

Effect of different nutrient decoupling strategies on aerobic polyhydroxybutyrate production and microbial community

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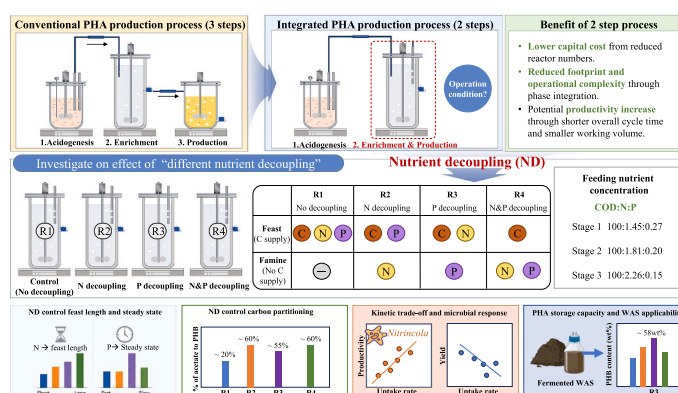
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HIGHLIGHTS

- Ammonium placement, not dose, controlled feast length in single-stage SBRs.
- Nitrogen limitation diverted 56–63% of feast carbon to PHB storage.
- Phosphate limitation raised PHB content mainly via cross-cycle carry-over.
- PHB productivity rose but yield fell with specific acetate uptake rate.
- *Nitriicola* dominated fast-uptake, high-storage communities on waste carbon.

GRAPHICAL ABSTRACT



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ABSTRACT

Polyhydroxybutyrate (PHB) production by mixed microbial cultures can recover wastewater carbon as a biodegradable polymer, but the effects of nutrient placement in single-stage nutrient-decoupling systems remain unclear. Four sequencing batch reactors were operated for 420 days under control, ammonium-decoupled, phosphate-decoupled, and combined-decoupled conditions at three nutrient concentrations with a fixed feast-to-famine ratio of 0.20. Ammonium placement, rather than total dose, primarily controlled feast length, while feast-phase phosphate availability accelerated stabilization. Feast-phase ammonium limitation directed 56–63% of acetate carbon to PHB, whereas phosphate limitation produced similarly high end-of-feast PHB contents partly through residual PHB carry-over. Famine-phase ammonium further promoted biomass growth using stored PHB. Across twelve steady-state conditions, PHB productivity increased with specific acetate uptake rate, whereas PHB yield decreased, indicating a productivity–yield trade-off linked to microbial community shifts. *Nitriicola* was strongly associated with high acetate uptake and PHB storage rates. Fed-batch accumulation reached 58 wt% PHB, and communities with high storage capacity on synthetic medium largely retained this phenotype on

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fermented waste activated sludge. These results identify phase-specific ammonium supply as a key operational lever for directing carbon between PHB storage and biomass growth.

1. Introduction

Polyhydroxybutyrate (PHB) is a biodegradable polyester with potential to replace petroleum-derived plastics, but its broader application remains limited by production cost. Conventional pure-culture fermentation typically requires refined carbon substrates and sterile operation, both of which increase process costs [1]. Mixed microbial culture (MMC) processes offer an alternative by eliminating the need for sterilization and enabling the use of waste-derived carbon sources, including those recovered from wastewater and waste activated sludge (WAS) [2]. This approach also shifts wastewater treatment from carbon removal toward resource recovery by converting organic carbon that would otherwise be oxidized during treatment or remain in residual sludge into a feedstock for value-added biopolymer production. Phosphorus is a finite and non-substitutable resource, and its recovery from wastewater, most often as struvite, is increasingly pursued to offset the depletion of phosphate rock and to limit eutrophication [3]. Withholding phosphate during the feast phase is therefore attractive, because it promotes PHB storage while remaining compatible with downstream phosphorus recovery, and because in fermented waste streams ammonium is typically abundant whereas phosphate can be selectively precipitated, making phosphate decoupling operationally realistic.

The established MMC route to PHB production generally consists of three steps. Waste organics are first fermented into volatile fatty acids (VFAs), a PHB-accumulating microbial community is then enriched in a sequencing batch reactor (SBR), and the enriched biomass is finally subjected to fed-batch accumulation to reach its maximum storage capacity. Community enrichment relies on alternating feast and famine conditions within each cycle. During the feast phase, externally supplied carbon is rapidly taken up and stored intracellularly as PHB, whereas during the famine phase the stored polymer supports maintenance and growth, thereby selecting organisms with a strong storage phenotype. Effective enrichment generally requires a sufficiently long famine phase. This is commonly expressed by the feast-to-famine (F/F) ratio, defined as the length of the feast phase divided by that of the famine phase within a cycle, and F/F ratios below approximately 0.25 have been reported to provide the famine period needed to select organisms with high storage capacity [4,5].

