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Ammonia impact on the selection of a phototrophic - chemotrophic consortium for polyhydroxyalkanoates production under light-feast / dark-aerated-famine conditions

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ABSTRACT

Phototrophic polyhydroxyalkanoate (PHA) production is an emerging technology for recovering carbon and nutrients from diverse wastewater streams. However, reliable selection methods for the enrichment of PHA accumulating purple phototrophic bacteria (PPB) in phototrophic mixed cultures (PMC) are needed. This research evaluates the impact of ammonia on the selection of a PHA accumulating phototrophic-chemotrophic consortium, towards the enrichment of PHA accumulating PPB. The culture was operated under light-feast/dark-aerated-famine and winter simulated-outdoor conditions (13.2 ± 0.9 °C, transient light, 143.5 W/m^2), using real fermented domestic wastewater with molasses as feedstock. Three ammonia supply strategies were assessed: 1) ammonia available only in the light phase, 2) ammonia always present and 3) ammonia available only during the dark-aerated-famine phase. Results showed that the PMC selected under 1) ammonia only in the light and 3) dark-famine ammonia conditions, presented the lowest PHA accumulation capacity during the light period (11.1 % g PHA/g VSS and 10.4 % g PHA/g VSS, respectively). In case 1), the absence of ammonia during the dark-aerated-famine phase did not promote the selection of PHA storing PPB, whereas in case 3) the absence of ammonia during the light period favoured cyanobacteria growth as well as purple sulphur bacteria with increased non-PHA inclusions, resulting in an overall decrease of phototrophic PHA accumulation capacity. The best PHA accumulation performance was obtained with selection under permanent presence of ammonia (case 2), which attained a PHA content of 21.6 % g PHA/g VSS (10.2 Cmmol PHA/L), at a production rate of 0.57 g PHA/L·day, during the light period in the selection reactor. Results in case 2 also showed that feedstock composition impacts the PMC performance, with feedstocks richer in more reduced volatile fatty acids (butyric and valeric acids) decreasing phototrophic performance and leading to acids entering the dark-aerated phase. Nevertheless, the presence of organic carbon in the aerated phase was not detrimental to the system. In fact, it led to the establishment of a phototrophic-chemotrophic consortium that could photosynthetically accumulate a PHA content of 13.2 % g PHA/g VSS (6.7 Cmmol PHA/L) at a production rate of 0.20 g PHA/L·day in the light phase, and was able to further increase that storage up to 18.5 % g PHA/g VSS (11.0 Cmmol PHA/L) at a production rate of 1.35 g PHA/L·day in the dark-aerated period. Furthermore, the light-feast/dark-aerated-famine operation was able to maintain the performance of the selection reactor under winter conditions, unlike non-aerated PMC systems operated under summer conditions, suggesting that night-time aeration coupled with the constant presence of ammonia can contribute to overcoming the seasonal constraints of outdoor operation of PMCs for PHA production.

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1. Introduction

Plastic pollution is currently one of the most urgent environmental concerns, and thus, new ecological and sustainable options for its management are needed (Kumar et al., 2020). Polyhydroxyalkanoates (PHA) are bio-based and biodegradable polymers, with similar properties to traditional plastics and have been explored as a potential alternative to non-biodegradable petroleum-based polymers. PHAs can be produced by many bacteria, which internally accumulate it as granules, for carbon and energy reserves (Adeleye et al., 2020).

Technology development within the field of PHA production has mostly targeted the use of aerobic systems, both with single strains or mixed microbial cultures. In the case of open mixed cultures operation, it allows the valorisation of complex waste streams, including agro-industrial (e.g. cheese whey, pulp/paper mill wastewater) and municipal wastes (organic fraction of solid wastes, activated sludge, domestic wastewater), as extensively revised in Gottardo et al. (2022) and Sabapathy et al. (2020). Recently, phototrophic mixed cultures (PMC) composed of microalgae-bacterial consortia rich in PHA producing purple phototrophic bacteria (PPB) have surged as a new technology in this field (Almeida et al., 2021; Fradinho et al., 2021; Fradinho et al., 2013). PPB are some of the most metabolically versatile microorganisms, being able to use light energy as a means to obtain ATP, and capable of removing organic carbon from waste streams under illuminated, anaerobic environments (Capson-Tojo et al., 2020). PHA production with PMCs facilitate a reduction of bioproduction costs by means of using sunlight as a free energy source, but also through the use of fermented waste streams rich in volatile fatty acids (VFA), which are the preferred substrates for PHA production (Bengtsson et al., 2008). To attain a high PHA production level, it is necessary to enrich PHA storing PPB in the PMC. This enrichment can be either obtained with the permanent presence of organic carbon (with the cellular reducing power balanced through PHA production) or through the feast and famine (FF) strategy, which is the approach also used in aerobic mixed culture systems. The transient availability of organic carbon, characteristic of the FF regime, allows the selection of bacteria that can accumulate PHA during the feast phase, and consume it for growth during the following substrate depletion (or “famine”) phase. At present, with this FF selection strategy, a PHA accumulation level of 30 % was reported for a PMC under simulated summer outdoor conditions (25 °C, 1.9 W/L, light/dark), using a fermented mixture of wastewater and molasses as feedstock (Almeida et al., 2021). The same study has revealed that prolonged periods of feast can enhance the growth and accumulation of PHA by PPB. However, the lack of oxygen production by microalgae during the dark phase prevented further growth of PPB on accumulated PHA, which implied that both the feast and famine phases had to be confined to the light period. As such, it is crucial to explore how the night period can be best operated to augment the overall PHA productivity.

The organic rich waste streams typically used for PHA production can have an extremely variable nutrient concentration (Rodriguez-Perez et al., 2018, Li & Wilkins, 2020), which can impact system performance. When the ammonia concentration is low, it is possible to uncouple the ammonia supply from the organic feeding stream and establish a selective pressure that separates the storage period from the growth period (Oliveira et al., 2017). In fact, studies with aerobic bacteria under the FF strategy reported a higher enrichment in PHA producers as well as higher PHA productivities with uncoupled nutrient feeding operation (Matos et al., 2021, Cruz et al., 2022). Similarly, studies of several single strains of purple bacteria reported that PHA accumulation is improved under ammonia limiting conditions (Sali & Mackey, 2021). However, there is still the need to evaluate the impact of when ammonia is fed during the selection of a PMC and its respective PHA production efficiency.

Furthermore, the prospect of using sunlight as a free energy source points towards the future implementation of PMC for PHA production in outdoor operating systems. However, outdoor conditions such as light intensity and temperature are subject to seasonal fluctuations which can have an impact on the culture performance. While studies on PPB for wastewater treatment have shown great adaptability of the PPB at low temperatures (Hülsen et al., 2016, Dalaei et al., 2019), studies with aerobic mixed cultures for PHA accumulation have shown that low temperatures negatively influenced the culture performance (Johnson et al., 2010). Additionally, light is a key factor affecting photosynthetic metabolism and low light intensity can directly impact performance (Fradinho et al., 2021). Therefore, the effect that low light and temperature have on the selection of a PMC for PHA production must be understood to successfully design and operate these systems during the winter months.

This work studies the impact of winter-simulated conditions on the PHA accumulation capacity of a PMC under FF operation, using a real fermented feedstock with low ammonia concentration. To overcome the adversities that winter conditions (lower light intensity and lower temperatures) may have on the culture performance, the full light period is proposed to be used as a feast phase to improve PPB growth. In addition, the dark phase was transformed into an active period through the supply of mechanical aeration, allowing PHA consumption by PPB in a dark-aerated-famine phase, making it independent of microalgae oxygenation. Therefore, the main focus of the study is the assessment of PMC selection and PHA productivity in the presence of ammonia at different phases of the cycle: 1) Light-phase only, 2) constant presence along the cycle and 3) only in the dark-famine aerated phase.

2. Methods

2.1. Phototrophic mixed culture operation

For 232 days, a sequencing batch reactor (SBR) simulating outdoor-winter conditions (143.5 W/m^2 light intensity, 13.2 ± 0.9 °C daily average temperature, temperature profile in Figure S4.A) and operational conditions (K_La of 0.0035 min^{-1}, 30 cm of liquid height) of a paddle wheel High Rate Algae Pond (HRAP) from El Torno WWTP (Chiclana de La Frontera, Spain) was operated with a working volume of 5.8 L (Figure S1). The reactor was inoculated with sludge from a PMC operated under a FF regime (Almeida et al., 2021). At a temperature of 13.2 ± 0.9 °C (profile in Figure S4.B) and a transient light cycle of 24 hours (12 h light / 12 h dark), the reactor was operated under FF conditions. During the 12 hour light period, the reactor was illuminated with a light intensity of 139.5 W/m^2 (0.82 W/L), provided by an external halogen lamp of 60 W. The SBR was pulse-fed with a real fermented mixture of wastewater with molasses during the initial hours of the light phase. The number of pulses and the interval between them were adjusted according to the feedstock concentration and performance of the culture. Likewise, the organic loading rate (OLR) was adjusted, as needed, to ensure that the feast phase was restricted to the light phase. During the 12 hour dark-period, aeration was achieved through an air pump connected to a disperser, to ensure complete consumption of any remaining carbon, and guarantee an aerobic famine dark phase for PHA consumption. The air pump operated with a maximum air flow of 0.36 L/min, and it was controlled by a set point established on the oxygen transmitter (M300 process, Mettler Toledo) that switched off aeration when the oxygen concentration reached 2 mg O_2/L. The reactor was operated with a K_La of 0.0035 min^{-1} in the light phase, through overhead continuous stirring, and with a HRT and a SRT of 6 days, established through mixed liquor withdrawal of 1 L at the end of the light phase.

