



Polyhydroxyalkanoates production in purple phototrophic bacteria ponds: A breakthrough in outdoor pilot-scale operation

J.R. Almeida ^{a,b}, E. Serrano León ^c, F. Rogalla ^c, J.C. Fradinho ^{a,b,*}, A. Oehmen ^{b,1}, M.A. M. Reis ^{a,b}

^a Associate Laboratory i4HB – Institute for Health and Bioeconomy, NOVA School of Science and Technology, NOVA University Lisbon, 2829-516 Caparica, Portugal

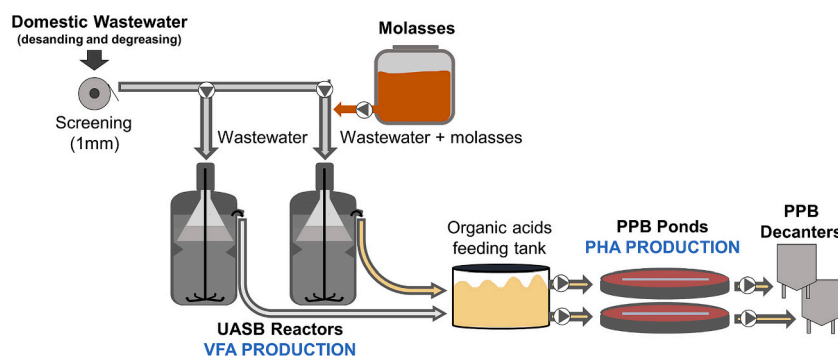
^b UCIBIO – Applied Molecular Biosciences Unit, Department of Chemistry, NOVA School of Science and Technology, NOVA University Lisbon, 2829-516 Caparica, Portugal

^c FCC Servicios Ciudadanos, Av. del Camino de Santiago, 40, edificio 3, 4ª planta, 28050 Madrid, Spain

HIGHLIGHTS

- Pioneering phototrophic PHA production in an outdoor pilot scale set-up
- Integrated system combining UASBs and retrofitted purple phototrophic bacteria ponds
- Successful translation of lab-scale PHA technology into outdoor pilot-scale systems
- Controlled OLR/VSS ratio and VFA profile are crucial for phototrophic PHA production.
- Outdoor operation showed promising PHA productivities, hitting 36 % g PHA/ g VSS.

GRAPHICAL ABSTRACT



ARTICLE INFO

Guest Editor: Maite Pijuan

Keywords:

Resource recovery
Polyhydroxyalkanoate (PHA)
Purple phototrophic bacteria ponds (PPB ponds)
Up-flow anaerobic sludge blanket reactor (UASB)
Pilot-scale
Outdoor operation

ABSTRACT

The versatile capacity of purple phototrophic bacteria (PPB) for producing valuable bioproducts has gathered renewed interest in the field of resource recovery and waste valorisation. However, greater knowledge regarding the viability of applying PPB technologies in outdoor, large-scale systems is required. This study assessed, for the first time, the upscaling of the phototrophic polyhydroxyalkanoate (PHA) production technology in a pilot-scale system operated in outdoor conditions. An integrated system composed of two up-flow anaerobic sludge blanket (UASB) reactors (for fermentation of wastewater with molasses), and two high-rate algal ponds retrofitted into PPB ponds, was operated in a wastewater treatment plant under outdoor conditions. UASB's adaptation to the outdoor temperatures involved testing different operational settings, namely hydraulic retention times (HRT) of 48 and 72 h, and molasses fermentation in one or two UASBs. Results have shown that the fermentation of molasses in both UASBs with an increased HRT of 72 h was able to ensure a suitable operation during colder conditions, achieving 3.83 ± 0.63 g COD_{Fermentative Products}/L, compared to the 3.73 ± 0.85 g COD_{Fermentative Products}/L achieved during warmer conditions (molasses fermentation in one UASB; HRT 48 h). Furthermore, the

* Corresponding author at: Associate Laboratory i4HB – Institute for Health and Bioeconomy, NOVA School of Science and Technology, NOVA University Lisbon, 2829-516 Caparica, Portugal

E-mail address: j.fradinho@campus.fct.unl.pt (J.C. Fradinho).

¹ Present address: School of Chemical Engineering, University of Queensland, Brisbane, QLD, 4072, Australia.

<https://doi.org/10.1016/j.scitotenv.2023.168899>

Received 31 August 2023; Received in revised form 7 November 2023; Accepted 24 November 2023

Available online 27 November 2023

0048-9697/© 2023 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

PPB ponds were operated under a light-feast/dark-aerated-famine strategy and fed with the fermented wastewater and molasses from the two UASBs. The best PHA production was obtained during the summer of 2018 and spring of 2019, attaining 34.7 % gPHA/gVSS with a productivity of 0.11 gPHA L⁻¹ day⁻¹ and 36 % gPHA/gVSS with a productivity of 0.14 gPHA L⁻¹ day⁻¹, respectively. Overall, this study showcases the first translation of phototrophic PHA production technology from an artificially illuminated laboratory scale system into a naturally illuminated, outdoor, pilot-scale system. It also addresses relevant process integration aspects with UASBs for pre-fermenting wastewater with molasses, providing a novel operational strategy to achieve photosynthetic PHA production in outdoor full-scale systems.

1. Introduction

Creation of economic value whilst performing resource recovery from waste streams is key to advance the implementation of sustainable resource management practices. In the last decade, purple phototrophic bacteria (PPB) have garnered renewed attention in the field of resource recovery applications due to their versatile capability for generating added-value bioproducts (e.g., polyhydroxyalkanoates (PHA), single cell proteins (SCP), hydrogen, carotenoids, fertilizers, co-enzyme Q10, and 5-aminolevulinic acid), which is an attractive feature when seeking the valorisation of waste and surplus streams (Capson-Tojo et al., 2020). PHA is a naturally occurring biopolymer that can be produced by several microorganisms as intracellular storage compounds. The biodegradability of PHA as well as their similar physical properties to conventional plastics, make this biopolymer a compelling alternative to substitute synthetic, petroleum-based polymers (Reddy et al., 2003). However, PHA production is currently associated with high production costs, mostly because of the high cost of carbon substrates, sterilization (in the case of single strain systems), and aeration requirements (in the case of aerobic cultures), as well as downstream cost for polymer extraction and purification (Reis et al., 2003; Fradinho et al., 2021a). In the search for lower-cost processes, PHA production using photosynthetic mixed cultures (PMC) enriched in PHA accumulating PPB have seen rising interest in literature studies. This emerging technology relies on PPBs capability to thrive in illuminated anaerobic environments as well as their remarkable ability to remove organic carbon from waste streams without requiring aeration (eliminating aeration and reducing substrate costs). Such features not only allows them to take advantage of sunlight as a free energy source, but also minimizes carbon dissipation through oxidation (typically associated to aerobic systems) and they therefore attain higher carbon recovery efficiency from waste streams (Fradinho et al., 2021b). Likewise, in PPB microorganisms, PHA storage can serve as a sink of reducing power or as a carbon source reserve, widening the selection strategies that can be applied in PMC to enrich them in PHA accumulating PPB. Up till now, such enrichment has been accomplished through the continuous presence of organic carbon, where PHA production was a means to maintain their internal cell redox balance (permanent feast selection) or through alternating periods of feast and famine of organic carbon, selecting bacteria that store PHA during the feast phase to consume it for growth during the famine phase (feast and famine selection) (Fradinho et al., 2013; Fradinho et al., 2016).

Despite the extensive knowledge gained thus far on PPB technologies most studies on phototrophic PHA production have been conducted at laboratory-scale and/or using artificial illumination (e.g., halogen lamps, infra-red lamps) (Fradinho et al., 2013; Allegue et al., 2022; Almeida et al., 2021; Luongo et al., 2017; Policastro et al., 2020), and very few were performed with real waste streams or even considered the simulation of outdoor conditions (e.g., transient light, temperatures). Nevertheless, promising results with waste streams have been attained under laboratory conditions with continuous illumination, where 20 % gPHA/g VSS was reported using fermented cheese whey (Fradinho et al., 2019) and fermented municipal waste (Luongo et al., 2017), and 42 % gPHA/g VSS was found utilizing fermented urban organic waste (Allegue et al., 2022). Prior lab-scale studies simulating outdoor conditions using fermented wastewater with molasses as feedstock have found a content

of 30 % gPHA/g VSS (Almeida et al., 2021) and 21.6 % gPHA/g VSS (Almeida et al., 2023) under simulated summer and winter conditions, respectively. Such findings demonstrate the feasibility of phototrophic PHA production using real waste, however, scale-up studies in real outdoor operating systems remains an unexplored area, and there is still the necessity to assess the full potential of the technology in such conditions. Under outdoor conditions, daily and seasonal fluctuations of sunlight and temperature are expected, which can impact the overall performance of PMC (Fradinho et al., 2021b). Furthermore, and since fermentation products such as volatile fatty acids (VFAs) are the preferable substrates to produce PHA (Sekoai et al., 2021), the assessment of a biorefinery concept that integrates waste fermentation with PHA production is needed, in a similar way as performed with aerobic mixed cultures (Morgan-Sagastume et al., 2015; Valentino et al., 2019).

With the aim of addressing these needs, this study evaluated for the first time an integrated system for phototrophic PHA production under real outdoor conditions at a pilot-scale level. Two outdoor high rate algae ponds (HRAPs), typically used for wastewater treatment, were retrofitted into PPB ponds targeting PHA production whilst valorising an organic rich stream (molasses) and using domestic wastewater as process water. Upstream to the PPB ponds, two up-flow anaerobic sludge blanket (UASB) reactors were used to ferment the molasses-supplemented domestic wastewater in order to produce VFAs, the precursors for PHA. Overall, this study focused on the assessment of the feasibility to transfer the phototrophic PHA production technology into an outdoor pilot-scale operated system and conduct an evaluation of the performance of an integrated process consisting of UASBs and PPB ponds.

2. Materials and methods

During one year and a half, an integrated system of UASB reactors and PPB ponds was operated under outdoor conditions, targeting the production of a VFA-rich stream and PPB biomass with a high PHA content, respectively. Fig. 1 presents the overall diagram of the process. Along the experimentation, adaptations and modifications of the operating conditions were made to promote PPB culture stability caused by environmental fluctuations. The specific operational parameters of the UASB reactors and the PPB ponds are discussed in the following sections and are summarized in Table 1 (schematically represented in Fig. S2, in the supplementary material).

2.1. UASB operation

Two 20 m³ UASB reactors (4.3 m high and 2.5 m in diameter) were operated to perform acidogenesis and produce VFA to be subsequently fed to the PPB ponds. The UASB reactors were operated in the wastewater treatment plant (WWTP) “El Torno” in Chiclana de la Frontera (36° 25'38.0" N – 6° 09'22.0" W). These reactors were built in fiberglass and had an internal triphasic separator (water, solids and gas) in the inner top-half. Each reactor had an external recirculation system to continuously measure pH and temperature as well as the redox potential using a CRI5303.99 probe and 5362 electrode, respectively.