The enrichment and accumulation functions can also be integrated within a single reactor through double growth limitation (DGL). In this approach, a growth-limiting nutrient is withheld during the feast phase to promote carbon storage and supplied during the famine phase to support growth on previously stored PHB [6]. This temporal separation of carbon and nutrient availability strengthens the selective pressure for storage and has achieved end-of-feast PHB contents of approximately 27–70 wt%, comparable to those obtained in conventional three-step systems while eliminating the need for separate accumulation reactors.

Operational factors governing conventional MMC enrichment have been studied extensively, including organic loading rate (OLR) [7], nitrogen [8] and phosphorus limitation [9], carbon source [10], temperature [11], and pH [12]. In contrast, integrated nutrient-decoupling systems remain comparatively underexplored. Available studies have mainly examined feeding strategy [13], ammonium decoupling [14], and OLR [15], leaving several fundamental aspects of phase-specific nutrient supply unresolved.

Three knowledge gaps are particularly important. First, the effect of nutrient decoupling has not been clearly separated from changes in the F/F ratio. Operational variables such as feed ammonium concentration [14] and OLR [15] alter feast duration and consequently famine duration, making it difficult to distinguish the direct effect of nutrient availability from the effect of feast and famine selection pressure. For

example, increasing OLR above 8.5 g COD/L/d was associated with lower end-of-feast PHB content as the feast phase became longer [14]. Second, previous integrated PHB production systems have focused mainly on ammonium decoupling, leaving phosphate decoupling and combined ammonium–phosphate decoupling largely unexplored. This is a critical limitation because phosphorus limitation has been shown in conventional enrichment systems to promote intracellular storage selection [9,16]. Phosphate decoupling is also more compatible with practical waste-derived feedstocks. Fermented waste streams commonly contain substantial residual ammonium, which is difficult to remove selectively, whereas phosphate can be readily separated by precipitation. Thus, phosphate-limited feast operation can be implemented more directly with real feedstocks, while nitrogen-limited feast operation is more dependent on additional pretreatment [3]. Phosphate decoupling and combined ammonium and phosphate decoupling remain largely unexplored, despite evidence from conventional enrichment systems that phosphorus limitation can also impose strong selection for storage. The relative roles of nitrogen and phosphorus, and whether their effects are enhanced when both are temporally decoupled, therefore remain unresolved. Third, little is known about how phase-specific nutrient availability restructures the microbial community. Consequently, the taxa selected under different nutrient-decoupling strategies and their associations with PHB storage kinetics, yield, and productivity have not been clearly established.

This study investigates how nutrient-decoupling strategy and feed nutrient concentration govern PHB production in integrated feast and famine SBRs while maintaining the F/F ratio at 0.20. Four reactors were operated under control, ammonium-decoupled, phosphate-decoupled, and combined ammonium and phosphate-decoupled conditions across three feed nutrient concentrations. Reactor performance was evaluated through acetate uptake and PHB storage kinetics, stoichiometric yields, carbon partitioning among storage, growth, and respiration, fed-batch storage capacity using synthetic medium and fermented WAS, and microbial community analysis. By fixing the F/F ratio, the study separates phase-specific nutrient effects from variation in famine duration and determines how the timing of nitrogen and phosphorus supply governs carbon allocation and microbial selection. The overall aim is to identify the nutrient-decoupling strategy that most effectively enriches a high-storage community and to link this phenotype to its underlying kinetic, stoichiometric, and microbial characteristics.

2. Materials and methods

2.1. Sequence Batch Reactor (SBR) operation with different nutrient concentration

2.1.1. Reactor configuration and dissolved-oxygen control

Four bubble-column SBRs (R1–R4), each with a working volume of 1.90 L, were inoculated with 1.20 L of enriched mixed microbial culture obtained from a previous study. The inoculum contained 1.73 g/L mixed liquor suspended solids (MLSS) and 33 wt% PHB at the end of the feast phase. The reactors were operated in sequential batch mode using a programmable logic controller (PLC), which maintained a fixed F/F ratio of 0.20 based on dissolved oxygen (DO) profiles. A sharp increase in DO was used to indicate carbon depletion and the end of the feast phase. The F/F ratio was fixed at 0.20 to provide a sufficiently long famine phase, thereby favoring microorganisms capable of storing PHB during feast and utilizing intracellular PHB during famine.

