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A comparative study of the treatment of dark fermentation effluent by purple-phototrophic bacteria and microalgae with focus on substrate to biomass conversion

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ABSTRACT

The treatment of dark fermentation effluents from food waste was evaluated in two photobioreactor systems: a purple phototrophic bacteria (PPB) reactor and a microalgae-bacteria consortium (MBC) reactor. Experiments were performed at hydraulic retention times (HRT) of 5 and 10 days (P1 and P2, respectively) to maximize biomass yield for wastewater valorization.

At the microbiological level, the PPB reactor exhibited a decrease in PPB abundance with longer HRTs, favoring other genera. In contrast, the MBC reactor showed a marked reduction in microalgae under both conditions, with PPBs predominating in P1 and a diverse microbial community in P2. The increase in HRT from 5 to 10 days improved pollutant removal but did not enhance biomass concentration, which stabilized at 0.61 ± 0.08 g/L (PPB) and 1.37 ± 0.16 g/L (MBC) at 5-day HRT.

The highest biomass yield (1.03 ± 0.07 $gC_{biomass}/gTOC_{removed}$) was achieved in the MBC reactor at 5-day HRT, where preferential consumption of lactate and butyrate occurred, leaving acetate less assimilated. Despite the lower overall pollutant removal at 5-day HRT (TOC: 56.0 ± 3.5 %, TN: 60.3 ± 9.0 %, PO_4^{3-}: 20.4 ± 7.4 %), this condition allowed for higher conversions of dissolved carbon into biomass rather than full mineralization. This trade-off is advantageous when targeting biomass valorization over complete pollutant removal, especially considering the commercial value of the residual organic acids. These results highlight the potential of short HRT operations in MBC systems for industrial application, enabling efficient resource recovery from fermentation effluents through selective assimilation, while maximizing biomass productivity and minimizing loss of valuable organics.

1. Introduction

The worldwide growing interest in H_2 has promoted dark fermentation (DF) as a potential ecological alternative for the treatment of complex organic waste, such as food waste or tequila vinasse, along with the generation of clean energy [1,2]. DF is a biological process that includes the hydrolysis and acidogenesis stages of anaerobic digestion, which produce H_2, CO_2 and volatile fatty acids (VFAs) from carbohydrate-rich substrates [3]. It is, therefore, an incomplete treatment process, which releases an effluent rich in energy-dense VFAs along with nitrogen (ammonia) and phosphorus (orthophosphate) compounds. This effluent requires further treatment before being released into the environment [4,5].

Several technologies are capable of partially exploiting the resources of this effluent. For instance, methanogenesis can utilise VFAs efficiently for CH_4 production [6], although it supports a poor nitrogen and phosphorus removal [4]. In contrast, microalgae-based photobioreactors could recover this P and N via assimilatory mechanisms in the form of biomass, but their potential to remove organic carbon from VFAs remains largely unexplored [4,7]. In this context, the symbiosis between microalgae and bacteria in high rate algal ponds (HRAPs) would allow the simultaneous removal and valorisation of nitrogen, phosphorous and VFAs in the form of biomass [5]. On the other hand, purple phototrophic bacteria (PPB), which exhibit a very versatile metabolism capable of assimilating VFAs, could also support an effective valorization of DF effluents [8]. Thus, PPBs can undertake

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photofermentation, a process by which they can use organic carbon as a source of carbon and infrared radiation (IR) as an additional source of energy, with the associated release of hydrogen and carbon dioxide [8]. Photofermentation allows PPB to support high biomass yields (1 g COD biomass/1 g COD consumed), which would be impossible to reach under classical chemoheterotrophic growth mode [8]. On the contrary, PPB can also consume an organic carbon source without additional IR energy supply in a classical heterotrophic metabolic process using electron acceptors such as oxygen or nitrate, which would reach less yield compared.

Both algal and PPB biomass can be used as a feedstock for the manufacture of biofertilizer, biostimulants or animal feed. In addition, this biomass can be employed as a source of proteins and other added-value compounds such as carotenoids (and other pigments), coenzyme Q10 or 5-aminovulemic acid, among others [8,9]. The value of these photoheterotrophic microorganisms is therefore significantly higher than that of their chemoheterotrophic counterparts (such as activated sludge), commonly used for the treatment of wastewater with high organic loads. Therefore, algal and PPB biomass holds a significant market value, although its industrial exploitation is not yet profitable to date due to their high production cost (3.0–4.0 $/kg$_{dry\ biomass}$) [9,10]. The roadmap towards reducing this high production cost involves the use of residual carbon and nutrient streams, open ponds and natural light as an energy source [8]. Besides, process operation must be adjusted in order to enhance photoheterotrophic growth over chemo-autotrophic one. Nevertheless, the performance of microalgae and PPB during the treatment of dark fermentation effluents has been scarcely investigated to date [4,5]. In this context, there is a research gap regarding the ability of these microorganisms to efficiently treat the effluent from this process directly in continuous photobioreactors. Due to the high concentration and diversity of pollutants present in this effluent, photosynthetic treatment could reduce the amount of required processing steps while simultaneously generating biomass as a value-added product.

This study comparatively assessed at laboratory scale the bioremediation performance and biomass production potential of microalgae-bacteria consortium (MBC) and PPB open ponds fed with DF effluents under different hydraulic retention times (HRT). The structure of the microbial populations prevailing in the ponds under steady state was also evaluated. The objective of this study is to determine the optimal HRT value to maximize biomass production in both systems, while characterizing the microbial profile of the resulting biomass for its potential industrial valorization.

2. Methodology

2.1. Substrate and inoculum

An artificial wastewater mimicking the characteristics of the effluent from a DF process treating real food waste was herein used (Table 1). The use of a simulated substrate allowed avoiding the natural variability of a real effluent, while eliminating dependence on the performance of an upstream reactor throughout the experiment. Due to the high VFA concentration of this effluent, a tenfold dilution was required to maintain the maximum organic loading rate (OLR) below 2 g COD/L-d [8] at the operational hydraulic retention times (HRTs) tested. The dilution of anaerobic digestion (AD) effluent (including those derived from dark fermentation) is typically implemented in order to reduce the turbidity and the concentrations of COD, ammonia and phosphorus prior microalgae cultivation [4,5,11]. The artificial wastewater exhibited the following final concentrations of organic acids (OAs): lactate (0.24 g/L), acetate (0.55 g/L), propionate (0.44 g/L), and butyrate (0.6 g/L), resulting in a total OA concentration of 1.83 g/L (0.86 g total organic carbon (TOC)/L). The nitrogen and phosphorous concentrations accounted for 0.128 g/L and 0.114 g/L, respectively. The artificial wastewater was prepared by modifying a standard mineral medium for microalgae cultivation [12], which resulted in the following final concentrations: K_2HPO_4 (0.64 g/L), NH_4Cl (0.49 g/L), $MgSO_4$ (9.77 mg/L), $CaCl_2$ (5.66 mg/L), $FeSO_4$ (0.65 mg/L), EDTA (0.2 mg/L), and yeast extract (0.2 mg/L). Additionally, 0.1 mL of a micronutrient solution was supplemented, containing: H_3BO_3 (2.8 g/L), $MnSO_4$ (1.45 g/L), Na_2MoO_4 (0.68 g/L), $ZnSO_4$ (0.104 g/L), and $Cu(NO_3)_2$ (30 mg/L). The pH of the artificial wastewater decreased to an average value of 3.77 ± 0.06 due to OA supplementation, which required the addition of 3.69 ± 0.23 mL of NaOH 6 M to reach a pH of 6.5 [13] before dilution. The average inorganic carbon (IC) concentration in the wastewater was 50 ± 4 mg/L, which corresponded to the CO_2 solubilization from the atmosphere.

