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Energy-efficient selection of high-yield PHA producers via microaerophilic uncoupled feeding

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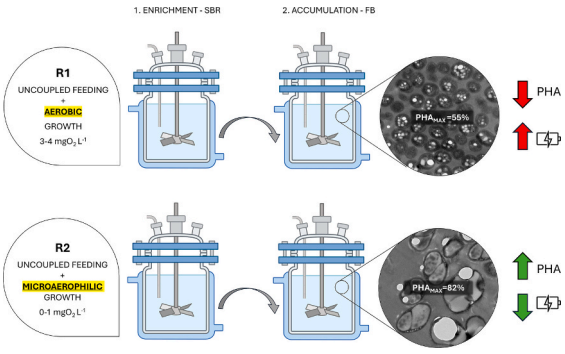
HIGHLIGHTS:

- Microaerophilic conditions boosted PHA content to 82%.
- PHA yield improved to 0.88 g COD_{PHA}/g COD_{VFA} under low-oxygen operation.
- Microaerophilia promoted ~ 50% higher polymer storage efficiency.
- Microaerophilia modified polymer composition and microbial communities.
- Aeration energy demand dropped by 32% compared to conventional aerobic operation.

GRAPHICAL ABSTRACT



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ABSTRACT

Uncoupled feeding (UnF), supplying organic carbon and nutrients separately, is a promising strategy to enrich polyhydroxyalkanoates (PHA)-accumulating mixed microbial communities (MMCs) and has shown higher productivity than conventional approaches. UnF enables improved oxygen control, which is particularly relevant for PHA production, where aeration constitutes a major operational cost. However, the effect of dissolved oxygen (DO) on PHA production remains unclear. To address this gap, this study investigates the effect of microaerophilic conditions on biomass enrichment during the growth phase of PHA-accumulating bacteria in an UnF regime. Reduced DO is expected to lower energy demands and mitigate bacterial decay and predation, which are typically intensified under aerobic conditions. Two lab-scale sequencing batch reactors (R1-R2) were operated under identical conditions, differing only in DO during the growth phase of PHA-accumulating bacteria: R1 was

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maintained under aerobic conditions (3–4 mg O_2 L^{-1}), while R2 was operated under microaerophilic conditions (0–1 mg O_2 L^{-1}). After 100 days of enrichment with a synthetic acetate-propionate influent, the microaerophilic reactor achieved a maximum PHA accumulation of 82 ± 11%, while the aerobic reactor reached 55.1 ± 0.3% (volatile suspended solids basis). Microaerophilic conditions promoted greater PHA accumulation, enhancing PHA yield of 17% (0.88 vs 0.75 $gCOD_{PHA}$ $gCOD_{VFA}^{-1}$) and storage capacity (49% increase), also affecting the polymer composition and both eukaryotic and prokaryotic community, while achieving an estimated 32% reduction in aeration energy demand compared to conventional aerobic operation. These findings show that integrating microaerophilia into UnF enrichments offers a simple, energy-efficient strategy for selecting MMCs with high PHA accumulation potential.

1. Introduction

Plastic materials are widely used in modern society due to their durability, pliability, and cost-effectiveness. However, concerns about hazardous additives (e.g., stabilisers and colorants), reliance on non-renewable resources, and slow degradation have raised questions about their long-term sustainability (Rajvanshi et al., 2023). As an alternative to conventional petrochemical-based plastics, bioplastics have been proposed as a more environmentally sustainable solution. They can reduce fossil fuels dependence, lower greenhouse gas emissions, minimise plastic waste accumulation, and improve end-of-life management (Kumar et al., 2024).

Polyhydroxyalkanoates (PHA), a diverse family of polyesters synthesized by microorganisms as intracellular carbon and energy storage materials, are gaining increasing attention as truly biodegradable bioplastics (Park et al., 2024). Among them, Poly(3-hydroxybutyrate) (PHB) is the most prevalent homopolymer. PHB is characterized by its stiffness and brittleness, along with a narrow processing temperature window. One effective approach to enhance the mechanical and thermal properties of PHA is the synthesis of copolymers, such as poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV). These copolymers consist of two or more comonomer units, like 3-hydroxyvalerate (HV) and 3-hydroxybutyrate (HB), which are randomly distributed along the polymer chain (Mai et al., 2024). PHA are typically produced by pure cultures grown on pure substrates in controlled environments. This process presents significant challenges due to the need for extensive sterilisation and complex contamination control, resulting in high costs (Jayalath and de Alwis, 2025). In recent years, substantial research has focused on the utilisation of mixed microbial cultures (MMCs) for PHA production (Zhang et al., 2024). MMCs offer advantages over pure cultures by employing synergistic dynamics among community members and a broad range of metabolic capabilities, enabling treatment of high organic loads or complex pollutants. This approach allows the use of waste instead of costly pure substrates, supporting a circular economy and reducing production costs (Ahuja et al., 2024). The production of PHA from organic waste and MMCs involves three key stages. First, the waste undergoes acidogenic fermentation, generating volatile fatty acids (VFA) that serve as the main precursors for polymer synthesis. Next, microbial cultures are enriched with PHA-storing bacteria, typically in sequencing batch reactors (SBRs) operating under feast-famine conditions, i.e., alternating periods of nutrient abundance and nutrient limitation, which selectively favour organisms capable of intracellular carbon storage. Finally, under optimised conditions, the biomass maximise the content of PHA, which is then extracted and purified for further use (Zhang et al., 2024).

The uncoupled feeding (UnF) strategy for the enrichment of PHA-accumulating microorganisms relies on the temporal separation of the addition of carbon (C) and nitrogen (N) or phosphorus (P) within the feast-famine regime: carbon is dosed at the beginning of the feast, namely accumulation phase, whereas N and/or P are supplied at the start of the famine, namely growth phase, only once the carbon has been fully depleted. The UnF was first proposed by Silva et al., (2017), with the aim of improving PHA productivity from MMCs and waste substrates, a key limitation of conventional feast-famine strategy. This approach enhances selective pressure towards PHA-accumulating organisms, while potentially simplifying the conventional three-step process of selection, production, and accumulation (Oliveira et al., 2017; Stouten, 2021). A crucial benefit of UnF concerns to oxygen management: by decoupling accumulation from microbial growth, the oxygen demand is more balanced over the cycle. This contributes to higher process stability, energy efficiency, and robustness under complex and high-load operating conditions (Stouten, 2021). Although aeration is one of the largest operational expenses in commercial PHA production (Zhang et al., 2026), UnF can optimize aeration by adjusting DO to each phase's metabolic demands, reducing costs and improving efficiency; however, little is known about how oxygen supply affects PHA production by MMCs under low DO conditions (De Donno Novelli et al., 2021).

To date, only a limited number of studies have examined the influence of DO on the process, concentrating mainly on traditional feast-famine regimes (Pratt et al., 2012;Coats et al., 2016; Wang et al., 2017; Castagnoli et al., 2024), highlighting the need for further research into oxygen dynamics in UnF. Most of the existing studies are primarily devoted to investigating microaerophilic PHA accumulation (Pratt et al., 2012; Pranav Kshirsagar, 2013; Wang et al., 2017; Blunt et al., 2018; Liu et al., 2023). In summary, literature data highlight the dual role of DO as both a stressor and a regulator of PHA metabolism. Low DO generally enhances yield and modulates polymer composition but may compromise productivity rates. Nevertheless, variable outcomes across substrates and microbial consortia underscore the complexity of oxygen control in mixed-culture PHA systems. Current knowledge on PHA enrichment mainly relies on fully aerobic conditions or microaerophilic conditions applied during the feast phase (Zhang et al., 2022). However, little is known about the effect of oxygen limitation specifically during the growth phase on the selection of efficient PHA-accumulating bacteria.