Synthetic wastewater was prepared every 2–3 days, depending on the cycle length, and stored at 4°C to minimize microbial growth before use. Air was continuously supplied at 2.5 L/min to maintain aerobic

conditions. No sludge settling phase was included; therefore, biomass was discharged with the effluent, and the hydraulic retention time (HRT) and sludge retention time (SRT) varied with the cycle length determined by the fixed F/F ratio. The reactors were operated at 28°C without pH control, and the pH ranged from 7.8 to 8.6 during operation. The reactor configuration is shown in Fig. 1.

2.1.2. Nutrient-decoupling strategies and cycle operation

The four reactors were operated with different nutrient-feeding strategies. R1 served as the control, in which carbon, nitrogen, and phosphorus were supplied together at the beginning of the feast phase. Each R1 cycle consisted of 2 min feeding of 600 mL synthetic wastewater, followed by aerobic feast, discharge of 600 mL mixed liquor at the end of the feast phase, and aerobic famine. No additional nutrient feeding was supplied during the famine phase. In R2 and R3, each cycle consisted of 2 min initial feeding, aerobic feast, discharge of 500 mL mixed liquor at the end of the feast phase, addition of 100 mL nutrient solution, aerobic famine, and final discharge of 100 mL mixed liquor. In R4, the cycle structure was the same as in R2 and R3, except that nitrogen and phosphorus solutions were supplied separately as 50 mL each, giving a total famine-phase nutrient-feeding volume of 100 mL. The volume exchange ratio was 31.5% per cycle in all reactors. The SBR operation regime is illustrated in Fig. 2.

2.1.3. Staged operation and feed composition

The reactor operation was divided into three stages to evaluate the effects of nutrient concentration and nutrient-decoupling strategy on PHB enrichment and accumulation. Stage 1 was operated from day 1 to day 177, Stage 2 from day 177 to day 347, and Stage 3 from day 347 to day 420. In Stage 1, the overall COD:N ratio was 100:1.45:0.27 in all reactors, and this was changed to 100:1.81:0.20 in Stage 2. In Stage 3, reactor-specific COD:N ratios were applied to further evaluate the effects of nutrient concentration and feeding distribution. Detailed wastewater compositions, nutrient concentrations, and COD:N ratios for the feast- and famine-phase feedings are provided in Table 1. Operational conditions were changed to the next stage only after the reactors reached steady state, defined by stable feast length, MLSS concentration, and PHB content.

Sodium acetate trihydrate ($\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$) was used as the

carbon source, ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$) as the nitrogen source, and potassium dihydrogen phosphate (KH_2PO_4) and dipotassium hydrogen phosphate (K_2HPO_4) at a 1:1 ratio as the phosphorus source. Micronutrients were added to the synthetic wastewater at the following concentrations: 40 mg/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 12.5 mg/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.307 mg/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.045 mg/L H_3BO_3 , 0.009 mg/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.054 mg/L KI, 0.036 mg/L $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.018 mg/L $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.036 mg/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.045 mg/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, and 3 mg/L EDTA. Thiourea was added to the feed at 20 mg/L to inhibit nitrification, as previously reported [17].

During reactor operation, effluent samples from the feast- and famine-phase discharges were collected to determine PHB content, MLSS, ammonium, and phosphate concentrations. After steady state was reached in each stage, cycle analysis was conducted to evaluate carbon and nutrient utilization and PHB storage dynamics over one complete SBR cycle.

2.2. Fed-batch experiment with the enriched culture from SBRs

Fed-batch experiments were conducted using the feed-on-demand method to evaluate the maximum PHB storage capacity of the enriched cultures from the one-stage SBRs [18]. At the end of the famine phase, 250 mL of mixed liquor from each steady-state SBR was transferred to a batch reactor and adjusted to a biomass concentration of 1.0 g/L. The cultures were aerated overnight at 3.0 L/min and 28°C to consume residual intracellular PHB and obtain the minimum PHB content before the accumulation test. Subsequently, 50 mL of synthetic carbon medium containing 15.0 g/L $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$, equivalent to 7.2 g COD/L, was added to each batch reactor.

The DO concentration was monitored using a DO meter (YSI 5100). When the DO concentration stabilized after carbon addition, carbon depletion was assumed, and an additional 50 mL of the same carbon-only medium was supplied. This feed-on-demand procedure was repeated until the DO concentration no longer changed after carbon addition, indicating that the culture had reached its maximum PHB storage capacity [19]. All fed-batch experiments were performed in duplicate using sludge collected from the same steady-state SBRs.