Table 1
Concentration values of the different compounds present in the feedstock.

Compound	Concentration
Lactate (g/L)	0.24
Acetate (g/L)	0.55
Propionate (g/L)	0.44
Butyrate (g/L)	0.6
K_2HPO_4 (g/L)	0.64
$MgSO_4$ (mg/L)	9.77
$CaCL_2$ (mg/L)	5.66
$FeSO_4$ (mg/L)	0.65
EDTA (mg/L)	0.2
Yeast extract (mg/L)	0.2
NH_4Cl (g/L)	0.49
Micronutrient Solution (mL)	0.1

Compound	Concentration (g/L)
H_3BO_3	2.8
$MnSO_4$	1.45
Na_2MoO_4	0.681
$ZnSO_4$	0.104
$Cu(NO_3)$	0.03

An aliquot of the cultivation broth of a 180 L HRAP fed with mineral salt medium and synthetic biogas was used ad inoculum in the microalgal-bacterial photobioreactor. This HRAP was an indoor pilot-scale system with 1.2 m^2 of illuminated surface, equipped with paddlewheel mixing (20 cm s^{-1}), operated under a 16 h:8 h light:dark cycle with 1316 μmol m^{-2} s^{-1} PAR supplied by LED lamps. The system was fed daily with 5 L of digestate supplemented with $NaHCO_3$/Na_2CO_3 to maintain inorganic carbon levels of 1000–1300 mgC L^{-1}, while synthetic biogas (70 % CH_4, 29.5 % CO_2, 0.5 % H_2S) was sparged at the connected absorption column to provide CO_2. The HRAP was run in zero-effluent mode with continuous biomass harvesting (29–89 g m^{-2} d^{-1})."The inoculum contained a monoculture of the microalgal species *Chloroides ellipsoideum*, with no predominant bacterial taxa (>1 % relative abundance). On the other hand, an aliquot of the cultivation broth of a PPB photobioreactor treating synthetic domestic wastewater stored at 4 °C was used as inoculum in the PPB photobioreactor. The dominant bacterial genera in this inoculum were *Rhodobacter*, *Rhodopseudomonas*, *Rhodocista* and *Dysgonomonas*.

2.2. Experimental set-up

HRAP-type photobioreactors were used for the cultivation of PPB and MBC. Both reactors consisted of a PVC cylindrical vessel (17 cm height × 16.5 cm of internal diameter) with 3.4 L of total capacity. The ponds featured an effluent outlet positioned at a height of 14 cm, setting a working volume of 3 L. PPBs are known to be sensitive to dissolved oxygen, which inhibits their photoheterotrophic metabolism. Therefore, the PPB pond was equipped with a poly methyl methacrylate (PMMA) cover to limit air diffusion into the cultivation broth, and thus prevent oxygen inhibition. PMMA was selected due to its high near IR (NIR) permeability [14]. The cover was coated with Ultralite 299 1.2 ND visible light filter film (Ehingen, Germany) and incorporated a septum to

supply the artificial wastewater. The filtered cover promoted the selective PPB growth, while suppressing other phototrophs such as microalgae and reducing the water evaporation losses. The outlet of the PPB pond was fitted with a water trap seal to prevent air intrusion into the system.

Both systems were illuminated using artificial light sources to enable photosynthesis. The MBC pond was provided with visible light LEDs (360–365 nm and 380–385 nm; 400 W m^{-2}) supplying 1000 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR) at the pond surface. Conversely, NIR light-emitting diodes (LEDs; INSTAR IN-905) were used in the PPB pond, delivering 120 W m^{-2} at the pond surface (around 850 μmol m^{-2} s^{-1}). The irradiation of both systems mimicked the NIR and visible irradiations provided by sunlight in outdoors ponds. Both of these light sources fulfilled the requirements for each microbial groups while minimizing energy consumption and heat emission. Magnetic stirring plates (LBX Instruments, S20 series) were used to maintain microbial suspensions in both ponds. Feeding was carried out using a multichannel peristaltic pump (DINKO Instruments, Spain). Air conditioning equipment was used to minimize room temperature variations due to seasonal change during the experiment execution.

2.3. Process operation and monitoring

The operation of both ponds lasted 90 days and was divided into two periods. In the first period (P1), the ponds were operated at an HRT of 5 days (equivalent to an OLR of 0.5 g COD/L-d) for 51 days (from April, 30th to June, 20th), while the ponds were operated at an HRT of 10 days (equivalent to an OLR of 0.25 g COD/L-d) for 39 days (from June, 20th to July, 29th) in the second period (P2). The values selected were based on the input influent and did not take into account the variating evaporation rates during the process operation. These conditions were selected based on the maximum recommended OLR of 2 g COD/L-d in PPB cultures, although lower OLRs were chosen in our 14-cm water level pond due to the low penetration of IR radiation in water [8].

The ponds were monitored twice per week by measuring the inlet and outlet concentrations of total organic carbon (TOC), inorganic carbon (IC), total nitrogen (TN), NH_4^+, NO_2^-, NO_3^-, PO_4^{3-}, SO_4^{2-}, lactate, acetate, propionate, and butyrate. Furthermore, the concentration of total and volatile suspended solids (TSS and VSS, respectively), along with the pH, temperature, dissolved oxygen (DO) concentration in the cultivation broths. The evaporation rate in the ponds was estimated by measuring the influent and effluent flow rates. Finally, microbial community sequencing of the cultivation broth was performed at the beginning and end of each experimental period in both ponds.

2.4. Analytical methods

2.4.1. Nutrients analysis

TOC, IC and TN concentrations were determined in a TOC-VCSH analyser (Shimadzu, Japan) coupled with a TNM-1 chemiluminescence module. NH_4^+ was determined using the Nessler method described by Posadas et al. (2017) [7] in an UV-2550 spectrophotometer (Shimadzu, Japan). The concentration of N-NO_2^-, N-NO_3-, PO_4^{3-} and S-SO_4^{2} were quantified by HPLC-IC (Waters 432, conductivity detector, USA) [7]. A Shimadzu HPLC (Model LC-2050C; Oregon, USA) equipped with an UV detector at 214 nm was used to measure the OAs concentration following the method and configuration described by Regueira-Marcos et al. (2024) [13].

2.4.2. Biomass

TSS and VSS were determined according to standard methods [15]. Biomass concentration was also estimated by optical density measurements in a spectrophotometer (Star Nano, BMG LACTECH, Germany), using a wavelength of 808 nm for PPBs and 650 nm for microalgae. All concentration reported accounted for the water evaporation in the ponds, except for the biomass concentrations in the ponds, as this value directly impact the transmittance of IR and PAR on both systems. It should be noted that while biomass concentrations were reported without evaporation adjustment to reflect the actual optical density and metabolic conditions inside the ponds, evaporation was considered when calculating biomass extraction and carbon yields, as described in Section 2.5.

2.4.3. Environmental parameters

The pH was assessed using an Eutech Cyberscan pH 510 (Eutech instruments, The Netherlands). Temperature and DO were determined employing an OXI 3310 oximeter (WTW, Germany). IR intensity in the PPBs pond was determined by using a PASPort PS-2148 (PASCO/USA) connected to an AirLink 097–769 (PASCO, USA) as wireless interface connector. PAR was measured by using a LI-250a light meter (LI-COR Biosciences, Germany) in the MBC pond (Fig. 1).