Along the SBR operation, 3 different stages were defined through the ammonia availability: 1) Ammonia present only in the light phase (carbon to nitrogen molar ratio, C/N = 11); 2) Ammonia always present in the light and dark phases (C/N = 6.7); and 3) Ammonia present only in the dark-famine phase (ammonia was fed after 4 hours in the dark-aeration period, C/N = 11). The C/N ratios were set to ensure N limitation in stage 1 and 3 and guarantee the constant presence of ammonia in the medium in stage 2. The mineral media feeding (ammonia and phosphate) was performed by dump feed, and the phosphate loading rate (PLR) was set at 0.3 Pmmol/L·day to ensure no phosphate limitation (Almeida et al., 2021). Table 1 shows the operational parameters along the 3 stages. The oxidative reduction potential (ORP) and pH were monitored, with the pH naturally maintained between 6 and 7 (more detail on the daily real-time monitoring data in supplementary material section 1.1).

2.2. Feedstock solution

The reactor was fed with a fermented mixture of domestic wastewater (pre-treated, de-sanded and degreased) supplemented with molasses supplied by the Guadalete sugar plant. The fermented solution was obtained at laboratory scale (F1, F2 and F3), as described in Almeida et al. (2021), and at demo scale (F4, F5, F6, F7 and F8), with a 20 m^3 up flow anaerobic sludge blanket reactor. The molasses (chemical oxygen demand (COD), $COD_T = 1038 \pm 83$ g O_2/L) addition to the municipal wastewater ($COD_T = 592 \pm 114$ mg O_2/L) augmented the chemical oxygen demand, allowing the production of an effluent rich in organic acids. Detailed information about the demo scale operation, and respective effluent characterization is available in the supplementary material (Supplementary material, section 2. and Table S1). The fermented mixture was regularly collected and frozen at a temperature of -20 °C. Before feeding to the SBR, the feedstock was defrosted and settled overnight (0.51 ± 0.29 g TSS/L) and posteriorly kept at 4 - 6 °C under continuous stirring. A detailed analysis of the feedstock concentration and composition used throughout this paper is shown in Table S2 and Table S3.

2.3. PHA accumulation tests (pulse-feed experiments)

The maximum PHA accumulation capacity of the PMC was evaluated through pulse-feed experiments. External reactors were operated with a working volume of 1 L at a temperature of 15 °C, and externally illuminated with a halogen lamp (60 W) with a light intensity of 1.9 W/L (~ two times the light intensity of the SBR). The illumination intensity was set considering the operation of an outdoor accumulation pond with half the height of the selector pond, and thus, with the double of the light intensity. The biomass for the accumulation tests was collected directly from the selector SBR. The withdrawal was performed at different cycle times to ensure that the accumulation tests were performed under the same conditions imposed to accumulate PHA in the SBR (presence and absence of ammonia). For the accumulation tests relative to Stage 2 (NH_4^+ always present in the SBR), the biomass was collected at the end of the cycle and directly used for the accumulation tests, since ammonia was constantly present. For the accumulation tests relative to Stage 3 (NH_4^+ present only at the dark-famine phase) the biomass was collected from the SBR withdrawal at the end of the feast phase before the feeding of ammonia, to ensure that no ammonia was present in the medium. The biomass was kept at room temperature without stirring until the test was performed (~12 h later). Feedstock was supplied by pulse-feeding, according to the pH profile (i.e. pulse addition in response to a pH increase), and controlling the pH at 5.5 – 6.5. Table 2 summarizes the operational conditions of each accumulation experimental trial.

2.4. Analytical methods

The organic acid, ethanol and sugar concentrations were determined by high performance liquid chromatography (HPLC) using a refractive index detector and an Aminex HPX-87H (Biorad) column. Sulphuric acid (0.01 N) was used as eluent, at a flow rate of 0.5 mL/min and 30 °C operating temperature. Total carbohydrates hydrolysable to glucose were determined using the method described by Lanham et al. (2012), and the polyhydroxyalkanoates content was determined by gas chromatography according to Lanham et al. (2013), both with small adjustments described in Fradinho et al. (2013). Ammonia and phosphate were analysed by a colorimetric method implemented in a Segmented Flow Analyser (Skalar 5100, Skalar Analytical, The Netherlands). Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS) were determined according to standard methods (APHA et al., 2012). The light measurements, in the selection and accumulator reactors, were performed by a Li-COR light metre LI-250 A equipped with a pyranometer sensor LI-200 SA. The evolution of the microbial culture

Table 1
Operational conditions (OLR, NLR, PLR, mineral medium feeding time, feedstock) along the SBR's process stages.

Stage	Adaptation	1	2	3
Duration (days)	**1 – 11**	**12 – 35**	**36 – 147**	**148 – 232**
NH_4^+ presence	–	Light phase	Light and Dark phases	Dark-famine phase
Nutrient feeding Time	–	Beginning of light phase	Beginning of light phase	Beginning of Dark-famine phase
NLR	0	0.8 ± 0.3	1.8 ± 0.1	1.0 ± 0.2
PLR	0	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0
OLR	7.4 ± 2.9	9.5 ± 1.6	12.3 ± 3.4	11.2 ± 2.6
Feedstock	F1	F1	F2, 3, 4, 5, 6	F 6, 7, 8

NLR – Nmmol/L·day, **PLR** - Pmmol/L·day; **OLR** – Cmmol/L·day.

Table 2
Operational conditions of the accumulation tests.

Stage	Tests	Conditions	Duration (h)	Biomass withdrawal time	FEED
2	**A**	Light; Ammonia presence	8	End of cycle	F2
	B	Light + Air Ammonia presence	8	End of cycle	F2
3	**C**	8 h Light + 8 h Dark-air; No ammonia	16	Dark period End of feast phase	F8
	D	Light; No ammonia Acetate and Propionate pulses	16	Dark period End of feast phase	Synthetic
	E	Light + Air; No ammonia	8	Dark period End of feast phase	F8
	F	Dark + Air; No ammonia	8	Dark period End of feast phase	F8

regarding its bacteriochlorophyll *a*+*b* (Bchl *a*+*b*) and chlorophyll *a* (Chl *a*) pigments was performed by ethanol extraction, as described in Almeida et al. (2021).

2.5. *Microbial community analysis*

The characterization of the microbial culture was performed by DNA sequencing and fluorescence in situ hybridization (FISH). The DNA extraction and sequencing was performed by DNA Sense (Aalborg,