To evaluate PHB accumulation under more practical feedstock conditions, VFA rich broth produced from waste activated sludge

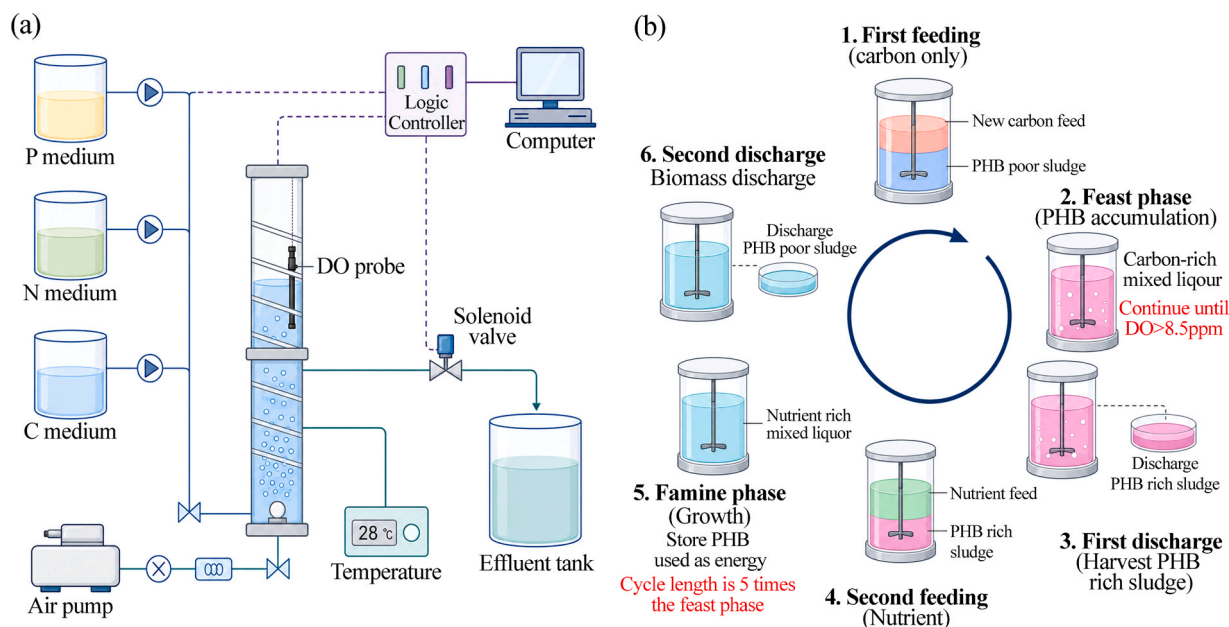


Fig. 1. Schematic diagram of the nutrient-decoupled feast-famine SBR system for one-stage PHB enrichment and accumulation: (a) laboratory-scale bubble-column reactor with separate C, N, and P feeding lines and DO-based process control; and (b) operating cycle consisting of carbon feeding, feast-phase PHB accumulation, PHB-rich sludge discharge, nutrient feeding, famine-phase growth, and final biomass discharge.