A 0.2 m³ tank was placed on top of each UASB reactor to store the pre-treated wastewater (Fig. S3) that was fed into the reactors. Wastewater pre-treatment consisted of desanding-degreasing and sieving by a

1 mm rotary filter. The pre-treated wastewater was then pumped to the tanks using two mono pumps (Mono® Noy C23K, $Q = 0\text{--}3 \text{ m}^3/\text{h}$). The feeding flowrate pumped into the UASB reactor (0.28 and $0.42 \text{ m}^3/\text{h}$), was defined as a function of the UASB HRT for each operational period (Table 1). The ascending velocity of the feed water was provided under a pulsed strategy, allowing it to reach 8.63 m/h (Corona et al., 2016).

Throughout the experimental period, the wastewater and molasses fed into the UASB reactors were analysed. The average and standard deviation of the main parameters characterized in each stream can be found in Tables S1 and S2, respectively.

The molasses was provided by a sugar factory “Azucarera Iberia S.L.” based in Jerez de la Frontera (Spain) and stored in a 12 m^3 fibre glass tank. The molasses fermentation was carried out in UASB 2, while UASB 1 performed the fermentation of the pre-treated wastewater. From January 2019 onwards molasses started to be fermented as well in UASB 1 (Table 1).

The effluent generated in the UASB reactors was collected and stored continuously in a 1 m^3 fiberglass tank. The proportional mixture of effluent rich in volatile fatty acids (UASB 2) and the effluent without volatile fatty acids (UASB 1) was assured by the inertia and turbulences of the effluent downflow from the UASB digesters through the upper-lateral part (3 m height). In this mixing tank, the potential 1 % or 0.5 % fermented molasses proportion was diluted 1:2 as result of the addition of the UASB 1 effluent or the pre-treated wastewater.

2.1.1. Initial optimization - start-up

Each of the UASB reactors was initially inoculated with 10 m^3 of anaerobic sludge from an anaerobic sludge digester of the Guadalete WWTP, in Jerez de la Frontera. The sludge was characterized via analysis of volatile suspended solids ($\text{VSS} = 15.67 \text{ g/L}$), specific methanogenic activity ($\text{SMA} = 0.043 \text{ g COD/g VSS}\cdot\text{d}^{-1}$) and sludge volumetric index ($\text{SVI} = 69.2 \text{ mL/g TSS}$).

Throughout 90 days, an adaptation period was maintained (October to December 2017) to assure consistent biomass growth and reactor effluent stability. During this period, both UASB reactors were operated under an HRT of 48 h, with $0.5 \pm 0.1 \text{ g COD/L}$. During this time pH in both reactors was kept around 7.2 ± 0.2 , indicative of the stability of the system and an optimal value considered for continuous operation (Mainardis et al., 2020).

After the adaptation period the operational conditions in UASB 1 were kept, while the UASB 2 feeding was increased to $10.4 \pm 0.8 \text{ g COD/L}$, corresponding to 1 % (v/v) molasses in the wastewater in UASB 2. Molasses dosing was carried out in the feed line of the pre-treated wastewater to the UASB 2 feeding tank (Fig. S2, UASB Start Up), using 2 mono pumps (Seepex, $Q = 0.1 \text{ m}^3/\text{h}$). However, the presence of remaining sugars in the UASB effluent led to a change of operational conditions, reducing the molasses proportion in the UASB 2 feeding to 0.5 % (v/v) (Table 1, March 2018). This change allowed to reduce the remaining sugar content and decrease the COD_T to a new stable concentration in the PPB Ponds influent of $5.5 \pm 1.4 \text{ g COD/L}$ during the whole experimental period (Phase I, Phase II and Phase III).

2.1.2. Reactor operation in Winter (Phase II)

As the UASB reactors were not thermally insulated, weather variations influenced their performance and high levels of sugars could be registered in the winter UASB effluents due to incomplete fermentation. To avoid such problems, during the cold period between January 2018 and March 2019, the HRT of both UASB reactors was increased from 48 h up to 72 h. This change in the HRT led to a reduction in the available effluent flow and could not provide sufficient influent to the PPB ponds downstream. To compensate for this flow reduction, molasses addition (0.5 % v/v) was extended to both UASB digesters and the dilution of the effluent rich in VFAs was performed using the same wastewater fed to the UASB reactors (Fig. S2, Phase II).

After the winter period, temperature and solar irradiation increased, which allowed restoration of the previous HRT (48 h) in the UASB reactors supplemented with molasses (Fig. S2, Phase III).

2.2. Ponds operations

2.2.1. Initial optimization - start-up

Two identical 9.6 m^3 HRAP were operated in parallel for 426 days under outdoor conditions. PPB ponds were inoculated in March 2018 with a microalgal mixotrophic culture (9.6 m^3 , 148 mg TSS/L , 127 mg VSS/L , $76 \text{ mg COD}_S/\text{L}$, $26 \text{ mg NH}_4^+/\text{L}$, $3.5 \text{ mg PO}_4^{3-}/\text{L}$) from a HRAP of the WWTP, supplied with urban wastewater and operated for 3 weeks under fed-batch conditions with sporadic addition of fermented wastewater. Although, soon after, ponds had to be emptied to roughly half

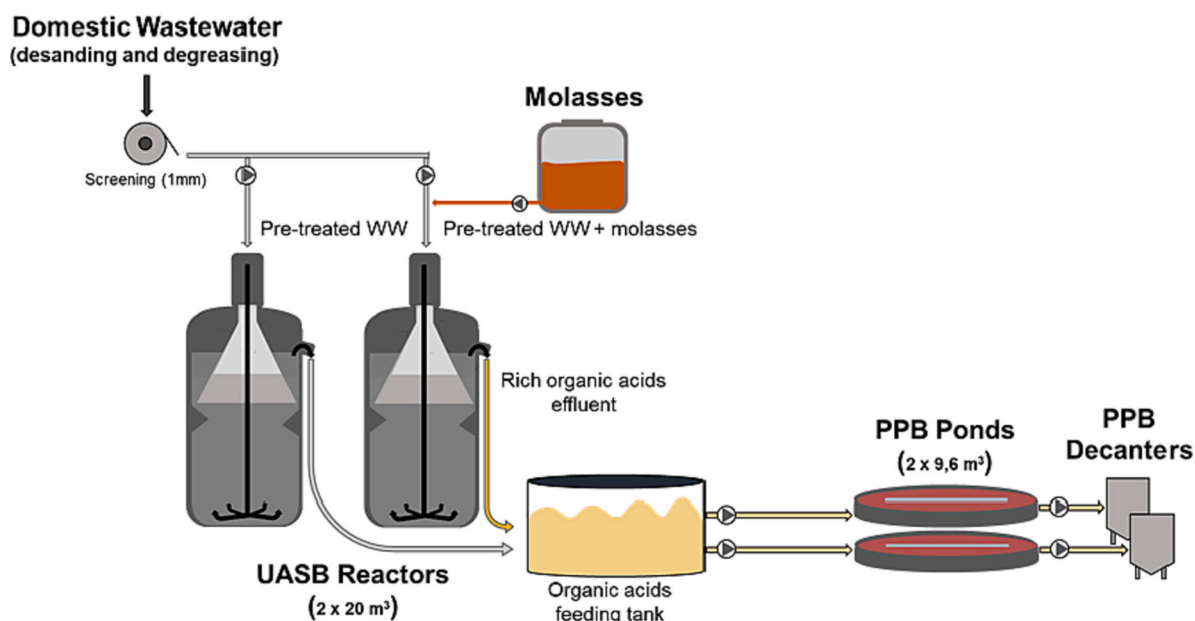


Fig. 1. General diagram of the operational process. A pictorial representation of the biomass evolution across the process can be found in Fig. S1. WW – Wastewater.

their content (= 15 cm) and refilled with fresh content from the operating pilot raceways, due to drastic pH drop caused by the phosphoric acid supplementation. While the pH of Pond 1 increased steadily, the pH of Pond 2 stayed low, and the previous procedure had to be repeated after 10 days (consult Fig. S4 to follow pH drop). After 3 weeks in batch mode operation, the supply of organic carbon (VFA rich effluents) was applied through pulse feeding. Organic load was then gradually increased along two weeks, until reaching a maximum of 8 daily pulses of 200 L each, in each pond, as described in Table 1. A submersible jet mixer (Caprari MAM11T2-400V) was placed in each pond as a stirring system. The ponds were operated under 24 h cycles and the PMC was selected under a light-feast/dark-aerated famine strategy according to Almeida et al. (2023) to enrich the ponds in PHA accumulating purple bacteria. The light-feast phase was ensured by the carbon pulse feeding during the initial hours of the day, starting at 9 am and spaced hourly. The pulse addition was ensured by two mono pumps (Seepex, Q = 0.5 m³/h) that pumped the stored effluent from the mixing tank of the UASB to the two ponds. To accomplish the dark-aerated famine phase, an aeration system was implemented in the night period to ensure PHA consumption by the PPB. This aeration was supplied via a pneumatic valve on the jet mixer that was activated after biomass harvesting (from 11 pm to 9 am) and allowed the entry of air into the jet mixer. The oxygen levels were not regulated until Phase II. An HRT of 6 days was implemented during spring/summer operation, and an HRT of 8 days in the winter period. This was set though the harvesting of biomass that was carried out before aeration started (around 10 pm). Pond harvesting was done using one Seepex mono pump (Q = 0.5 m³/h) in each pond. The harvested volume was 1.2–1.6 m³/day, according to the 8 or 6 day HRT setpoint, respectively. Harvested biomass was collected in a 2 m³ fiberglass decanter for each pond.

Ammonium (N) and phosphate (P) concentrations were kept between 30 and 100 mg/L in the pond, assuring non-limiting nutrients concentrations (Almeida et al., 2023). Dosing was done at the end of the pulsed VFA feeding phase via addition of liquid Brenntag urea TEC GF ([CH₄N₂O] = 43 %) and Brenntag phosphoric acid TEC GF ([H₃PO₄] = 75 %). To avoid a drastic pH drop, phosphoric acid addition was performed through a multidosage strategy that was dependent on the pH, to avoid the pH level going below 6. pH was monitored and varied between

6 and 7 during the 24 h cycle. The pH profile and overview of the pond's operation can be found in Fig. S5 of the supplementary material.

Weather parameters during the experimental period (sun irradiance, rain and temperature) were collected from the Agroclimatic Information Network of Andalusia (RIA) web site (Red de Información Agroclimática de Andalucía (RIA), 2018 and 2019).