The novelty of the present study lies in applying microaerophilic conditions during the growth phase of PHA-accumulating bacteria in the enrichment process, unlike conventional PHA selection strategies which are generally fully aerobic or microaerophilic during feast phases. To the best of our knowledge, this is the first work targeting the growth phase with controlled oxygen limitation to enhance PHA enrichment. Microaerophilic conditions, defined as DO concentrations between 0 and 1 mg O_2 L^{-1}, are hypothesized to provide a dual benefit. On one hand, they reduce aeration requirements and thus energy demand; on the other hand, because DO is a critical substrate for microbial growth and maintenance, its availability during the growth phase may trigger specific physiological responses that influence microbial interactions. These responses can, in turn, affect competitive dynamics and shape dominance patterns in ways consistent with resource-ratio theory, intrinsic growth rates, and other ecological frameworks. Furthermore, low DO would also contribute to decrease bacterial decay and predation, processes that are intensified under fully aerobic conditions (Munz et al., 2011; Castagnoli et al., 2024).

In this sense, the aim of this work was to investigate the impact of microaerophilic growth phase on biomass enrichment, under the hypothesis that such conditions would selectively promote PHA-storing bacteria while simultaneously lowering operational energy costs. By

shifting the conventional paradigm of aerobic enrichment, this approach offers new insights into optimising PHA production systems for greater sustainability and efficiency.

2. Materials and methods

2.1. SBRs: setup and operating conditions

Two SBRs (R1 and R2) of a maximum working volume of 9.3 L each were used to perform the experiment. Both were equipped with a mixer (Dutchi NL, 200 rpm), aeration system, thermal jacket, dosing/sampling points, specific probes for DO and pH (Oxysens 237,150 Hamilton and Hach 52 03, respectively), and a pumping system. A PLC (Siemens TD 200) regulated the activation/shutdown of the feed and purge pumps, aeration and mixing, determining the sequence of the various phases of the reactor cycle (Fig. 1A).

The cycle of 12h consisted specifically in carbon feeding, accumulation phase, settling, supernatant discharge, idle, nutrients feeding, growth phase, purge of the biomass, idle. Fig. 1B shows the cycle diagram. A clarification of the terminology used is required, as the two phases of UnF have historically been referred to simply as feast and famine. The "*accumulation phase*" corresponds to the period during which conditions favour the accumulation of intracellular reserves, immediately after the addition of rapidly biodegradable carbon, and before nutrients are supplied. This phase has a fixed duration and corresponds to the interval in which microbial energy-storage pathways may be activated. Within the accumulation phase, the "*feast*" refers to the subset of time during which external carbon is available. In contrast, the "*famine*" is the period of the cycle after the feast phase, during which microorganisms have limited or no access to readily biodegradable external carbon, and bacteria rely on previously stored reserves to survive. The "*growth phase*" occurs after the accumulation phase and is characterised by the availability of nutrients for bacteria that have already accumulated reserves. This phase also has a fixed duration and precedes the biomass purge at the end of the cycle.

The feeding solution consisted of 80% of acetate and 20% of

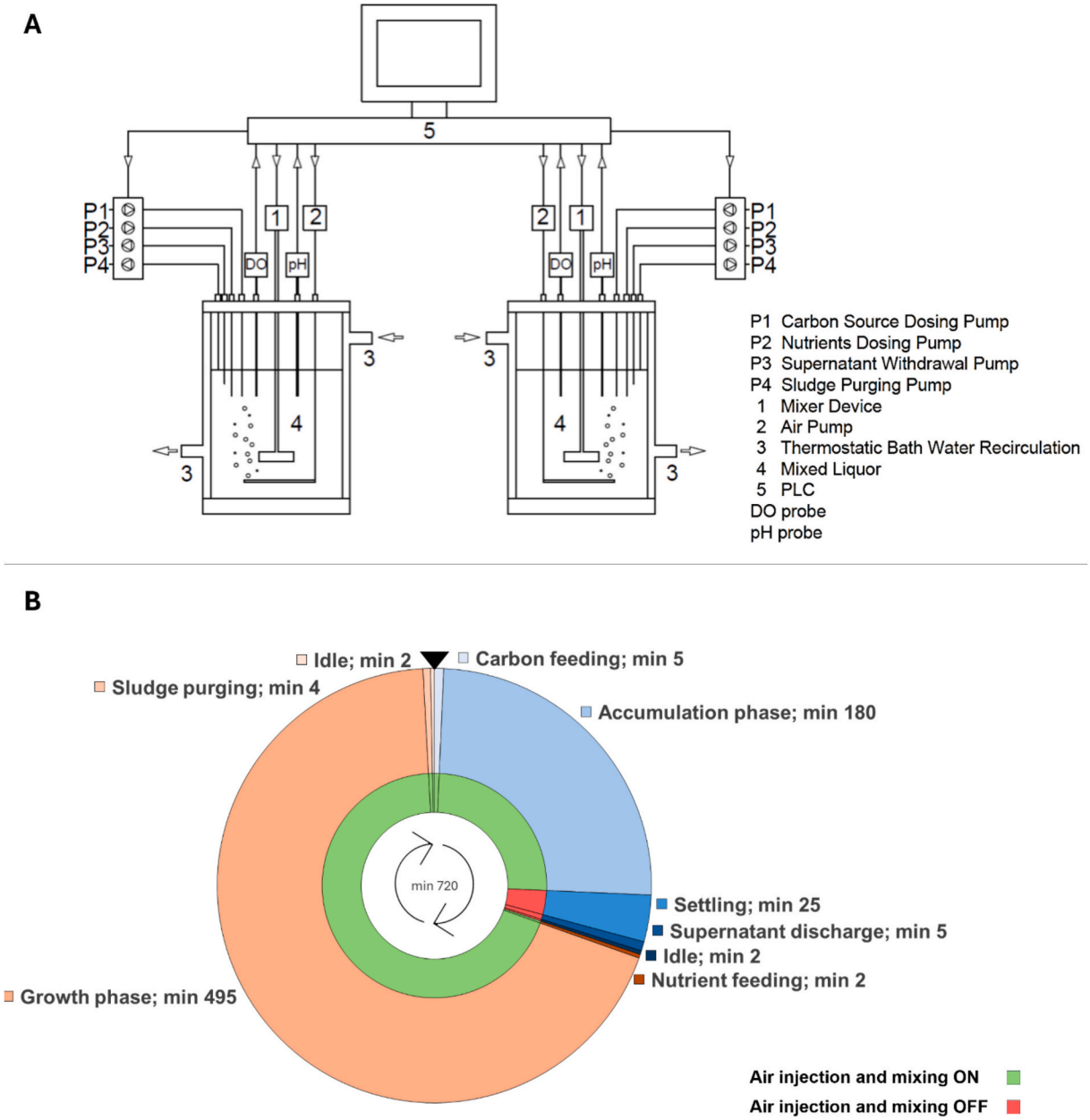

Fig. 1. Schematic of the reactor setup (A) and operational cycle (B).

propionate (on COD basis), dosed like sodium acetate and sodium propionate. The nutrients solution was adapted from Palmeiro-Sánchez et al., (2016). Allylthiourea was added to inhibit nitrification.

Temperature was maintained at 25°C via recirculating distilled water within the reactor's outer jacket using an immersion thermostat (Termotronic – 100, Selecta) and a thermal bath. The pH was not externally controlled but free to change and naturally maintained in a range reported to be favourable to the selection of PHA accumulating bacteria (≥7.5, in particularly between 8.8 and 9.2) by Montiel-Jarillo et al., (2017) and Zhang et al., (2022).