2.4.4. Sequencing

DNA extraction and sequencing (amplicon) was performed in triplicate following the methodology described by Shafana Farveen et al. (2025) [16]. Microalgae taxonomic analysis was performed by microscopic identification as described by Marín et al. (2021) [17].

2.5. Data treatment

Steady-state conditions were defined as the period when the outlet concentrations of TOC, TN and biomass showed fluctuations below 10 % over at least two consecutive HRT cycles (10 days at 5 days HRT and 20 days at 10 days HRT). Only data from these steady-state periods were considered for the calculation of average values and removal efficiencies presented in this work. Error values on tables and text, and error bars on figures, indicates standard deviation of the observed values. Presented results are based on descriptive statistics and observed trends, without implying formal statistical significance.

The biomass yield (BY) and COD yield (CODY) in each pond was calculated following Eqs. (1) and (2):

$$BY = \frac{Q_{out} * B_C}{(Q_{in} * TOC_{in}) - (Q_{out} * TOC_{out})} \quad (1)$$

$$CODY = \frac{Q_{out} * B_{COD}}{(Q_{in} * COD_{in}) - (Q_{out} * COD_{out})} \quad (2)$$

where Q_{out} and Q_{in} stands for the effluent and influent flow rate (L/day); B_C represents the carbon present in the biomass, calculated based on the biomass concentration in the pond multiplied by the standard carbon content of bacterial and microalgae biomass (0.53 $g_C/g_{Biomass}$, following the $C_5H_7O_2N$ formula); B_{COD} stands for the biomass COD equivalent estimated with a COD/biomass ratio of 1.42 $g_{COD}/g_{biomass}$; TOC_{in} and TOC_{out} stands for the TOC concentrations in the influent and effluent, respectively; while COD_{in} and COD_{out} account for the COD concentrations (by transforming OAs into COD equivalents) in the influent and effluent, respectively.

The empirical liquid-gas mass transfer coefficients for the undissociated form of the OAs were calculated based on eq. 3, as described by Estrada et al. (2014) [18]. The mass flow of OAs loss by volatilization was calculated based on eq. 4:

$$\frac{k_L a_{OA}}{k_L a_{O_2}} = \frac{\left(\frac{1}{Vm_{OA}}\right)^{0.4}}{\left(\frac{1}{Vm_{O_2}}\right)^{0.4}} \quad (3)$$

where $k_L a_{O2}$ and $k_L a_{OA}$ (h^{-1}) stands for the empirical mass transfer coefficient of oxygen and organic acids in the pond; and *Vm* stands for the molecular volume of each compound at the boiling point, (i.e. oxygen (Vm_{O2}) or a determined OA (Vm_{OA}))

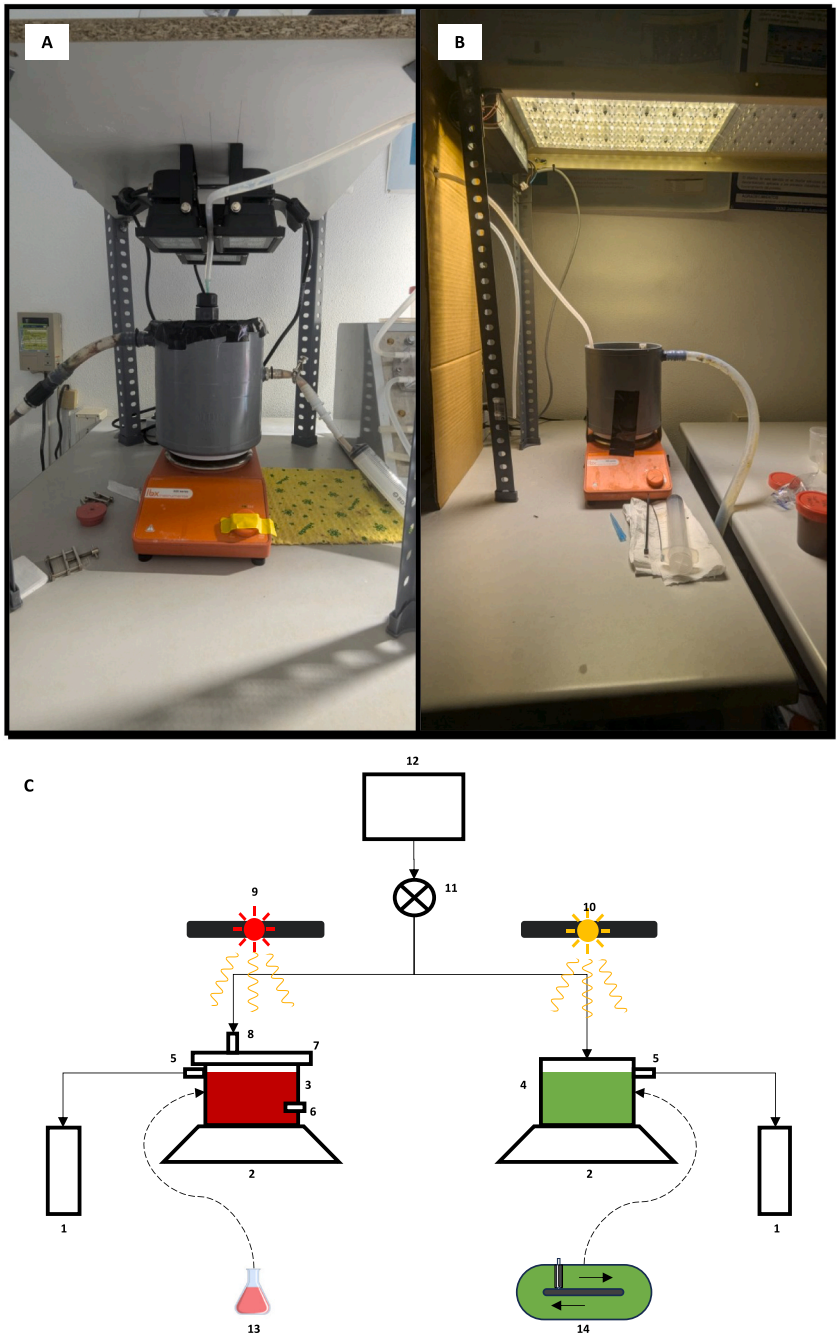

Fig. 1. A) Picture of the PPB pond system. B) Picture of the MBC pond system. C) Diagram of the experimental design used in the study: 1) Effluent tank; 2) Magnetic stirring plates; 3) PPB pond; 4) MBC pond; 5) Outlet port; 6) Sampling port; 7) PPB pond cover; 8) Inlet port; 9) NIR LEDs; 10) PAR LEDs; 11) Pump; 12) Influent tank; 13) PPB inocula, grown on OA-rich substrate in an Erlenmeyer flask; 14) MBC inocula, taken from a 180 L HRAP treating urban wastewater.

$$OA_{vol} = \sum_{ace:pro:but} Vk_L a_{OA} \left(\frac{C_{OA\ out}}{1 + 10^{pH - pK_a}} \right) \quad (4)$$

where OA_{vol} stands for the mass of volatilized carbon as dissociated organic acids; V stands for the working volume of the pond, pH accounts for the average pH value under steady state; and pKa represents the dissociation constant of the corresponding OA. The total carbon balance in the ponds was calculated using eq. 5:

$$C_{in}Q_{in} = C_{out}Q_{out} + OA_{vol} + Q_{out}\frac{X_C}{BY} \left(\quad (5)$$

C_{in} and C_{out} stand for the influent and effluent OA concentration; X accounts for the biomass concentration (g/L) in the pond.