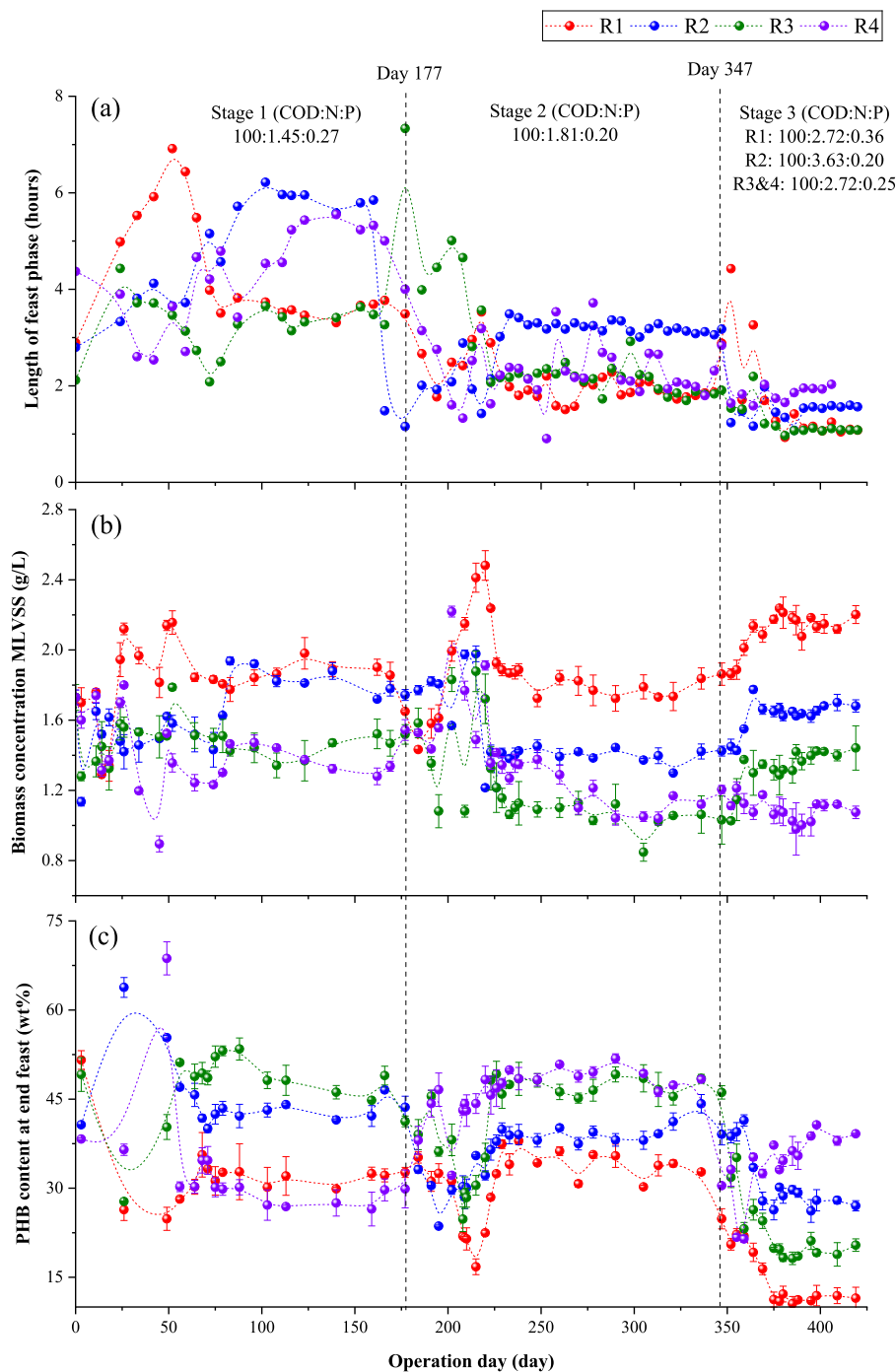


Fig. 2. Changes in (a) feast-phase length, (b) biomass concentration as MLVSS, and (c) PHB content at the end of the feast phase during nutrient-decoupled SBR operation. R1, non-decoupled control; R2, nitrogen decoupling; R3, phosphorus decoupling; and R4, combined nitrogen and phosphorus decoupling. Vertical dashed lines indicate stage transitions on days 177 and 347.

fermentation was also used in the fed-batch experiments. The sludge fermenter was operated at a working volume of 1.5 L, an SRT of 5 days, a stirring speed of 400 rpm, a temperature of 35°C, and pH 6.

The fermented sludge was centrifuged at $10,000 \times g$ for 15 min to obtain the supernatant. Subsequently, 0.40 g of aluminum sulfate ($\text{Al}_2(\text{SO}_4)_3$) was added to 1.0 L of the supernatant to precipitate phosphate. The solution was then centrifuged again at $11,000 \times g$ for 15 min, and the resulting supernatant was filtered through a 47 mm glass microfiber filter (Whatman™) to remove residual suspended solids.

The prepared VFA-rich broth contained 8.1 g COD/L, including 2149 mg/L acetic acid, 1049 mg/L propionic acid, 1214 mg/L butyric

acid, 1079 mg/L valeric acid, 107 mg/L hexanoic acid, and 936 mg/L ammonium. Phosphate was not detected after aluminum sulfate treatment. The prepared broth was then used for fed-batch PHB accumulation following the same feed-on-demand procedure described for the synthetic carbon medium.

2.3. DNA extraction and sequencing

Genomic DNA was extracted from 0.25 g of enriched sludge collected from the SBRs using the DNeasy® PowerSoil® Pro Kit (Qiagen, Manchester, UK), according to the manufacturer's instructions. The

Table 1

Wastewater compositions with different carbon and nutrient medium for feast and the famine phase in four reactors across three stages.