The aeration system operated at a flow rate of 5 NL min^{-1}, which was adjusted and measured using a rotameter (Model 2150, Tecfluid, Sant Just Desvern). Air was supplied through a perforated silicone tube diffuser, closed in a ring and placed at the base of the reactor. Aeration was controlled in on–off mode during the growth phase, whereas in the other phases air was either continuously supplied without control or turned off (Fig. 1B). Specifically, during the accumulation phase, DO was not controlled and during the feast remained low in both reactors (0.5 ± 1.5 mg L^{-1} in R1 and 1.1 ± 2.1 mg L^{-1} in R2), before increasing towards saturation levels after external carbon uptake (6.6 ± 1.9 mg L^{-1} in R1 and 6.3 ± 1.6 in R2). During the growth phase, DO was maintained within two setpoints (3–4 mg O_2 L^{-1} in R1 and 0–1 mg O_2 L^{-1} in R2). Overall, following an initial stabilisation period, DO regulation was effective and stable. In approximately 5 min in R1 and 10 min in R2, the DO reached the setpoint (3.7 ± 0.6 mg L^{-1} in R1 and 0.5 ± 0.4 mg L^{-1} in R2), with slight exceedances and fluctuations observed. In R2, DO remained within the target range for 93% of the operating time. In R1, DO more frequently fell outside the defined range; however, since it remained above 2 mg L^{-1}, deviations from the set range were not considered critical given the aerobic nature of the process.

Except for DO levels in the growth phase, all other operational parameters set were identical for both reactors (Table 1), which then ran for 100 days. To ensure reliable DO monitoring and to prevent biofilm formation on reactor walls, diffusers, and probes, the reactors were cleaned every 2–3 days.

The SBRs were inoculated with pre-enriched biomass, originally collected from a mixed domestic and industrial tannery wastewater treatment plant and previously enriched with PHA-accumulating bacteria under alternating nutrient limitation conditions. NaCl was added to both feeding solutions to achieve a chloride concentration of 3 g Cl^{-} L^{-1}, thereby maintaining the salinity level of the inoculum.

2.2. Processes monitoring strategy and analytical methods

Continuous monitoring of DO and pH profiles enabled daily verification of the system's proper functioning. The system was periodically evaluated through ammonium measurements at the end of the cycle, according with the reactors stability observed. Since ammonium represented the limiting nutrient in the process, the concentration at the end of the cycle (from 600 to 720 min) was expected to remain below 0.5 mg N L^{-1}. Comprehensive cycle analysis and specific accumulation tests (see section 2.4) were performed on the 100th experimental day to assess the overall system behaviour and the maximum PHA storage capacity of the enriched culture, respectively. The cycle analyses included measurements of total organic carbon (TOC), ammoniacal nitrogen (N-NH_4^+), orthophosphate (P-PO_4^{3-}), and Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) in addition to standard parameters such as DO and pH. For TOC, N-NH_4^+, and P-PO_4^{3-} analyses, samples were filtered through 0.22 μm membranes and subsequently analysed. TOC was quantified using a Multi N/C 2100S analyser (Analytik Jena) equipped with a nondispersive infrared detector (NDIR). Ammoniacal nitrogen was determined using Hach kits (LCK 303 or LCK 304, depending on concentration range), while orthophosphate was analysed with a Phosphax sc analyser (Hach). Total suspended solids (TSS) and volatile suspended solids (VSS) were quantified both in the purged sludge at the end of the cycle and in the discharged supernatant, following the Standard Methods (APHA, 1998). Based on VSS content, the sludge volume index (SVI) was determined over time to assess sludge settleability.

For PHBV analysis (which will hereafter be referred to simply as PHA in the following sections), sludge samples were withdrawn from the reactor, centrifuged (10,000 rpm, 20 min), frozen at −80 °C, lyophilised, and stored. PHBV quantification was performed following a modified Werker et al., (2008) method, which uses acidic alcoholysis of dried biomass with butanol and concentrated HCl, with hexane as a co-solvent, to hydrolyse and derivatize storage products and membrane lipids. The digested samples were then analysed via gas chromatography with a flame ionization detector (GC-FID) to quantify HB and HV fractions. Duplicate spot analysis revealed a 5% margin of error. In addition to these measurements, further analyses were performed to characterise the biomass. Biomass selected in R1 and R2 was observed through a Hitachi H-7000 transmission electron microscope (TEM), after proper sample preparation.

Table 1
Main set operational parameters of the reactors.

Variable	Aerobic	Microaerophilic
Reactor	R1	R2
DO-growth (mg O_2 L^{-1})	3–4	0–1
DO-accumulation (mg O_2 L^{-1})	0–8	
Exp. days (d)	100	
Sludge Retention Time – SRT (d)	4	
Hydraulic Retention Time – HRT (d)	1.2	
Volumetric Exchange Ratio – VER (%)	41	
Organic Loading Rate – OLR (g O_2 L^{-1} d^{-1})	1	
C:N:P (mass basis)	100:9:3	

2.3. Data elaboration

The PHA content was calculated in terms of percentage of VSS on mass basis, with VSS representing the sum of PHA and biomass [PHA (wt.%) = 100*(g PHA $gVSS^{-1}$)] as reported by Oliveira et al., (2017). The maximum PHA content stored by the MMC (PHA_{MAX}, wt.%) was defined as the maximum percentage of PHA on VSS after the accumulation test (Matos et al., 2021). Data were analysed using descriptive statistics (PHA_{MAX} values represent the mean of the last two accumulation measurements ± standard deviation), and results are presented as descriptive comparisons only. The PHA yield ($Y_{PHA/S}$) with respect to the substrate utilised (S) was calculated as $Y_{PHA/S}$ = g COD_{PHA} produced g COD_{VFA} consumed $^{-1}$. The COD_{PHA} was calculated using the PHA_{MAX} and the conversion factors from oxidation stoichiometry (1.67 g COD g HB^{-1} and 1.92 g COD g HV^{-1}). The cumulative COD added at the second-to-last spike, prior to reaching PHA saturation in the cells and concluding the experiment, corresponded to the COD_{VFA} consumed (Laumeyer et al., 2025). Volumetric PHA productivity (g PHA $L^{-1}h^{-1}$) was calculated as the ratio of cumulative produced PHA (ΔPHA) in 1 L working volume of the accumulation reactor per unit of time (Carvalho et al., 2022).

Based on the ΔPHA produced in the accumulation step and the total substrate provided in both enrichment and accumulation , an overall yield factor was calculated (Y_{global}), which takes into account the entire process as a whole: the daily PHA production and the daily substrate supplied during the enrichment and accumulation steps.

Potential energy savings of operating the bioreactor under microaerophilic conditions (DO = 0–1 mg L^{-1}) versus aerobic conditions (DO = 2–3 mg L^{-1}) were estimated based on average DO values (0.5 mg L^{-1} for R2, 2.5 mg L^{-1} for R1). Assuming identical biomass oxygen uptake rates (OUR) in both reactors, a conservative assumption supported by steady-state measurements, the lower DO setpoint reduces the required aeration rate. The change in aeration demand was calculated using empirical kLa-airflow relationships (Chen et al., 2020; Garcia-Ochoa and Gomez, 2009), and energy savings were estimated assuming

blower power scales linearly with airflow.