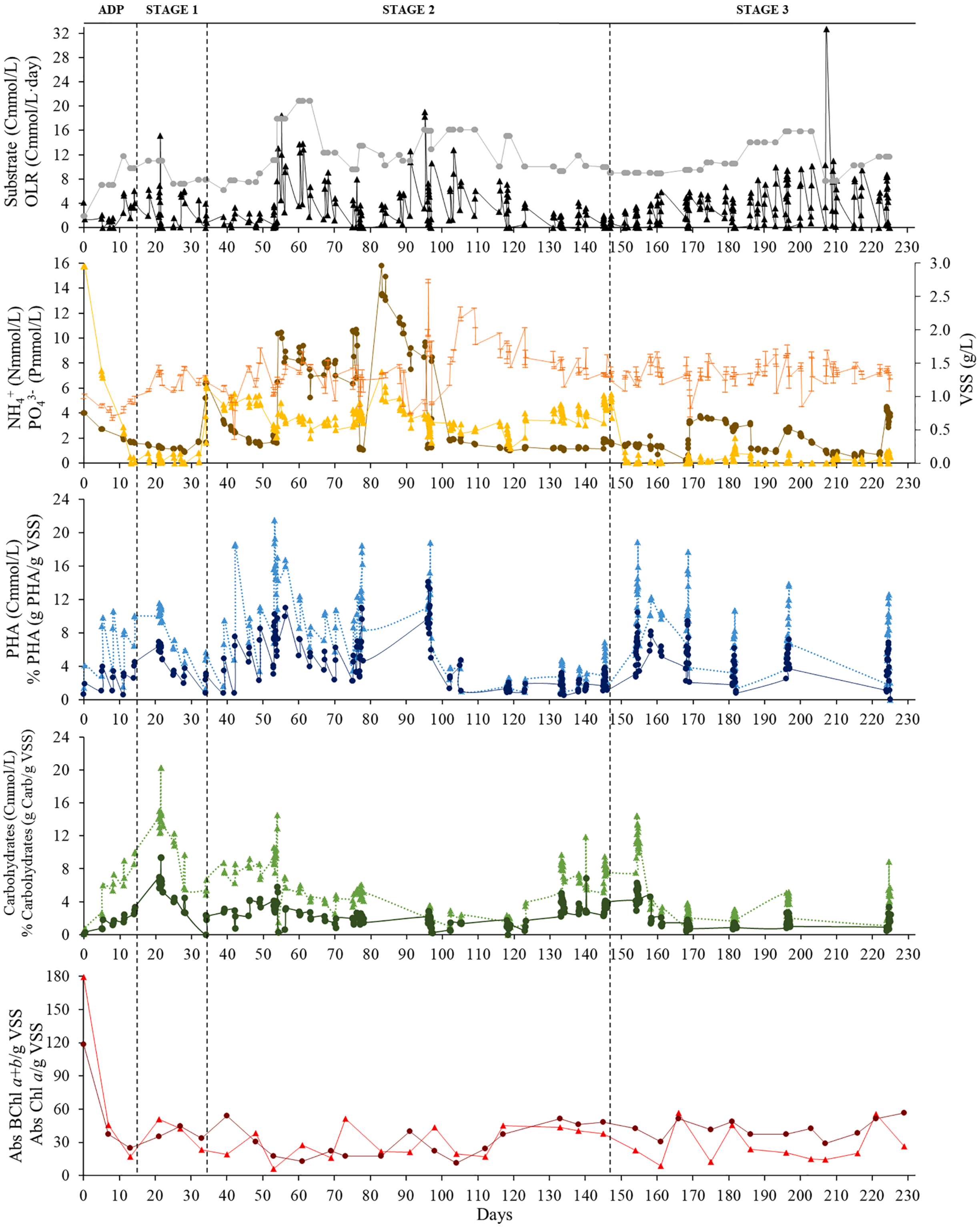

Fig. 1. Sequencing batch reactor profile along the 232 days of operation. (▲) Total carbon substrate; (●) Organic loading rate (OLR); (▲) Ammonia (NH_4^+); (●) Phosphate (PO_4^{3-}); (▬) Volatile Suspended Solids (VSS); (▲--) Polyhydroxyalkanoates content (% PHA/VSS); (●) Polyhydroxyalkanoates (PHA) concentration (Cmmol/L); (▲--) Total Carbohydrates content (% Carbs/VSS); (•) Total Carbohydrates concentration (Cmmol/L); (▲) Abs BChl a+b / g VSS (●) Chl a / g VSS.

Denmark) on a MiSeq (Illumina, USA). The biomass samples for this analysis were collected from the reactor, at different operational conditions, and stored at -20 °C. The protocol followed was similar to that described in Almeida et al. (2022), with minor alterations (detailed in the supplementary material, section 7).

The morphology and respective abundance of individual populations of the microbial culture was analysed in parallel through FISH. Sludge biomass samples were previously fixed with 4% paraformaldehyde (PFA), or ethanol as described by Nielsen et al. (2009). Detailed information on the probes used is described in supplementary material (section 7). Complementary to the FISH analyses, the intracellular PHA granules were observed through Nile blue staining, according to Bengtsson et al. (2008). The Nile blue staining samples and the fresh biomass samples were observed with an Olympus BX51 epifluorescence microscope.

2.6. Calculation of kinetic and stoichiometric parameters

The biomass PHA content and the total carbohydrate content were determined in terms of percentage of VSS on a mass basis, considering that VSS consists of active biomass (X), PHA and total carbohydrates. Active biomass was calculated by subtracting PHA and total carbohydrates from VSS. The standard biomass chemical formula applied for mixed microbial cultures, $CH_{1.8}O_{0.5}N_{0.2}P_{0.02}$, was used for active biomass calculations.

When NH_4^+ was not limiting, the specific biomass growth rate (μ, in day^{-1}) was determined through the uptake rate of ammonium, divided by the respective N:C molar biomass ratio of 0.2, according to the above-mentioned biomass chemical formula, and divided by the initial active biomass concentration.

Average specific PHA and carbohydrate production rates ($\overline{q}_{PHA}$, $\overline{q}_{Carbs}$ in Cmmol/Cmmol X·day) were calculated dividing the respective change in concentration as a function of time during the feast phase, per initial active biomass concentration.

The overall PHA productivity, in g PHA/L·day_feast phase, was determined by dividing the amount of PHA produced during the feast phase by the duration of the feast phase (day) and by the respective reactor volume (L). Since the feast phase could extend from the light to the dark period, PHA productivity was also separately determined for the feast-light and feast-dark phases, in g PHA/L·day_light phase and g PHA/L·day_dark phase, based on the PHA produced during the feast hours in each phase. The PHA productivity in the accumulation tests was calculated in a similar way, where the operational time of the accumulation test replaced the feast phase time.

The average specific substrate uptake rate ($-\overline{q}_S$, in Cmmol/Cmmol X·day) for each feedstock component was determined through an organic carbon mass balance per initial active biomass concentration. The sum of each individual uptake rate was used to calculate the total substrate uptake rate, namely, the sum of lactate, acetate, butyrate, propionate and valerate concentrations (organic acids, in terms of Cmmol/L) plus ethanol and sugar (glucose and fructose) concentrations.

The yields of PHA, carbohydrates and active biomass per substrate ($Y_{PHA/S}$, Cmmol PHA/Cmmol S; $Y_{Carbs/S}$, Cmmol Carbs/Cmmol S; $Y_{X/S}$, Cmmol X/Cmmol S) were calculated by dividing the volumetric production rates of PHA, carbohydrates and growth rate by the substrate uptake rate. The total yield (Y_{total}, Cmmol PHA+Carbs+X/Cmmol S) was calculated by the sum of the yields of PHA, carbohydrates and active biomass per substrate uptake rate.

The feast to famine ratio (F/F) was determined by dividing the number of hours of the feast phase by the hours of the famine phase.

The relative abundance of purple bacteria was obtained by dividing the sum of the percent content of *Rhodobacter, Rhodopseudomonas, Bosea, Chromatiaceae, Rhodospirillaceae*, and *Hyphomicrobiaceae* by 100.

The ratios between the absorbence of Bacteriochlorophyll $a+b$ or absorbence of Chlorophyll a per volatile suspended solids (Abs BChl $a+b$/gVSS; Abs Chl a/gVSS), were calculated by dividing the extracted pigments absorbence (775 nm and 665 nm respectively) per the total grams of biomass used in the analysis.

3. Results

3.1. Photosynthetic mixed culture development overview

A sequencing batch reactor was operated for 232 days under winter outdoor-simulated conditions and a feast and famine regime, using real fermented feedstock. After inoculation, the reactor had an adaptation period of 11 days, to acclimatize the culture to the presence of aeration in the dark phase and to limit the ammonia to the light phase. Fig. 1 presents the profile of the PMC along the 3 operational stages, and Table 3 summarizes the respective parameters analysed. Across the 3 stages with different ammonia feeding strategies, the reactor was operated with a stable OLR, and a constant concentration of biomass around 1.4 ± 0.2 g VSS/L was maintained. Irrespectively of the ammonia feeding approach applied to the system in the SBR, the microbial culture was able to accumulate PHA (Table 3). However, the lowest values of PHA accumulation were registered at stage 1 and at the

Table 3
PMC characterization and respective variation at each operational stage.

		Stage 1 (Light ammonia) 12nd to 35th day	Stage 2 (Always ammonia) 36th to 147th day	Stage 3 (Dark-famine ammonia) 148th to 232nd day
(1)	**VSS** (g/L)	1.4	1.5 ± 0.3	1.4 ± 0.1
(2)	**Active Biomass (X)** (g/L)	0.8 ± 0.2	1.1 ± 0.3	1.2 ± 0.1
(3)(*)	**NH_4^+** (Nmmol/L)	0.7 ± 0.2	4.4 ± 1.1	1.1 ± 0.5
(3)(**)	**PO_4^{3-}** (Pmmol/L)	1.5 ± 0.2	4.9 ± 3.9	2.6 ± 0.9
	OLR (Cmmol/L·day)	9.5 ± 1.6	12.3 ± 3.4	11.2 ± 2.6
(4)	**PHA**			
	(Cmmol/L)	6.7	7.0 ± 3.6	8.5 ± 1.6
	(% g PHA/g VSS)	11.2	12.9 ± 7.2	15.3 ± 3.3
(4)	**Carbohydrates**			
	(Cmmol/L)	9.4	4.1 ± 1.0	3.0 ± 2.0
	(% g Carbs/g VSS)	20.3	9.9 ± 3.1	6.5 ± 4.7
	F/F	1.3	1.0± 0.4	1.5± 0.6

(1) Before biomass withdrawal; **(2)** After carbon feedstock feeding; **(3)** After nutrient feeding; **(4)** Maximum values registered during full cycles monitoring, Stage 1 n=1; Stage 2 n=4; Stage 3 n=4; (*) The effluent contained ammonium only in stage 2; (**)The effluent contained phosphate in all stages, minimum value registered at the end of the cycles was 21 mg P/L;

end of stage 2 (Fig. 1).