3. Results

3.1. Microbial communities

Based on the microbiological analysis (Fig. 2), at least 90 % of the bacteria present in the inoculum of the PPB pond belonged to the purple photosynthetic bacteria group (as Non-Sulfur PPB). The majority (>50 %) belonged to the genus *Rhodobacter*, followed by *Rhodopseudomonas* and *Rhodocista*. On the other hand, over 75 % of the sequenced genome in the inoculum of the MBC pond belonged to chloroplasts, presumably belonging to microalgae of the specie *Chloroides ellipsoideum* (previously *Chlorella elipsoidea*). The remaining microbiome consisted of undetermined genera that failed to exceed the 1 % relative abundance (RA)

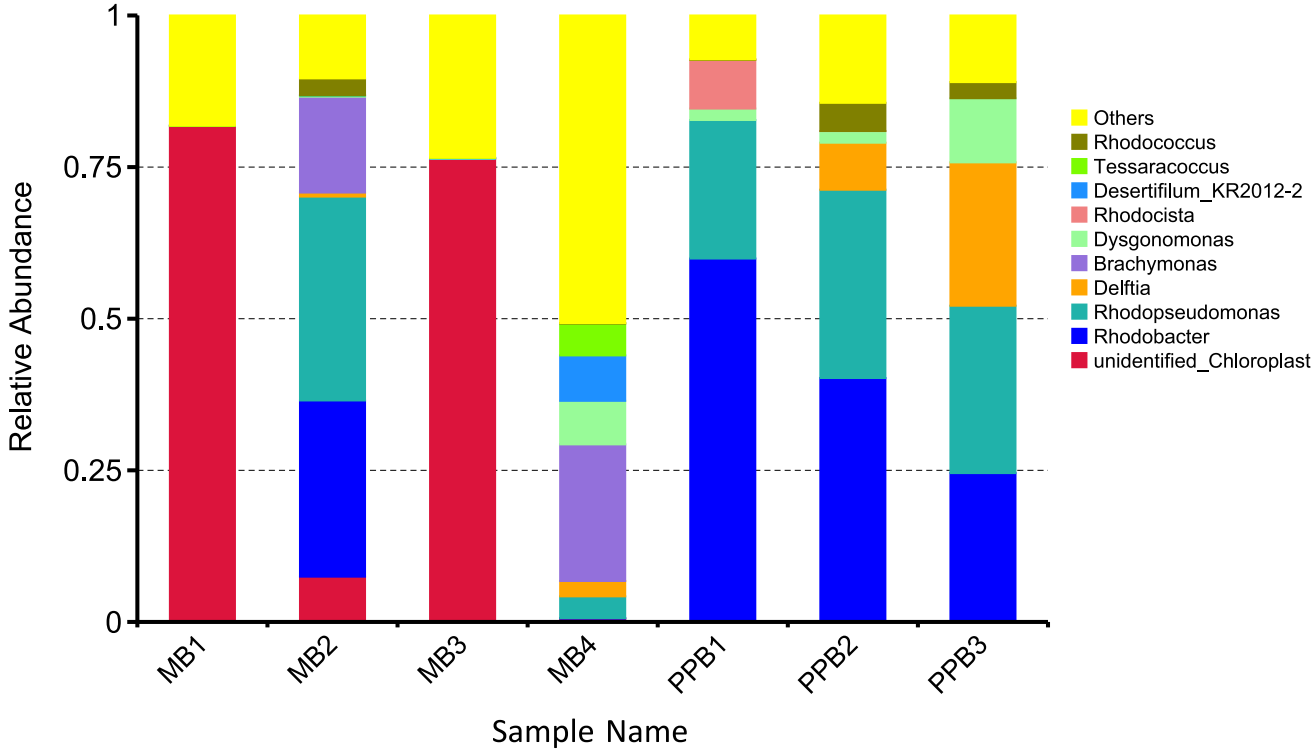

Fig. 2. Bar plot of the relative abundance of the 10 main microbial genera of both ponds at different operational moments. From left to right: MB1, start up of the MBC pond on P1; MB2, end of P1 in the MBC pond; MB3, start up of the MBC pond in P2; MB4, end of P2 in the MBC pond; PPB1, start up of the PPB pond; PPB2, end of P1 in PPB pond; PPB3, end of P2 in PPB pond. "Others" colour corresponds to the sum of genera under the 1 % limit of relative abundance.

threshold.

The evolution of the PPB bacterial community structure in the PPB pond when the HRT increased showed a sharp decrease of *Rhodobacter* species (≈ 25 % RA in P2), together with the maintenance of the abundance of *Rhodopseudomonas* genus and the disappearance of the *Rhodocista* genus (RA < 1 %), which was partially replaced by *Rhodococcus* genus. Although these 4 genera are non-sulfur PPBs, the first 3 belong to the group of alpha-proteobacteria (facultative anaerobic organisms), while *Rhodococcus* belongs to the actinobacteria group, which is constituted only by strict aerobic organisms [19]. *Rhodobacter* and *Rhodopseudomonas* are very similar genera able to degrade OAs, the latter having a more versatile metabolism, which probably enabled its maintenance regardless of the HRT [20–22]. On the other hand, *Rhodocista* includes species whose adaptive advantage lies in their survival in more extreme conditions (such as extreme pH and temperatures) [23,24], thus their replacement by the other PPB species was feasible under standard favorable environmental conditions. The prevalence of *Rhodobacter* at low HRTs and its displacement by *Rhodopseudomonas* at long HRTs was also observed by Alloul et al. (2019) [25] in a photobioreactor fed with VFAs. Beyond the PPBs group, the appearance of bacteria of the genus *Delftia* (RA ≈ 25 % in P3) and the development of the genus *Dysgonomonas* was recorded in the PPB pond. *Delftia* is a genus of non-fermentative and generally aerobic bacteria, although they can employ external acceptors such as nitrate [26,27]. Given the absence of nitrate and nitrifying bacteria in the PPB pond, this genus could only subsist by consuming the O_2 diffusing from the atmosphere into the cultivation broth (despite the presence of the pond cover limited this O_2 diffusion), similarly to *Rhodococcus* species [19]. This O_2 would enable the aerobic consumption of OAs, preventing it from accumulating in the media. Conversely, even if it was low, the longer exposure time at P2 fostered the diffusion of O_2 into the cultivation broth, thus favoring the growth of this aerobic genera over PPBs. *Delftia* are also characterized by their phosphorus accumulation ability, so they may play an important role in the system on the removal of this nutrient [26,27]. *Dysgonomonas* is, in turn, a genus of anaerobic bacteria (strict or facultative) able to grow on complex substrates (starches, hemicelluloses…) [28,29]. Despite members of the *Dysgonomonas* genus are not capable of handling OAs as a carbon source, their growth has been previously reported in cultures with VFAs as the only carbon source [30]. In our particular study, *Dysgonomonas* might feed on detritus and cellular remains of other species, as well as on cell exopolysaccharides.