2.4. Accumulation tests

The accumulation tests were carried out in a fully aerated, 1.4 L total volume fed-batch reactor (FB), monitored continuously by a DO probe (S423/C/OPT/T by Chemitec Srl), in order to evaluate the maximum storage capacity of R1 and R2 biomasses. The air was supplied continuously through a compressor (flow rate = 1.8 NL min^{-1}) and diffused through a porous stone submerged in the mixed liquor. Aeration also served to keep the biomass mixed, since the accumulation reactor was not equipped by a stirrer. No pH monitoring/control was applied. The biomass purged (0.9 L) in R1 and R2 at the end of the cycle was collected and then subjected to a series of spikes of feeding in the FB reactor. Previously, the biomass in the SBR was settled, and the supernatant removed. The biomass was washed twice and resuspended in a mineral medium free of macronutrients (nitrogen and phosphorus), but containing micronutrients in the same concentration than that during the enrichment. After that, the accumulation test was started following standard procedures reported in the literature (Papa et al., 2020; Mineo et al., 2023). DO monitoring is crucial in accumulation tests, as spikes are added based on the DO profile until no further DO drop occurs, indicating that the biomass has reached its maximum PHA storage capacity (Argiz et al., 2020). The spikes of concentrated organic matter (120 g COD L^{-1}) contained both acetate and propionate, in the same proportions as the feeding in the enrichment step (80–20% on COD basis), and the same salinity (3 g Cl^{-} L^{-1}). Each spike (2 mL) dosed about half carbon source in terms of concentration than a single feeding in parent reactors (0.2 g COD L^{-1}). Samples were taken for TOC and PHA analysis just before spikes and at the end of the test. Before and after the test, TSS and VSS concentrations were measured.

2.5. Microbiota analysis

The mixed liquors of the reactors were sampled during the enrichment step on the experiment days 1, 8, 17, 36, 65, 81 and 117. DNA was extracted from the samples using DNeasy PowerSoil Pro kit (Qiagen, Germany). Subsequently, the V3-V4 region of the 16S rRNA gene was amplified using the primers pair Pro341FB (Klindworth et al., 2013; Mazzoli et al., 2020) and 805R. In addition, the total DNA was sequenced using Illumina NovaSeq X for the samples collected on the experiment days 81 and 117 to obtain further insight into the entire microbial community. Genera capable of accumulating PHA were recognised according to the literature.

3. Results and discussion

3.1. Mixed microbial communities' enrichment

Throughout the enrichment step, the DO and pH dynamics provided clear indications of system performance. Following each substrate pulse, DO systematically declined while pH increased during the initial accumulation phase. DO then rose to saturation before nutrient additions and the onset of the growth phase. During the growth phase, both DO and pH decreased, with DO remaining within the imposed operational range and pH staying low until the next cycle. Over time, the characteristic saw-tooth pattern of DO during the growth phase became progressively less frequent, signalling a gradual decrease in oxygen consumption (Fig. 2).

DO and pH monitoring further indicated that maintaining the target microaerophilic conditions was initially challenging. During the first 44 days (referred to as "*stabilisation phase*"), the operating conditions of the system rendered microaerophilic DO control intermittently ineffective, specifically, during experimental days 3–6, 21–25, 31–37 and 41–43 and recovering on its own without any external interventions. End-of-cycle analyses indicated pronounced fluctuations in residual ammonium,

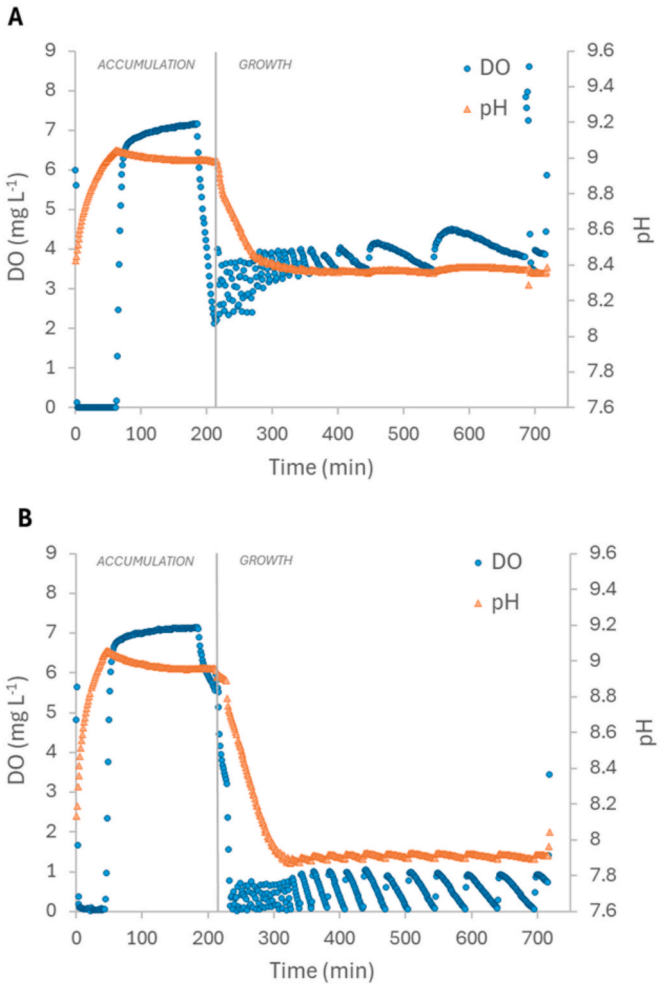

Fig. 2. DO and pH trends over the typical operating cycle during enrichment in R1 (A) and in R2 (B), reflecting microbial activity and system performance.

concomitant with a decline in biomass concentration. The low growth on the accumulated substrate (limited PHA and biomass), combined with oxygen transfer from the atmosphere via mixing, may have prevented the maintenance of the DO setpoints. Once PHA consumption rates increased during the growth phase, no rise in end-of-cycle residual ammonium was observed and the system effectively behaved as microaerobic.

No significant difference in the duration of the feast was observed between the two reactors (62 ± 20 min in R1 and 59 ± 19 min in R2), which may also be influenced by notable variability in the data. The pH measurements were more consistent, with minimum and maximum pH values reported for each cycle. While the maximum pH was comparable in both reactors (R1 = 9.1 ± 0.1, R2 = 9.1 ± 0.2), the minimum pH was lower in R2 (~7.9 ± 0.2 vs 8.3 ± 0.2 in R1) after the stabilisation phase (after day 44).

The lower pH may be attributed to reduced CO_2 stripping due to lower aeration during the growth phase, resulting in a more acidic environment.

Once operational stability was reached, the behaviour of the two communities was examined in detail over the course of a single operational cycle on day 100 (Fig. 3). The two biomass cultures exhibited similar behaviour, with both accumulating approximately 20–25% PHA at the end of the feast and subsequentially growing on the storage carbon. Differences in monomer dynamics, however, were noted (which are described in greater detail in the sections 3.3 and 3.4). Finally, given that DO might significantly influence the microbial community structure