2.2.2. Winter adaptation (Phase II)

As in the UASB reactors, the operation of the PPB reactors was adjusted in the winter months during the experimentation period. As shown in Table 1, the number of feed pulses was reduced from 8 to 6 and the aeration system was set to automatically control the DO levels below 2 mg O₂/L.

2.3. Analytical methods

Once a week, samples of pre-treated wastewater, the effluent from each UASB, a mixture of both effluents from the 1 m³ feed tank and effluents from each PPB pond were collected, stored in plastic canisters at -4 °C and transferred to the laboratory for analysis.

A Hach Lange DR 3900 RFID 320–1100 nm spectrometer and dedicated kits were used to determine the total nitrogen (LCK238), ammonium (LCK305), phosphate and total phosphate (LCK349) and the total and soluble chemical oxygen demand (COD_T and COD_S, respectively) (LCK214).

Total suspended solids (TSS) and volatile suspended solids (VSS) were determined accordingly to method 2540 D from APHA standard methods (APHA, 2012).

Pond samples were collected for cycle monitoring of organics, ammonia, phosphate and PHA. Unfiltered supernatant and biomass pellets were collected and frozen, and posteriorly sent to NOVA School of Science and Technology for analysis. The supernatant was then filtered (0.2 μm) and the biomass pellets were lyophilized for PHA analysis.

Volatile fatty acids, ethanol and sugar concentrations were determined by high performance liquid chromatography (HPLC) using a refractive index detector and an Aminex HPX-87H (Biorad) column. The eluent used was sulphuric acid (0.01 N), at a flow rate of 0.5 mL/min and an operating temperature of 30 °C.

Table 1
Operational conditions of the UASB reactors and the PPB ponds across all stages.

| | MONTHS | UASB | | | PPB Ponds | | | |
|---|----------|---------|------------------|--------|-----------|----------------|----------------|----------------------|
| | | HRT (h) | Molasses (% v/v) | | HRT (day) | Feeding Pulses | Night Aeration | Oxygen level control |
| | | | UASB 1 | UASB 2 | | | | |
| UASB | Oct 2017 | 48 | 0 | 0 | - | - | - | - |
| | ... | | | | | | | |
| | Dec 2017 | | | | | | | |
| Start Up | Dec 2017 | 48 | 0 | 1 | - | - | - | - |
| | ... | | | | | | | |
| | Feb 2018 | | | | | | | |
| PHASE I PPB pond Start-up and Summer operation | Mar 2018 | 48 | 0 | 0.5 | 6 | 1 → 8 | Yes | No |
| | Mar 2018 | | | | | | | |
| | Oct 2018 | | | | | | | |
| PHASE II Overcoming winter | Oct 2018 | 48 | 0 | 0.5 | 6 | 8 | Yes | No |
| | ... | | | | | | | |
| | Dec 2018 | | | | | | | |
| PHASE III Spring 2019 | Jan 2019 | 72 → 48 | 0.5 | 0.5 | 8 | 6 | Yes | Yes |
| | ... | | | | | | | |
| | Mar 2019 | | | | | | | |
| | Mar 2019 | 48 | 0.5 | 0.5 | 8 → 6 | 6 → 8 | Yes | Yes |
| | Apr 2019 | | | | | | | |

HRT – Hydraulic retention time.

Ammonia and phosphate were analysed by a colorimetric method implemented in a segmented flow analyser (Skalar 5100, Skalar Analytical, The Netherlands). Polyhydroxyalkanoate content was determined by gas chromatography according to Lanham et al. (2013), with small adjustments described in Fradinho et al. (2013).

Sludge volume index (SVI) was measured by adding 1 L of PPB pond activated sludge to a graduated cylinder and letting it settle. Biomass volumes were measured after 10 and 60 min.

2.4. Calculation of kinetic and stoichiometric parameters

The biomass PHA content was determined in terms of percentage of VSS on a mass basis (g PHA/g VSS). Biomass productivity, in g VSS/m²·day, was attained by multiplying the VSS concentration per the volume withdrawn per day (1600 L) and dividing it by the pond's area (32 m²). The overall PHA productivity, in g PHA L⁻¹ day⁻¹, was determined by multiplying the maximum percentage of PHA attained per the VSS concentration, multiplied by the total volume withdrawn per day (1600 L) and divided by the total volume of each pond (9600 L). The sludge volume index (SVI), in mL/g, was measured by dividing the biomass volume (mL/L), after 60 min, per the respective TSS

concentration (g/L). The ratio of the COD of fermentative products (COD_{FP}) per the soluble COD (COD_S), COD_{FP}/COD_S, was attained by dividing the COD_{FP} (in g COD/L) per the COD_S (in g COD/L).

3. Results and discussion

3.1. UASB performance

The initial 90 days of the start-up period were used to acclimatize the UASB's inoculum to the operational conditions of the reactors (HRT 48 h) and to the low COD charged wastewater (0.5 ± 0.1 g COD/L) (Table 2, October to December 2017). The long extension of the start-up was due to technical issues with the molasses piping (blockage and breaking due to molasses' high viscosity), delaying the molasses feeding into UASB 2.

From December 2017 to December 2018, UASB 1 fermented only wastewater, delivering an effluent with no more than 0.11 ± 0.02 g COD_S/L and a pH of 7.1 ± 0.1 (Table 2, UASB 1). On the other hand, the UASB 2 organic load was increased in December 2017 to 10.4 ± 0.8 g COD/L·day as a result of the mixture of 1 % molasses with wastewater. Although the organic load of UASB 2 could meet methanogens' needs

Table 2
UASB 1 and 2 and PPBs feeding tank variations.

| | PHASE | MONTHS | UASB settings | | pH | TSS | VSS | COD _T | COD _S |
|--------------------|---------------------|---------------------|----------------|-----------------|----------------|----------------|------------------|------------------|------------------|
| | | | HRT | Molasses | | | | | |
| | | | (h) | (% v/v) | | | | | |
| | | | | | | (mg/L) | (g COD/L) | | |
| Start up | | Oct 2017 → Dec 2017 | 48 | – | 7.0 (±0.1) | 43 (±20) | 38 (±15) | 0.22 (±0.04) | 0.12 (±0.03) |
| | | Dec 2017 → Feb 2018 | 48 | – | 7.1 (±0.1) | 42 (±4) | 38 (±2) | 0.21 (±0.04) | 0.11 (±0.02) |
| | | Mar 2018 | | | 6.9 (±0.5) | 39 (±14) | 36 (±12) | 0.19 (±0.05) | 0.09 (±0.02) |
| UASB 1 effluent | I | Mar 2018 → Oct 2018 | 48 | – | 7.0 (± 0.5) | 50 (± 33) | 43 (± 27) | 0.17 (± 0.04) | 0.08 (± 0.02) |
| | II | Oct 2018 → Dec 2018 | 48 | – | 6.9 (±0.8) | 31 (±13) | 27 (±10) | 0.14 (±0.04) | 0.08 (±0.02) |
| | | Jan 2019 → Mar 2019 | 72 | 0.5 | 4.6 (±0.4) | 377 (±118) | 348 (±109) | 5.46 (±1.76) | 4.63 (±1.52) |
| III | Mar 2019 → Apr 2019 | 72 → 48 | 4.7 (± 0.2) | | 398 (± 165) | 371 (± 152) | 4.98 (± 1.11) | 4.17 (± 1.30) | |
| Start up | | Oct 2017 → Dec 2017 | 48 | – | 7.3 (±0.5) | 59 (±15) | 53.2 (±23.4) | 0.19 (±0.03) | 0.09 (±0.02) |
| | | Dec 2017 → Feb 2018 | 48 | 1 | 4.5 (±0.3) | 542 (±33) | 483.1 (±11) | 22.3 (±4.60) | 20.12 (±4.26) |
| | | Mar 2018 | 48 | | 4.3 (±0.2) | 613 (±96) | 553 (±86) | 18.43 (±2.18) | 15.85 (±2.85) |
| UASB 2 effluent | I | Mar 2018 → Oct 2018 | 48 | 0.5 | 4.4 (± 0.2) | 513 (± 171) | 478 (± 148) | 13.7 (± 2.8) | 11.6 (± 2.6) |
| | II | Oct 2018 → Dec 2018 | 48 | | 4.5 (±0.6) | 574 (±328) | 530 (±301) | 10.22 (±5.46) | 7.47 (±4.16) |
| | | Jan 2019 → Mar 2019 | 72 | | 4.2 (±0.3) | 769 (±286) | 705 (±261) | 7.30 (±2.86) | 6.21 (±2.52) |
| III | Mar 2019 → Apr 2019 | 72 → 48 | 4.6 (± 0.4) | 1044 (± 289) | 931 (± 285) | 9.1 (± 0.8) | 7.8 (± 1.2) | | |
| PPB ponds influent | I | Mar 2018 → Oct 2018 | | | 4.8 (± 0.6) | 264 (± 119) | 246 (± 100) | 5.6 (± 1.5) | 4.5 (± 1.7) |
| | II | Oct 2018 → Dec 2018 | | | 4.9 (±0.9) | 243 (±92) | 223 (±84) | 5.29 (±1.61) | 4.53 (±1.31) |
| | | Jan 2019 → Mar 2019 | | | 4.7 (±0.6) | 303 (±97) | 283 (±88) | 5.03 (±1.95) | 4.45 (±1.78) |
| III | Mar 2019 → Apr 2019 | | | 4.6 (± 0.1) | 434 (± 120) | 406 (± 111) | 4.7 (± 0.9) | 3.8 (± 1.0) | |

(COD loads between 8 and 57.8 g COD/L-day; HRT 6–8 days; Lo et al., 1991), the operation under a stricter HRT of 48 h was expected to stimulate acidogenesis. It is worth noting that several studies on acidogenic fermentation using sugarcane molasses have HRTs lower than 24 h for a similar OLR. However, all of these studies were conducted at controlled temperatures exceeding 30 °C (Chang et al., 2011; More et al., 2023; Albuquerque et al., 2007). In contrast, in the present study, UASB reactors were operated without temperature control and were exposed to ambient temperatures, which were generally lower. Consequently, slower biochemical reaction rates were expected due to the lower operational temperatures (Khan et al., 2016; Liu et al., 2023) leading to the decision to implement a 48 h HRT.