In the MBC pond, a fraction (~10 % in P1; <1 % in P2) of the sequences was classified as "unidentified chloroplasts." This result is commonly interpreted as a proxy for green microalgae (mainly Chlorophyta), as chloroplasts are derived from cyanobacteria and share conserved 16S regions that are amplifiable by universal bacterial primers [31]. However, it is important to highlight that this relative abundance value does not accurately reflect the true contribution of microalgae to the total biomass in the pond. On one hand, microalgal cells possess chloroplasts with multiple genome copies, but they also feature thick cell walls and complex intracellular structures that reduce DNA extraction efficiency and amplification performance, leading to a systematic underrepresentation in sequencing results [32–34]. On the other hand, the size and mass of a single microalgal cell can exceed that of a typical bacterial cell by several orders of magnitude, which entails that even a modest relative abundance of green microalgae may actually represent a dominant fraction of the total biomass concentration [35]. Altogether, these factors suggest that the observed percentage of chloroplasts in metagenomic profiles should be interpreted as a minimum threshold for microalgal presence, and that their actual contribution to biomass may be substantially higher. Even in P2, where their relative abundance was lower than 1 %, the presence of microalgae in the pond was clearly visible to the naked eye, mostly in the form of aggregated flocs. Under the microscope, these aggregates were composed of a microalgae nucleus surrounded by bacteria. Microalgae were also observed as single free-live cells in the cultivation medium under the microscope. A similar finding was reported by Fradinho et al. (2013) [36] in a 4.4 L sequence batch reactor (SBR) inoculated with MBC and

fed with acetate at 6 h HRT.

Interestingly, the HRT determined the bacterial genera that proliferated alongside the microalgae in the MBC pond. Paradoxically, the main bacterial group that proliferated at HRT of 5 days were PPBs, which accounted for more than 50 % of the bacterial relative abundance. The presence of PPBs in the inoculum was previously unknown, and the possibility of cross-contamination between systems is highly unlikely, though not entirely impossible. In this regard, Fradino et al. (2013) [36] also observed the development of PPBs with microalgae in a SBR inoculated with a MBC and operated at 6 h of HRT. The distribution of the PPB genera in the MBC pond under steady state at 5 days of HRT was very similar to that observed in the PPB pond under P2, with *Rhodobacter* and *Rhodopseudomonas* as the dominant genera, followed by *Rhodococcus*. The genus *Brachymonas* proliferated—another aerobic beta-proteobacterium with a metabolic profile very similar to *Delftia* [37]. Therefore, the MBC pond proved to be an efficient technology for producing both microalgal and PPB biomass, especially considering that the total biomass concentration in this system was 2-folds higher than that achieved in the PPB pond. This suggests that in non-axenic cultures, PPBs can proliferate using DF effluent from food waste even in open systems exposed to the atmosphere (despite O_2 being an inhibitor of their bacteriochlorophylls) and under PAR lighting (which favors microalgae and cyanobacteria in a competitive environment) [8]. In contrast to P1, the significant presence (RA > 1 %) of PPBs in P2 in the MBC pond was limited to the genus *Rhodopseudomonas*, which represented less than 5 % of the total relative abundance. Notably, over 50 % of the total microbial community in the MBC pond at 10 days of HRT was composed of genera whose individual relative abundances were below 1 %, indicating a marked taxonomic dispersion among the functional degraders in the system. Among the dominant genera, *Brachymonas* was the most prevalent (RA ≈ 20 %), followed by *Delftia* and *Dysgonomonas*, both of which have been previously discussed in terms of their metabolic roles. A particularly interesting finding was the proliferation of the cyanobacterial genus *Desertifilum*, capable of performing oxygenic photosynthesis [38]. Its presence suggests a potential contribution to the system's aerobic micro-niches by providing oxygen to support coexisting aerobic bacteria. Additionally, the genus *Tessaracoccus* emerged as a relevant taxon. These Actinobacteria are facultative anaerobic chemoorganotrophs known for their ability to accumulate intracellular polyphosphate in wastewater treatment systems, suggesting they may have contributed significantly to phosphorus removal under these conditions [39,40].

3.2. Environmental factors

The pH in the cultivation broth of both ponds increased compared to the pH of the influent (6.5), reaching 7.40 ± 0.05 (P1) and 7.38 ± 0.07 (P2) in the PPB pond, and 8.07 ± 0.07 (P1) and 8.19 ± 0.09 (P2) in the MBC pond (Fig. 3). Thus, both ponds experienced an alkalinization process, primarily due to the removal of OAs (pKa ≤ 5) present in the medium [41]. The higher pH values prevailing in the MBC pond were likely attributed to a higher total OA removal and the photosynthetically mediated CO_2 capture [42]. Despite the occurrence of photosynthetic activity, both ponds exhibited nearly null DO concentrations (≤ 0.05 mg O_2/L) under both operational periods. This was mainly caused by the active aerobic heterotrophic activity of the microbial communities present in the cultivation broths of both ponds, described in previous section 3.1. In this regard, the increase in HRT allowed more time for oxygen to dissolve in the culture broth and preventing the washout of slow-growing microalgae species, therefore enhancing the growth of aerobic microbes on both ponds. These DO concentrations remained sufficiently low (≤ 2 mg O_2/L) to prevent the complete oxidation of the organic compounds or NH_4^+ present in the DF effluent [7]. The temperature of the ponds increased from P1 to P2 in both systems as a result of higher water retention time at 10 days, rising from 20.9 ± 0.9 to 23.6 ± 1.4 °C in the PPB pond and from 22.1 ± 1.0 to 25.2 ± 1.1 °C in the MBC pond. The temperature was higher in the MBC pond due to the greater heat radiation emitted by the LEDs compared to the IR lamps. The temperature rise, even though limited, likely impacted the evolution of the microbial profile of both ponds, enhancing the predominance of genera adapted to higher temperatures. Also, this temperature rise resulted in a significant increase in the evaporation rate, rising from 8.7 ± 3.8 % to 18.0 ± 7.2 % in the PPB pond, and from 31.6 ± 5.2 % to 59.2 ± 10.2 % in the MBC ponds. In this context it should be highlighted that the presence of a cover in the PPB pond significantly reduced water evaporation in this system compared to the MBC pond.

3.3. Carbon and nutrient removal

The steady state removals of organic carbon, nitrogen and phosphorous increased when the HRT was increased from 5 to 10 days (Table 2, Fig. 4). Thus, the HRT rise entailed an increase in TOC removals from 52.6 ± 3.3 % to 72.6 ± 3.4 % in the PPB pond; and from 56.0 ± 3.5 % to 79.8 ± 4.1 % in the MBC pond, during P1 and P2, respectively. On the other hand, the increase in IC concentration was higher in the cultivation broth of the PPB pond (≈ 0.1 g/L) with respect to the cultivation broth of the MBC pond (≈0.05 g/L), likely due to the