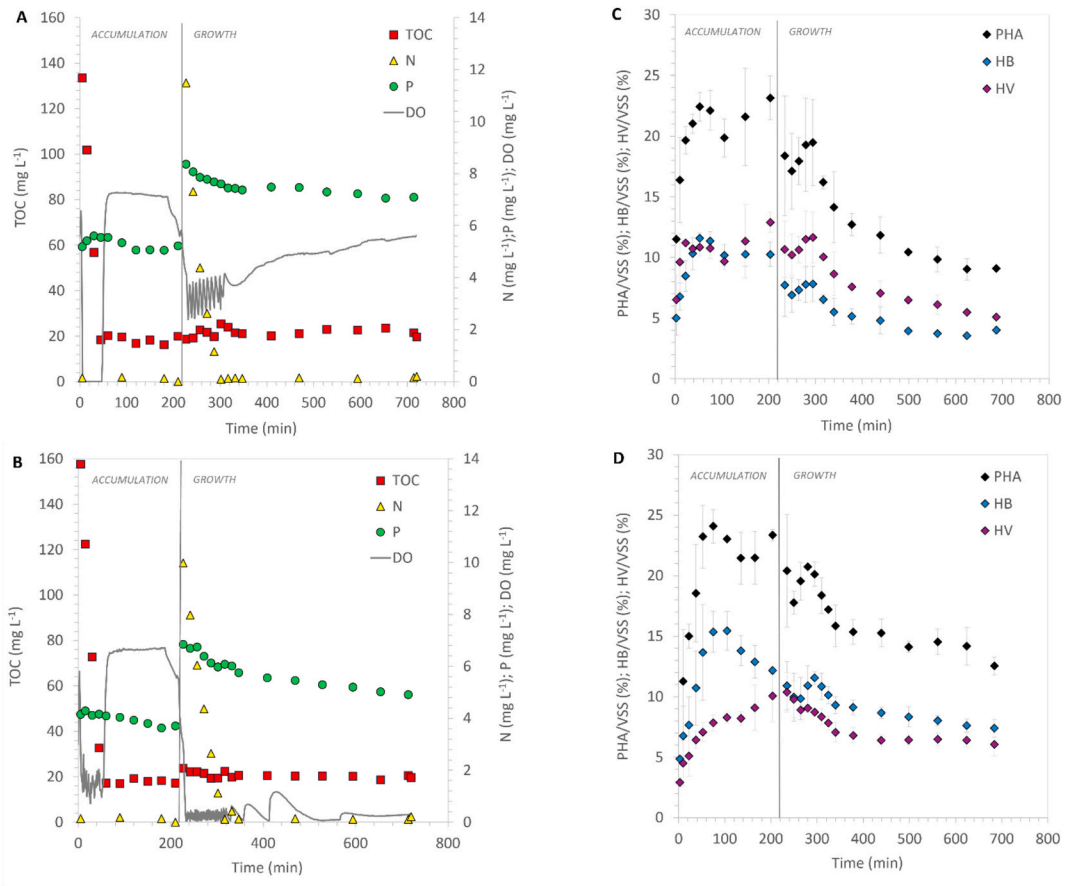

Fig. 3. Temporal dynamics of key compounds throughout the operational cycle at steady state conditions: TOC, N, P and DO in R1 (A) and R2 (B) – PHA, HB and HV in R1 (C) and in R2 (D).

in activated sludge, with potential consequences for sludge settleability (Zhang et al., 2020), the characteristics of the sludge were analysed along the experiment. Throughout the study, the biomass in R2 became visibly more compact and developed a pink coloration, whereas in both reactors the sludge retained a floc-like structure, exhibited a pronounced tendency to adhere, and settled well (SVI < 150 mL g^{-1}). On day 100, the final SVI values were 61 mL g^{-1} in R1 and 106 mL g^{-1} in R2.

3.2. Ecological analysis of microbial communities

The predominant phyla detected in both reactors were Proteobacteria and Bacteroidota. At the genus level, an increase in the relative abundance of *Paracoccus* was observed in both reactors, while *Azoarcus* remained among the predominant genera in both systems throughout the experiment (Fig. 4A). Both genera are renowned PHA accumulators. Other PHA-accumulating taxa were identified in both reactors included *Amaricoccus*, *Flavobacterium* and, at lower abundance, *Thauera* (Fig. 4A). Notably, the proportion of 16S reads classified as originating from PHA-accumulating genera accounted for approximately 75% of the total identified reads in both reactors (Fig. 4A). The communities of the two reactors both underwent marked changed over time but slightly diverged in their overall composition, as revealed by the PCoA (Fig. 4B). Most of the community shift occurred before day 64 in both reactors, given the substantially lower variance explained by PC2 compared to PC1. In particular, the abundance of few taxa resulted significantly different between the matured communities (from day 64 onwards) of two reactors. Among these were genera known to be able of PHA accumulation: *Flavobacterium* was more abundant in R2, whereas *Planktosalinus*, *Amaricoccus*, *Thauera* and *Hydrogenophaga* were more abundant in R1. The total DNA sequencing data corroborate the 16S rRNA results in terms of the main genera enriched in both reactors. Furthermore, total DNA sequencing revealed that differences between the two communities are also observable at the species level, with *Amaricoccus* with reads identified as *Amaricoccus HAR-UPW-R2A* and *Amaricoccus solimangrovi* predominating in R1, whereas species of *HGW-Betaproteobacteria* clades were more abundant in R2. Regarding the genus *Paracoccus*, the species *Paracoccus denitrificans* was more abundant in R2, where it accounted for an average of 0.15% of the estimated microbial community across the R2 samples, compared to 0.10% in R1. As expected, most of the microbial community was mostly composed of eubacteria in both reactors (over 99.7%) while less than 0.2% of the DNA originated from eukaryotes. TEM images suggest a lower number and smaller size of eukaryotes in R2 compared to R1, yet DNA sequencing indicates no clear overall reduction in their relative abundance. Nevertheless, notable shifts in community composition are observed: R2 is strongly enriched in *Diploscapter* compared to R1, and other nematodes such as *Anisakis* and *Brugia* are also more abundant. However, the identification of the latter nematodes could derive from misclassification of *Diploscapter* DNA (Di Gloria et al., 2025). Unlike other metazoan, many nematodes can tolerate short periods of anoxia (Van Voorhies and Ward, 2000), thereby obtaining a selective advantage over other eukaryotes in microaerophilic conditions. In addition, *Exophiala*, a fungal genus capable of degrading PHA (Brtnicky et al., 2025), was the most abundant fungus in both reactors. In summary, the total relative abundance of PHA-accumulating bacteria was comparable between the reactors, but differences were observed in the dominant taxa. Microaerophilic conditions reduced the relative abundance of known PHA accumulators such as *Amaricoccus* and *Thauera*, but other PHA-accumulating taxa appeared more tolerant of such conditions. In particular, *Paracoccus* and *Azoarcus* were the two most abundant genera

