can transform solar energy into chemical energy, producing O_2 and organic matter. In turn, bacteria may use the O_2 for respiration, decompose the organic compounds, and supply CO_2 and growth-promoting factors and vitamins (B12, B1 and B7) that boost microalgal growth [58–60].

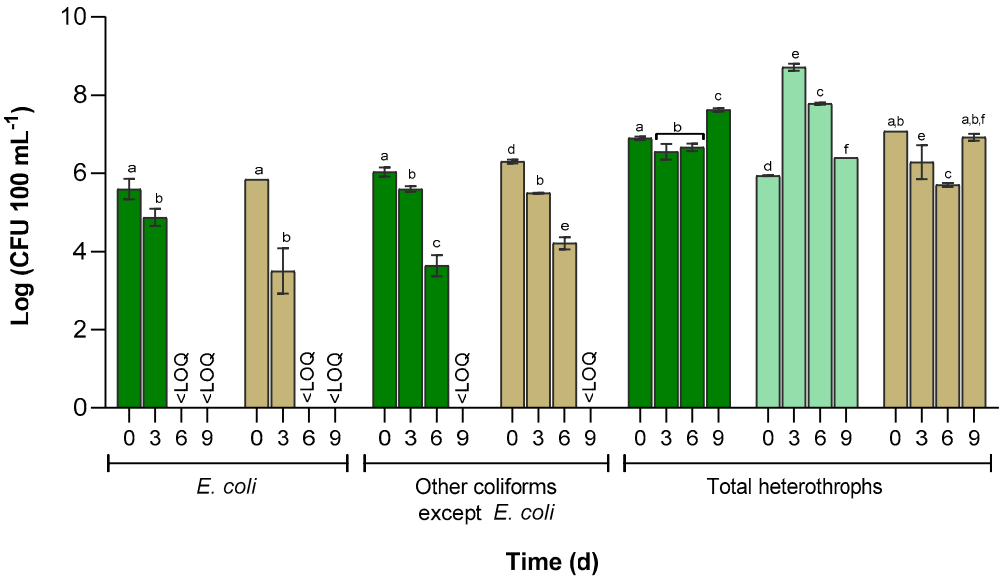

Figure 7. Quantification of *E. coli*, other faecal coliforms except *E. coli*, and total heterotrophs over time (d) for each PBR: ■ CV (*C. vulgaris* + UWW); ■ C+ (*C. vulgaris* + autoclaved UWW); and ■ C− (raw UWW). Results are expressed in log (CFU 100 mL^{-1}) (average ± standard deviation). Bars from the same group of bacteria with a common superscript letter are not statistically different (two-way ANOVA followed by Tukey post hoc test $p > 0.05$).

It should not be neglected that other operational characteristics related to microalgal growth may have had a role in the bacterial abundance during the experiments. Light exposure, particularly from photosynthetic active radiation (PAR), has been described as having disinfection effects on water due to its ability to penetrate in water depth. For example, over 99% of visible light at 556 nm can penetrate 8 cm, while shorter wavelengths (UV-B or UV-A) are retained in the first 2.5 cm [18]. Still, the responses of different bacteria to light damage vary significantly; while inactivation of *E. coli* occurs through endogenous mechanisms promoted by UV-B and UV-A, for other bacteria like *E. faecalis,* it occurs by PAR [18]. Additionally, a light–dark photoperiod of 24:0 showed a low reduction in *E. coli* abundance, while for *E. faecalis* it had the opposite effect [4]. Moreover, the presence of oxygen produced by microalgae may have a role in the reduction of *E. coli* abundance [4,56].

3.4. Abundance of Antibiotic Resistance Genes

Due to the non-cultivability nature of most water's bacterial organisms, total bacterial abundance was evaluated by quantifying the housekeeping gene 16S rRNA. Additionally, the abundance of *intl*1 and ARGs (*sul*1 and bla_{TEM}) was quantified in all PBRs (Figure 8). Results show that at the beginning of the experiment, in both the CV and C− PBRs, the abundance of total bacteria was at least 3 log higher than the ones quantified by cultivable methods (Figures 7 and 8, respectively). However, this difference was much higher in the C+ PBR, 4.1 logs, indicating a high abundance of non-cultivable bacteria in consortia with *C. vulgaris*, which was kept stable during the 9 d of the experiment. At the end of the experiment, the ratio of abundance of the 16S rRNA gene and total heterotrophs was reduced in the CV, and slightly in the C− PBR, possibly due to the release of algae metabolites that favored the cultivability of some UWW bacterial groups.