Nevertheless, the evolution of UASB 2 to an acidogenic regime was confirmed from December 2017 on, through a fast pH decrease from 7.3 ± 0.5 to 4.5 ± 0.3 , as well as by the increase of volatile suspended solids in the effluent from 53.2 ± 23.4 mg VSS/L to 483.1 ± 11 mg VSS/L (Table 2, UASB 2, October to December 2017 and December 2017 to February 2018), which suggested the growth of acidogenic bacteria. In addition, the reduction of the pH down to 4.5 is well known to inhibit methanogens (Steinbusch et al., 2009; Hernández-Mendoza and Buitrón, 2014; Guarda et al., 2023) and therefore favour the growth of acidogenic bacteria. Under the operation of 1 % molasses with the wastewater, VFA production was detected, however, a high concentration of sugars in the UASB 2 effluent was observed, representing 30 % of the total carbon available (Table S3, February 2018), revealing an incomplete fermentation of the molasses. Therefore, the organic load of the UASB 2 was decreased to 0.5 % (v/v) molasses with wastewater to avoid the presence of sugar in the PPB ponds feedstock (since sugars are not PHA precursors for PPB and may promote the growth of fermentative organisms in the ponds, Almeida et al., 2021). The decrease of molasses from 1 to 0.5 % (v/v) ensured an effluent without the presence of sugars during phase I (Table S3, March to October 2018), achieving a content of fermentative products around 3.73 ± 0.85 g COD_{FP}/L, corresponding to 93.89 ± 29.97 Cmmol/L (Fig. 2 and Table S3, Phase I and II) in the PPB ponds influent, with butyrate and valerate representing more than 50 %

of the total VFAs composition. Previous research on sugarcane molasses fermentation reported similar final concentrations of VFAs (Chang et al., 2011; More et al., 2023). However, it is important to note that when considering the composition of the fermentate, earlier studies achieved higher concentrations of acetic acid, accounting for 50 % to 70 % of the total VFAs produced. This difference can likely be attributed to the pH conditions during operation. Some previous studies maintained higher pH levels (with a pH of 6.2 in Chang et al., 2011 and a pH of 9.5 in More et al., 2023), whereas the present study observed a lower pH of 4.5. Nevertheless, a COD_{FP}/COD_S ratio around 0.71 ± 0.15 (Fig. 2 and Table S3), was attained during the UASB 2 operation in Phase I, which could eventually allow the growth of a non-PHA-storing side population. A higher value of the COD_{FP}/COD_S ratio is an important parameter to achieve when considering a feedstock for the selection of a PHA-accumulating consortia (Morgan-Sagastume et al., 2015; Moretto et al., 2020), since increased COD_{FP}/COD_S ratios will ensure a higher VFA content in relation to other residual substrates, promoting the growth of PHA accumulating organisms over non-accumulating ones, as previously observed by Albuquerque et al. (2010).

3.1.1. UASB adaptation to the winter and spring periods

Once entering the winter period (Phase II, October 2018 to December 2018), a gradual decrease of the fermentative products content (2.83 ± 1.00 g COD_{FP}/L and 57.38 ± 31.05 Cmmol/L, Fig. 2 and Table S3) as well as the COD_{FP}/COD_S ratio (0.47 ± 0.20 , Fig. 2 and Table S3) were observed, indicating a loss of efficiency of the fermentative system at colder temperatures (Fig. S6.A). Therefore, to ensure a proper fermentation of the molasses during phase II, the HRT of both UASB reactors was increased from 48 to 72 h. Also, to generate a greater volume of VFA rich influent for the PPB ponds that could compensate the UASB's HRT increase, UASB 1 started to be fed with wastewater supplemented with 0.5 % of molasses, mimicking the conditions carried out in the UASB 2 reactor. Similar to the transition that occurred in UASB 2 during Phase I, the molasses addition to the UASB 1 reactor generated a change in the effluent composition, with a pH drop from 6.9

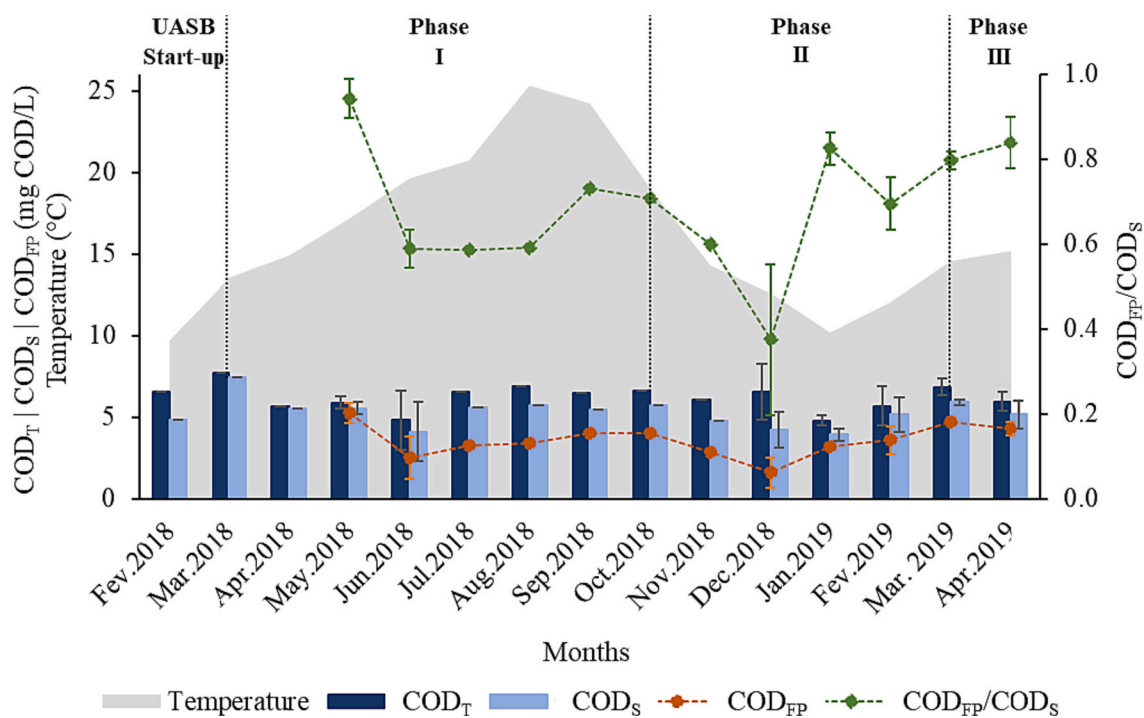


Fig. 2. PPB ponds influent. COD_T – total COD; COD_S – soluble COD; COD_{FP} – fermentative products COD (fermentative products – acetate, propionate, ethanol, butyrate isovalerate and valerate); COD_{FP}/COD_S – ratio of fermentative products COD per soluble COD; Temperature – average temperature registered during each month.

± 0.8 to 4.6 ± 0.4 , an increase in the effluent's VSS from 31 ± 13 to 377 ± 118 mg VSS/L, and as expected, an increase in the soluble organic content from 0.08 ± 0.02 to 4.63 ± 1.52 g COD_S/L (Table 2, UASB 1, Phase II).

In the UASB 2 reactor, the change in HRT from 48 to 72 h did not have an impact on the pH, which remained stable through all periods. However, a 34 % increase in TSS and VSS concentrations were observed (Table 2, UASB 2, Phase II), which could be due to an accumulation of biomass inside the UASB 2 reactor, since no biomass wastage of the reactor was carried out (Van Lier et al., 2010). The return to an HRT of 48 h in the spring period (Phase III) did not change the biomass accumulation trend in the digester. TSS and VSS concentration in the UASB 2's effluent increased again by 36 % and 32 %, respectively, between phases II and III. Expectably, the augmentation in solids was also detected in the influent fed to the PPB ponds, with increases of 25 % TSS and 27 % VSS after the change in HRT from 48 to 72 h (Table 2, PPB ponds inlet, Phase II) and 43 % in both TSS and VSS after the HRT was decreased from 72 h back to 48 h (Table 2, PPB ponds inlet, Phase III). This trend highlighted the need to establish a sludge wastage system in the UASB, as suggested by Cavalcanti et al. (1999) and Serrano et al. (2018), to avoid possible sludge clogging and sludge leaks to the effluent. Although such wastage was not implemented to avoid operational instabilities, it should be tested in future operational tests.

The effluent COD of the UASB 2 reactor decreased during the winter period (HRT 48 to 72 h; COD_T 29 % and COD_S 18 %) as a result of the reduction in the molasses fed to the reactor. However, the lower values achieved were compensated by operating UASB 1 with molasses, which ensured similar contents of COD_S and COD_{FP} in the PPB ponds inlet throughout the operation under an HRT of 72 h, namely 4.45 ± 1.78 g COD_S/L and 3.83 ± 0.63 g COD_{FP}/L (Phase II), comparatively to the operation with an HRT of 48 h, 4.53 ± 1.31 g COD_S/L and 3.73 ± 0.85 g COD_{FP}/L (Phase I). Furthermore, once the HRT was readjusted back to 48 h (Phase III), the COD_T and COD_S values in the UASB 2's effluent recovered, with an augmentation of 25 % and 26 %, respectively (Table 2, UASB 2, Phase II vs III).

Moreover, by the end of February 2019, a desirably high concentration of organic acids was achieved, with values higher than 80 Cmmol/L (3.16 ± 0.17 g COD_{FP}/L) consistently attained, with several days above 100 Cmmol/L (4.24 ± 0.73 g COD_{FP}/L) (Fig. 2 and Table S3). This time a higher COD_{FP}/COD_S ratio of 0.81 ± 0.04 was attained (Table S3), which was considered suitable for the efficient selection of a PHA-accumulating consortia, has previously discussed. Additionally, at this time, the preferable organic acids of purple bacteria, acetic and propionic, represented more than 50 % of the carbon source, which was a significant increase from the levels in the summer of 2018 that ranged only from 15 % - 30 % (Table S3). Once again, the VFA concentrations obtained were comparable with the ones achieved in molasses fermentation studies with a similar OLR (Chang et al., 2011; More et al., 2023). Propionic acid represented more than 30 % of the overall acids, which is higher than the propionic acid content attained by UASB studies fed with molasses (around 10 %) (Chang et al., 2011; More et al., 2023; Albuquerque et al., 2007).

3.2. Retrofitting of HRAP into PPB ponds

Retrofitting of the HRAPs into PPB ponds started with the inoculation of the two ponds with microalgae sludge (March 2018). After 1 month of stabilization (April 2018), the ponds were fed with fermented wastewater with molasses and supplemented with P and N to promote the growth of purple bacteria. Due to the pH drop, pond 2 required more time to stabilise as compared to pond 1. Nevertheless, the feeding of nutrients (ammonia and phosphate) and organic carbon allowed the growth of purple bacteria, and a gradual evolution in the biomass characteristics was visually observed through the colour alteration of the biomass from the ponds. The initial green colour of the culture

suggested a dominance of algae (March 2018, Pond 1 and 2), which evolved to a red-purple culture, suggesting the increased presence of purple bacteria in both ponds (June 2018 in Pond 1 and July 2018 in Pond 2) (see Fig. S7, from March to May 2018). This enrichment in purple bacteria coincided with increases in the biomass concentration (Pond 1– 2.51 ± 0.23 g VSS/L; Pond 2– 2.22 ± 0.33 g VSS/L) and with the maximum irradiance and temperature registered in Chiclana in 2018 (Fig. 3).

