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Alkalinity, and Not the Oxidation State of the Organic Substrate, Is the Key Factor in Domestic Wastewater Treatment by Mixed Cultures of Purple Phototrophic Bacteria

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Abstract: Domestic wastewater treatment by purple phototrophic bacteria (PPB) is based on the assimilative uptake of organics and nutrients into the bacterial biomass. Thereby, it strongly depends on the carbon/nutrients ratio of the wastewater. The physiological COD/N/P ratio for PPB growth in domestic wastewater makes the addition of an external organic carbon source necessary in order to allow for an efficient process. However, PPB need a source of alkalinity (as CO_2) to grow on reduced organics that serves as an electron acceptor since biohydrogen production (an alternative electron sink) is inhibited by ammonium. A preliminary experiment showed that high nutrients-loading wastewater was limited by CO_2 imbalance, leading to poor removal efficiencies. Subsequently, the effect of the oxidation state of the organics added as external organic carbon sources to PPB reactors treating low nutrients-loading domestic wastewater has been analyzed. Three organics were used as additives to PPB development in four consecutive batches: acetate (more oxidized), ethanol and butyrate (more reduced). The PPB population was settled and the general performance under the three situations, in terms of organics, N and P assimilation, and growth kinetics was not significantly different irrespective of the external organic carbon source. The reactors were dominated by PPB, though reduced organics allowed for dominance of *Rhodopseudomonas palustris*, whereas oxidized organics caused co-dominance of *R. palustris* and *Rhodobacter capsulatus*. Thereby, alkalinity (as bicarbonate), and not the oxidation state of the organics, is the key parameter for the efficient treatment of domestic wastewater by PPB.

Keywords: purple phototrophic bacteria; emerging anaerobic processes; phototrophic mechanisms; partition-release-recovery

1. Introduction

Purple phototrophic bacteria (PPB) are a wide group of anoxygenic phototrophs that are highly metabolically versatile. This versatility led PPB to be proposed as key actors in a novel platform for anaerobic wastewater treatment with resource recovery [1]. Photoheterotrophy is probably their most

interesting metabolic pathway as it entails organics and nutrients assimilation into biomass with almost complete recycle of C and electrons, thereby not producing CO_2. These bacteria use preferentially low energy infrared light, contrary to other photosynthetic microorganisms as algae and cyanobacteria. Incident photons (from light) generate an electron transport chain (ETC) and proton gradient and allow energy capture as adenosine tri-phosphate (ATP) [2]. Both the Tricarboxylic Acid (TCA) and the Calvin-Bensom-Bassham (CBB) cycles are involved in the central metabolism. The TCA cycle produces CO_2 when the organic substrate is assimilated and provides electrons to fuel the ETC. The CBB cycle synthesizes organic molecules (anoxygenic photosynthesis), where CO_2 is extraordinarily efficient as an electron acceptor. McKinlay and Harwood have demonstrated that "photoheterotrophic growth is associated with a challenge in maintaining redox balance" and that CO_2 fixation in the CBB is a fundamental feature of metabolism in photoheterotrophic species [3]. They confirmed that CO_2 fixation was necessary to dissipate the excess of electrons when a more reduced substrate than biomass, such as butyrate, is used. An alternative electron sink is H_2, which can be produced via the nitrogenase complex [4]. Hydrogen production is strongly inhibited by ammonium and so is not an option in the case of typical wastewater sources containing ammonium, as domestic wastewater (DWW) [5]. Therefore, without the option of additional CO_2 fixation, PPB should theoretically be unable to grow on a substrate more reduced than biomass.

Recently, PPB have been proposed for domestic wastewater treatment (DWWT) as a single-stage process that is able to simultaneously reduce C, N, and P parameters below discharge limits through assimilative (not oxidative) uptake [6]. However, full nutrients recovery from DWW by PPB requires modifying the COD/nutrients ratio in order to align with the physiological COD/N/P ratio for PPB growth, which is around 100/7.1/1.8 [7]. There are two valid strategies for achieving this. The first strategy is to reduce nutrients from the original source, e.g., by separate collection [8]. This strategy works well irrespectively of the characteristics of the DWW but depends on a full modification of the sewerage system. The second strategy consists of adding an external organic carbon source [6]. This can be done by supplying commercial organics (less sustainable); supplying organics from other organic waste sources (implying nearness to the waste source); or by internal recycling of organics from PPB biomass through bacterial solubilization and subsequent separation of the organics from the nutrients matrix, e.g., by thermal hydrolysis, thermal liquefaction, thermophilic fermentation, or cavitation [9]. However, typical organics extracted by these ways are normally reduced (as ethanol, propionate, and butyrate), and therefore the re-assimilation of these organics into PPB would need an extra source of electron acceptors. The heterogeneous composition of DWW leads to thinking that other oxidized organics and inorganics, mainly alkalinity, could provide the conditions to allow PPB growth on reduced organics. However, this axiom has never been demonstrated experimentally.

The aim of this work is to investigate the effect of the redox state of the external organic carbon source added to DWW in order to assess the PPB growth capability. The role of alkalinity in the assimilation of reduced organics is also analyzed. Finally, variations in PPB mixed community dynamics are studied based on the organics supplied to the DWW. We conclude that bicarbonate alkalinity is a strong factor to check in the scale-up of the PPB technology for domestic wastewater treatment. The mixed cultures of PPB can evolve irrespectively from the oxidation state of the organic source if there is enough bicarbonate alkalinity to allow for the release of the excess of electrons.

2. Materials and Methods

2.1. Source of Wastewater

Two sources of domestic wastewater were used in this study, a low COD/nutrients ratio (DWW-1) and high COD/nutrients ratio (DWW-2). The DWW-1 was acquired from a pilot DWWTP located in the University Rey Juan Carlos, Móstoles, Spain. Average characteristics were as follows (mg L^{-1}, n = 5, SD in brackets): TCOD = 968 (72), SCOD = 427 (107), NH_4^+-N = 40 (7), PO_4^{3-}-P = 6 (2), alkalinity (as HCO_3^-) = 831 (50), with a COD/N/P ratio of 100/10 (3)/1.5 (0.8). The DWW-2 was sourced from a

municipal WWTP located in Mostoles, Spain. Average characteristics were as follows (mg L^{-1}, n = 3, SD in brackets): TCOD = 302 (13), SCOD = 159 (7), NH_4^+-N = 116 (7); PO_4^{3-}-P = 9 (2); alkalinity (as HCO_3^-) = 553 (78), with a COD/N/P ratio of 100/73 (1)/5.4 (1.2). Raw wastewater was used directly without prior settling.