In stage 1, ammonia was fed and present only during the light phase, and a maximum accumulation of 11.2% g PHA/g VSS (6.7 Cmmol/L; 60HB:40HV; HB as short abbreviation for 3-hydroxybutyrate monomer and HV as short abbreviation for 3-hydroxyvalerate monomer) was reached (Fig. 1). However, no PHA consumption was observed during the famine phase (Figure S6 and Table S4), which led to the absence of a PHA accumulation/consumption profile during this stage. Stage 2 was characterized by the constant presence of ammonia and registered the highest PHA storage in the selection reactor across all ammonia settings, 21.6% g PHA/g VSS (10.2 Cmmol/L; 75HB:25HV) (Fig. 1). However, by the end of the stage, a low PHA content was verified, 4.7% g PHA/g VSS (3.0 Cmmol/L; 54HB:46HV) (Fig. 1), under the operation with a feedstock with a high concentration of sugars (F4 and F5, 29% and 14% of the total organic carbon, respectively). Regarding stage 3, the presence of ammonia was restricted to the dark-famine phase, and the PMC was able to achieve a maximum PHA content of 17.8 % g PHA/g VSS (11.1 Cmmol PHA/L; 68HB:32HV) (Fig. 1) in the selection reactor.

The HB and HV content of the PHA was observed to oscillate according to the feedstock composition. An increased HB content was obtained under a higher total concentration of acetate, butyrate, ethanol and lactate in the feedstock, while a greater HV content was attained under a higher total concentration of propionate and valerate (Figure S5). However, this tendency was not observed when the feedstock had a high sugar content (F4 and F5; Table S3).

Furthermore, different period lengths were applied for each ammonia condition, where stage 1 was the shortest operating period due to the absence of a PHA accumulation/consumption profile, showing that PHA accumulators did not display a selective advantage over the other microorganisms. Stage 2 was the longest operational stage, mostly due to the entrance of a feedstock with sugars (non-PHA precursors), necessitating evaluation of the process performance with and without sugar. Finally, stage 3 was operated for about 80 days, matching the operating period of stage 2 with a sugar-free feedstock.

Since the highest PHA accumulation was attained under the constant presence of ammonia (stage 2) and with ammonia in the dark-famine period (stage 3), a deeper analysis of the PMC selection and PHA accumulation capacity with these conditions was performed and presented in the subsequent sections.

3.1.1. *Ammonia always present in the light and dark phases*

The continuous presence of ammonia during the cycle was applied in stage 2. Fig. 2 presents two cyclic profiles of stage 2, with different feedstocks (F2 and F3, Table S3), while Table 4 indicates the kinetic and stoichiometric parameters measured in these cycles.

When the culture was fed with the F2 feedstock solution (Table S3) at an OLR of 11.1 Cmmol/L·day, the feast phase was approximately 6 hours (F/F = 0.34) (Fig. 2.A). In contrast to what was observed in Stage 1, under a similar feedstock composition (Figure S6), the culture presented an improved storage/consumption profile of PHA. In the light

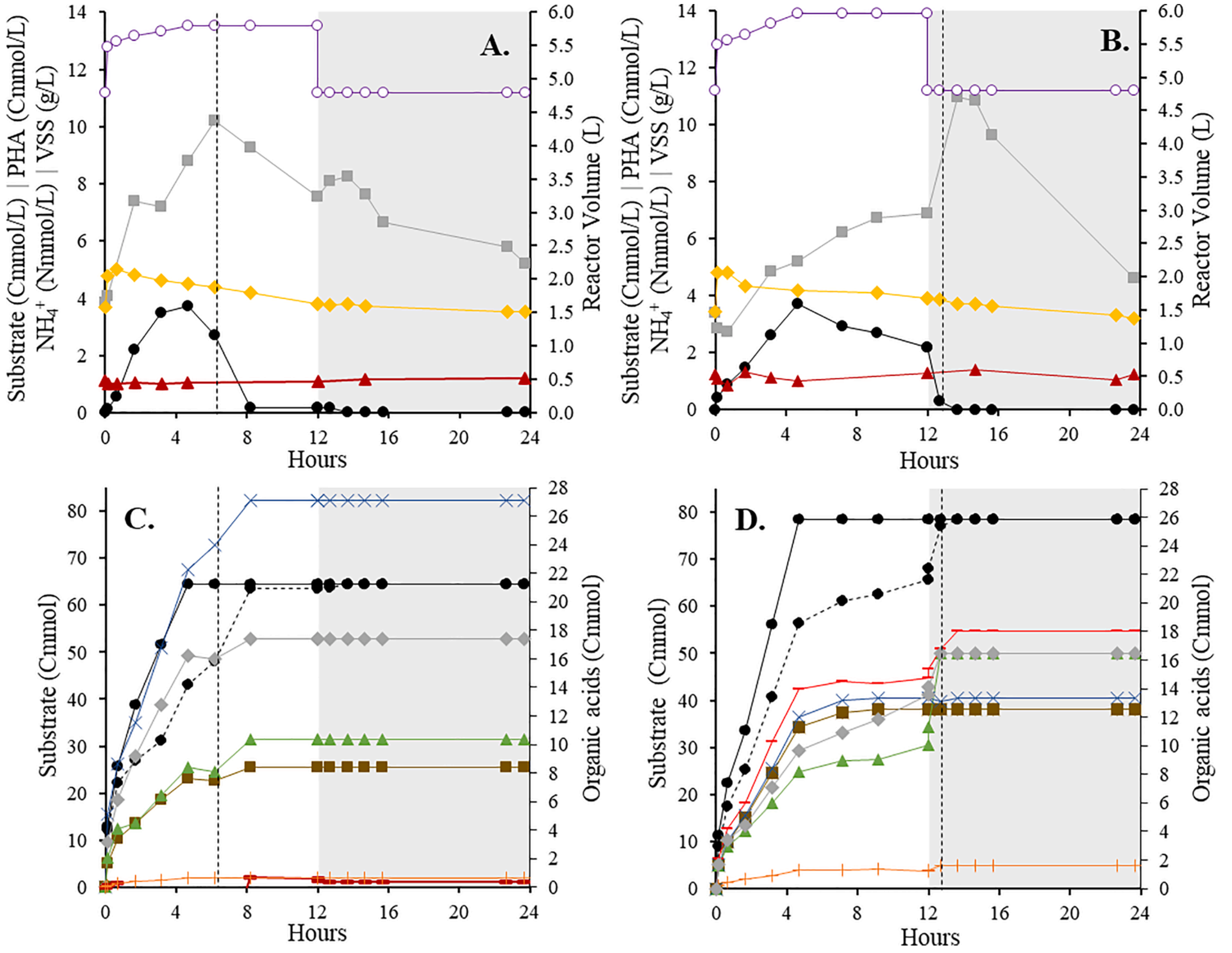

Fig. 2. Photosynthetic mixed culture profile in Stage 2 under constant presence of ammonia. [ ] Light period; ▒ Dark aerated period; (—) End of feast phase; A. General profile of SBR cycle, on 53rd day, with feedstock F2; B. General profile of SBR cycle, on 77th day, with feedstock F3; (○) Reactor volume; (■) Polyhydroxyalkanoates (PHA) concentration; (●) Total carbon substrate; (◆) Ammonia (NH$_4^+$); (▲) Volatile Suspended Solids (VSS); C. Cumulative profiles of chart A.; D. Cumulative profiles of chart B.; (●) Substrate feeding; (-●-) Substrate consumption; (×) Acetate consumption; (◆) Butyrate consumption; (▲) Valerate consumption; (■) Propionate consumption; (+) Lactate consumption; (▬) Ethanol consumption.

Table 4
Operational conditions, kinetic and stoichiometric parameters of the PMC with the continuous presence of ammonia.