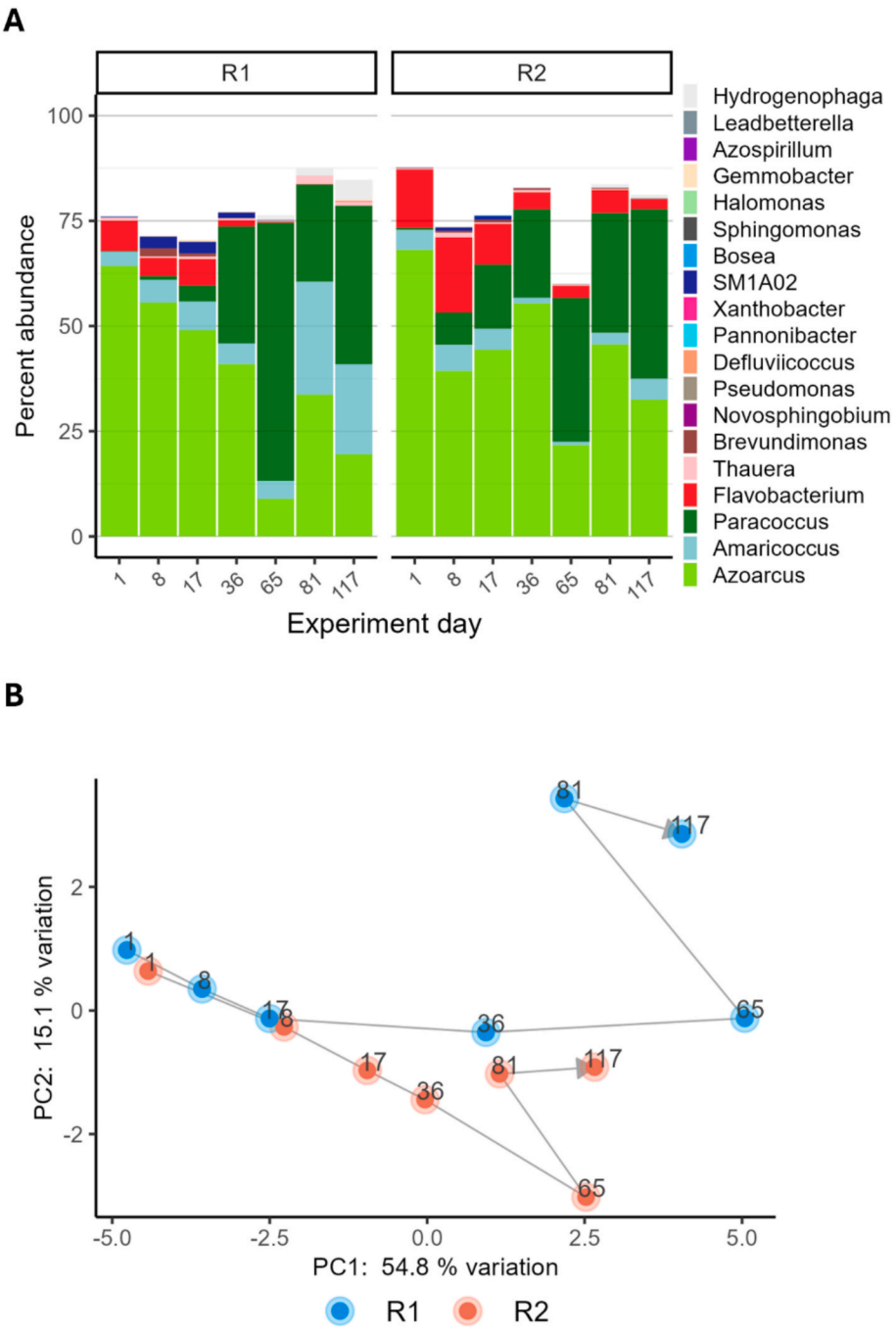

Fig. 4. Temporal dynamics of microbial communities. (A) Bar plot showing the relative abundances of the predominant PHA-accumulating genera identified. (B) PCoA of microbial communities across the experimental timeline of the two reactors. Experimental days are indicated along the X-axis of the bar plot and as numbers on each point in the PCoA.

in the mature communities of both reactors. Assuming that their dominance reflects higher metabolic adaptability, the majority of PHA may have been produced by these two taxa.

At species level, the reactor R2 exhibited a higher abundance of *Paracoccus denitrificans* compared to R1. Although this species can grow facultatively under anaerobic conditions by using the denitrification pathway, the absence of nitrification in the reactor suggests that this scenario may be unlikely. Nevertheless, *P. denitrificans* can also perform efficiently under microaerophilic conditions, owing to adaptations that maintain the efficiency of oxidative phosphorylation, such as the use of a high-affinity cbb_3-type cytochrome oxidase as a terminal respiratory oxidase (Baker et al., 1998).

3.3. Evaluation of maximum PHA accumulation capacity and polymers composition

Once steady state conditions were reached on both reactors, accumulation tests were performed with the MMC of both reactors to evaluate the maximum PHA accumulation capacity of the biomass. Fig. 5 shows the PHA, HB and HV along the accumulations test in R1 (Fig. 5A) and in R2 (Fig. 5B). The biomass cultivated in R2 had a 49% higher PHA accumulating capacity (PHA_{MAX} = 82 ± 11%) than the biomass cultivated in R1 (PHA_{MAX} = 55.1 ± 0.3%). The HV:HB ratio at the end of the test performed with the biomass cultivated in R1 (0.41 ± 0.02) was 65% higher than that in R2 (0.25 ± 0.03). Therefore, enrichment involving a

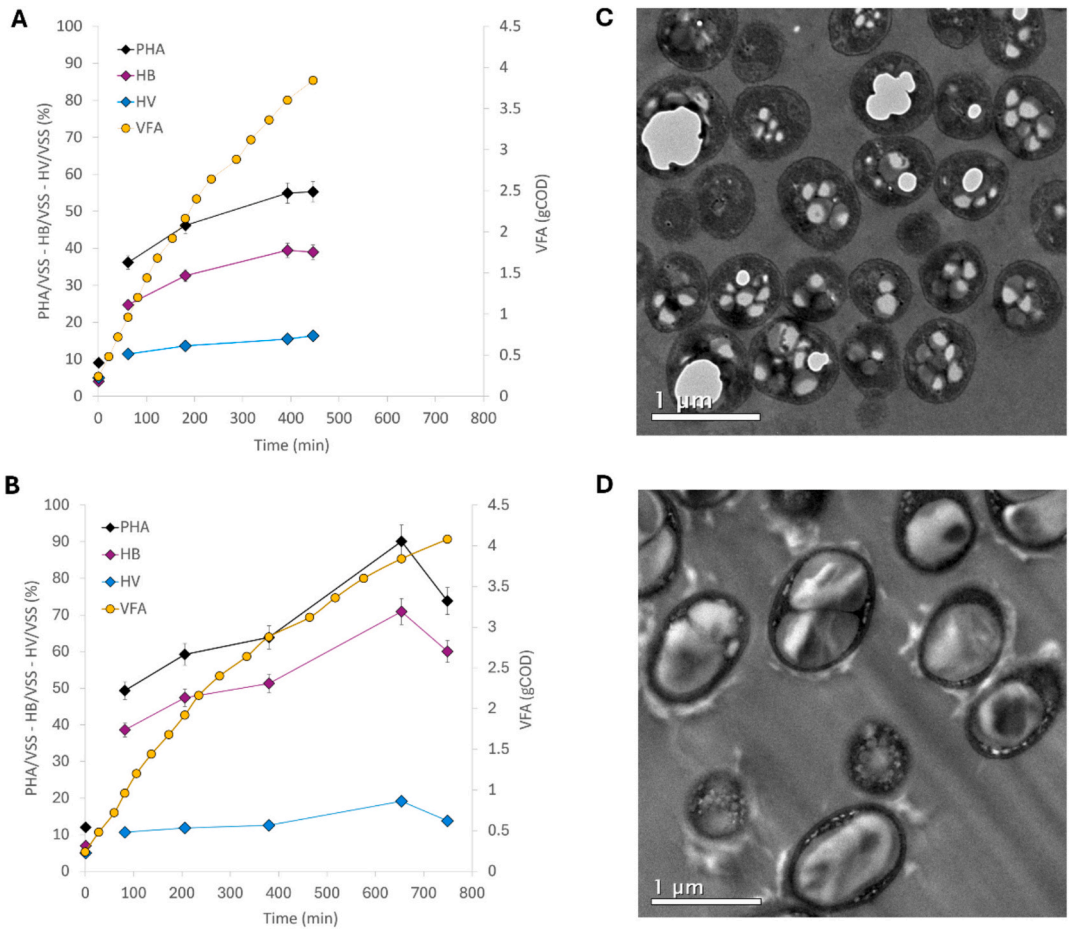

Fig. 5. Trends of PHA accumulation tests in R1 (A) and R2 (B) on experimental day 100 and TEM images of the biomass at the end of the test in R1 (C) and in R2 (D).