2.2. Mixed Enrichment Culture of Purple Phototrophic Bacteria

A mixed PPB culture was enriched from the DWW-1 above-described. The wastewater was supplemented with 2 gCOD/L of acetic acid. Ammonium chloride and sodium dihydrogen phosphate were added to reach a COD/N/P ratio of 100/7.1/1.8. The culture was enriched in 10 L ISO glass bottles during 7 days in anaerobic conditions, pH 6.5 and at room temperature, illuminated with infrared lamps (Philips BR125 IR 150W), and covered with VIS/UV filters (ND 1.2 299, Transformation Tubes, Banstead, UK), providing 70 W/m^2 of near infrared (NIR) irradiance. Following enrichment, the culture was continuously stirred and refreshed weekly with 90% fresh wastewater under the above described conditions.

2.3. Experimental Design

2.3.1. Preliminary Tests on the Effect of Alkalinity on Domestic Wastewater Treatment by Purple Phototrophic Bacteria

A preliminary experiment focused on the role of alkalinity over the PPB metabolism during DWW treatment was firstly performed. The experiment compares the two strategies for enabling DWW by PPB: (1) nutrients removal in origin and (2) addition of an external organic carbon source, both focused on tuning the COD/N/P ratio to align with the physiological one of PPB. In the first strategy, N is removed by alkaline stripping, whereas P is removed by $FeCl_3$ precipitation, thus emulating reduction in origin that essentially decreases nutrients content of the DWW [10]. In the second strategy, a complex artificial organic source was added to the DWW that was composed by a 1:1:1:1 mixture of acetate, propionate, butyrate, and ethanol (in COD terms). Subsequently, the wastewater was inoculated with 100 mgVSS/L of the PPB mixed enrichment culture and pH was adjusted to 6.5 in both cases. The experiments were accompanied with two controls where both types of raw wastewater were used with no modifications. All the experiments were performed in triplicated 100 mL serum bottles in anaerobic conditions for around 2 days as described elsewhere [7]. Resulting initial concentrations of TCOD, SCOD, TSS, VSS, NH_4^+-N and PO_4^{3-}-P in the serum bottles are shown in Table 1.

Table 1. Initial concentrations (with 95% confidence intervals) of main parameters in preliminary experiments (in mg/L).

	TCOD *	SCOD	TSS *	VSS *	NH_4^+-N	PO_4^{3-}-P
DWW-1						
Control	363	197 ± 2	250	245	32 ± 1	3.7 ± 0.2
(1) *Nutrients removed*	372	205 ± 5	240	200	17 ± 2	2.3 ± 0.2
(2) *Substrate added*	495	305 ± 10	260	230	30 ± 2	3.8 ± 0.2
DWW-2						
Control	472	124 ± 10	221	195	88 ± 9	7.4 ± 0.1
(1) *Nutrients removed*	410	128 ± 9	160	142	11.9 ± 0.2	3.7 ± 0.1
(2) *Substrate added*	1701	1039 ± 144	261	235	92 ± 8	7.9 ± 0.1

* Some parameters were measured just once.

2.3.2. Effect of Additional Organics with Different Redox State on Domestic Wastewater Treatment by Purple Phototrophic Bacteria

The central experiment of this study consisted of an analysis of the long-term effect caused by the addition of external organic carbon sources with different oxidation states on the development

of a mixed culture of PPB in DWW. In order to avoid limitations derived from a lack of alkalinity, the study was performed with the DWW-1. Three anaerobic 10 L reactors covered with UV/VIS light filters (ND 1.2 299, Transformation Tubes) were used for biomass enrichment. Each of the reactors was magnetically stirred and illuminated continuously with infrared lamps (Philips BR125 IR 150W) providing around 70 W/m^2 of NIR light (750–1200 nm). Each reactor was closed with a special lid fitted with both a sampling line with tubing submerged in the liquid, and a gas line connecting the headspace to a gas airbag filled with N_2 to maintain anaerobic conditions and atmospheric pressure in the headspace.

The reactors were not inoculated, and the phototrophic mixed culture was developed from the inner microorganisms already present in the DWW, following the recommendations by Hülsen, Barry, Lu, Puyol, Keller and Batstone [6]. The reactors were operated in batch mode by supplying DWW and external organic carbon sources once per week to achieve a COD/N/P ratio of 100/7.1/1.8, for a total of 4 enrichment rounds. Initial pH was fixed to 6.5. 9 L of culture broth was discarded at the end of each round before adding fresh DWW and external organic carbon source up to 10 L. Reactor's walls were cleaned at the end of each round and the attached biomass was mixed thoroughly with the rest of the culture before refreshment in order to minimize the biofilm growth. Organic substrates tested were Acetate (Reactor 1, R1), Ethanol (Reactor 2, R2), and Butyrate (Reactor 3, R3). Acetate is more oxidized than PPB biomass, thereby producing CO_2 during normal PPB metabolism. Ethanol and butyrate are more reduced than biomass, and therefore PPB theoretically needs to fix CO_2 in order to assimilate these substrates because the presence of ammonium in DWW inhibits the nitrogenase complex [3]. The main difference between the reduced organics is that butyrate can directly enter the TCA cycle (via butyryl-CoA), whereas ethanol follows a previous pathway to be oxidized to acetate before entering the TCA cycle. Sampling was performed daily for measuring DWW characteristics and VIS/IR spectra, whereas biomass was sampled at the beginning and end of each round for microbial characterization.

2.4. Analytical Methods

The TCOD, SCOD, NH_4^+-N and PO_4^{3-}-P were measured by using specific kits (Merck, Darmstadt, Germany). TSS/VSS were measured according to Standard Methods. Volatile fatty acids (VFAs) and ethanol were measured by HPLC/IR. Samples were filtered by using a 0.45 µm syringe filters in order to determine soluble components (SCOD, NH_4-N PO_4^{3-}-P and VFAs). Biomass absorption spectra (400–950 nm) were analyzed by using a JASCO V-630 UV-VIS (Madrid, Spain). Biomass growth was monitored at 660 nm. A linear response between the absorbance at 660 nm and the VSS values was ensured due to the planktonic nature of the enrichment cultures, where the calibration curve was determined to be Abs660 = 0.0021·VSS (mg/L) (R^2 = 0.989). More details about analytical procedures can be found at [11].