Chart		**A**	**B**		
Feedstock		**F2**	**F3**		
F/F		0.34	1.1		
OLR		11.12	13.53		
X (Cmmol/L)		37.92	44.96		
X (g/L)		0.93	1.11		
Phase		Light feast	Light feast		Dark-aerated feast
			0 – 7h	*7 – 12h	
- $q_{Substrate}$	**Lactate**	n.p.	0.02	0.00	0.06
	Acetate	0.51	0.20	n.p.	n.p.
	Propionate	0.16	0.19	n.p.	n.p.
	Ethanol	0.00	0.23	0.01	0.23
	Butyrate	0.34	0.17	0.06	0.37
	Valerate	0.17	0.14	0.03	0.82
	Total	1.05	0.95	0.09	1.48
q_{PHA}		0.81	0.34	0.09	1.30
μ		0.23	0.36	0.01	0.19
$Y_{PHA/S}$		0.71**	0.36	0.85	0.84**
$Y_{X/S}$		0.02	0.38	0.12	0.13
PHA Productivity		0.57	0.20		1.35

OLR – Cmmol/L·day; **qS, qPHA** – Cmmol/Cmmol *X*·day; **PHA Productivity** – g PHA/L·day; **μ** - day^{-1}; $Y_{PHA/S}$ – Cmmol PHA/Cmmol S; $Y_{X/S}$ – Cmmol *X*/Cmmol S; n.p. – not present; (*) depletion of acetate; (**) considering the carbohydrates consumption as substrate.

phase, a maximum of 21.6% g PHA/g VSS (10.2 Cmmol/L; 75HB:25HV) at a productivity of 0.57 g PHA/L·day was achieved, and PHA storage was preferred over biomass growth ($Y_{PHA/S}$= 0.71; $Y_{X/S}$= 0.02). In the famine phase (between 8 and 24 hours in Fig. 2.A), this PHA was then consumed for biomass growth ($Y_{X/PHA}$= 0.74).

In Fig. 2.B, the culture was fed with a different feedstock solution (F3, Table S3), with a lower acetate content. To overcome the lack of acetate in the medium (a preferable VFA of PPB as observed in Almeida et al. (2021), Fradinho et al. (2014) and Table 4) two extra pulses were fed to the reactor with this feedstock, leading to a higher OLR (13.53 Cmmol/L·day), and the feast phase increased up to 12.7 hours.

Despite the efforts to maintain acetate availability, acetate or propionate were not available in the reactor after 7 hours of operation (Fig. 2.D). At that same time, the uptake rates of the remaining ethanol and organic acids decreased until the end of the light phase, leading to the passage of organic carbon into the dark-aeration phase. Once in the presence of aeration, the uptake rates of the remaining substrates increased (Table 4). Despite the similar substrate uptake rate during the light period with the different feedstock compositions, the PHA accumulation rate decreased with the F3 feedstock. Indeed, more of the substrate was driven towards growth with feedstock F3 as compared to F2 (Table 4). The passage of organics to the dark period likely led to PHA production by the aerobic bacterial population present in the culture, which can attain high PHA production rates (Duque et al., 2014, Oliveira et al., 2017). A PHA content of 18.5% PHA/VSS (11.0 Cmmol PHA/L; 60HB:40HV) at a production rate of 1.30 Cmmol PHA/Cmmol *X*. day was achieved in the test shown in Fig. 2.B. Notably, a low yield for the biomass growth was registered during the dark-feast period, indicating a strong storage preference over growth, as observed in Table 4.B ($Y_{PHA/S} = 0.84$; $Y_{X/S} = 0.13$).

Overall, and independently from the feedstock, the culture was able to attain global yields of approximately 0.9 Cmmol PHA+Carbs+*X* /Cmmol S during the feast phase. The higher substrate uptake rates registered during the light phase belonged to acetate and propionate (0.51 and 0.16 Cmmol/Cmmol *X*·day, respectively).

By the end of stage 2, two feedstocks with higher sugar and lactate concentrations were fed to the reactor (F4 with 29% of sugar and F5 with 23% of lactate and 14% of sugar, in total carbon content, Table S3). At the same OLR (Table S7), a general decrease in PHA accumulation was observed (4.7% g PHA/g VSS, 3.0 Cmmol/L; 54HB:46HV), leading the culture to accumulate carbohydrates (Figure S7 and Table S5).

3.1.2. Ammonia in the dark-aerated phase

Stage 3 evaluated the presence of ammonia only during the dark-famine period, and its effect on the PHA accumulation capacity of the microbial culture. Fig. 3 presents the PMC profile during this stage (additional profile presented in Figure S8 and both cycles are characterized in Table S7 that summarizes the stoichiometric parameters of the culture).

At a similar OLR as stage 2, the feast phase increased up to 13 h hours (Fig. 3 and Figure S8). During the light period, the substrate uptake rate was 3.8 times lower (Table S7) as compared with stage 2. Bacteria were not able to grow without ammonia in the light phase, explaining the decrease in the substrate uptake rate, where the substrate uptake was

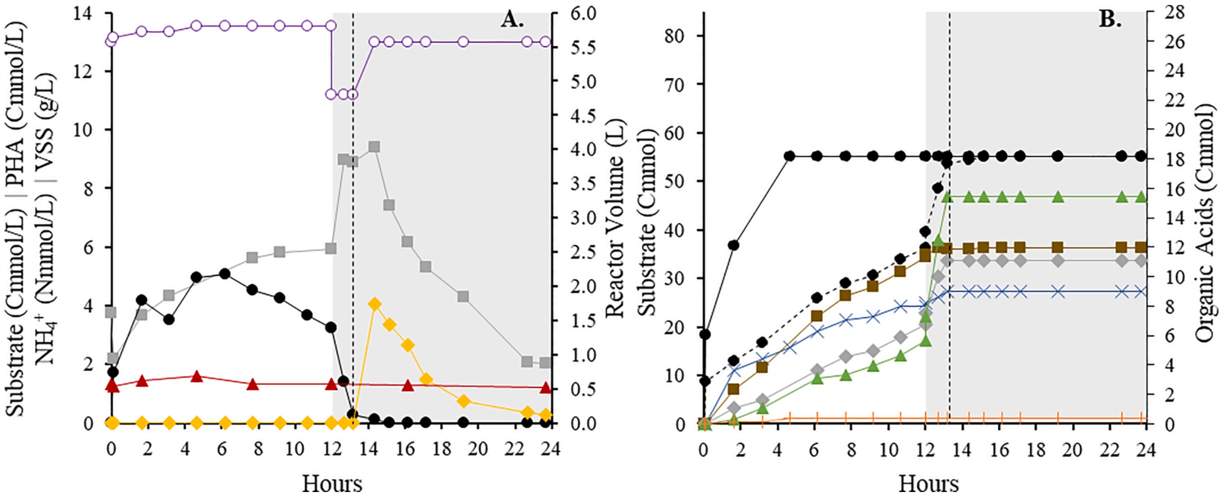

Fig. 3. Culture profile along a 24 hours cycle at Stage 3, with ammonia only present in the famine period of the dark-aerated phase, at 168th day of operation with feedstock F6. [ ] Light period; ▨ Dark aerated period; (—) End of feast phase; A. (○) Reactor volume; (■) Polyhydroxyalkanoates (PHA) concentration; (•) Total carbon substrate; (◆) Ammonia (NH_4^+); (▲) Volatile Suspended Solids (VSS); B. Cumulative profiles; (•) Substrate feeding; (-•-) Substrate consumption; (×) Acetate consumption; (◆) Butyrate consumption; (▲) Valerate consumption; (■) Propionate consumption; (+) Lactate consumption.

mostly directed to PHA and carbohydrate accumulation ($Y_{PHA/S}$= 0.57; $Y_{Carbs/S}$= 0.16). The PHA accumulation during the light phase was 10.4% PHA/VSS (6.0 Cmmol/L; 49HB:51HV) at a production rate of 0.16 Cmmol PHA/Cmmol X·day. Characteristically, the dark-aerated phase presented the greatest PHA accumulation rate and productivity across all stages, 0.88 Cmmol PHA/Cmmol X·day and 1.43 g PHA/L·day respectively, with a maximum accumulation content of 17.8% PHA/VSS (9.4 Cmmol/L; 47HB:53HV).

3.2. PHA accumulation performance

PHA accumulation was studied using pulse-feed tests in stages 2 and 3 at higher light intensity (2.3 times higher than the winter condition illumination applied to the selection reactor), since studies have indicated that short-term increases to the light intensity can improve phototrophic PHA accumulation (Almeida et al., 2021; Fradinho, et al 2019).

In stage 2, accumulation tests were performed with the biomass withdrawn from the selection reactor at the end of the cycle. The PMC accumulation capacity was tested a) with light only, to infer the phototrophic bacteria accumulation capacity (Fig. 4.A) and b) with light plus aeration, to assess the simultaneous accumulation of phototrophic and aerobic bacteria (Fig. 4.B and Table S6).