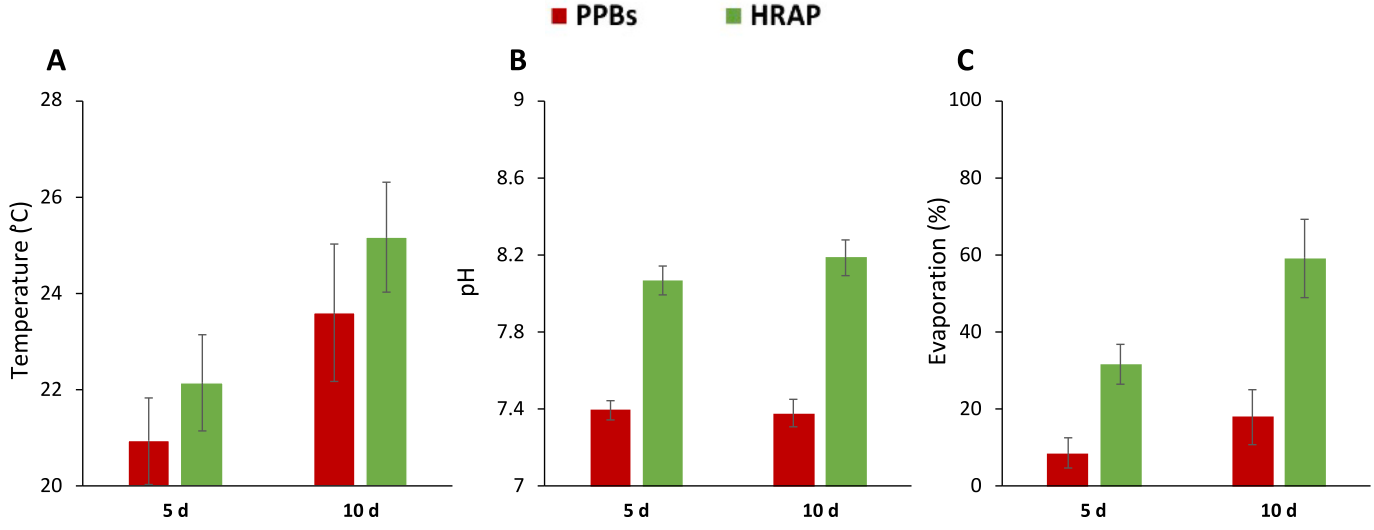

Fig. 3. Bar plot of the average values of temperature (A), pH (B) and evaporation (C) under steady state condition for environmental factors.

Table 2
Steady state effluent values of the key parameters used for the evaluation of the performance of the PPBs and HRAP ponds. Concentrations are adjusted according to evaporation percentages, except for biomass (g/L).

Reactor	PPB		MBC	
HRT (days)	5	10	5	10
TOC (mg/L)	406.7 ± 28.6	234.5 ± 29.6	377.5 ± 29.9	173.4 ± 35.3
IC (mg/L)	100.0 ± 9.04	114.1 ± 15.6	49.88 ± 0.01	42.59 ± 14.3
TN (mg/L)	70.96 ± 2.79	60.78 ± 5.79	59.59 ± 11.4	20.18 ± 4.52
N-NH_4^+ (mg/L)	67.97 ± 3.7	56.93 ± 7.14	47.14 ± 5.65	23.99 ± 8.69
PO_4^{-3} (mg/L)	0.28 ± 0.01	0.27 ± 0.01	0.28 ± 0.03	0.13 ± 0.03
Biomass (g/L)	0.61 ± 0.08	0.61 ± 0.04	1.37 ± 0.16	1.23 ± 0.22
Biomass production (g/d)	0.31 ± 0.04	0.14 ± 0.01	0.53 ± 0.06	0.14 ± 0.03
BY ($gC_{biomasa}/g_{TOC\ removed}$)	0.65 ± 0.05	0.43 ± 0.02	1.03 ± 0.07	0.39 ± 0.12

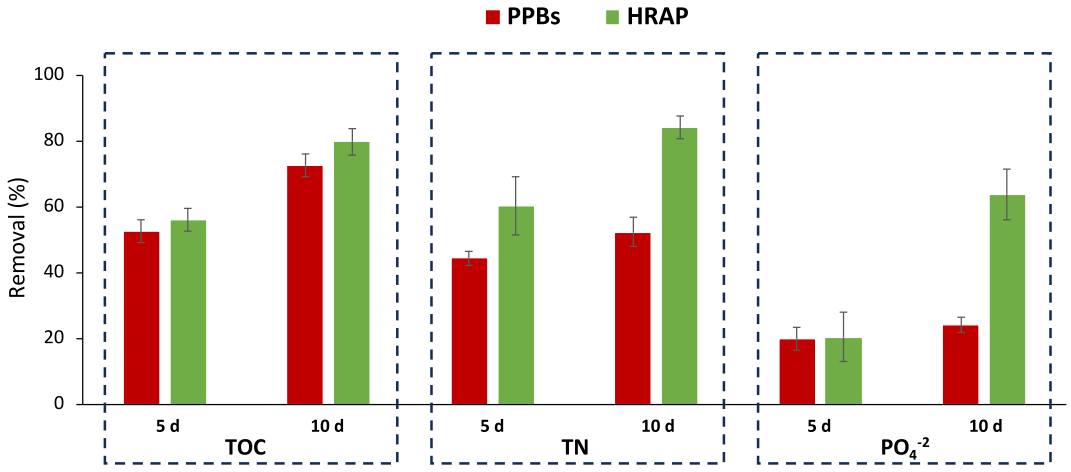

Fig. 4. Bar plot of the average removal efficiencies under steady state condition for TOC, TN and PO_4^{-3}.

active photosynthetic fixation of IC mediated by microalgae. Similarly, the increase in HRT from 5 to 10 days led to an increase in TN removal from 44.3 ± 2.2 % to 52.3 ± 5.6 % in the PPB pond; and from 60.3 ± 4.4 % to 84.2 ± 3.5 % in the MBC pond, at P1 and P2, respectively. Ammonium concentrations differed slightly from TN concentrations (≈6.5 %) in both ponds during steady state. This difference was probably due to analytical errors, which, together with the absence of nitrates and nitrites, ruled out the occurrence of nitrifying processes or other forms of nitrogen in the system, except in the case that nitrates and nitrites are consumed simultaneously as they are produced. However, the possibility of ammonia nitrification and the subsequent reduction of nitrates and nitrites into N_2 cannot be ruled out [7].

Steady state PO_4^{-3} removals also rose from 19.9 ± 3.5 % to 24.6 ± 3.4 % in the PPB pond, and from 20.4 ± 7.4 % to 46.4 ± 13.6 % in the MBC pond, during P1 and P2, respectively. This increase in phosphorus removal related directly to the greater development of polyphosphate-accumulating genera such as *Delftia* or *Tessarococcus*, even though it is impossible to quantitatively estimate the contribution of these genera to the total phosphorus removal. Additionally, chemical precipitation mechanisms may also have contributed to phosphorus removal. The metabolic activity of phototrophs typically leads to an increase in pH (from 6.5 on the feed to 7.4 in the PPB pond and above 8.0 in the MBC pond), which can promote the precipitation of phosphate with cations such as Ca^{2+}, Mg^{2+}, or Fe^{3+} naturally present in the medium or released through microbial activity. This abiotic pathway may have complemented biological uptake, particularly under the higher pH values observed in P2 [21,43]. The average concentrations of phosphate the effluent surpassed the maximum limit of 0.7 mg/L required by current European regulations [44]. In literature, most PPB systems operate under influent N:P ratios of 5:1, with average removal efficiencies of 53 % for nitrogen and 58 % for phosphorus [8]. Therefore, our PPB pond matched the average nitrogen removal while down-performed on phosphorus removal, probably due to the low N:P ratio of the feed (1.2:1). Finally, Liu et al. (2016) [45] reported even higher removals of TOC (73–90 %), TN (60–90 %) and phosphorus (85–95 %) in a *Rhodobacter palustris*-based photobioreactor fed with acid food industry wastewater rich in VFAs at different HRTs (96–24 h), obtaining an optimal HRT of 48 h. It is important to highlight that, generally, it is very difficult to quantitatively determine the effect of microbiological profile variations with HRT on pollutant removal, due to the large number of variables involved in the overall process.