microaerophilic growth phase suggests an influence on both the maximum storage capacity and the polymer composition. PHA measurements were complemented with TEM images of the biomass collected at the end of the test (Fig. 5C and Fig. 5D). In both biomasses, inclusions of PHA were observed as clear, subcircular structures within the cells. Notably, the most prominent white circles with sharp boundaries did not appear to be PHA inclusions. Instead, they may correspond to areas where the embedding matrix was damaged during sample sectioning. Their frequent occurrence in regions with higher PHA content may be due to the greater material density in these areas, which makes them more prone to damage during cutting. The biomass in R1 was rich in PHA-accumulating bacteria of various morphologies (round or elongated), which contained numerous small PHA inclusions: as whitish, roughly circular structures within the bacterial cells (Fig. 5C). TEM images of the R2 biomass, similar to those of R1, show a large number of PHA-accumulating bacteria of various morphologies. However, a major distinction lies in the size of the PHA inclusions. Biomass grown under microaerophilic famine conditions contained larger PHA inclusions that almost completely filled the bacterial cells. Interestingly, very small inclusions were also observed adjacent to the larger ones (Fig. 5D). Bacteria in R2 were on average larger ($\geq$ 1 µm) than those in R1 (0.5–1 µm), which may be associated with the internal pressure generated by PHA accumulation. In fact, when imaged during the enrichment step, cells with comparable PHA contents display similar dimensions.

The substrate is generally considered the primary factor determining the nature and basic composition of the polymer as the substrate defines the type of PHA and the monomeric precursors (Wongsirichot, 2024). Nevertheless, polymer composition is determined by a combination of factors, including microbial, genetic, and operational parameters, which act together with the substrate (Bodhe et al., 2024; Wongsirichot, 2024). Identifying the co-factors that influence polymer composition beyond the substrate is crucial for developing strategies to precisely tailor PHA properties for specific biotechnological applications. Prior to our study, Blunt et al., (2018) indicated that oxygen transfer rate (OTR) is a key determinant of PHA characteristics. A reduction in oxygen transfer not only alters the incorporation pattern of monomers but could also lead to an increase in polymer molecular weight. Oxygen availability during synthesis therefore exerts an integrated effect on both PHA composition and quality. Our study demonstrates that controlling DO during biomass enrichment can alter polymer composition, independently of the substrate, both during the enrichment and in the subsequent accumulation step. These results reveal a novel strategy to steer PHA properties via oxygen management, with important implications for process optimisation in MMCs for PHA production. From Fig. 5A and B, it is immediately evident that in this study the main difference between R1 and R2 polymer composition lies in HB accumulation; at the end of the test HV levels were comparable between the two reactors (HV_R1 = 16% vs HV_R2 = 14%), however HB accumulation was markedly higher in R2 (HB_R1 = 39% vs HB_R2 = 60%). This difference in terms of HB accumulation alters the HV:HB ratio (lower in R2), which in turn influences the thermo-mechanical properties of the final polymer determining the polymer application (Cho et al., 2025).

Consistent differences in polymer composition were already apparent during the biomass enrichment. Throughout the experimental period, the HV:HB ratio remained consistently higher in R1 than in R2, with the difference between the two reactors increasing over time. Interestingly, in the accumulation step, the final HV:HB ratio was lower

(0.42 in R1; 0.23 in R2) compared to the enrichment step in both reactors the same day, both at end of feast (0.95 in R1 and 0.58 in R2) and at end of famine (1.55 in R1 and 0.76 in R2). In contrast, Coats et al., (2016) observed the opposite trend: the average HV:HB was markedly higher in the PHA accumulation reactor (1.3 ± 0.9) compared with the biomass enrichment reactor (0.6 ± 0.2).

Furthermore, the HV:HB ratio at the end of the famine was higher than at the end of the feast, especially in R1. This pattern suggests that it increases predominantly after the feast phase. Examining the cycle analysis on day 100, the HB and HV trends are particularly noteworthy (Fig. 3). In R1, HV and HB accumulated in similar amounts during the initial phase. However, as the cycle progresses, HV became predominant, leading to a higher HV content than HB during the growth phase. Conversely, in R2, HB accumulation was favoured from the very beginning. A notable observation is the dynamic interplay between the monomers, especially in R2: a decrease in HB appeared to correspond to an increase in HV at the end of the accumulation phase, after feast phase, once all the organic substrate had been assimilated. This observation suggests that extending the accumulation phase could potentially influence the polymer composition. However, no comparable monomer dynamics were observed during the growth phase, where apparently similar conditions prevailed.

3.4. Proposed mechanisms and hypotheses for PHA accumulation

At the end of the accumulation test, reactor R2 exhibited a higher PHA storage capacity. In both accumulation tests, the first PHA analysis was performed immediately before the fourth carbon spike, after a total of 0.72 g COD had been dosed. At that time, the PHA content in R1 was 36%, compared to 49% in R2, indicating that a larger fraction of the supplied carbon was directed toward PHA storage in R2 from the very beginning of the test (Fig. 5). This behaviour would also be expected during the enrichment step (Fig. 3); however, no significant differences were observed between the two reactors in this stage. The biomass in R1 and R2 accumulated roughly the same amount of PHA over the same period (20–25%). This pattern suggests that between PHA 25% and 35%, the uptake of VFAs and/or their conversion into PHA by the biomass cultivated in R1 begin to slow down.

The oxidation of VFA to acetyl-CoA and propionyl-CoA is regulated by the cellular [acetyl-CoA]/[CoA] and [NADPH]/[$NADP^+$] ratios. The PHA pathway is known to act as a cellular mechanism for channelling excess acetyl-CoA and for regenerating $NADP^+$ from NADPH. When PHA approaches saturation, intracellular PHA levels can modulate (and inhibit) PHA synthase activity, causing PHA synthesis to slow and leading to accumulation of acetyl-CoA and NADPH, and NADPH accumulation is associated with decreased Tricarboxylic Acid (TCA) cycle activity. In summary, when the cell is approaching PHA saturation, both the PHA pathway and the TCA cycle slow down (Pardelha et al., 2012; Chen et al., 2016). In R2 microaerophilic conditions or the alternation between microaerophilic and aerobic conditions may improve regulatory effects associated with intracellular PHA accumulation and direct more effectively the substrate toward storage, although slowed by overfull inhibition. This hypothesis suggests a more efficient use of energy resources by the bacterial cell in R2.

During the growth phase of the enrichment step, a difference was detected between R1 and R2 in the extent and timing of PHA consumption (Fig. 3). Mostly in R1, PHA consumption began with a delay relative to the onset of nitrogen availability and the majority of PHA was consumed after nitrogen depletion (7.1% out of 9.3% of PHA). In R2 the post-nitrogen depletion consumption was lower than in R1 and represented a smaller although not negligible fraction of the total consumption (4.7% out of 7.9% of PHA).

Several possible explanations can be considered, including nitrogen accumulation phenomena in R1, or the potential use of storage products alternative to PHA, combined with differing maintenance energy requirements. In particular, it is not to be excluded that cells require less energy for maintenance under microaerobic conditions. Therefore, a larger proportion of substrate could potentially be directed toward PHA synthesis, which may result in higher yield factors and increased PHA accumulation. These ideas remain speculative and would require further investigation to be confirmed.