Additionally, sludge volume index (SVI) analysis performed during this period (July 2018) showed a SVI_{60min} of 42.2 ± 29.9 mL/g in pond 1, while pond 2 presented a SVI_{60min} of 58.5 ± 5.4 mL/g (Fig. S9 and Table S4). Both ponds presented low SVI (lower than 150 mL/g) after sedimentation, indicating a sludge with good settling characteristics, which would facilitate biomass recovery in the settling step for the subsequent downstream processing (e.g. PHA extraction).

Furthermore, though there was a decrease of light intensity in October and November, the ponds were still dominated by a red-purple colour (indicative of purple bacteria). Nevertheless, the culture started to become unstable, with the appearance of floating algae that stayed at the surface, shadowing the organisms underneath (Fig. S7, October and November 2018). From November onwards, the ponds were subjected to much lower irradiance and lower and less stable influent COD levels, leading to a loss in biomass concentration (Pond 1– 0.98 ± 0.36 g VSS/L; Pond 2– 0.94 ± 0.24 g VSS/L). Furthermore, the ponds were no longer red-purple (Fig. S7), indicating diminishment of the purple bacteria population. To overcome these changes, an aeration control system was applied during the famine phase, controlling the DO at a maximum of 2 mg O₂/L. This change was applied to lower energy consumption and prevent excessive proliferation of aerobic bacteria. After UASB operation was improved by the end of February 2019 (Table S3), and after the operation of the PPB ponds stabilized with the increased organic acid content, the red-purple colour became more accentuated with higher bacteriochlorophyll content, indicating the recovery of PPBs in the ponds (March 2019, Fig. S7 and S10).

Overall, the results suggested that biomass was conditioned more by the light irradiance than by the feedstock concentration. While during summer, autumn, and winter 2018, similar feedstock concentrations were observed (Fig. 3), the VSS concentration presented lower levels during the autumn and winter of 2018, when lower light irradiance was available. Light irradiance has been found to be a key factor for PPB proliferation (Fradinho et al., 2021b), and the results of this study suggest that it is likely to be the limiting factor controlling the phototrophic biomass growth rate.

3.3. PHA accumulation performance

The PHA accumulation performance of the biomass from the PPB ponds were evaluated in Phase I and Phase III. During Phase II, such evaluation was not conducted, since the system was unstable due to the adjustments made to UASB operation that led to instability of the ponds. Fig. 4 presents the PMC profile in the months of July, August, and October (2018), and the respective feedstock composition.

Table 3 presents the stoichiometric and kinetics parameters and productivities across the operation of the ponds.

Results indicate that in July, the culture of pond 1 was accumulating PHA during the daytime and consuming it during the night aerated period. It achieved a PHA content of 34.7 % g PHA/g VSS (HB:HV C-molar composition of 36:64) and a VSS concentration of 1.9 ± 0.4 g VSS/L, resulting in a system productivity of 0.11 g PHA L⁻¹ day⁻¹ (Table 3). In the case of pond 2, an accumulation of 5.7 % g PHA/g VSS (HB:HV C-molar composition of 62:38) was registered. Despite presenting a biomass concentration of 2.1 ± 0.5 g VSS/L, comparable to pond 1, pond 2 seemed to have a one-month delay in relation to pond 1, which started to show growth of purple bacteria earlier than pond 2. Also, an interesting difference between the two ponds is the HV content in the produced PHA, with 64 % HV and 38 % HV in pond 1 and 2,

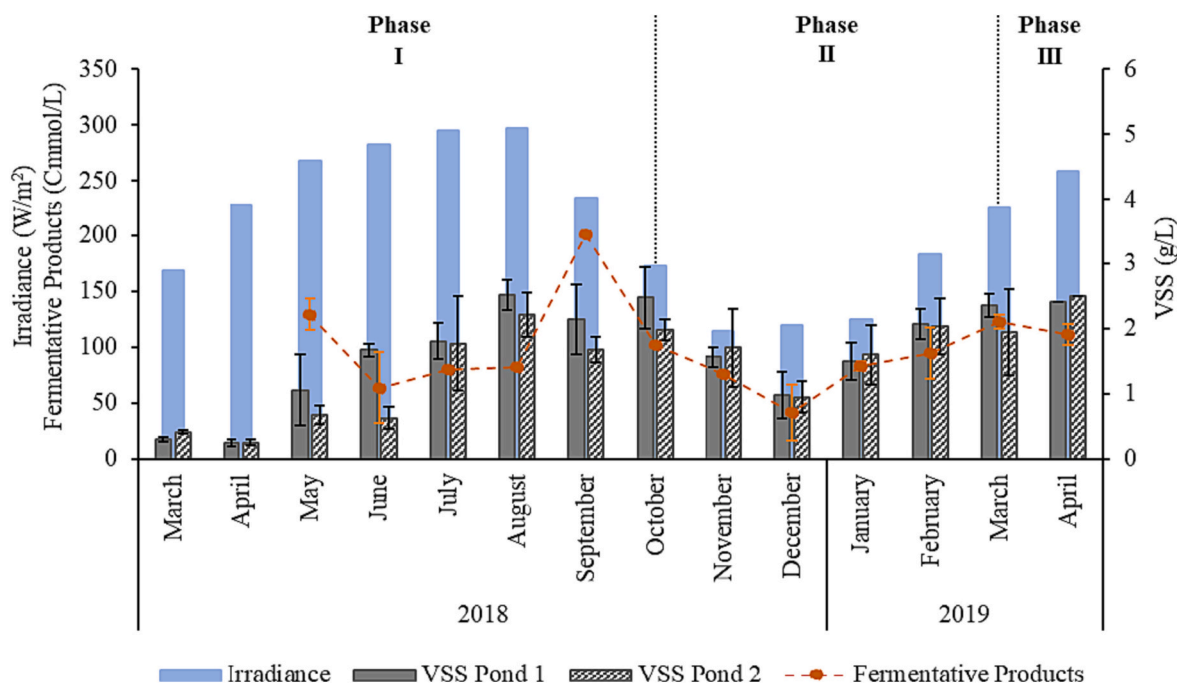


Fig. 3. Profile of the biomass concentration, irradiance, and influent concentration of fermentative products to the ponds. Complete profile of irradiance, rain, and temperature fluctuation in Fig. S6 and complete profile of VSS and TSS in Fig. S8.

respectively. Fig. 4 shows that in July, the influent was very rich in valeric acid, a compound that favours HV monomers production. The culture of pond 1 consumed all the organic acids in the light phase, while in pond 2, a high content of organic acids passed to the night phase, which was mostly valeric acid (later consumed in the night-aerated period, Fig. S11.A.2). This suggests that at this time of operation, the PHA producers in ponds 1 and 2 had different carbon preferences, which was reflected in the polymer composition and possibly, on its capacity to accumulate PHA. Nevertheless, correlations between the microbial population and respective substrate preferences should be conducted in future studies, for better understanding of the microbial culture response to eventual feedstock fluctuations and further advance process optimization.

In August, the culture in pond 2 achieved up to 21.7 % g PHA/g VSS (HB:HV C-molar composition of 73:27), while in pond 1 it achieved 14.5 % g PHA/g VSS (HB:HV C-molar composition of 39:61) (Fig. 4). The increase in pond 2 accumulating capacity was expected, since it had a one-month delay in the appearance of purple bacteria in relation to pond 1. In the case of pond 1, the low PHA accumulation is very likely due to an organic carbon load that could not meet the culture requirement. With a VSS concentration of 2.7 ± 0.3 g VSS/L, higher than the 1.9 ± 0.3 g VSS/L obtained in July, pond 1 presented just residual amounts of ethanol (< 0.5 mM C) and almost no organic acids were detected (Fig. S11.B.1). As such, with carbon limitation, the culture could not increase the PHA content. In pond 2, butyric and valeric acids were leftover carbon sources that passed to the night phase (Fig. S11.B.2), again indicating the low preference for these compounds. These results suggest that higher productivities could have been achieved if acetic and propionic had been the prevalent acids in the influent. During these months, biomass was also collected for microscopic observations and the PHA containing biomass could be visualized by microscopy (Fig. S12), corroborating the analytical data.

In October, results indicated that both ponds were behaving very similarly with respect to PHA accumulation. PHA contents of 9.5 % g PHA/g VSS (HB:HV C-molar composition of 46:54) and 12.5 % g PHA/g VSS (HB:HV C-molar composition of 30:70) were registered in pond 1 and 2, respectively, and at this stage no organic carbon was passing to the night phase. Once again, the culture of pond 1 seemed to be limited

in organic carbon (residual values < 1 mM C) and in pond 2, butyric and valeric were the acids measured in the pond. This likely impacted the PHA productivity and stresses the importance of a controlled OLR/VSS ratio and a favourable influent acid composition. Indeed, despite the higher OLR of the ponds (Table 3) than reported by previous studies on PHA production with PMC selected under simulated outdoor conditions at lab-scale (9 Cmmol/L-day in Almeida et al., 2021; 11.1 Cmmol/L-day in Almeida et al., 2023), the obtained ratio of OLR/VSS in the PPB ponds was lower (6.7 ± 0.4 OLR/VSS in this study; 8.6 OLR/VSS in Almeida et al., 2021; 10.3 OLR/VSS in Almeida et al., 2023) as well as the level of PHA accumulation attained (26.1 % g PHA/g VSS in Almeida et al., 2021; 21.6 % g PHA/g VSS in Almeida et al., 2023).

After the winter period and UASB stabilization, the ponds were again stable and the reappearance of the reddish colour in the ponds indicated the enrichment of PPB (Phase III, Fig. S7). Fig. 5 presents the feedstock composition and the PHA content obtained from the ponds in April 2019. The UASB alterations led to a higher concentration of fermentative products (Fig. 5 and Table S3) in 2019, with 50 % content of the preferable organic acids of purple bacteria, acetic and propionic (contrary to summer 2018 that ranged only from 15 % - 30 %; Fig. 4 and Table S3). Pond 1 did not go further than a 6.9 % g PHA/g VSS content, despite the constant presence of organic acids in the medium (Fig. S13), suggesting a poor selection of PHA accumulating microorganisms. On the other hand, Pond 2 achieved a PHA content of 36 % g PHA/g VSS (HB:HV C-molar composition of 17:83) and presented a VSS concentration of 2.3 g VSS/L, resulting in a system productivity of 0.14 g PHA $L^{-1} day^{-1}$ (Table 3).