3.4. Biomass production and yield

Despite the higher overall pollutant removal observed at P2 in both systems, the increase in HRT exerted no impact on biomass production in the PPB pond, whose concentration remained constant at 0.61 g/L (Fig. S1). In the MBC pond, biomass concentration was comparable between periods, changing from 1.37 ± 0.16 to 1.23 ± 0.22 g/L. Therefore, regardless of the higher pollutant removal, the reduction in HRT entailed a severe drop in BY in both systems (Table 2), especially in the MBC pond, where BY decreased from 1.03 ± 0.07 to 0.39 ± 0.12 $g_{biomass}/g_{TOC\ removed}$. The high BY of 1.0 $g_{biomass}/g_{TOC\ removed}$ recorded in the MBC pond at P1 was attributed to the occurrence of CO_2 fixation by photoautotrophic growth by microalgae along with TOC heterotrophic degradation. Compared to other studies, Liu et al. (2016) [45] obtained high PPB (*R. palustris*) biomass concentrations of 2.3–2.6 g/L in a PBR fed with a food industry wastewater with high VFA concentrations at different HRTs (96–24 h). On the other hand, Ren et al. (2018) [4] obtained an average biomass concentration of 1.12 g/L, with *Scenedesmus* sp. as the main microalgae specie, by depleting the OAs in the influent wastewater (but not the ethanol).

This inconsistency between nutrient removal and biomass growth derives from a net loss of carbon and nutrients in the ponds. This net loss of carbon accounted for 57 and 81 mg C/d in the PPB pond; and from −8 (this value resulting from external CO_2 fixation) and 119 mg C/d in the MBC pond, at P1 and P2, respectively. This loss could likely be attributable to the increase in water retention in the pond in P2. In both systems, the inlet TOC is entirely composed of organic acids, of which 88.7 % (*w/w*) correspond to the VFAs (acetate, propionate, and butyrate) [46]. Therefore, it could be plausible to hypothesize (as an initial hypothesis to be confirmed or rule out) that a significant portion of TOC removal was caused by volatilization rather than microbial assimilation. However, the abiotic conditions of the systems ruled out a significant contribution of this mechanism. Hence, the extent of acid volatilization highly depends on the OA concentration in the cultivation broth under steady state, the pH of the medium and the liquid-gas mass transfer coefficient (k_La) of each OA, which was not measured in our system but can be estimated using Eq.3 and the k_La of oxygen. At the pH values prevailing in the ponds (7.4–8.2), the fraction of undissociated OA (pKa ≈ 4.8) [46] was negligible, which together to the low OA concentrations observed under steady state, excluded volatilization as a significant OA removal mechanism (Eq. 4). Indeed, k_La between 400 and 4000 h^{-1} would be required depending on the OA to explain the net carbon losses recorded in the MBC pond (between 25 and 35 %). However, most HRAP studies with paddlewheel agitation have experimentally measured k_La for O_2 close to 1.0 [43,47–50]. In this context, OA mineralization during cellular respiration followed by CO_2 stripping from the pond could explain these net carbon losses. Thus, the drift of the microbial community towards non-photosynthetic aerobic genera at P2 likely fostered this phenomenon, contributing to reduction of the BY in the ponds. This was especially pronounced in the MBC pond at P2 due to the high genera biodiversity observed, mostly composed of non-phototrophic genera. These findings indicate that longer HRTs not only favored oxygen diffusion and nutrient depletion but also promoted the selection of microbial communities dominated by non-phototrophic aerobic genera. This shift likely led to a change in dominant metabolic pathways, from biomass synthesis towards carbon mineralization via respiration, explaining the observed decrease in biomass yield. In summary, the fate of organic acids in both systems can be described as a combination of: 1) residual OAs detected in the effluent, 2) negligible volatilization losses, 3) assimilation into biomass as estimated from the carbon yield, and 4) mineralization through cellular respiration and subsequent CO_2 stripping.

On the other hand, the higher temperatures and increased evaporation in P2 favored the loss of nitrogen as NH_3 gas in the ponds [51]. This phenomenon was more pronounced in the MBC pond due to its higher pH (> 8.0) and temperatures, and to the absence of a photobioreactor cover to limit mass exchange with the atmosphere. In contrast, phosphates cannot be lost through evaporation, but their precipitation with divalent or trivalent cations (e.g., Ca^{2+}, Mg^{2+}, Fe^{3+}) was likely enhanced under alkaline conditions (pH ≥ 7.5–8.5) [52].

In brief, the MBC pond model clearly supported a better biomass production, reaching a concentration of 1.37 g/L (2.25 times higher than the PPB pond) and a daily production rate of 0.53 ± 0.06 g/d, compared to 0.31 ± 0.04 g/d in the PPB pond. Biomass in the MBC pond was primarily composed of microalgae followed by PPBs, which makes it a high-value biomass with promising potential for downstream applications or commercialization [8,53]. Therefore, the best-performing model for biomass production from dark fermentation effluent was the MBC-type system operated at an HRT of 5 days. On the other hand, the 10 d HRT condition allowed higher pollutant removal, mostly related to carbon mineralization through respiration, nutrient losses through stripping and precipitation, reduced light availability, and microbial community shifts. Altogether, these mechanisms represent potential metabolic bottlenecks limiting biomass conversion at longer HRTs.

Beyond its high productivity, the composition of the MBC biomass (dominated by microalgae and PPBs) confers significant added-value for downstream biorefinery applications. Microalgae are a promising source of proteins, pigments (i.e., carotenoids) and biofertilizers, while PPBs are known for accumulating bioplastics precursors such as PHB under nutrient-limited conditions. Although PHB accumulation was not quantified in this study, the high C/N ratio of the dark fermentation effluent could favor such metabolic pathways, as previously reported in PPB systems. Therefore, future research should explore the co-production of PHBs in MBC systems to expand the techno-economic potential of this valorization strategy.

3.5. Organic acids

The removal of OAs increased upon increasing the HRT in both ponds (Table 3; Fig. 5; Fig. S2). Thus, the increase in HRT from 5 to 10 days entailed an increase in the removal of lactate from 91.2 ± 0.8 % to 97.3 ± 1.6 %, from 38.0 ± 6.3 % to 57.7 ± 6.0 % for acetate, from 69.7 ± 2.8 % to 91.1 ± 3.2 % for propionate, and from 38.5 ± 2.8 to 74.3 ± 6.4 % for butyrate. In the MBC pond, the switch from P1 to P2 involved an upsurge in the removals from 95.0 ± 1.3 % to 98.4 ± 0.96 % for lactate, from 24.9 ± 10.0 % to 50.5 ± 15.5 % for acetate, from 56.8 ± 7.6 % to 92.2 ± 1.1 for propionate, and from 85.0 ± 8.7 % to 99.0 ± 0.98 % for butyrate. The small differences observed between TOC and total OA removal (average absolute difference ~ 3.5 %) were within the combined analytical uncertainty of both measurements.