Illumina NGS and DGGE

DNA was extracted from PPB samples at the end of the enrichment cycles by Kit UltraClean Microbial DNA Isolation (Mobio, Inc., Solana Beach, CA, USA). Purified DNAs were quantified by Picogreen and (0.5–3 ng) of DNA was used for a first amplification of the 16SrRNA gene using primers (5′-ACACTGACGACATGGTTCTACACCTACGGGNGGCWGCAG-3′ and 5′-TACGGTAGCAGAGA CTTGGTCTGACTACHVGGGTATCTAATCC-3′) which amplify the V3-V4 region of 16S. A second reamplification was performed with primers (5′-AATGATACGGCGACCACCGAGATCTACACTG ACGACATGGTTCTACA-3′ and 5′-CAAGCAGAAGACGGCATACGAGAT-[10 nucleotides barcode]-TACGGTAGCAGAGACTTGGTCT-3′) of the Access Array Barcode Library for Illumina Sequencers (Fluidigm). These primers contained a 5′ oligonucleotide tail used to allow sequencing in Illumina Mi-Seq PCR. Amplicons were denatured prior to be seeded on a flowcell, where clusters were formed and sequenced using a "MiSeq Reagent Kit v3", in a 2 × 300 pair-end sequencing run on a MiSeq sequencer. For 16S sequencing, reads were mapped against the GreenGenes database using current applications of Base Space (Metagenomics 16S, Illumina, San Diego, CA, USA). DNA samples were also

performed by denaturing gradient gel electrophoresis (DGGE) according to Molina, et al. [12]. PufM gene (photosynthetic reaction center M subunit) were utilized as molecular marker and amplified using the primers set M-150F and M-572R [13]. DGGE bands were excised and processed as followed by de las Heras et al. 2020 [14] to confirm the most abundant strains identified with Illumina MiSeq.

2.5. Data Handling and Statistical Procedures

Observed biomass yield (mgVSS/mgCOD) was calculated accounting with the biomass production in each experiment (in mg VSS) in relation to the total organic substrate consumption (in mg COD). The maximum specific growth rate (μ_{max}, 1/d) was calculated as the slope of the linear regression between Ln of absorbances (at 660 nm) in the exponential growth phase and the experimental time. Confidences intervals for μ_{max} (at 95%) were calculated from the standard error estimated in the linear regression from triplicate measurements.

Two-way analysis of variance (ANOVA) was performed over response-dependent variables resulted from the central experiment in this work in order to analyze the significance of the differences between data means from these variables. These variables were μ_{max}, COD/N and COD/P uptake ratios, and observed biomass yield (Y_{OB}). Differences were considered significant for a p-value < 0.05. The previous assumptions of normality and homoscedasticity were checked by the Shapiro-Wilk and the Levene's tests, respectively.

From abundance and identification of the strains by Illumina, community diversity (Shannon-Wiener and Simpson index), community richness, and Pielou Evenness Index were calculated. The similarity among whole samples was computed using the Bray-Curtis distance with the function vegdist in the R studio package Vegan [15] using the index similarity coefficient. The dendrogram was generated using the method of unweighted pair group with arithmetic mean (UPGMA) with the function hclust of the stats package in R studio (version 1.2.5033) at 1% position tolerance.

3. Results and Discussion

3.1. Preliminary Tests

The performance of the batch tests in the preliminary analysis is depicted in Figure 1, where the two strategies for enabling DWW by PPB are compared. It is noteworthy that the first strategy works well independently from the type of wastewater. In the experiments using both DWW-1 and DWW-2, the reduction of nutrients before the experimental setup allowed the PPB cultures to uptake the SCOD, N and P until achieving discharge limits (e.g., <125 mgCOD/L, <10 mgN/L, <1 mgP/L). Besides, the absolute values of SCOD, N and P uptake were not significantly different to those obtained by the control experiment in both cases ($p > 0.05$ in all cases based on two-tailed t-tests), suggesting that the microbial process is being performed by the same community. Thereby, it can be inferred that the initial reduction of nutrients does not affect the biomass development and it is a suitable strategy for enabling efficient DWW by PPB. However, it involves a complete modification of the sewerage system and therefore is a high-cost option that may be suitable in a long-term strategy [16].

The second strategy implies the addition of an external organic carbon source to improve N and P uptake by the PPB community. This strategy only works if COD/nutrients ratio is not too high, otherwise a lot of inorganic carbon (as CO_2) is required for assimilative uptake of reduced organics. Once CO_2 is depleted or becomes unavailable, the PPB biomass is not able to assimilate organics anymore. This was the case with the DWW-2 experiment, the main results of which are shown in Figure 1a. Initial total alkalinity (bicarbonate + carbonate) of this wastewater was 553 ± 40 mg/L. According to the initial composition of the wastewater and the addition of the external organic carbon sources [7,17], the bicarbonate needs for the assimilation are 256 mg/L. However, at the final pH (around 9), most of the carbonate might become precipitated (thus bio-unavailable). Final alkalinity is 105 mg/L, suggesting that it strongly limited the organics uptake. This was evidenced by the final composition of the VFAs in the media, where more than 50% was composed by butyrate, which indeed

is the most CO_2 demanding substrate of those used as additional carbon source in this experiment. Moderate COD/nutrients ratio reduces considerably the CO_2 needs for reduced organics uptake, as was the case with DWW-1. Figure 1b shows a COD assimilation high enough to comply with discharge limits in the experiment where the external organic carbon source was added. Initial alkalinity of this wastewater was 831 ± 50 mg/L. According to the initial composition of the wastewater and the addition of the external organic carbon sources [7,17], CO_2 fixation needs to be 79 mg/L of alkalinity. Despite the potential carbonate precipitation caused by the final high pH (around 8.5), the alkalinity was high enough for maintaining reduced organics uptake. In fact, only in the case of DWW-1 was it possible to achieve discharge values of COD, N and P (<125 mgCOD/L, <10 mgN/L, <1 mgP/L). Therefore, in short-term experiments it seems that alkalinity is the limiting factor for the PPB to be able to assimilate organics. In any case, it is important to know the system's performance when alkalinity is not limiting, and thus long-term experiments were conducted to study the effect of different organics on the behavior of PPB mixed cultures.