Stage	Reactor	Total COD:N:P ratio	Feast phase			Famine phase	
			COD ^a (g/L)	N ^a (mg/L)	P ^a (mg/L)	N ^a (mg/L)	P ^a (mg/L)
Stage 1 Day 1–176	R1	100:1.45:0.27	3.6	52.2	9.78	0.00	0.00
	R2			0.00	9.78	52.2	0.00
	R3			52.2	0.00	0.00	9.78
	R4			0.00	0.00	52.2	9.78
Stage 2 Day 177–346	R1	100:1.81:0.20	3.6	65.2	7.29	0.00	0.00
	R2			0.00	7.29	65.2	0.00
	R3			65.2	0.00	0.00	7.29
	R4			0.00	0.00	65.2	7.29
Stage 3 Day 347–420	R1	100:2.72:0.36	3.6	97.9	13.1	0.00	0.00
	R2			65.3	7.29	65.3	0.00
	R3			97.9	1.82	0.00	7.29
	R4			0.00	0.00	97.9	9.11

^a COD from CH₃COONa•3 H₂O, N from (NH₄)₂SO₄, P from 50% of KH₂PO₄ and 50% of K₂HPO₄

extracted DNA was purified using the NucleoSpin™ gDNA Clean-up kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) to obtain DNA with a 260/280 ratio above 1.8. DNA concentration was measured using a NanoDrop™ ND-1000 spectrophotometer (Thermo Fisher Scientific, USA).

Microbial community analysis was performed by 16S rRNA gene amplicon sequencing described by Heo [20]. Briefly, the V3 to V4 region was amplified using the pro341F and pro805R primers and sequenced on an Illumina MiSeq platform using 2 × 300 bp paired-end sequencing. Sequences were processed using QIIME2 version 2022.2 with DADA2 denoising and VSEARCH filtering, and taxonomy was assigned using a Naive Bayes classifier trained on the SILVA 132 database. Taxa were expressed as relative abundances based on total sequence reads.

Alpha and beta diversity analyses were performed at a rarefaction depth of 33,000 reads. Alpha diversity was evaluated using ACE, Pielou's evenness, Shannon diversity, and Faith's phylogenetic diversity indices. Beta diversity was assessed using Jaccard distance to compare qualitative differences in microbial community structure. The resulting distance matrix was visualized by principal coordinate analysis (PCoA) using Origin 2023 (OriginLab, USA).

2.4. Analytical methods

MLSS, sludge volume index after 30 min settling (SVI₃₀), ammonium, and phosphate were measured according to Standard Methods [21]. PHB and VFAs were quantified by gas chromatography (GC). The detailed PHB analytical procedure was described in our previous study [22].

For VFA analysis, sludge samples were transferred to centrifuge tubes and centrifuged at 8500 rpm, equivalent to 7916 × g, for 15 min. The supernatant was filtered through a 0.8 μm polyethersulfone (PES) membrane syringe filter. Then, 0.9 mL of the filtrate was transferred to a 1.5 mL vial, followed by the addition of 0.1 mL of formic acid. The vial was loaded into an AOC-20i auto-injector (Shimadzu, Japan) and analyzed using a GC-2010 gas chromatograph (Shimadzu, Japan) equipped with a BP21 (FFAP) capillary column with a length of 30 m, an internal diameter of 0.25 mm, and a film thickness of 0.25 μm. Helium was used as the carrier gas, and nitrogen was used as the makeup gas. The injector and detector temperatures were set at 235°C and 250°C, respectively. The oven temperature was initially set at 60°C, increased to 230°C at 10°C/min, and then held at 230°C for 3 min.

2.5. Calculation of stoichiometric parameters

The F/F ratio was calculated as the length of the feast phase divided by the length of the famine phase in the same cycle. The PHB content of the sludge (wt%) was calculated as the PHB mass divided by the dry sludge mass (eq 1).

$$\text{PHBcontent(wt\%)} = \frac{\text{PHB amount(g)}}{\text{dry mass of a sample(g)}} \times 100 \quad (1)$$

The volumetric PHB productivity in the feast and famine phases, $r\text{PHB}_{\text{fst}}$ and $r\text{PHB}_{\text{fam}}$, was calculated per cycle from the PHB content and the effluent biomass concentration (eq 2, eq 3).

$$r\text{PHB}_{\text{fst}}(\text{mgPHB} / \text{L} \cdot \text{h}) = \frac{\text{PHB}_{\text{efst}}(\text{wt\%}) \times \text{MLSS}_{1\text{st}}(\text{g/L}) \times 1000}{\text{Cycle length(h)} \times 100} \quad (2)$$

$$r\text{PHB}_{\text{fam}}(\text{mgPHB} / \text{L} \cdot \text{h}) = \frac{\text{PHB}_{\text{efam}}(\text{wt\%}) \times \text{MLSS}_{2\text{nd}}(\text{g/L}) \times 1000}{\text{Cycle length(h)} \times 100} \quad (3)$$

Where PHB_{efst} and PHB_{efam} are the PHB contents at the end of the feast and famine phases, and $\text{MLSS}_{1\text{st}}$ and $\text{MLSS}_{2\text{nd}}$ are the biomass concentrations of the first (post-feast) and second (post-famine) discharges.