In general, results showed that both ponds became enriched in PHA producing purple bacteria and that the cultures followed the behaviour previously predicted in similar studies performed at laboratory scale, with a PMC selected under outdoor simulated conditions and under a light feast/dark-aerated famine strategy (Almeida et al., 2023). The best outcomes were achieved in July 2018 and April 2019, with 0.11 g PHA $L^{-1} day^{-1}$ and 0.14 g PHA $L^{-1} day^{-1}$ (Table 3), respectively, which are very promising results for a first approach to the pilot operation of this technology. The obtained productivities were higher than the results achieved at laboratory scale by Almeida et al., 2023 (0.05 g PHA $L^{-1} day^{-1}$). Nevertheless, the pilot pond was operated in summer conditions

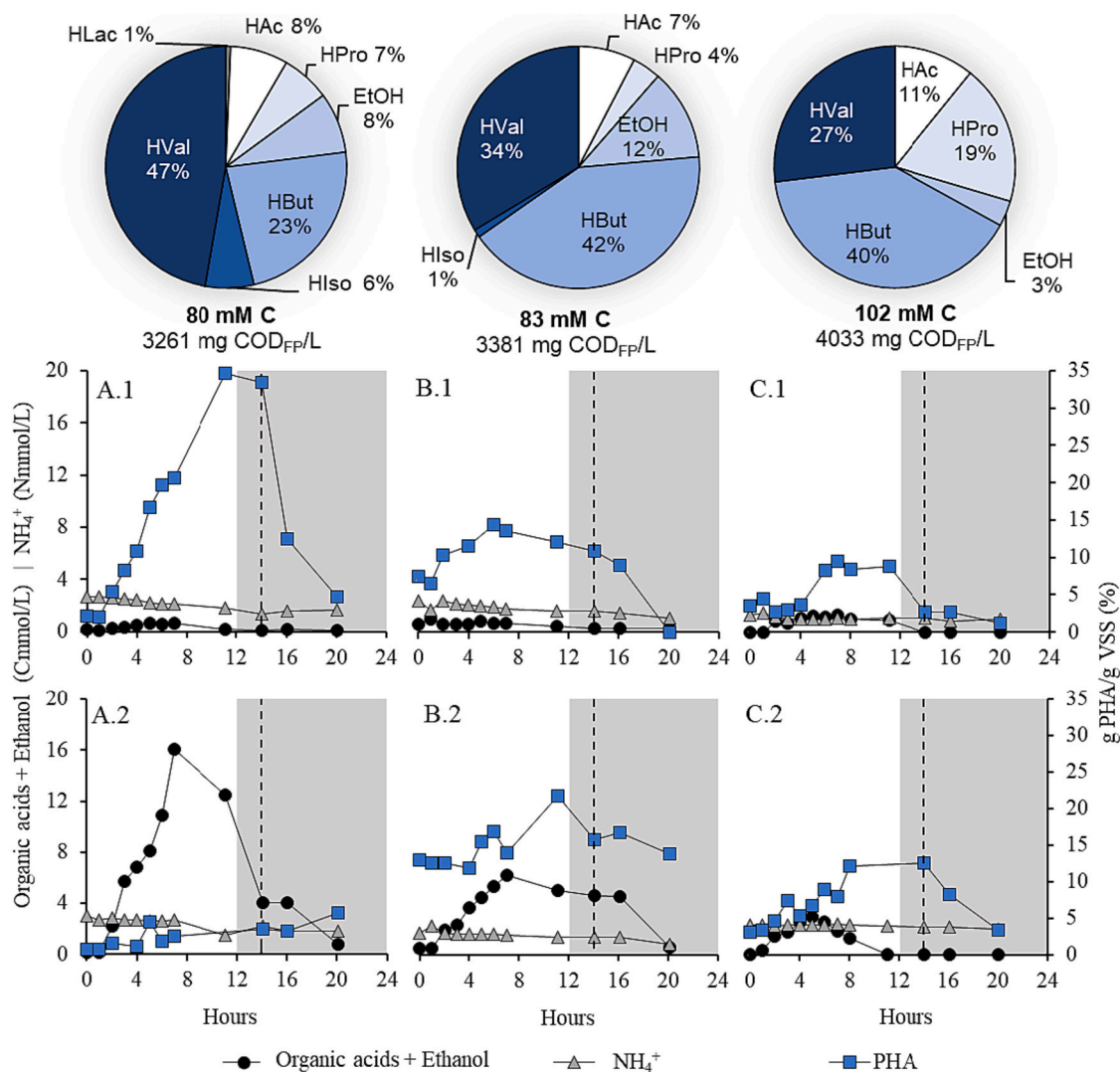


Fig. 4. PMC accumulation profile and respective feedstock composition and concentration. A. July 2018; B. August 2018; C. October 2018; 1. Pond 1; 2. Pond 2; □ Day period without aeration; ■ Night period; (—) Aeration start; HLac - lactate; HAc - acetate; HPro - propionate; EtOH - ethanol; HBut - butyrate; Hlso - isovalerate; HVal - valerate; COD_{FP} - Fermentative product's COD.

with temperatures between 15 °C – 30 °C (Fig. S6) and volumetric light intensities of 2000 W/m³, while the lab tests were conducted in winter conditions (13.2 ± 0.9 °C; 820 W/m³). Additionally, and when considering the maximum PHA contents obtained, 34.7 % and 36 % g PHA/g VSS, these results are in close alignment with the 42 % g PHA/g VSS content reported by a previous research on PHA production with PMC using fermented urban organic waste (Allegue et al., 2022). Though, it is noteworthy that such outcome was attained with a PMC selected under a permanent feast regime in a laboratory-scale membrane photobioreactor. Furthermore, it is evident that the PHA outcomes of the ponds were close to the 40 % g PHA/g VSS threshold established for a cost-effective downstream polymer recovery process (Bengtsson et al., 2017; Werker et al., 2018). Yet, several factors can lead to improved results in the future, namely a better control of the OLR/VSS ratio and the influent composition, both of which can likely lead to improved PHA productivities in the ponds. Results indicated that pond 1 was often limited by organic carbon and an increase of the influent carbon concentration would be beneficial. As well, results from pond 2 indicated that purple bacteria likely preferred acetic and propionic acids, and therefore, the operation of the fermenter should be tuned according to favour the production of these VFAs. In fact, after the feedstock composition and organic loading rate had stabilized (April 2019,

springtime), the selected PMC presented a productivity increase of 20 % in relation to the best productivity results achieved in July 2018 (summertime). This result not only corroborates the increased PHA production with purple bacteria with a controlled OLR and feedstock composition, but also that in outdoor conditions, PHA production is not limited to only the summer periods, but it is also achievable during springtime. Nevertheless, further optimization of the acidogenic fermentation should be considered, not only to maximize the PHA accumulation by the PMC, but also to ensure the economic feasibility of the process. For instance, previous acidogenic fermentation studies with sugarcane molasses have shown that higher acetic acid contents can be attained at pH 6 as compared to pH 5 (Albuquerque et al., 2007). Therefore, higher pH levels might be considered in future studies in order to increase the concentration of acetic and propionic acids, and thus, the growth and PHA production of PPB. Additionally, when considering the overall setup, a decrease in operational costs may be achieved by lowering the molasses dilution with the domestic wastewater, while tuning the HRT and the pH of the UASB for complete and efficient fermentation of the feedstock (Khan et al., 2016). Moreover, due to the adjustments of the UASB reactors during the winter period, further information is still required on the performance of the PPB ponds during wintertime. Previous reports have highlighted the capacity of

Table 3

Summary of the PHA content and volumetric productivities of VSS and PHA attained with the pilot ponds during the months of July, August and October of 2018, April of 2019.

| | | | OLR | | g PHA/ g VSS | Productivity | |
|--------|------|---------|-------------|----------------|-----------------|---|---|
| | | | Cmmol/L.day | VSS g/L | | g PHA L ⁻¹ day ⁻¹ | g VSS L ⁻¹ day ⁻¹ |
| Pond 1 | 2018 | July | 13.34 | 1.9 (± 0.3) | 0.35 | 0.11 | 0.31 |
| | | August | 13.78 | 2.7 (± 0.3) | 0.14 | 0.07 | 0.45 |
| | 2019 | October | 14.98 | 2.4 (± 0.5) | 0.09 | 0.04 | 0.40 |
| | | April | 17.35 | 2.4 (± 0.1) | 0.07 | 0.03 | 0.41 |
| Pond 2 | 2018 | July | 13.34 | 2.1 (± 0.5) | 0.06 | 0.02 | 0.34 |
| | | August | 13.78 | 2.3 (± 0.4) | 0.22 | 0.08 | 0.38 |
| | 2019 | October | 14.98 | 2.1 (± 0.4) | 0.13 | 0.04 | 0.35 |
| | | April | 17.35 | 2.3 (± 0.0) | 0.36 | 0.14 | 0.38 |

OLR – considers the total content of acetate, propionate, ethanol, butyrate, isovalerate and valerate detected by HPLC.

maintaining the PHA accumulation performance of phototrophic mixed cultures under simulated winter outdoor conditions (Almeida et al., 2023), nevertheless, further studies will be necessary to evaluate the possibility of operating outdoor ponds throughout the full year, which is a fundamental step to the economical sustainability of the process. Overall, such findings suggests that the production of PHA in retrofitted PPB ponds should be pursued further in the future, and that the technology of PHA production with photosynthetic systems was successfully transferred from lab scale to pilot-scale.

3.4. Implications for industrial PHA production by PMCs

The technology of resource recovery through phototrophic PHA production was, for the first time, implemented in an outdoor pilot-scale operational system, and results have shown that the implementation of PHA production in outdoor operating systems using fermented real waste is achievable. The first step towards the implementation of the technology in industry was accomplished, however when prospecting future on-site implementation, there are several factors that must be considered. Some important factors include the process design and/or infrastructure retrofit, the type of feedstock to be used within the process and also the water demand and usage within the process.

This study was conducted in a WWTP, an industry that may possess open raceway ponds for wastewater treatment that could be retrofit to

PHA production by PPB. Such infrastructure facilitates the growth of PPB in PMCs and represents a potentially viable means of implementing phototrophic PHA production in WWTPs. Nevertheless, the application of this technology in other industries (e.g., food industry with organic rich waste/surplus streams) without pre-existing infrastructure is also possible. Although initial investment would be necessary, limitations tied to the design of existing structures would be removed and the construction of reactors with a high ratio of volume to illuminated surface (such as tubular reactors or flat panels) would be possible, enabling the enhancement of the overall productivity of the PMC system (Fradinho et al., 2021b). When considering operations with diluted feedstocks (e.g. low organic carbon or nutrient content), a membrane photobioreactor configuration would allow the uncoupling of the HRT from the SRT, enabling a higher fluid inflow into the reactor without the washout of the biomass. Such set-ups could also be taken into consideration for PHA production. In fact, previous reports presented very promising PHA levels (42 % g PHA/g VSS) with a PMC operated in a membrane photobioreactor and fed with fermented urban organic waste (Allegue et al., 2022).