PPBs preferentially consumed lactate followed by propionate, with acetate and butyrate as the least preferred substrates. This consistent preference for lactate likely results from the widespread presence of lactate dehydrogenases and monocarboxylate transporters in both PPBs and associated heterotrophic bacteria, which allow for a rapid conversion of lactate to pyruvate (a central metabolite in energy and biosynthetic pathways). This route is metabolically more direct than the assimilation of longer-chain VFAs such as butyrate or propionate, which require additional enzymatic activation steps [5,25,54]. The preferential consumption of propionate over acetate and butyrate by PPBs was previously observed by Alloul et al. (2019) [25]. Conversely, the MBC pond supported considerably higher removals of butyrate compared to propionate and acetate. The preferential consumption of some OAs has been previously reported in microalgae [4,55]. In this regard, while the ability to consume acetate has been reported for most of heterotrophic microalgae, the consumption of lactate, propionate and butyrate has been more rarely observed [5]. Noteworthy, literature studies have consistently described the low degradability of butyrate (≈5 %) by most microalgae species [4,5,55]. Therefore, the high butyrate removals measured in the MBC were likely mediated by PPB metabolism in P1 or by aerobic bacterial metabolism in P2. In another study, Fradinho et al. (2014) [56] reported that acetate was the preferred OA in algal-bacterial batch photobioreactors when fed alone, while its presence in a 4:1:1 mix of acetate, propionate and butyrate boosted the consumption of the latter 2 at the expenses of slowing the degradation of the former, which would act as a co-substrate in the mix. While the OA mix herein tested was not so biased towards acetate, it is possible that lactate partially played this role in the ponds, as most of its degradation steps involve the

Table 3
Final OA concentrations and COD yields measured under steady state conditions in both ponds. Concentrations are adjusted according to evaporation percentages.

Reactor	PPB		MBC	
HRT (days)	5	10	5	10
Lactate (g/L)	0.02 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.0 ± 0.0
Acetate (g/L)	0.34 ± 0.03	0.23 ± 0.03	0.41 ± 0.05	0.27 ± 0.09
Propionate (g/L)	0.13 ± 0.01	0.04 ± 0.01	0.19 ± 0.03	0.03 ± 0.0
Butyrate (g/L)	0.37 ± 0.02	0.15 ± 0.04	0.09 ± 0.05	0.01 ± 0.01
Total (g/L)	0.86 ± 0.04	0.43 ± 0.07	0.71 ± 0.12	0.32 ± 0.09
CODY ($g_{biomass\ COD}/g_{COD\ removed}$)	0.59 ± 0.04	0.35 ± 0.02	0.78 ± 0.06	0.32 ± 0.05

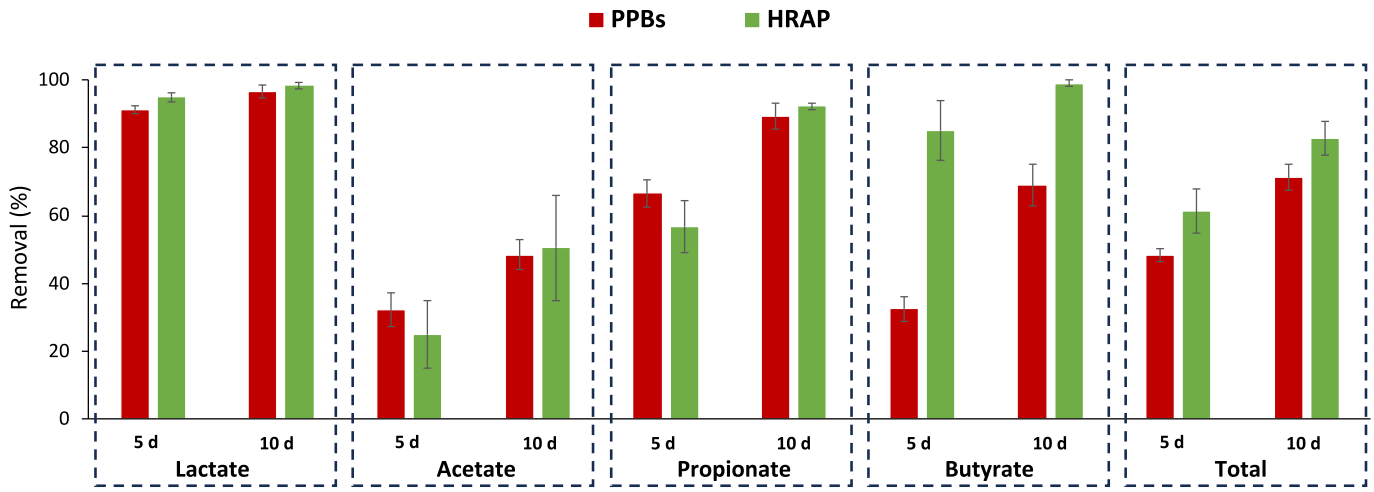

Fig. 5. Bar plot of the average removal efficiencies under steady state condition for organic acids.

intermediate formation of acetate, thus favoring the consumption of propionate and butyrate in the mix [54].

The CODY presented lower values than those observed for the BY (Table 3). This difference was due to the preferential removal of the most reduced OAs (propionate and butyrate) compared to less reduced ones (i.e. acetate), rendering a higher COD loss per unit of carbon. In the PPB pond, the CODY value at P1 (0.59 ± 0.04 $g_{biomass\ COD}/g_{COD\ removed}$) was closer to BY owing to the low removals of butyrate compared to those of acetate and lactate. The increase in butyrate and propionate consumption in the PPB pond at P2 resulted a larger decrease in CODY (0.35 ± 0.02 $g_{biomass\ COD}/g_{COD\ removed}$) compared to BY ($0.43 \pm 0.02$ $g_{biomass}/g_{TOC\ removed}$). On the other hand, CODY dropped from 1.03 ± 0.07 $g_{biomass\ carbon}/g_{carbon\ removed}$ to 0.78 ± 0.06 $g_{biomass\ COD}/g_{COD\ removed}$ at P1, and to 0.32 ± 0.05 $g_{biomass\ COD}/g_{COD\ removed}$ at P2 (comparable to BY) in the MBC pond. The CODY value recorded at P2 was comparable to the average 0.8 $g_{biomass\ COD}/g_{COD\ removed}$ achieved with enriched PPB cultures in non-sterile conditions [8]. Nevertheless, it is important to note that this reported average yield includes systems designed with higher exposure of the culture to light radiation [8]. In our pond system, the deepest part hardly receives any light radiation, especially in the PPB pond, due to the low transmittance of IR in water [57]. This transmittance is further reduced as the microorganisms grow, blinding the infiltration of radiation into the system.

4. Conclusions

This study assessed the performance of PPB and microalgae-bacteria systems for the treatment of dark fermentation effluents, with a focus on the influence of HRT. Increasing HRT from 5 to 10 days improved pollutant removal in both systems but did not enhance biomass concentration, which remained highest at 5-day HRT, particularly in the MBC pond (1.37 ± 0.16 g/L). Under this condition, the MBC system achieved the highest biomass yield (1.03 ± 0.07 $gC_{biomass}/gTOC_{removed}$), supported by a microbial community dominated by microalgae and PPBs. In contrast, longer HRTs favored microbial diversity and heterotrophic genera, likely contributing to increased mineralization and reduced biomass yield. Organic acid removal profiles showed consistent lactate preference, followed by butyrate in the MBC and propionate in the PPB system.

These findings identify the MBC reactor operated at 5-day HRT as the most promising configuration for biomass-oriented valorization. Despite the lower pollutant removal, this condition preserved valuable residual organics and favored their conversion into biomass rather than full degradation. The system's high productivity, compatibility with diluted waste streams, combined with the use of open-pond designs suggest strong potential for industrial scalability and integration into cost-effective wastewater valorization strategies.

CRediT authorship contribution statement

Lois Regueira-Marcos: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Octavio García-Depraect:** Writing – review & editing, Visualization, Supervision, Project administration, Methodology, Conceptualization. **Raúl Muñoz:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2025.104306.

Data availability

Data will be made available on request.

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