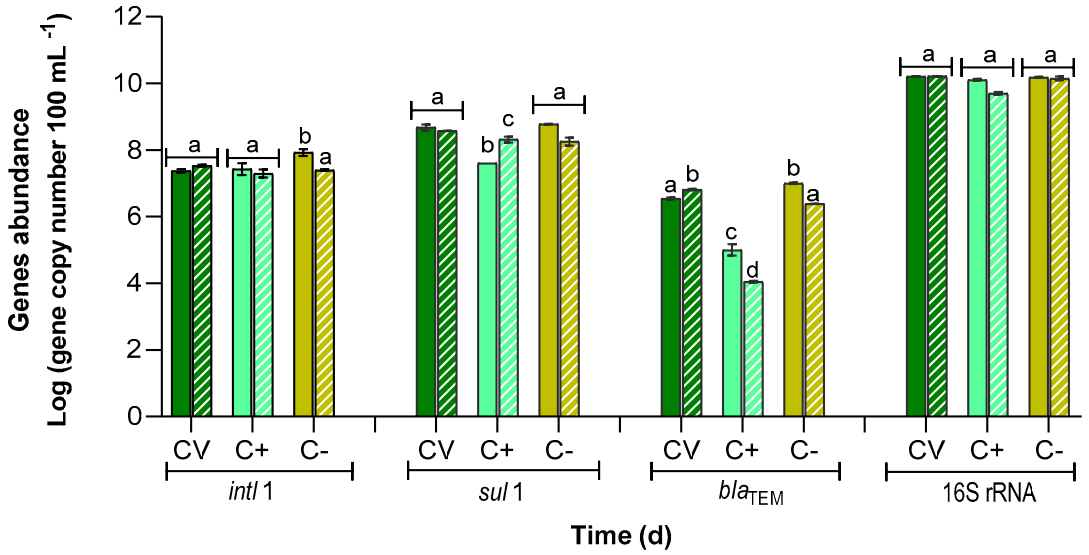

Figure 8. The abundance of the 16S rRNA, *intl*1, and antibiotic resistance genes (*sul*1 and bla_{TEM}) detected in each PBR: ■ CV (*C. vulgaris* + UWW); ■ C+ (*C. vulgaris* + autoclaved UWW); and ■ C− (raw UWW), at the beginning of the experiment (full colored bars) and after 9 d of treatment (striped bars). Results (average ± standard deviation) are expressed in a log (gene copy number 100 mL^{-1}). Bars of the same gene with the same letters are not statistically different (two-way ANOVA followed by Tukey post hoc test $p < 0.05$).

The *intI*1 gene encoding the class 1 integrons integrase, found in pathogenic gram-negative and commensal bacteria of humans and domestic animals, is commonly used as a marker for anthropogenic pollution [61]. Moreover, this gene is usually related to ARGs, namely *sul*1 since *intI*1 encodes for an integrase responsible for the insertion and excision of exogenous gene cassettes [62]. The ARGs bla_{TEM} and *sul*1 are related to beta-lactams and sulfonamides resistance, respectively [32,63]. The reduction of *intI*1 and ARGs can occur via the removal of living cells containing these genes and the loss of ARG-containing plasmids [24,62].

Although the *C. vulgaris* inoculum used in this study did not have an environmental origin, it was revealed to be non-axenic and possibly in a consortium with bacteria carrying *intl*1, *sul*1, and bla_{TEM} genes, with an abundance of 7.4 ± 0.2, 7.6 ± 0.1 and 5.0 ± 0.2 log (gene copy number 100 mL^{-1}), respectively (C+, Figure 8).

In terms of microalgae's ability to reduce *intl*1 and ARGs from UWW, *C. vulgaris* appears to not have any significant ($p < 0.05$) effect in terms of log reduction, RRG and prevalence of these genes (CV and C+, Figure 8, Table 4). In contrast, AMS from the C− PBR may have contributed to the reduction of the *intl*1 (0.5 log reduction, $p < 0.05$) and bla_{TEM} (0.6 log reduction, $p < 0.05$) genes abundance after 9 d, corresponding to percentages of RRG of 70.4 ± 9.2%, 69.2 ± 7.5%, and 75.7 ± 1.6%, for *intl*1, *sul*1, and bla_{TEM} respectively (Figure 8, Table 4). Additionally, a significant decrease in the prevalence of the bla_{TEM} gene after 9 d was registered in the C-PBR (Table 4).