An ideal feedstock for PMC technology would be fermented organic streams rich in VFAs and depending on the characteristics of the waste stream available on site (e.g., organic content), the resources for the fermentation step can be locally or externally sourced. In WWTPs, a high amount of wastewater sludge is produced, and its narrow disposal routes

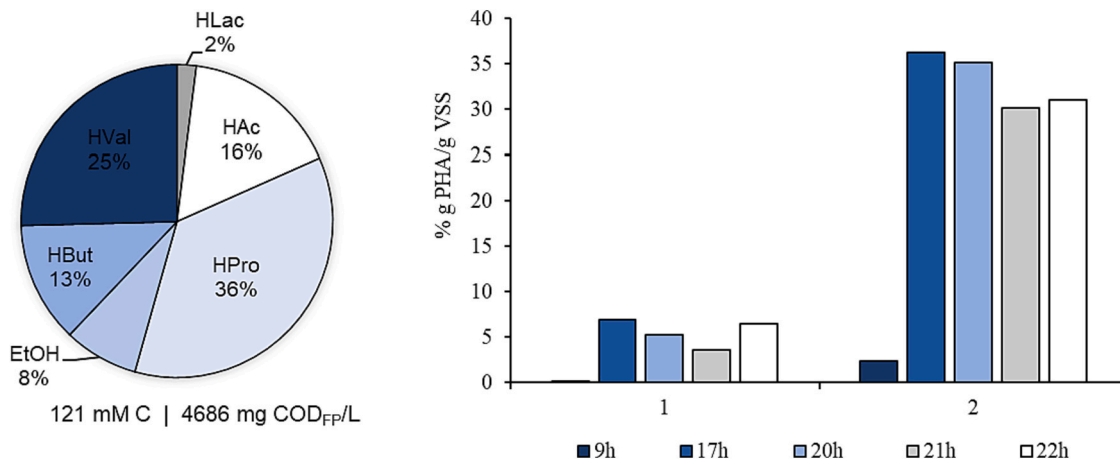


Fig. 5. PHA content obtained in both ponds in April 2019 and respective feedstock concentration and composition. 1. Ponds 1; 2. Pond 2; HLac - lactate; HAc - acetate; HPro - propionate; EtOH - ethanol; HBut - butyrate; HVal - valerate; COD_{FP} - Fermentative product's COD.

are a major problem for WWTP (Gherghel et al., 2019), so valorisation of sewage sludge by fermenting it into VFAs can be an interesting approach that some have suggested to be viable (Presti et al., 2021; Tayou et al., 2022). However, external feedstock sources could also be considered, such as animal waste, agriculture waste and even food waste, where the fermentation of such streams have been extensively reviewed by Sekoai et al. (2021), Mengqi et al. (2021) and Battista et al. (2022), respectively.

Furthermore, it's crucial to consider the increasing demand for fresh water and its imminent scarcity within PMC technology. In the context of this particular study, domestic wastewater was employed as the water source, where the PMC demonstrated its capability to grow and produce PHA. The fact that PPB could indeed thrive in systems with wastewater was expected, given that these microorganisms have been frequently studied for their wastewater treatment capabilities (Puyol et al., 2019; Hülsen et al., 2022; Sepúlveda-Muñoz et al., 2022). Furthermore, PPB have often been found in saline environments, however, the understanding of PPB's performance under high salinity conditions is still limited. Recent studies have reported that PPB are readily adaptable to wastewater treatment with high salinities (Hülsen et al., 2019), and can also produce PHB when growing in high salinity environments (Carlozzi et al., 2020). This highlights the opportunity of utilizing seawater or saline waste streams in PMC processes enriched in PPB for phototrophic PHA production.

4. Conclusions

The potential for scaling up phototrophic PHA production technology in an outdoor pilot-scale system was evaluated through the performance of an integrated setup comprised of two UASB reactors (responsible for the fermentation of wastewater with molasses), and two outdoor PPB ponds for PHA production using light-feast/dark-aerated-famine operation. The study revealed successful outdoor PHA production with the PMC enriched in PPB, achieving promising productivities across the summer and spring seasons. Control of OLR/VSS ratio in the PPB ponds and the VFA profile from the anaerobic pre-fermentation step were identified as key parameters impacting PHA production by the PMC outdoors. Overall, the transition of phototrophic PHA production technology to a larger operational scale was successful, prospecting future implementation of the technology.

CRedit authorship contribution statement

J.R. Almeida: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **E. Serrano León:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft. **F. Rogalla:** Resources, Project administration, Funding acquisition. **J.C. Fradinho:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – review & editing, Visualization, Supervision, Project administration. **A. Oehmen:** Conceptualization, Writing – review & editing, Supervision, Project administration. **M.A.M. Reis:** Conceptualization, Validation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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.

Data availability

Data will be made available on request.

Acknowledgements

This work was supported by national funds from FCT - Fundação para a Ciência e a Tecnologia, I.P., in the scope of the project UIDP/04378/2020 and UIDB/04378/2020 of the Research Unit on Applied Molecular Biosciences - UCIBIO and the project LA/P/0140/2020 of the Associate Laboratory Institute for Health and Bioeconomy - i4HB. Likewise, the INCOVER project has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (grant agreement n° 689242). J.R. A. also acknowledges the financial support of FCT - Fundação para a Ciência e a Tecnologia through the Ph.D. grant DFA/BD/8201/2020.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2023.168899>.

References

- Albuquerque, M.G.E., Eiroa, M., Torres, C., Nunes, B.R., Reis, M.A.M., 2007. Strategies for the development of a side stream process for polyhydroxyalkanoate (PHA) production from sugar cane molasses. *J. Biotechnol.* 130 (4), 411–421. <https://doi.org/10.1016/j.jbiotec.2007.05.011>.
- Albuquerque, M.G.E., Torres, C.A.V., Reis, M.A.M., 2010. Polyhydroxyalkanoate (PHA) production by a mixed microbial culture using sugar molasses: effect of the influent substrate concentration on culture selection. *Water Res.* 44, 3419–3433. <https://doi.org/10.1016/j.watres.2010.03.021>.
- Allegue, L.D., Ventura, M., Melero, J.A., Puyol, D., 2022. Unraveling PHA production from urban organic waste with purple phototrophic bacteria via organic overload. *Renew. Sustain. Energy Rev.* 166, 112687 <https://doi.org/10.1016/j.rser.2022.112687>.
- Almeida, J.R., Serrano, E., Fernandez, M., Fradinho, J.C., Oehmen, A., Reis, M.A.M., 2021. Polyhydroxyalkanoates production from fermented domestic wastewater using phototrophic mixed cultures. *Water Res.* 197, 117101 <https://doi.org/10.1016/j.watres.2021.117101>.
- Almeida, J.R., Serrano, E.L., Corona, E.L., Fradinho, J.C., Oehmen, A., Reis, M.A.M., 2023. Ammonia impact on the selection of a phototrophic - chemotrophic consortium for polyhydroxyalkanoates production under light-feast/dark-aerated-famine conditions. *Water Res.* 244, 120450 <https://doi.org/10.1016/j.watres.2023.120450>.
- APHA/AWWA/WEF, 2012. *Standard methods for the examination of water and wastewater*. Standard Methods 541.
- Battista, F., Strazzera, G., Valentino, F., Gottardo, M., Villano, M., Matos, M., Silva, F., Reis, M.A.M., Mata-Alvarez, J., Astals, S., Dosta, J., Jones, R.J., Massanet-Nicolau, J., Guwy, A., Pavan, P., Bolzonella, D., Majone, M., 2022. New insights in food waste, sewage sludge and green waste anaerobic fermentation for short-chain volatile fatty acids production: a review. *J. Environ. Chem. Eng.* 10 (5), 108319 <https://doi.org/10.1016/j.jece.2022.108319>.
- Bengtsson, S., Werker, A., Visser, C., Korving, L., 2017. PHARIO. Stepping Stone to a Value Chain for PHA Bioplastic Using Municipal Activated Sludge. STOWA, report 2017-15, Amersfoort, The Netherlands.
- Capson-Tojo, G., Batstone, D.J., Grassino, M., Vlaeminck, S.E., Puyol, D., Verstraete, W., Kleerebezem, R., Oehmen, A., Ghimire, A., Pikaar, I., Lema, J.M., Hülsen, T., 2020. Purple phototrophic bacteria for resource recovery: challenges and opportunities. *Biotechnol. Adv.* 43 <https://doi.org/10.1016/j.biotechadv.2020.107567>.
- Carlozzi, P., Di Lorenzo, T., Ghanotakis, D.F., Touloupakis, E., 2020. Effects of pH, temperature and salinity on P3HB synthesis culturing the marine *Rhodovulum sulfidophilum* DSM-1374. *Appl. Microbiol. Biotechnol.* 104, 2007–2015. <https://doi.org/10.1007/s00253-020-10352-1>.
- Cavalcanti, P.F.F., Medeiros, E.J.S., Silva, J.K.M., van Haandel, A., 1999. Excess sludge discharge frequency for UASB reactors. *Water Sci. Technol.* 40 (8), 211–219. [https://doi.org/10.1016/S0273-1223\(99\)00628-9](https://doi.org/10.1016/S0273-1223(99)00628-9).
- Chang, S., Li, J., Liu, F., 2011. Continuous biohydrogen production from diluted molasses in an anaerobic contact reactor. *Front. Environ. Sci. Eng.* 5 (1), 140–148.
- Corona, E.L., Santiago, J.R., Boizán, M.F. (inventors), FCC Aqualia, S.A. (assignee). 2016. Pulse wastewater injection and mixing device and wastewater injection method for anaerobic reactors. Madrid (ES) 28050 - EP3009408B1.
- Fradinho, J.C., Domingos, J.M.B., Carvalho, G., Oehmen, A., Reis, M.A.M., 2013. Polyhydroxyalkanoates production by a mixed photosynthetic consortium of bacteria and algae. *Bioreour. Technol.* 132, 146–153. <https://doi.org/10.1016/j.biortech.2013.01.050>.
- Fradinho, J.C., Reis, M.A.M., Oehmen, A., 2016. Beyond feast and famine: selecting a PHA accumulating photosynthetic mixed culture in a permanent feast regime. *Water Res.* 105, 421–428. <https://doi.org/10.1016/j.watres.2016.09.022>.
- Fradinho, J.C., Oehmen, A., Reis, M.A.M., 2019. Improving Polyhydroxyalkanoates production in phototrophic mixed cultures by optimizing accumulator reactor operating conditions. *Int. J. Biol. Macromol.* 126, 1085–1092. <https://doi.org/10.1016/j.ijbiomac.2018.12.270>.