When comparing both tests, the increase in the PHA accumulation capacity was noteworthy with aeration. In the light test shown in Fig. 4.A, the culture obtained a 17.7% PHA/VSS (11.1 Cmmol PHA/L; 68HB:32HV) content at a production rate of 0.62 Cmmol PHA/Cmmol X·day. Despite the relatively high activity of the phototrophic microorganisms, the culture was under permanent limitation of acetate (Fig. 4.A) and was not able to achieve a maximum PHA storage capacity during the 8 hours of the test. On the other hand, in the light-aeration test shown in Fig. 4.B, the culture was able to achieve a maximum accumulation of 38.9% PHA/VSS (14.6 Cmmol PHA/L; 63HB:37HV) after 4 hours, at a rate of 3.48 Cmmol PHA/Cmmol X·day, with constant detection of all organic acids. In both studies, the presence of ammonia allowed the growth of biomass in parallel to PHA production ($\mu_a = 0.29$ day^{-1}; $\mu_b = 0.56$ day^{-1}). Nevertheless, the loss of organic carbon by oxidation to CO_2 in the light-aerated test was quite significant,

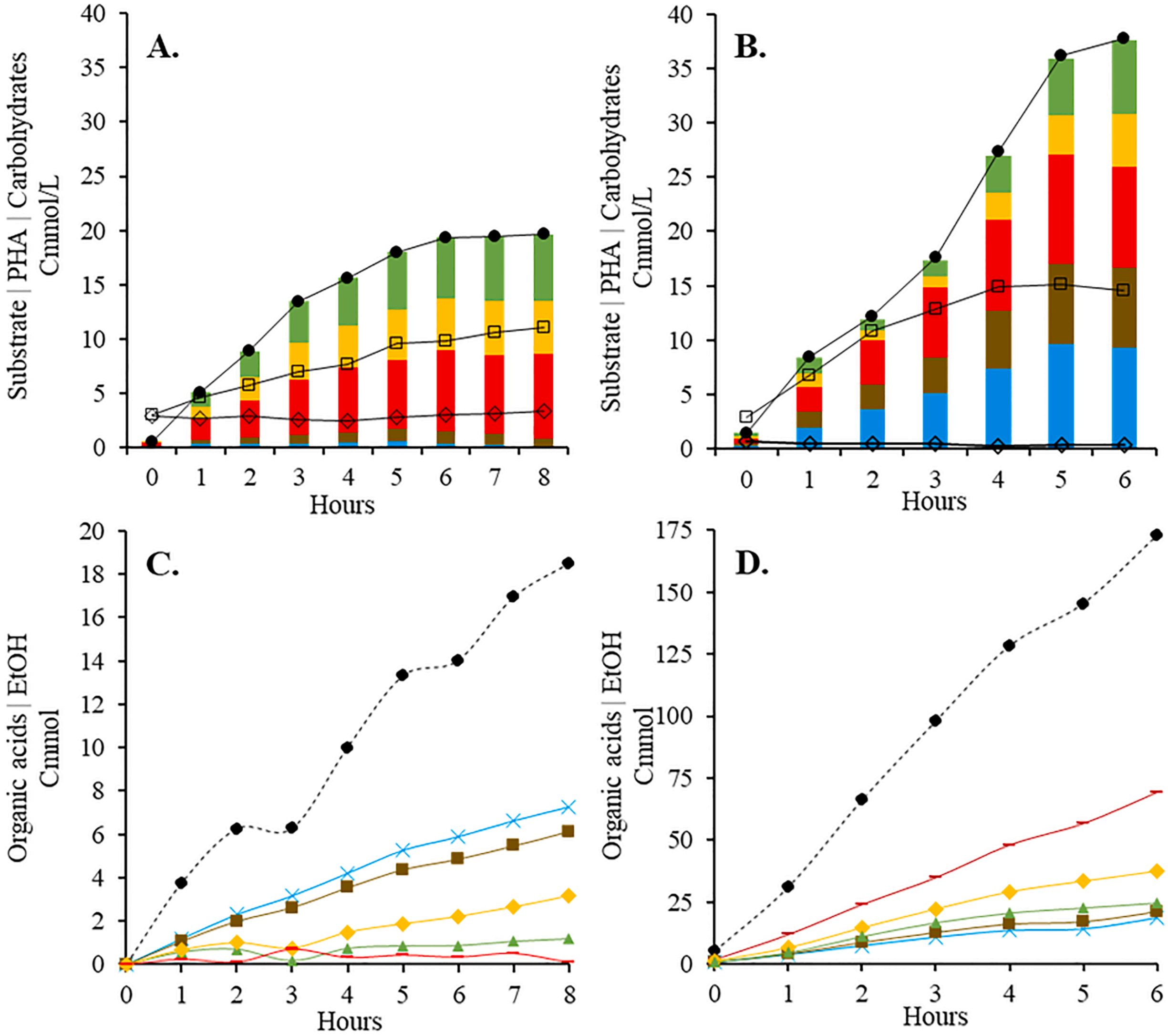

Fig. 4. Accumulation tests of a PMC selected in Stage 2 under permanent presence of ammonia, performed under higher light intensity (1.8 W/L) and feedstock F3. A. Light accumulation test at the 75th day; B. Light-aerated accumulation test at the 96th day. (□) PHA concentration; (•) Substrate concentration; (◇) Total carbohydrates concentration; 🟦 Acetate; 🟨 Butyrate; 🟩 Valerate; 🟫 Propionate; 🟥 Ethanol; C. Cumulative consumptions of chart A.; D. Cumulative consumptions of chart B.; (-•-) Substrate; (×) Acetate; (◆) Butyrate; (▲) Valerate; (■) Propionate; (▬) Ethanol.

representing a loss of 80% of carbon ($Y_{\text{total}} = 0.20$ Cmmol/Cmmol S), while in the test with just light the total yield was 0.99 Cmmol/Cmmol S.

Additionally, the culture showed a preference for acetate and propionate (PPB preferences) in the illuminated test (Fig. 4.C), while in the light-aerated test (Fig. 4.D), ethanol, butyrate and valerate were the preferred carbon sources. These were likely preferred by the aerobic bacteria, since they were the typical substrates left-over at the beginning of the dark-aerated phase of the selection reactor. In addition, studies have shown that PHA producing aerobic mixed microbial cultures have preference for longer chain VFA due to the higher carbon retrieved per energy consumed during substrate uptake (Wang et al., 2017; Portela--Grandío et al., 2021, Pardelha et al., 2012).

In stage 3 accumulation tests, the biomass was withdrawn at the end of the feast phase before ammonia feeding, allowing minimization of the PHA accumulation time as well as improving PHA accumulation due to growth limitation. Several accumulation conditions were performed (**test c:** 8 h light + 8 h dark-aeration, **test d:** 16 h light with synthetic feeding of acetate and propionate, **test e:** 8 h light-aeration and **test f:** 8 h dark-aeration; as shown in Table 3), and the respective culture profiles and efficiency rates can be found in the supplementary material (Figure S10 and Table S8). In accumulation tests with light only (initial 8 h of test c and full test d), purple bacteria were not able to increase their PHA content during the light period even with a synthetic feed of acetate and propionate (c. 9.3 % PHA/VSS and d. 10.6 % PHA/VSS), instead showing carbohydrate accumulation (c. 7.3% g Carbs/g VSS in 8 hours; d. 10.6 % g Carbs/g VSS in 16 hours). Since the tests were performed with biomass withdrawn at the end of the feast-phase, it is possible that purple bacteria had already achieved their maximum PHA accumulation in the selection reactor. Nevertheless, the PHA content attained during stage 3 accumulation tests with light only was around half of the PHA attained in corresponding tests of stage 2 (Fig. 4.A). In tests with aeration (last 8 h of test c, test e and test f, Figure S10), the PHA production rate and the yields of PHA per substrate consumed ($Y_{\text{PHA/S}} = 0.88 \pm 0.06$) were improved compared to the previous stage, achieving the highest rate observed in this study of 5.73 Cmmol PHA/Cmmol X·day. The maximum PHA content of 49.6% PHA/VSS (50.2 Cmmol/L; 39HB:61HV) was observed in this test after 4 hours of accumulation (Table S8 and Figure S10).

3.3. Microbial community evolution

Microbial community analyses were performed in stage 2 and stage 3 to evaluate the impact of the different selection strategies on the PMC selection, as well as to follow the culture content of PPB versus non-phototrophic aerobic microorganisms. Such analysis was not performed for stage 1, since the bacteria were not growing on the accumulated PHA, and the strategy applied to the PMC did not have a selective advantage for the PHA accumulators over other microorganisms. The most abundant taxonomic groups identified by DNA sequencing is shown in Table S10 of the supplementary material. Overall, photosynthetic microorganisms (*Rhodobacter, Rhodopseudomonas, Bosea, Chromatiaceae, Rhodospirillaceae, Hyphomicrobiaceae* and *Cyanobacteria*) represented the majority of the bacterial culture population, followed by the non-phototrophic aerobic microorganisms (*Paracoccus, Rhizobium, Kaistia, Xanthobacter, Comamonadaceae, Zoogleа*, and *Caulobacterales*) (Stage 2: 57 ± 15% vs. 32 ± 13%; Stage 3: 79 ± 9% vs. 8 ± 1%).