The obtained results are in line with similar studies. As observed by Cheng et al. [37] with *Galdieria sulphuraria*, AMS treatment also reduced *intl*1 abundance, indicating that the microalgal treatment could reduce the spread of resistance. Additionally, AMS's ability to reduce bla_{TEM} abundance was 0.1 log higher when compared to the *Chlorella sorokiniana-Brevundimonas basaltis* consortium, as studied by Silva Rodrigues et al. [40]. Recently, Tang et al. [24] observed that a *C. vulgaris -B. licheniformis* consortium was able to promote *sul*1 reduction of abundance due to the efficient removal of exogenous plasmids carrying ARGs such as *sul*1. They observed that microalgal cells (i) absorbed and endocytosed the exogenous plasmids carrying *sul*1, with no affection of the microalgae cell nucleus, (ii) EPS promoted and increased the adsorption of exogenous ARG-containing plasmids, and

(iii) endocytosis increased with a larger time (more than 6 d) of contact between microalgae and plasmids [24]. This study [24] was performed in synthetic media instead of WW, with a controlled microbial population, and with the different operating conditions of temperature (30 °C), light exposure (120 µmol m^{-2} s^{-1}), and cultivation time (10 d). However, in a real scenario using real WW, there are no guarantees that the same mechanisms may occur straightforwardly due to the complexity of WW composition, and the unlikeliness of the occurrence of intact plasmids in the environment. Nevertheless, the capacity of microalgae to adsorb extracellular DNA fragments containing ARGs should be studied in the future.

Table 4. Relative abundance (prevalence) of *intI*1 and resistance genes (*sul*1 and bla_{TEM}) at the beginning (T0) and at the end (T9) of the experiment, and percentage of the reduction of resistance genes after the treatment (RRG), determined for each PBR. For each gene, values (average ± standard deviation) with the same superscript letters are not statistically different ($p < 0.05$).

PBR	*intI*1 Prevalence		RRG (%)	*sul*1 Prevalence		RRG (%)	bla_{TEM} Prevalence		RRG (%)
	T0	T9		T0	T9		T0	T9	
CV	$(1.5 \pm 0.1) \times 10^{-3\,a}$	$(2.0 \pm 0.1) \times 10^{-3\,a}$	$-40.0 \pm 6.1^{\,a}$	$(3.0 \pm 0.1) \times 10^{-2\,a}$	$(2.3 \pm 0.1) \times 10^{-2\,a}$	$22.3 \pm 13.9^{\,a}$	$(2.2 \pm 0.1) \times 10^{-4\,a}$	$(4.0 \pm 0.3) \times 10^{-4\,b}$	$-85.6 \pm 4.9^{\,a}$
C+	$(2.2 \pm 1.0) \times 10^{-3\,a,b}$	$(4.0 \pm 1.5) \times 10^{-3\,a,b}$	$27.2 \pm 54.1^{\,b}$	$(3.1 \pm 0.1) \times 10^{-3\,a}$	$(4.2 \pm 0.1) \times 10^{-2\,a}$	$-422.6 \pm 113.5^{\,b}$	$(8.1 \pm 2.4) \times 10^{-6\,c}$	$(2.2 \pm 0.1) \times 10^{-6\,d}$	$89.2 \pm 5.1^{\,b}$
C−	$(5.6 \pm 1.2) \times 10^{-3\,b}$	$(1.8 \pm 0.3) \times 10^{-3\,a,c}$	$70.4 \pm 9.2^{\,c}$	$(3.9 \pm 0.1) \times 10^{-2\,a}$	$(1.3 \pm 0.1) \times 10^{-2\,a}$	$69.2 \pm 7.5^{\,c}$	$(6.6 \pm 0.1) \times 10^{-4\,e}$	$(1.7 \pm 0.2) \times 10^{-4\,a}$	$75.7 \pm 1.6^{\,c}$

Due to the promising results of AMS in the C− PBR, the treated water was tested for its stability in terms of 16S rRNA, *intI*1, and ARG content (Figure 9), envisioning a possible treated water reutilization scenario. Storage revealed a decrease of 16S rRNA abundance (Figure 9A), as well as a reduction of *intI*1, *sul*1, and bla_{TEM} abundance, with log reductions of 1.0, 0.7, and 2.5, respectively, when compared to values obtained at the end of the experiment (T9), corresponding to an RRG of 97.1, 94.2 and 99.9% from the beginning of the assay (T0) up to the end of the storage period (ST5d). Microalgae surface adsorbed bacteria co-precipitation might have had a role in this reduction, probably by a reconfiguration of the bulk water microbial composition [14].