- Fradinho, J.C., Carvalho, V.C.F., Reis, M.A.M., 2021a. New phototrophic factories for resource recovery. In: Moura, J.J.G., Moura, I., Maia, L.B. (Eds.), *Enzymes for Solving Humankind's Problems*. Springer, Cham. https://doi.org/10.1007/978-3-030-58315-6_13.
- Fradinho, J.C., Allegue, L.D., Ventura, M., Melero, J.A., Reis, M.A.M., Puyol, D., 2021b. Up-scale challenges on biopolymer production from waste streams by purple phototrophic Bacteria mixed cultures: a critical review. *Bioresour. Technol.* 327, 124820 <https://doi.org/10.1016/j.biortech.2021.124820>.
- Gherghel, A., Teodosiu, C., Gisi, S., 2019. A review on wastewater sludge valorisation and its challenges in the context of circular economy. *J. Clean. Prod.* 228, 244–268. <https://doi.org/10.1016/j.jclepro.2019.04.240>.
- Guarda, E.C., Costa, E., Gil, C., Amorim, C.L., Galinha, C.F., Duque, A.F., Castro, P.M.L., Reis, M.A.M., 2023. Acidogenic fermentation of brewers' spent grain monitored through two-dimensional fluorescence spectroscopy. *ACS Sustain. Chem. Eng.* 11 (19), 7398–7406. <https://doi.org/10.1021/acssuschemeng.3c00316>.
- Hernández-Mendoza, C.E., Buitrón, G., 2014. Suppression of methanogenic activity in anaerobic granular biomass for hydrogen production. *J. Chem. Technol. Biotechnol.* 89 (1), 143–149. <https://doi.org/10.1002/jctb.4143>.
- Hülsen, T., Hsieh, K., Batstone, D.J., 2019. Saline wastewater treatment with purple phototrophic bacteria. *Water Res.* 160, 259–267. <https://doi.org/10.1016/j.watres.2019.05.060>.
- Hülsen, T., Züger, C., Gan, Z.M., Batstone, D.J., Solley, D., Ochre, P., Porter, B., Capson-Tojo, G., 2022. Outdoor demonstration-scale flat plate photobioreactor for resource recovery with purple phototrophic bacteria. *Water Res.* 216, 118327 <https://doi.org/10.1016/j.watres.2022.118327>.
- Khan, M.A., Ngo, H.H., Guo, W.S., Liu, Y., Nghiem, L.D., Hai, F.I., Deng, L.J., Wang, J., Wu, Y., 2016. Optimization of process parameters for production of volatile fatty acid, biohydrogen and methane from anaerobic digestion. *Bioresour. Technol.* 219, 738–748. <https://doi.org/10.1016/j.biortech.2016.08.073>.
- Lanham, A.B., Ricardo, A.R., Albuquerque, M.G.E., Pardelha, F., Carvalheira, M., Coma, M., Fradinho, J., Carvalho, G., Oehmen, A., Reis, M.A.M., 2013. Determination of the extraction kinetics for the quantification of polyhydroxyalkanoate monomers in mixed microbial systems. *Process Biochem.* 48, 1626–1634. <https://doi.org/10.1016/j.procbio.2013.07.023>.
- Liu, H., Wang, F., Wang, Z., Wu, D., Xing, T., Kong, X., Sun, Y., 2023. Impact of pH, temperature, and hydraulic residence time on the acidogenic fermentation of fruit and vegetable waste and microbial community analysis. *J. Chem. Technol. Biotechnol.* 98 (3), 819–828. <https://doi.org/10.1002/jctb.7289>.
- Lo, K.V., Chiu, C., Liao, P.H., 1991. Anaerobic treatment of molasses wastewater I. Start-up and steady-state performance. *Can. Agric. Eng.* 33, 295–301.
- Luongo, V., Ghimire, A., Frunzo, L., Fabbriano, M., d'Antonio, G., Pirozzi, F., Esposito, G., 2017. Photofermentative production of hydrogen and poly- β -hydroxybutyrate from dark fermentation products. *Bioresour. Technol.* 228, 171–175. <https://doi.org/10.1016/j.biortech.2016.12.079>.
- Mainardis, M., Buttazzoni, M., Goi, D., 2020. Up-flow anaerobic sludge blanket (UASB) technology for energy recovery: a review on state-of-the-art and recent technological advances. *Bioengineering* 7 (2), 43. <https://doi.org/10.3390/bioengineering7020043>.
- Mengqi, Z., Shi, A., Ajmal, M., Ye, L., Awais, M., 2021. Comprehensive review on agricultural waste utilization and high-temperature fermentation and composting. *Biomass Conversion and Biorefinery* 2023 (13), 5445–5468. <https://doi.org/10.1007/s13399-021-01438-5>.
- More, P.P., Chavan, A.A., Sharma, M.B., Lali, A.M., 2023. Biobased volatile fatty acids (VFA) production via anaerobic acidogenesis of sugar processing industry effluent. *Environ. Technol.* 44 (8), 1179–1189. <https://doi.org/10.1080/09593330.2021.1996472>.
- Moretto, G., Russo, I., Bolzonella, D., Pavan, P., Majone, M., Valentino, F., 2020. An urban biorefinery for food waste and biological sludge conversion into polyhydroxyalkanoates and biogas. *Water Res.* 170, 115371 <https://doi.org/10.1016/j.watres.2019.115371>.
- Morgan-Sagastume, F., Hjort, M., Cirne, D., Gérardin, F., Lacroix, S., Gaval, G., Karabegovic, L., Alexandersson, T., Johansson, P., Karlsson, A., Bengtsson, S., Arcos-Hernández, M.V., Magnusson, P., Werker, A., 2015. Integrated production of polyhydroxyalkanoates (PHAs) with municipal wastewater and sludge treatment at pilot scale. *Bioresour. Technol.* 181, 78–89. <https://doi.org/10.1016/j.biortech.2015.01.046>.
- Policastro, G., Luongo, V., Fabbriano, M., 2020. Biohydrogen and poly- β -hydroxybutyrate production by winery wastewater photofermentation: effect of substrate concentration and nitrogen source. *J. Environ. Manage.* 271, 111006 <https://doi.org/10.1016/j.jenvman.2020.111006>.
- Presti, D., Cosenza, A., Capri, F.C., Gallo, G., Alduina, R., Mannina, G., 2021. Influence of volatile solids and pH for the production of volatile fatty acids: batch fermentation tests using sewage sludge. *Bioresour. Technol.* 342, 125853 <https://doi.org/10.1016/j.biortech.2021.125853>.
- Puyol, D., Monsalvo, V.M., Marin, E., Rogalla, F., Melero, J.A., Martínez, F., Hülsen, T., Batstone, D.J., 2019. Purple phototrophic bacteria as a platform to create the next generation of wastewater treatment plants: Energy and resource recovery. In: Olivares, J.A., Puyol, D., Melero, J.A., Dufour, J. (Eds.), *Wastewater Treatment Residues as Resources for Biorefinery Products and Biofuels*. Elsevier, pp. 255–280.
- Red de Información Agroclimática de Andalucía (RIA). (2018 and 2019). Estación Meteorológica de Jerez de la Frontera. <https://www.juntadeandalucia.es/agriculturaraypesca/ifapa/riaweb/web/estacion/11/2>.
- Reddy, C.S.K., Ghai, R., Rashmi, Kalia, V.C., 2003. Polyhydroxyalkanoates: an overview. *Bioresour. Technol.* 87 (2), 137–146. [https://doi.org/10.1016/S0960-8524\(02\)00212-2](https://doi.org/10.1016/S0960-8524(02)00212-2).
- Reis, M.A.M., Serafim, L.S., Lemos, P.C., Ramos, A.M., Aguiar, F.R., Van Loosdrecht, M.C.M., 2003. Production of polyhydroxyalkanoates by mixed microbial cultures. *Bioprocess Biosyst. Eng.* 25, 377–385. <https://doi.org/10.1007/s00449-003-0322-4>.
- Sekoai, P.T., Ghimire, A., Ezeokoli, O.T., Rao, S., Ngan, W.Y., Habimana, O., Yao, Y., Yang, P., Fun, A.H.Y., K.O., Daramola, M.O., Hung, C., 2021. Valorization of volatile fatty acids from the dark fermentation waste streams—a promising pathway for a biorefinery concept. *Renew. Sustain. Energy Rev.* 143, 110971 <https://doi.org/10.1016/j.rser.2021.110971>.
- Sepúlveda-Muñoz, C.A., Hontiyuelo, G., Blanco, S., Torres-Franco, A.F., Muñoz, R., 2022. Photosynthetic treatment of piggery wastewater in sequential purple phototrophic bacteria and microalgae-bacteria photobioreactors. *J. Water Process Eng.* 47, 102825 <https://doi.org/10.1016/j.jwpe.2022.102825>.
- Serrano, E.L., Vargas-Machuca, J.A.P., Corona, E.L., Arbib, Z., Rogalla, F., Fernández, M.B., 2018. Anaerobic digestion of municipal sewage under psychrophilic conditions. *J. Clean. Prod.* 198, 931–993. <https://doi.org/10.1016/j.jclepro.2018.07.060>.
- Steinbusch, K.J.J., Arvaniti, E., Hamelers, H.V.M., Buisman, C.J.N., 2009. Selective inhibition of methanogenesis to enhance ethanol and n-butyrate production through acetate reduction in mixed culture fermentation. *Bioresour. Technol.* 100 (13), 3261–3267. <https://doi.org/10.1016/j.biortech.2009.01.049>.
- Tayou, L.N., Lauri, R., Incocciati, E., Pietrangeli, B., Majone, M., Micolucci, F., Gottardo, M., Valentino, F., 2022. Acidogenic fermentation of food waste and sewage sludge mixture: effect of operating parameters on process performance and safety aspects. *Process Saf. Environ. Prot.* 163, 158–166. <https://doi.org/10.1016/j.psep.2022.05.011>.
- Valentino, F., Moretto, G., Lorini, L., Bolzonella, D., Pavan, P., Majone, M., 2019. Pilot-scale polyhydroxyalkanoate production from combined treatment of organic fraction of municipal solid waste and sewage sludge. *Ind. Eng. Chem. Res.* 58 (27), 12149–12158. <https://doi.org/10.1021/acs.iecr.9b01831>.
- Van Lier, J.B., Vashi, A., Van Der Lubbe, J., Hefferman, B., 2010. Anaerobic sewage treatment using UASB reactors: engineering and operational aspects. In: Fang, H.H.P. (Ed.), *Environmental Anaerobic Technology, Applications and New Developments*, pp. 59–89. https://doi.org/10.1142/9781848165434_0004.
- Werker, A., Simon Bengtsson, S., Korving, L., Hjort, M., Anterrieu, S., Alexandersson, T., Johansson, P., Karlsson, A., Karabegovic, L., Magnusson, P., Morgan-Sagastume, F., Sijstermans, L., Tietema, M., Visser, C., Wypkema, E., van der Kooij, Y., Deeke, A., Uijterlinde, C., 2018. Consistent production of high quality PHA using activated sludge harvested from full scale municipal wastewater treatment – PHARIO. *Water Sci. Technol.* 78 (11), 2256–2269. <https://doi.org/10.2166/wst.2018.502>.