Another interesting aspect concerns the role of phasins. In literature it is reported that phasins play a key role in regulating the surface-to-volume ratio of PHA granules by binding to their surface and controlling granule size and morphology. This prevents granule coalescence, maintaining multiple small granules per cell, as seen in wild-type strains. In contrast, phaP deletion strains, lacking phasins, accumulate PHB as a single large granule, lowering the surface-to-volume ratio and reducing the area available for PHA synthase activity, which may limit both synthesis rate and total PHB accumulation (York et al., 2001). In the present study, PHA granules appeared notably coalesced, which may suggest that microaerophilic conditions influenced phasin function. Reduced phasin activity could have promoted granule fusion, resulting in fewer, larger granules per cell and a lower surface-to-volume ratio. However, the net effect of granule coalescence on PHB accumulation remains unclear, as concentrating polymer synthesis in fewer granules could potentially enhance total polymer accumulation, despite possibly reducing local catalytic efficiency.

Another possible explanation is that the capacity for PHA accumulation may be influenced by the physical space available within the cell. This is supported by the observation that cells increase in size during the accumulation, suggesting that cell enlargement could accommodate higher amounts of polymer. According to Zhang et al., (2018), cell wall flexibility-which determines overall cell rigidity-is a critical factor influencing intracellular accumulation of PHB in *Escherichia coli*. Cell wall flexibility enhances PHB accumulation through two main mechanisms. First, it increases intracellular space: a rigid cell wall, primarily composed of peptidoglycan, stabilises cell shape and limits volumetric expansion. In contrast, weakened walls generate larger, more elastic cells, allowing greater volumetric expansion and PHB accumulation up to 93% content in dry cell weight. Second, flexibility reduces competition for carbon. Repressing wall synthesis genes redirects carbon flux toward PHB enhancing polymer yield.

These observations suggest the intriguing possibility that microaerophilic conditions, or alternation between microaerophilic and aerobic conditions, might influence PHB accumulation through changes in cell wall rigidity. However, this remains a speculative hypothesis, as the observed effects could also result from the selection of species or subclones better suited to polymer accumulation under these conditions. Further research will be needed to clarify whether oxygen availability directly affects the biophysical properties of the cells or primarily shapes the microbial population composition.

3.5. Critical assessment of operational outcomes

The different aeration conditions created sufficiently distinct environments to produce observable differences in biomass structure and behaviour. Since the apparent DO affinity (K_{DO}) for PHA-accumulating bacteria growing on PHA has been estimated at 0.15 ± 0.05 mg O_2 L^{-1} by Wang et al., (2019), even an average concentration of 0.3 mg O_2 L^{-1} was sufficient to affect the selection process. Biomass cultivated under microaerophilic conditions exhibited a markedly higher PHA content of 82%, corresponding to a 49% increase, thereby outperforming not only the control reactor but also benchmark values commonly reported in the literature. Johnson et al., (2009), for example, achieved PHA contents of up to 90% in fed-batch systems under growth-limiting conditions, using a culture enriched over four years on acetate as the sole substrate, thereby producing only PHB. Likewise, Carvalho et al., (2022) obtained biomass capable of accumulating up to 84% PHA from a synthetic mixture of acetate, propionate, butyrate, and valerate, despite the substantial salinity of the medium (30 g NaCl L^{-1}). The highest PHA content reported to date, 90.7%, was achieved by Coats

et al., (2016) using real fermented dairy manure and a pulsed-feeding strategy that maintained continuous feast conditions. Even when comparing with such optimised systems, the performance observed under microaerophilic conditions in the present study remains particularly noteworthy. Overall, microaerophilic cultivation favoured $Y_{PHA/S}$, which reached 0.88 in R2 compared with 0.75 in R1 (COD-basis), representing a 17.3% improvement. However, the higher accumulation yield does not directly correspond to an increase in volumetric productivity. While volumetric productivity was slightly higher in R1 (0.24 g PHA $L^{-1}h^{-1}$ versus 0.19 g PHA $L^{-1}h^{-1}$ in R2, a 20.8% reduction), this parameter alone does not fully capture the broader process advantages offered by microaerophilia. Indeed, when the two systems are compared through the lens of overall process yield (Y_{global}), the benefit of microaerophilic operation becomes more evident: 0.40 in R2 versus 0.33 in R1, corresponding to a 21.2% increase. Although rarely reported, these overall yields are consistent with literature values (0.33–0.38), as reported by Paul et al., (2020). This evidence indicates that, despite a modestly lower volumetric productivity, the microaerophilic configuration may ultimately enable more efficient substrate-to-product conversion at the process scale.

Finally, when operating with a blower featuring adjustable airflow and using conservative values of C*=9 mg O_2 L^{-1} and 2.5 mg O_2 L^{-1}, the estimated energy reduction in terms of kWh during the enrichment step corresponds to 32%. This represents an additional and non-negligible advantage, further supporting the potential benefits of microaerophilic operation.

The findings of this study may have potential relevance for industrial PHA production. Applying microaerophilic conditions during the growth phase appeared to increase PHA accumulation while reducing oxygen supply, which could lower aeration energy demand, one of the main operational costs in industrial bioreactors and a key factor for scale-up. This approach is conceptually scalable and is compatible with existing SBR, representing a relatively simple, low-cost modification that could be implemented even in existing systems without structural changes, requiring only operational control of oxygen levels and nutrient feeding. The observed higher PHA accumulation could potentially facilitate subsequent polymer extraction, and this strategy may provide an additional means to influence polymer composition, offering avenues for further investigation and process optimisation.

While the findings suggest potential benefits for industrial PHA production, several limitations should be considered. One key limitation is that the energy savings observed at the reactor scale need to be evaluated in the context of the entire production process, including downstream processing and aeration in other stages. Additional potential challenges include process stability under prolonged low-oxygen conditions and possible operational trade-offs, such as slower biomass growth or extended start-up times. These aspects remain to be further investigated to fully assess the feasibility, scalability and robustness of this approach in industrial applications.

4. Conclusions

After 100 days of enrichment in a lab-scale SBR fed with synthetic effluent, the microaerophilic reactor (0–1 mg O_2 L^{-1}) reached a PHA_{MAX} of 82 ± 11%, corresponding to a 49% increase in accumulation compared to the fully aerobic condition (3–4 mg O_2 L^{-1}). These results show that microaerophilic conditions during the growth phase enhance PHA accumulation, increasing both yields (~20%) and maximal storage capacity, while the aerobic reactor still maintains robust PHA production. Operating under low-oxygen conditions also substantially reduced aeration energy demand (by 32%), highlighting the potential for more energy-efficient processes. Importantly, this approach relies solely on operational control of oxygen levels and does not require any hardware modifications. Furthermore, microaerophilic conditions modified polymer composition, particularly the HV:HB ratio, suggesting a potential means to tailor polymer properties, and influenced both eukaryotic and prokaryotic microbial communities, including PHA-accumulating taxa, with minimal impact on settleability. Overall, microaerophilic enrichment represents a simple, promising strategy to simultaneously boost intracellular PHA content, optimise polymer characteristics, and reduce operational costs, providing a sustainable approach for improving PHA production in MMCs. Future studies will focus on elucidating the underlying mechanisms responsible for the observed improvements in PHA accumulation and polymer properties, to better understand the physiological and ecological responses driving these results.

CRediT authorship contribution statement

Serena Falcioni: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Alessio Castagnoli:** Writing – review & editing, Investigation, Formal analysis. **Ajchareeya Manmeen:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Elia Biagini:** Writing – review & editing, Formal analysis, Data curation. **Leandro Di Gloria:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Matteo Ramazzotti:** Writing – review & editing, Validation, Supervision. **María Eugenia Suárez-Ojeda:** Writing – review & editing, Validation, Supervision. **David Gabriel:** Writing – review & editing, Validation, Supervision, Resources, Project administration. **Giulio Munz:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

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Data availability

Data will be made available on request.

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