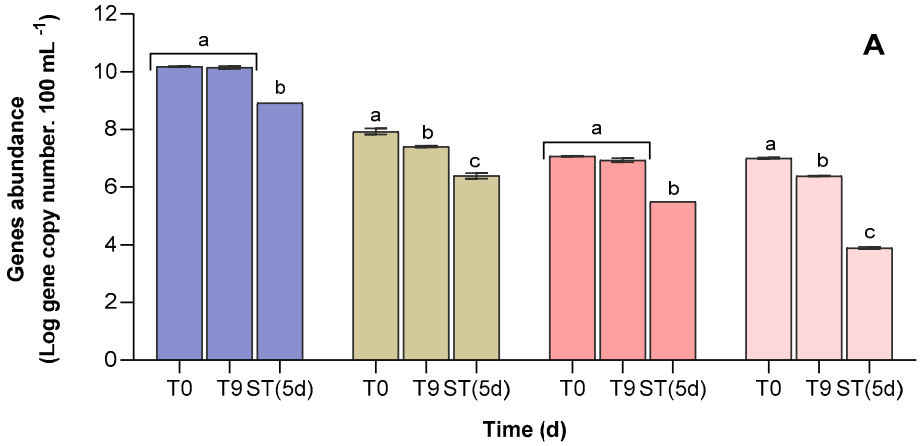

Figure 9. *Cont.*

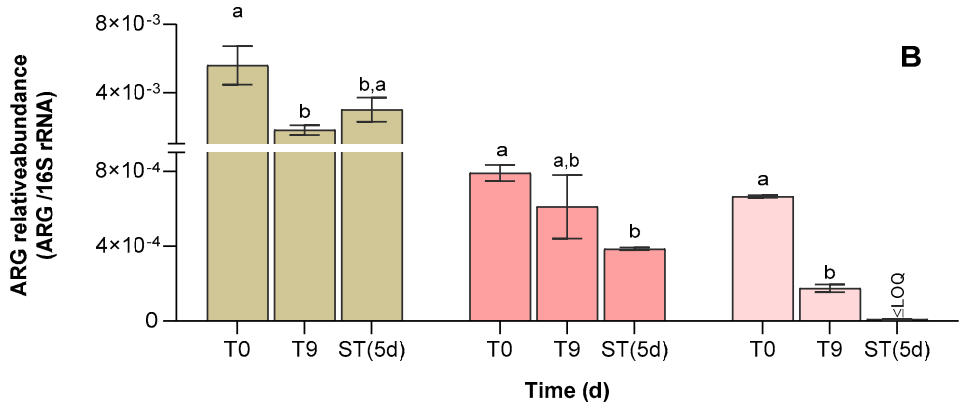

Figure 9. Abundance of the 16S rRNA, *intl*1and the antibiotic resistance genes *sul*1 and bla_{TEM} (**A**) and their relative abundance (prevalence) (**B**) in the C− (raw UWW) PBR over time and after 5 d of water storage (ST5d). Results (average ± standard deviation) of abundance are expressed in gene log (gene copy number 100 mL^{-1}) and genes' relative abundance is normalized according to 16S rRNA. Bars of the same gene with the same letter are not statistically different (two-way ANOVA followed by Tukey post hoc test $p < 0.05$).

4. Conclusions

In conclusion, the use of microalgal systems as a polishing treatment for UWW seems to be successful. This study demonstrated that the growth of both *C. vulgaris* and AMS in UWW promotes the removal of conventional pollutants such as PO_4–P and the reduction of *E. coli* and other coliforms' abundance. However, when comparing specific parameters of both microalgal systems, the use of AMS seems to be more promising due to the high (i) growth rate (μ_{max} of 0.687 ± 0.065 d^{-1}), and biomass productivity (9.0 ± 0.3) × 10^4, (ii) removal rate of PO_4-P (c.a. 92%), (iii) reduction of *intI*1 (0.5 log) and bla_{TEM} (0.6 log) genes' abundance after 9 d, and (iv) reduction of *intl*1 (1 log), *sul*1 (0.5 log), and bla_{TEM} (2.5 log) genes' abundance and their prevalence in treated water stored for 5 d. For future work, it should be interesting to identify the microalgal and bacterial species in the AMS system. Moreover, monitoring the AMS species and wastewater composition, namely in terms of nitrogen and phosphorus, would be helpful in understanding the dynamics of this system. As discussed throughout the present work, abiotic factors such as light intensity have a main role in both microalgal growth and the reduction of pathogens' proliferation, namely in conjugation with pH. Hence, studies considering the light intensity and pH ranges would be helpful in optimizing the potential of AMS. Moreover, due to the scarcity of information related to the microalgal mechanisms involved in ARGs reduction of abundance and prevalence in real wastewater, future studies should focus on these events.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app13074266/s1, Figure S1. Pearson correlation of all analyzed variables obtained for each PBRs: (A) CV (*Chlorella vulgaris* + UWW); (B) positive control, C+ positive control (*C. vulgaris* + autoclaved UWW); and (C) negative control, C− (raw UWW). Variables: pH; HA: humic acid concentration; COD: chemical oxygen demand concentration; MCC: microalgal cell concentration; PO_4–P: Phosphate-phosphorus concentration; OFC: other faecal coliforms except *Escherichia. coli* concentration; Ec: *E. coli* concentration; TH: Total heterotrophs concentration. White squares correspond to non-determined values.

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

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