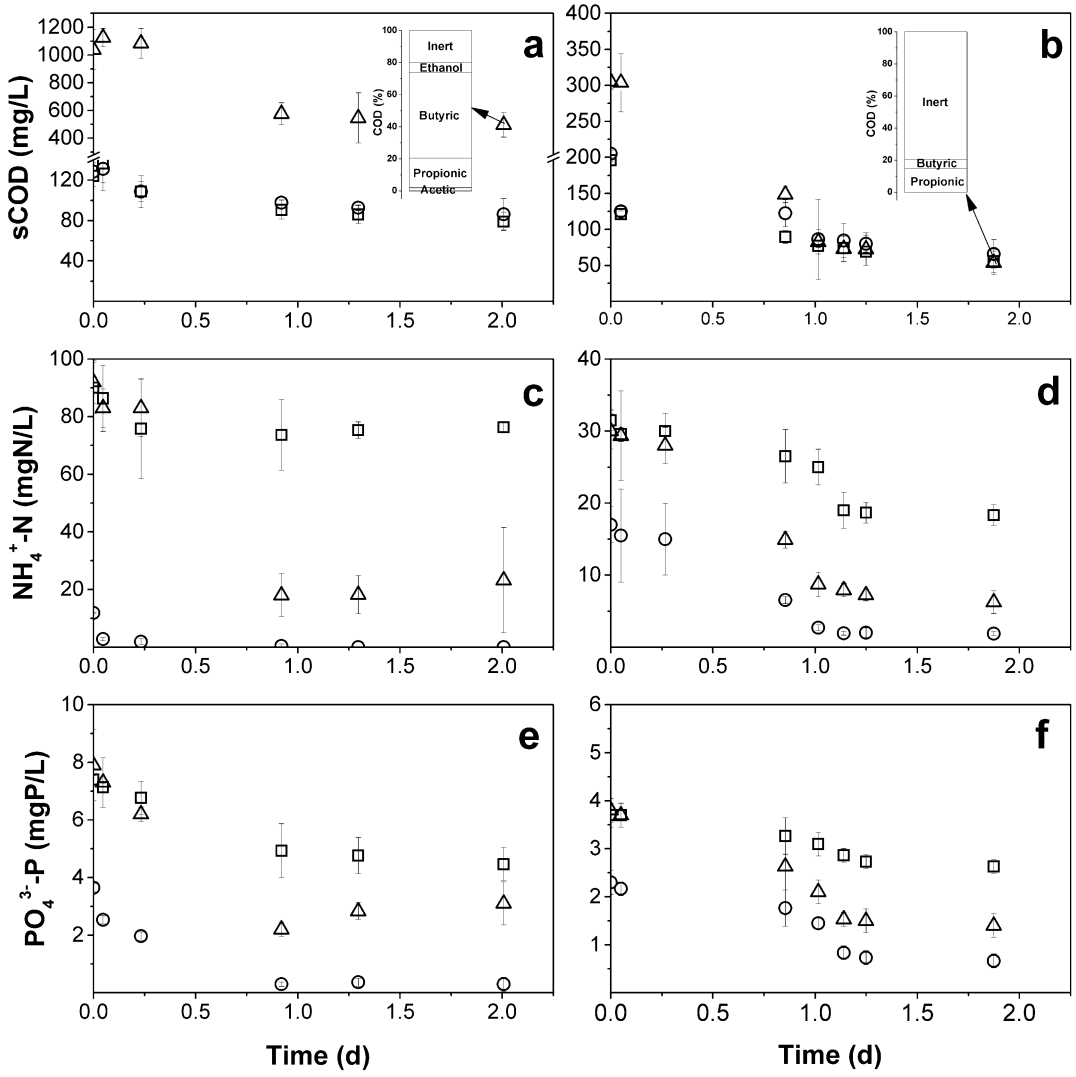

Figure 1. Time course of soluble COD, NH_4^+-N and PO_4^{3-}-P in the preliminary experiments using low COD/nutrients ratio (DWW-2, panels (**a**,**c**,**e**)) and high COD/nutrients ratio (DWW-1, panels (**b**,**d**,**f**)) domestic wastewater, respectively, in different conditions: using original COD/nutrients ratio (squares), or modifying this ratio by previous nutrients removal (circles) or by adding external organic carbon source (triangles), in order to comply with physiological COD/nutrients ratio. Inserts in panels (**a**,**b**) are COD removal of the final sample.

3.2. Process Performance Based on the Oxidation State of the Additional Organic Substrate

The organics and nutrients assimilation were analyzed in long-term experiments. In the first enrichment round, all the reactors needed around 3 d to develop a stable microbial community. The appearance of specific absorbance peaks at wavelengths of 590, 805, and 865 nm corresponds to bacteriochlorophyll (Bchl) *a* [18]. Also, the increase in absorbance at 660 nm (optical density -OD- for bacterial growth) strongly correlated with that at the Bchl *a* absorbance wavelengths (Figure 2a–c). These results are indicative of strong enrichment in PPB, which will be analyzed in detail later on this work. From this day onwards, the PPB cultures began to assimilate the organics and nutrients while the biomass was growing (Figure 2d–f). Subsequent enrichments caused the assimilation process to be active from the beginning. COD/N/P assimilation ratios are depicted in Table 2. As shown, N partitioning was relatively constant in enrichment Rounds 2–4, irrespective of the addition of the external organic carbon source. Similar N removal was obtained, around 75% in all cases. P partitioning was also similar for all the external organic carbon sources, but somehow varied between rounds, and was particularly elevated during Round 2. This could be due to the difference in suspended solids composition of the DWW during Round 2 due to heavy rain, which may cause shading and insufficient IR radiation. Under high shading conditions, there is lower IR energy available and PPB biomass may accumulate P as poly-P, as has been previously suggested [19]. This caused an increase in luxury P removal from around 45–50% in the Rounds 1, 3 and 4 to 65–70% in the Round 2, irrespective to the external organic carbon source.