Specific rates were normalized to active biomass, expressed on a carbon-mole (Cmol) basis assuming a biomass formula of CH_{1.8}O_{0.5}N_{0.2} (24.6 g/Cmol). The active biomass concentration, X_r , is the reactor MLSS corrected for its PHB content. The specific acetate uptake rate (q_{Ac}) and specific PHB storage rate (q_{PHB}) in the feast phase were calculated from eq 4 and eq 5, with acetate (C₂H₄O₂, 30.0 g/Cmol) and PHB (C₄H₆O₂, 21.5 g/Cmol) converted to a Cmol basis [23].

$$q_{\text{Ac}}(\text{Cmol} / \text{Cmol} \cdot \text{h}) = \frac{\Delta \text{Acetate}(\text{g/L}) * 24.6}{X_r(\text{g/L}) \times \text{Feastlength(h)} \times 30.0} \quad (4)$$

$$q_{\text{PHB}}(\text{Cmol} / \text{Cmol} \cdot \text{h}) = \frac{\Delta \text{PHB}_{\text{fst}}(\text{g/L}) \times 24.6}{X_r(\text{g/L}) \times \text{Feastlength(h)} \times 21.5} \quad (5)$$

Where $\Delta \text{Acetate}$ is the acetate consumed and $\Delta \text{PHB}_{\text{fst}}$ is the PHB stored during the feast phase. The feast storage yield reported in the text is the ratio $q_{\text{PHB}}/q_{\text{Ac}}$.

The specific ammonium and phosphate uptake rates were calculated from eq 6 and eq 7. Ammonium and phosphate were measured and are expressed on an elemental basis (NH₄⁺-N, 14.0 g/mol; PO₄³⁻-P, 30.97 g/mol).

$$q_{\text{NH4}}(\text{mmol N} / \text{Cmol} \cdot \text{h}) = \frac{\Delta \text{NH}_4^+ - \text{N}(\text{g/L}) \times 24.6 \times 1000}{X_r(\text{g/L}) \times \Delta t(\text{h}) \times 14.0} \quad (6)$$

$$q_{\text{PO4}}(\text{mmol P} / \text{Cmol} \cdot \text{h}) = \frac{\Delta \text{PO}_4^{3-} - \text{P}(\text{g/L}) \times 24.6 \times 1000}{X_r(\text{g/L}) \times \Delta t(\text{h}) \times 30.97} \quad (7)$$

Where $\Delta \text{NH}_4^+ - \text{N}$ and $\Delta \text{PO}_4^{3-} - \text{P}$ are the changes in ammonium and phosphate concentration over the interval Δt between two measured points. Because nutrients were fed in both phases, the ammonium and

phosphate uptake rates were calculated separately for the feast and famine phases.

The observed PHB yield on acetate ($Y_{\text{PHB}/\text{Ac}}$) was calculated for each phase from the harvested PHB and the acetate supplied (eq 8, eq 9), with PHB and acetate on a Cmol basis as above.

$$Y_{\text{PHB}} / \text{Ac, fam} (\text{PHB Cmol} / \text{Acetate Cmol}) = \frac{\text{PHB}_{\text{efst}} (\text{wt}\%) \times \text{MLSS}_{1\text{st}} (\text{g/L}) \times V_{\text{discharge}} (\text{L}) \times 30.0}{\text{Acetate}_{\text{initial}} (\text{g/L}) \times V_{\text{reactor}} \times 21.5 \times 100} \quad (8)$$

$$Y_{\text{PHB}} / \text{Ac, fst} (\text{PHB Cmol} / \text{Acetate Cmol}) = \frac{\text{PHB}_{\text{efst}} (\text{wt}\%) \times \text{MLSS}_{2\text{nd}} (\text{g/L}) \times V_{\text{discharge}} (\text{L}) \times 30.0}{\text{Acetate}_{\text{initial}} (\text{g/L}) \times V_{\text{reactor}} \times 21.5 \times 100} \quad (9)$$

Where V_{reactor} is the working volume, $V_{\text{discharge}}$ is the discharge volume (0.5 L in both phases), and $\text{Acetate}_{\text{initial}}$ is the initial acetate concentration in the reactor.