In stage 2, when the feast phase was restricted to the light period, the culture presented an enhanced content of PPB (77%), while non-phototrophic aerobic bacteria represented 15% of the population (Table S10, 53rd day). On the other hand, in feedstock operation with more reduced VFAs (i.e. a higher content of valerate and butyrate), the feast phase extended into the dark-aerated phase and the non-phototrophic aerobic bacteria content increased. At that time, the population was evenly divided into PPB and non-phototrophic aerobic bacteria, 42 ± 3 % and 39 ± 5%, respectively (Table S10, 96th and 145th day). During feast-phases confined to the light period, *Hyphomicrobiaceae* (68%) were the dominant population, followed by *Rhizobium* (10%), while when feast phases extended into the dark-aerated period, a higher content of *Rhodobacter* (22 ± 10%) and *Chromatiaceae* (13 ± 6%) was observed along with *Paracoccus* (7 ± 0.2%), *Rhizobium* (25 ± 7%), and *Comamonadaceae* (2 ± 0.1%). In stage 3, PPB still represented the majority of the microbial population (53 ± 2%), though sequencing results showed an increase in the Cyanobacteria phylum content (27 ± 7%) and a considerable drop in the non-phototrophic aerobic microorganisms' abundance (8 ± 2%) (Table S10). In stage 3, *Rhodobacter, Rhodopseudomonas, Chromatiaceae* and *Hyphomicrobiaceae* were identified within the PPB, and *Paracoccus, Rhizobium* and *Comamonadaceae* were found within the non-phototrophic aerobic bacteria (Almeida et al., 2022, Pereira et al., 2019).

Furthermore, microscopic observation of Nile blue staining and fluorescence in situ hybridization (FISH) were performed to complement the DNA sequencing data. In fact, microscopic observations and Nile blue staining analysis conducted for Stage 3 accumulation tests c. (8 h light + 8 h dark-aeration) and d. (16 h light with synthetic feeding of acetate and propionate) revealed the presence of a large community of coccus-shaped purple bacteria that was unable to accumulate PHA in either phototrophic or aerobic conditions (Figure S11). Instead, these microorganisms showed accumulation of non-PHA inclusions, which could possibly be sulphur granules. Further examination through FISH analysis revealed that these microorganisms belonged to the *Gammaproteobacteria* class (Figure S12) and were likely members of the *Chromatiaceae* family, identified through DNA sequencing results (Table S11).

4. Discussion

4.1. Ammonia impact on PMC selection and PHA productivity

The assessment of ammonia availability on the PMC selection and PHA production efficiency was performed along the operational stages. Independently of the ammonia feeding strategy applied to the system, a constant biomass concentration was observed along each operational stage. Despite the fact that no growth limitation by ammonia was observed during stage 2, not all the ammonia fed to the system was consumed along the cycle, since there was ammonia leakage into the effluent. This was likely due to the similar OLR applied across all operational stages, where the low variability in the biomass concentration observed was also expected, considering the limited organic carbon content inherent to feast and famine systems (Fig. 1 and Table 3).

In stage 1, ammonia was fed and present only during the light phase (Table 1) to favour the growth of PPB over the chemotrophic microorganisms, which were ammonia limited during the dark-aerated phase. However, no PHA storage/consumption profile was observed. This behaviour was probably due to the absence of ammonia in the famine phase, since the culture could not grow on the accumulated PHA (Figure S6 and Table S4). The presence of ammonia in the famine phase was concluded to be necessary for the consumption of PHA to occur, and to allow the further growth of PHA accumulating bacteria, thus ensuring a proper PHA storage/consumption profile. Since stage 1 did not provide PHA accumulators a selective advantage over other microorganisms, the reactor operational conditions were adjusted in stage 2.

In stage 2, the presence of ammonia in the light-feast and dark-famine periods (Table 1) was intended to boost PPB growth not only in the light phase, but also through PHA consumption during the dark-famine aerated phase, thus ensuring a PHA production/consumption profile. Overall, the best PHA accumulation performance was achieved with the constant presence of ammonia throughout both phases (Fig. 1 and Table 4). Independently of the feast phase length or the feedstock composition, the microbial culture was able to maintain its PHA accumulation capacity (Table 4) in stage 2, showing good adaptability. Likewise, the PMC demonstrated the capacity to consume most of the organic content during the light period (Fig. 2), lowering aeration requirements during the night. On the other hand, when a less preferable feedstock for PHA production by PPB was applied (higher content of reduced compounds, such as butyrate and valerate, Table 1 and Table S3, further discussed in section 4.2), the resulting feast phase extended to the dark period and led to increased growth of aerobic bacteria, which ensured total carbon depletion and led to further PHA production (Fig. 2.B and Table 4.B).

In stage 3, ammonia was present only during the dark-famine aeration period (Table 1). With this strategy it was intended to study the uncoupling of organic carbon from ammonia to favour the growth of photosynthetic PHA accumulating microorganisms. It was expected that only the bacteria that were able to accumulate PHA during the light phase would have the possibility to grow in the dark-aerated phase when the ammonia was finally available. However, a loss in PHA accumulation was found during the light period in stage 3(Table S7). It was hypothesized that the lower PHA productivity observed in the light phase could be related to the higher content of reduced VFAs (i.e. butyrate and valerate) in the feedstock during stage 3 (Table 1 and Table S3). However, acetate and propionate were always present during the light phase (Figure S9), which might suggest that the decrease of the PHA productivity could be related to the absence of ammonia during the light phase.

In fact, under the ammonia-limiting conditions observed in the light phase, nitrogenase activity could be triggered. The nitrogenase enzyme is commonly present in photosynthetic microorganisms and has the capability to fix nitrogen, reducing it to ammonia and hydrogen (Masepohl & Kranz, 2009). Both nitrogen fixation and PHA accumulation processes compete for reducing power, however, a higher ATP-demand is required for nitrogen fixation, as well as anaerobic conditions. Since low ORP was measured during the light-feast phase without ammonia (around -250 mV, data not shown), hydrogen production could be occurring and competing with PHA accumulation. However, the extent of this competition was not observable, due to the open configuration of the reactor operation.

On the other hand, the loss of PHA accumulation capacity during the light period could also be related to the change of the bacterial culture population (Table S10). Despite some reports indicating that some cyanobacteria are able to accumulate PHA under nutrient limitation and the presence of organic substrates (e.g. glucose and acetate) (Drosg et al., 2015; Troschl et al., 2017; Arias et al., 2020), they only attain low PHA content (around 10% cdw), which might justify the decrease in the PMC's PHA accumulation capacity during the light period. In addition, increased non-PHA inclusions were observed in microorganisms belonging to the Chromatiaceae family (Figure S11 and Figure S12.). This family encompasses a significant group of purple sulphur bacteria known for their unique ability to internally store sulphur granules (Imhoff, 2014; Pfenning & Trüper, 1992). Thus, the observed accumulation of non-PHA inclusions during this stage could be linked to sulphur accumulation. Nevertheless, these microorganisms have also been found to be capable of accumulating PHA (Almeida et al., 2022) and were also likely contributing to PHA storage in stage 2. However, PMC selection with ammonia uncoupling in stage 3 did not favour PHA storage in these microorganisms, thereby leading to a lower PHA accumulation capacity during the light period.

Overall, results showed that the selection of a photosynthetic mixed culture with ammonia uncoupled from the carbon feeding did not promote the selection of PHA accumulating phototrophic bacteria. The *Cyanobacteria* growth as well as the *Chromatiaceae* members predisposition to accumulate non-PHA inclusions instead of storing PHA led to an overall decrease of the PHA accumulation capacity during the light period. Instead, the ammonia uncoupling selected non-phototrophic aerobic PHA-accumulating bacteria that showed enhanced PHA productivity (higher productivity than registered across all stages) and were responsible for the majority of the PHA accumulation during this stage.

4.2. Importance of feedstock composition

Since stage 2 was the SBR's longest operational stage (111 days, Table 1), besides the assessment of the ammonia effect on culture selection, the performance of the culture under different feedstock solutions (F2 to F6, Table S3) was also evaluated.