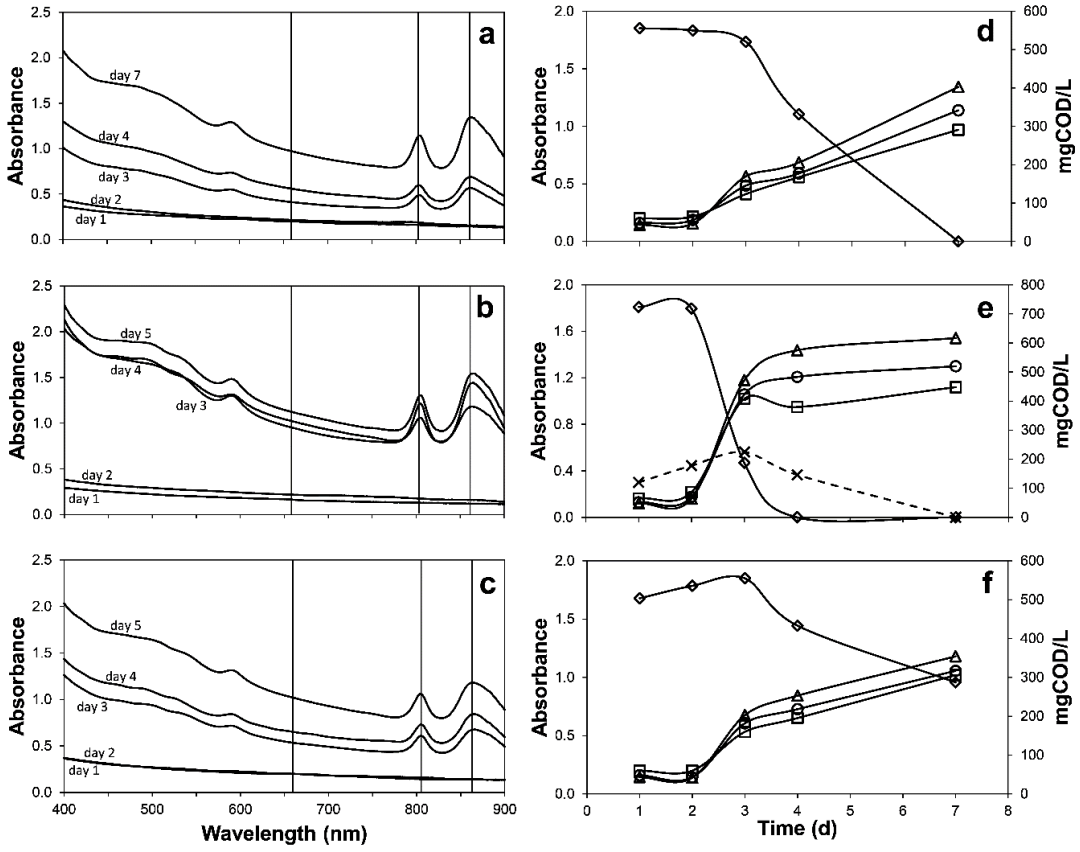

Figure 2. Round 1 VIS/NIR absorption spectra, and time course of absorbance at 660 (squares), 805 (circles), 864 (triangles) nm and substrate concentration (diamonds, in COD) for R1 (acetate, (**a**,**d**)), R2 (ethanol, (**b**,**e**)) and R3 (butyrate, (**c**,**f**)), respectively. Crosses in panel e show acetate evolution.

Table 2. Main growth parameters for the PPB mixed cultures upon each feed round in reactors R1 (acetate), R2 (ethanol), and R3 (butyrate).

Reactor/Round	COD/N/P Uptake Ratio	Observed Biomass Yield (mgVSS/mgSCOD)
R1—Acetate Feed		
Round 1	100/6.49/0.57	0.61
Round 2	100/8.07/1.28	0.34
Round 3	100/9.78/0.64	0.61
Round 4	100/6.63/0.61	0.97
R2—Ethanol Feed		
Round 1	100/4.60/0.43	0.88
Round 2	100/8.57/1.59	0.78
Round 3	100/8.07/0.68	0.39
Round 4	100/6.69/0.84	0.53
R3—Butyrate Feed		
Round 1	100/5.73/0.46	0.81
Round 2	100/10.56/2.19	0.75
Round 3	100/7.33/0.87	0.61
Round 4	100/9.45/1.01	0.74

There were some differences between the reactors in terms of substrate usage. In R1 (Figure 2d), rapid biomass growth is seen to start on 2nd day, but the consumption of acetate begins between the 3rd and 4th day, which suggests that another substrate (i.e., a component present in the DWW) is used preferentially. A similar trend is observed in R3 for butyrate (Figure 2f). In R2, however, the rapid biomass growth begins at the same time as ethanol consumption. Ethanol is not used directly for growth by PPB because acetic acid production is also observed and follows a different trend (like R1 and R3). Syntrophic oxidation of ethanol to acetic acid and hydrogen is proposed according to [7]. The acetic acid production was below the predicted by the stoichiometry of that process, and therefore it is suggested that acetate was consumed in parallel with the metabolization of ethanol.

The biomass growth over the four cycles was also analyzed. Figure 3 shows the time course of absorbance at 660, 805, and 865 nm using acetate (R1), ethanol (R2) and butyrate (R3) as external organic carbon sources. A maintained enrichment in PPB is observed in all cases, as evidenced by the concomitant increase of Abs at 805 and 865 with the increase of OD values. Indeed, ratios between Abs 865/660 and Abs 805/660 started from lower than 1 (indicating low PPB content) and ended in values higher than 1.1 and 1.3 for Abs 805/660 and Abs 865/660, respectively, along the four cycles. This is a clear indication that the biomass evolved from a non-phototrophic to a phototrophic consortium [14], and this consortium was maintained during the whole experimental setup. Generally, R2 (ethanol feed) seems to be more efficient in biomass growth during the Rounds 1 and 2, resulting in a higher Abs at 660, 805, and 865 nm compared to R1 and R3. This is also evidenced by a higher biomass yield during the Round 1 (see Table 2). During the Round 3 and 4, the R1 (acetate feed) was more efficient in growth and achieved the highest biomass yield values. This indicated that the enrichment was initially favoring heterogeneous metabolism (represented by ethanol), whereas more specific metabolism, represented by acetate, was enhanced in long-term operation and culture stabilization, which is also indicative of a strong enrichment [7]. A comparative analysis of growth kinetics was therefore performed to analyze with more detail these differences.

The specific growth rates were calculated from OD_{660} values (Figure 4). High values of μ_{max} (0.75–1.1 1/d) were observed at Round 1. These values subsequently decreased during the following rounds until achieving an equilibrium at around 0.4 1/d, which are like those observed in literature for pure cultures of PPB [3,20]. This decrease responds to the adaptation of the consortium to mixed-culture conditions, where bacteria compete for the substrate, and this usually derives in a decrease of the specific growth rate in benefit of the growth efficiency [21]. However, it seems that the different substrates did not cause an effect on the values of the μ_{max}. These observations were checked by a

two-way ANOVA analysis (Table 3), which confirmed that consecutive rounds caused a significant effect on μ_{max} values, whereas the factor substrate was not significant at 95% confidence. Thereby, it was statistically confirmed that the oxidation state of the external organic carbon source does not affect the growth kinetics of the PPB mixed cultures during DWW treatment. A similar analysis was conducted for the nutrient's uptake (N and P) and the apparent biomass yield.

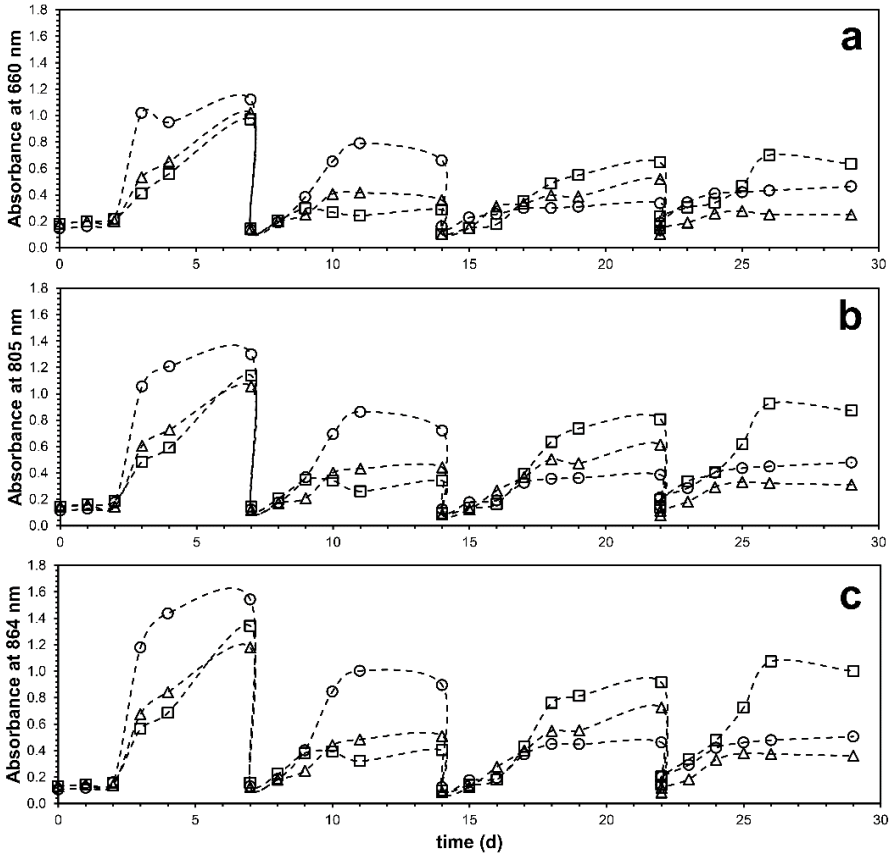

Figure 3. Time course of biomass growth expressed as absorbance at 660 (**a**), 805 (**b**), and 865 (**c**) nm in reactors R1 (acetate, squares), R2 (ethanol, circles) and R3 (butyrate, triangles).

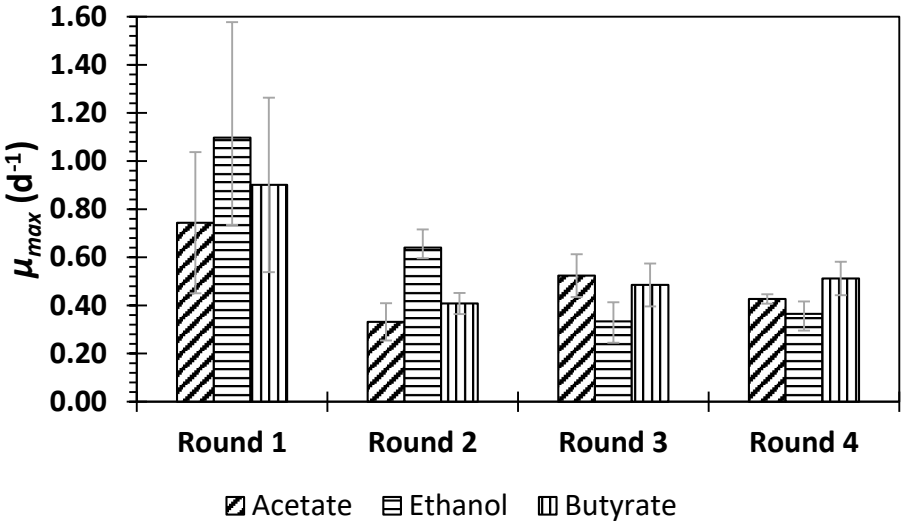

Figure 4. Specific growth rate values (μ_{max}) calculated for the different rounds in the reactors R1 (acetate), R2 (ethanol), and R3 (butyrate). Error bars are 95% confidence intervals from estimated values.

Table 3. ANOVA analysis on the effect of the addition of the external organic carbon source and the experimental round on the main growth parameters of the mixed cultures of purple phototrophic bacteria.