An explicit preference for less reduced VFAs (i.e. acetate and propionate) during light-periods was observed in the selection reactor and accumulation tests (Table 4 and Table S6), while more reduced VFAs (butyrate and valerate) were the preferable substrate in the dark-aeration period (Table 4 and Table S6). A possible explanation for this culture behaviour is that under higher reductive stress (light phase), the culture has a higher propensity to consume the less reduced compounds, such as acetate and propionate, since their degree of reduction ($\kappa_{\mathrm{HAc}} = 4$; $\kappa_{\mathrm{HPro}} = 4.67$) is lower in relation to the biomass and the PHA typically produced by these VFAs ($\kappa_{\mathrm{Biomass}} = 4.3$; $\kappa_{\mathrm{PHB}} = 4.5$; $\kappa_{\mathrm{PHV}} = 4.8$), as opposed to butyrate and valerate ($\kappa_{\mathrm{HBut}} = 5$; $\kappa_{\mathrm{HVal}} = 5.2$). For this reason, purple bacteria tend to consume the less reduced compounds (e.g. acetate and propionate), storing PHA to balance their internal redox potential. Similarly, the consumption of more reduced compounds is favoured in tandem with less reduced VFAs and PHA production for cell redox balancing, explaining the higher uptake rates of ethanol, valerate and butyrate observed while acetate was available (Table 4.B). In fact, a similar behaviour was observed by Fradinho et al. (2014), showing that acetate acted as a co-substrate, improving the uptake rate of butyrate.

Furthermore, the presence of sugars and ethanol in the feedstock also led to a negative impact on the PHA production during the light period, stimulating growth instead (Table S5 and Table 4). A higher content of ethanol in the feedstock led to a decreased PHA production rate and increased biomass growth rate (F3, Table 4.B), strongly suggesting that ethanol was being taken up for biomass growth instead of PHA accumulation. In fact, despite ethanol being mentioned as a PHA precursor in aerobic bacteria (Beccari et al., 2002, Duque et al., 2014), ethanol was not considered a precursor for PHA production in PMCs high in *Rhodopseudomonas palustris*, even though its presence improved the overall PHA accumulation (Montiel-Corona & Buitrón, 2022). On the other hand, sugars are not precursors for PHA production, and a high concentration of sugars in the feedstock (F4 and F5, Fig. 1) led to a drastic decrease in PHA production and a shift towards carbohydrate accumulation (F4 and F5, Fig. 1).

Moreover, the feedstock composition also had an important role on the polymer composition. HB monomer production is typically associated to the uptake of carbon sources within an even number of carbon atoms, such as acetate and butyrate (Fradinho et al. 2014; Wang et al. 2018), and reports with aerobic mixed microbial cultures have also indicated that lactate and ethanol act as HB precursors (Jiang et al. 2011; Lee et al. 2022; Beccari et al. 2002). On the other hand, HV monomers are correlated with carbon sources that have an odd number of carbon atoms, such as propionate and valerate (Fradinho et al. 2014; Wang et al. 2018). As expected, a feedstock composed of various organic acids resulted in the production of a co-polymer with an HB and HV content that fluctuated according to the feedstock composition (Figure S5). However, when sugars were present, the HB content was higher than the content of even numbered carbon sources in the feed (Figure S5). It is possible that during this period, sugar fermentation was resulting in HB precursors and contributing to the preferential accumulation of this monomer.

Overall, PHA productivity was strongly constrained by the feedstock composition, since the content of substrates less preferred by PPB (butyrate, valerate, ethanol and even sugars) represented over 50% of the total feedstock composition from stage 2 onwards (Table S3), leading to an average PHA accumulation of 10% g PHA/g VSS across all stages (Table 3). While high OLR is needed to achieve high PHA accumulation, an increase in OLR was not feasible in these circumstances, because the passage of organics to the dark-aerated phase would stimulate the growth of non-phototrophic aerobic microorganisms and threaten PPB dominance. Therefore, in the operation of PMC for PHA production, fermentative processes should be optimized to ensure higher content of acetate and propionate in the feedstock to boost PHA accumulation by PPB. Likewise, feedstock composition must be controlled to ensure reproducibility of the polymer produced. Nevertheless, the selected PMC was capable of adapting to different feedstock compositions and still maintained efficient PHA production as compared to previous work operated with a real fermented feedstock (Almeida et al. 2021) (further discussed in section 4.3).

4.3. Operational system overview

A dynamic operational strategy for PMC systems was proposed based on a light-feast/dark-aerated-famine process, for the purposes of improving the performance of the photosynthetic community expected under winter operational conditions (lower light intensity and lower temperatures). The present system was operated under simulated winter outdoor conditions (0.8 W/L; 13.2 ± 0.9 °C) and used a real fermented mixture of domestic wastewater and molasses as feedstock. Almeida et al. (2021) studied a 12 h Light/12 h dark non-aerated open system simulating summer outdoor conditions (1.9 W/L; 25 °C). When comparing the winter and summer SBRs, similar PHA productivities during the feast phase of the cycle were obtained, 0.57 (Table 4.A) and 0.52 g PHA/L·day, respectively, suggesting that winter SBR operation with longer feast phases and dark aeration was able to maintain the PHA productivity of the system. Furthermore, results showed that this operational mode facilitates the bacterial culture adaptation to less favourable feedstocks that contain a high content of less preferable substrates for PHA production by PPB. In this scenario, aeration could be applied in the accumulation step to ensure improved PHA accumulation, also offering an alternative process option to apply when light intensities are limited (e.g. the winter season, cloudy days).

Another important factor to be considered is the effect of the aerobic phase on the purple bacteria culture. Some studies mention that with oxygen present, PPB can be outcompeted by aerobic microorganisms due to the comparatively slower growth rate of PPB under aerobic conditions (Capson-Tojo et al., 2021). This is mostly because PPB are not able to synthesize the photosynthetic pigments responsible for light harvesting and energy transformation aerobically, being obligated to shift from a photoheterotrophic to a chemoheterotrophic growth mode, where energy is mostly derived from aerobic respiration (Huang et al., 2001, Bauer et al., 2003; Zhu et al., 1986).

When comparing the results of pigment production with a photosynthetic culture without an aeration phase (such as the inoculum utilized in this work, Almeida et al., 2021), it is possible to verify a drop in the Bchl *a*+*b* pigments (Figure S14), however, pigments were consistently present across all stages, indicating the continued presence of PPB in the culture (Figure S14).

The prevalence of PPB over non-phototrophic aerobic microorganisms observed through DNA sequencing analysis can be explained by the availability of organic carbon in the system. The feast phase was mostly restricted to the anaerobic light phase where purple bacteria could grow photoheterotrophically and accumulate PHA, and the growth of aerobic microorganisms was limited due to the low amount of organic carbon that reached the dark-aeration period. A wastewater treatment study with a combined daily-illuminated-anaerobic/night-aerobic system fed continuously during the day showed that organic carbon was present during the night-aerobic time, leading to a lower relative abundance of PPB (0.2 - 0.3) as compared to optimal PPB operation (0.5) (Hülsen et al., 2022). In this study, a higher relative abundance of PPB was obtained (0.5 ± 0.2, Table S10) with the constant presence of ammonia, supporting the hypothesis that the minimisation of organic carbon during the night-aeration period ensures a competitive advantage for PPB over non-phototrophic aerobic microorganisms.

Finally, for future process implementation, operation in real outdoor conditions subject to seasonal variations should be tested. Since the microbial culture is dependant on light availability, it would be expected that under winter conditions (shorter periods of light, lower light intensity, and lower temperatures) the photosynthetic population would be less efficient. By taking advantage of the full light period as a feast phase, growth and PHA accumulation by PPB will be expanded. Moreover, the existence of a dark-aeration period will ensure further growth on the PHA accumulated by PPB, and complete COD depletion. Combining an extended feast-phase with the dark-aerated period could effectively combat the slower rates of the photosynthetic culture under winter conditions. On the other hand, during the summer season (longer periods of light, higher light intensity, and higher temperatures) the system will achieve higher efficiency during the light period, perhaps not requiring dark-aeration. Overall, process operation under light-feast/dark-famine aeration with the constant presence of ammonia is capable of overcoming winter conditions and can ensure good performance of photosynthetic PHA production systems along the seasons.

5. Conclusion

The impact of different ammonia feeding strategies on the selection of a PHA producing phototrophic-chemotrophic consortium operated under light-feast/dark-aerated-famine was evaluated. The system operated with ammonia always present in the light and dark periods presented the best outcome, where both phototrophic and chemotrophic microorganisms' performance was improved. Furthermore, the light-feast/dark-aerated-famine operation led to the selection of a consortium capable of maintaining PHA production efficiency under winter conditions (low light availability, low temperatures), compared to summer conditions (higher light intensities, higher temperatures), as well as being capable of adapting to different feedstock compositions. Overall, the results strongly suggest that the application of light-feast/dark-aerated-famine operation in future outdoor systems will lead to successful PHA production and overcome the changing seasonal conditions.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Editor of Water Research - M. A. M. Reis Associated editor of Water Research - A. Oehmen.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2023.120450.

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