Source of Variance on μ_{max}	**SS ***	**df**	**MS**	**F**	***p*-Value**	**F Crit**
Substrate	0.022	2	0.011	0.532	0.613	5.143
Round #	0.491	3	0.164	7.934	0.016	4.757
Error	0.124	6	0.021			
Total	0.637	11				
Source of Variance on *COD/N*	**SS**	**df**	**MS**	**F**	***p*-Value**	**F Crit**
Substrate	3.333	2	1.666	0.972	0.431	5.143
Round #	20.179	3	6.726	3.925	0.073	4.757
Error	10.283	6	1.714			
Total	33.794	11				
Source of variance on *COD/P*	**SS**	**df**	**MS**	**F**	***p*-Value**	**F Crit**
Substrate	0.267	2	0.134	2.841	0.136	5.143
Round #	2.469	3	0.823	17.499	0.002	4.757
Error	0.282	6	0.047			
Total	3.018	11				
Source of Variance on $Y_{X/S}$	**SS**	**df**	**MS**	**F**	***p*-Value**	**F Crit**
Substrate	0.059	2	0.030	0.252	0.785	5.143
Round #	0.276	3	0.092	0.778	0.548	4.757
Error	0.709	6	0.118			
Total	1.045	11				

* SS: sum of squares; df: degrees of freedom; MS: mean squares; F: Fisher distribution value; *p*-value: probability of F </= Fcrit; Fcrit: critical value of F for accepting the Null Hypothesis.

Nutrients uptake was analyzed during the four rounds. As shown in Figure 5, simultaneous N and P uptake was generally observed during the whole experimental setup, irrespective of the addition of the external organic carbon source. This is indicative of the preferential usage of nutrients for growing, which is per the non-limiting and substrate-abundance conditions of the experiments. These conditions have been described as the optima for PPB-based processes [22]. The preeminent only exception was observed during Round 2. The fastest P uptake during the first hours of that feed round might be related to the preferential usage of P for building up polyphosphate because of low energy availability [19], as described previously. In any case, N uptake was more efficient than P uptake irrespective to the substrate used, as evidenced by a COD/N ratio that was very close to the theoretical optima (100/7.2, [7], see Table 2). Indeed, two-way ANOVA indicated that neither the substrate nor the round significantly affected the COD/N ratio. This entailed a high N removal, with final NH_4^+-N concentrations around 10 mgN/L, which are within discharge limits. P uptake was generally below the COD/P optima of 100/1.8 (see Table 2). However, with the previously mentioned exception found at Round 2, the COD/P ratio was increasing while the cultures were becoming more specialized, as evidenced by the significant effect of the round number on the COD/P ratio (Table 3). An increase in phosphorus uptake is indicative of an efficient usage of energy, where excess of ATP produced during the exponential growth phase is used for poly-phosphate production when the culture enters into a stationary growth phase [19]. In any case, neither N nor P uptake ratios were significantly different based on the addition of the external organic carbon source within a 95% confidence. This means that the oxidation state of the additional organic source did not affect the nutrients uptake of the PPB culture.

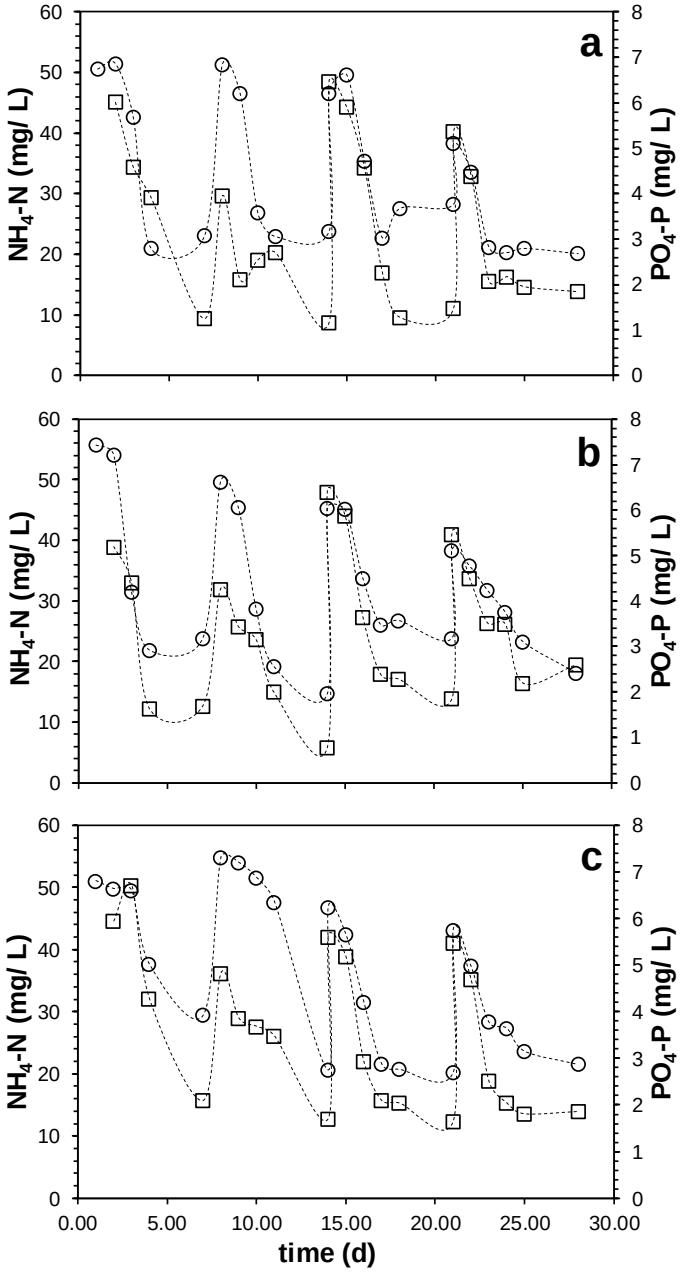

Figure 5. Time course of ammonium-N (squares) and phosphate-P (circles) in the reactors R1 (acetate, (**a**)), R2 (ethanol, (**b**)) and R3 (butyrate, (**c**)).

Finally, biomass yield values shown in Table 2 had a somehow high variability within 0.34–0.97 gVSS/gCOD, which are within typical values found in the literature for PPB mixed cultures [23]. These values were not significantly related, neither to the addition of the external organic carbon source nor to the feed round, at 95% confidence (Table 3). Again, this means that the oxidation state of the external organic carbon source did not cause a difference in the efficiency of the transformation of the substrate into biomass by the PPB mixed cultures developed in this work. Despite several works concluding that PPB behaves differently depending on the organic substrate [7,24], this work sustains that these metabolic-based differences do not pose a large difference in the overall performance of the mixed culture. In a recent paper, it has been concluded that controlling the general control parameters in wastewater treatment (like pH, oxygen, and alkalinity) is an efficient way to improve the performance of PPB-based wastewater treatment [25].

3.3. Effect of Organic Susbtrates on Microbial Community Development

The Initial microbial consortium has been growing during serial experimental rounds under selective pressure such as low infrared light and a solely organic carbon source (acetate, butyrate, and ethanol) favoring the enrichment and isolation of PPB [26]. The Illumina and DGGE identification confirmed that the most PPB abundant during all enrichment experiments were *Rhodobacter capsulatus* (94% similarity, Gen Bank Number AH000921.2) and *Rhodopseudomonas palustris* (100% similarity, Gen Bank Number AB015977.1). Results showed that the substrate type influences bacterial diversity (Figure 6) [27]. Although species related to *R. palustris* were able to grow under all 3 types of carbon sources and during all serial experimental rounds, there were two differentiated groups. One group (Initial and T1-Acetate) dominated by other bacteria showing high species richness and diversity (Table 4), whereas another group (T1 ethanol and butyrate; T4 Acetate, ethanol, and butyrate) was dominated by species related to *R. capsulatus* and *R. palustris* with lower species richness, bacterial diversity, and evenness. The oxidation state of the organic substrate affected bacterial diversity. Indeed, some *Rhodobacter* ssp. species were displaced by *Rhodopseudomonas spp.* when they grew on reduced organic carbon sources such as ethanol and butyrate. A previous work reported that a Rubisco-mutant of *R. palustris* was unable to grow on acetate, whereas a Rubisco-mutant of *Rhodobacter sphaeroides* (*R. sphaeroides*) was able by using the ethylmalonyl-CoA pathway as a way to reduce CO_2 [28,29]. Under reduced substrate feed, *R. palustris* is more efficient in using the CBB cycle, as in the case with butyric acid and ethanol. However, under acetic acid feed, *Rhodobacter* sp. can use the ethylmalonyl-CoA pathway, which allows competing with *Rhodopseudomonas* sp. This could explain the differential presence of bacteria depending on the substrate.

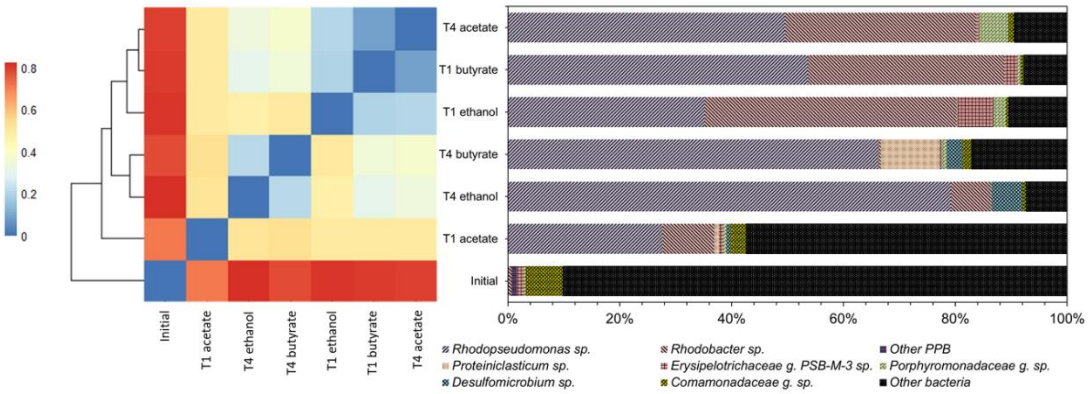

Figure 6. Hierarchical Clustering Heatmap Plot of dendrograms using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method. The heatmap represents a grid of colored points where each color represents a gradient of dissimilarity. The right panel shows the proportion of the most abundant genera found in the inoculum and in the photobioreactors upon the first and the fourth enrichment rounds, as resulted from Illumina 16S rRNA gene analysis.

Table 4. Community diversity indexes, community richness and Pielou Evenness Index at the first and fourth feed rounds with different carbon sources such as acetate, ethanol and butyrate.

Sample Name	Shannon-Wiener	Simpson's Diversity	Species Richness	Pielou Index (J')
Initial	3.83	28.84	188.00	0.51
T_1acetate	3.99	11.22	219.00	0.51
T_1ethanol	1.65	3.00	42.00	0.29
T_1butyrate	1.27	2.42	33.00	0.25
T_4acetate	1.44	2.74	41.00	0.27
T_4ethanol	0.97	1.57	31.00	0.20
T_4butyrate	1.65	2.20	58.00	0.28

Other ancillary species non-related with PPB were detected in low proportions and evolved mainly in the last feed rounds, especially in R2 and R3. The presence of some anaerobic fermentative bacteria belonging to genera *Proteiniclasticum*, *Desulfomicrobium*, and family *Porphyromonadaceae* indicated a transition of dominance in reactors fed with reduced substrates (R2 and R3). This might indicate bacterial decay that may become a substrate for these ancillary fermentative communities. These kinds of community dynamics during dominance transitions have been previously observed in other PPB-based photobioreactors [14].

4. Conclusions

It has been demonstrated that the general performance of the PPB mixed cultures was not affected by the oxidation state of the external organic carbon source. This contrasts with the strong effect of alkalinity availability shown in the preliminary experiment. Specific conclusions are:

- The similarity of growth parameters (μ_{max} and biomass yield), and the COD, N, and P removal efficiencies strongly suggest that the oxidation state of the external organic carbon source is not an impediment for the development of active PPB biomass in DWW treatment.
- High alkalinity supports the electron dissipation in PPB systems, allowing PPB to grow on reduced organics as butyrate and ethanol. Therefore, alkalinity, and not the oxidation state of the organic substrate, is the key limiting factor for PPB systems in DWW treatment.
- *Rhodopseudomonas palustris* prevails in reduced organics (butyrate and ethanol), whereas this species and *Rhodobacter capsulatus* co-dominate wastewater environments in oxidized organics (as acetate).

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