3. Results and discussion

3.1. Nutrient decoupling and feed concentration regulate feast length, biomass retention, and PHB accumulation

Feast length was a key operational variable in this system because the fixed feast-to-famine (F/F) ratio of 0.20 linked feast duration to famine duration, total cycle time, and consequently the OLR and SRT. Changes in feast length were therefore evaluated at steady state under each nutrient concentration and decoupling strategy (Table 1, Fig. 2).

Feast length decreased progressively as the feed COD:N ratio changed across the three stages, from an average of 4.5 h in Stage 1–2.6 h in Stage 2 and 1.3 h in Stage 3 (Table 2). The largest reduction occurred between Stages 1 and 2, when the change in feed ratio from 100:1.45:0.27–100:1.81:0.20 shortened the feast phase by 1.4–2.7 h across the reactors. The further increase in nutrient concentration in Stage 3 resulted in smaller reductions of 0.5–1.6 h. This diminishing response suggests that feast length was particularly sensitive to the COD ratio under strongly nitrogen-limited conditions. Similarly previous study reported a relatively stable feast fraction at COD:N ratios of

100:12:0.6 and 100:4.7:0.6, followed by a pronounced increase at 100:3.0:0.3, indicating a threshold below which nitrogen limitation substantially prolongs the feast phase [8]. Because ammonium concentration increased by 25% whereas phosphate concentration decreased by 25% between Stages 1 and 2, the observed shortening of the feast phase was attributed primarily to increased nitrogen availability.

The timing of ammonium supply had a stronger influence on feast length than its total dose. When ammonium was available during the feast phase (R1 and R3), feast length was 23–69% shorter than in reactors where ammonium was withheld until the famine phase (R2 and R4) (Table 1). R1, which received both nitrogen and phosphorus during the feast phase, and R3, which received nitrogen but not phosphorus, exhibited similar feast lengths. This indicates that ammonium availability, rather than phosphate availability, primarily governed feast duration. This pattern is consistent with the prolonged feast phases previously observed under ammonium-limited but phosphate-replete conditions [24].

In contrast, phosphate availability during the feast phase primarily affected the time required to reach steady state. R3 and R4, in which phosphate was supplied during the famine phase and only residual phosphate remained available in the subsequent feast, required 131–141 days to stabilize, compared with 56–91 days for R1 and R2, where phosphate was readily available during the feast phase (Table 2). A similar delay under phosphate limitation was reported [9], where biomass required approximately 50 days to stabilize under phosphate-limited conditions compared with 20 days under phosphate-sufficient conditions. The more rapid stabilization achieved when phosphate was available during the feast phase may be particularly advantageous for practical operation with variable real feedstocks, where rapid microbial adaptation to changes in substrate composition is desirable.

The shortening of the feast phase with increasing feed nutrient concentration also altered OLR and SRT through its effect on total cycle length. The average OLR across the four reactors increased from 1.71 to 3.28 and 5.47 g COD/L•d from Stages 1–3, whereas SRT decreased by 1.2–2.1 d between Stages 1 and 2 and by a further 0.4–1.3 d between Stages 2 and 3. Ammonium-decoupled reactors (R2 and R4) consistently exhibited lower OLRs and longer SRTs than reactors receiving ammonium during the feast phase (R1 and R3), directly reflecting their longer

Table 2
Effect of feast length on OLR, MLSS, SRT, F/M Ratio, and PHB content at end feast and famine phase.

Stage	Reactor	Feast length	OLR	MLVSS _{efst}	MLSS _{efam}	SRT	F/M ratio	PHB _{efst} content	PHB _{efam} content
Unit		hour	gCOD/L•d	g/L	g/L	day	gCOD/gMLVSS•day	wt%	wt%
1	R1	3.77	1.93	1.86	1.26	3.11	1.04	32.2	20.4
		±0.2						±0.0	± 0.3
	R2	5.85	1.24	1.78	0.95	4.82	0.84	46.5	2.3
		±0.1						±1.0	± 0.2
	R3	3.27	2.23	1.47	0.75	2.69	1.82	49.0	33.8
		±0.1						±0.6	±0.5
	R4	5.00	1.45	1.34	0.94	4.13	1.30	29.7	2.9
		±0.3						±1.3	±0.1
2	R1	1.87	3.90	1.84	1.24	1.54	2.12	32.7	15.3
		±0.3						±1.1	±0.1
	R2	3.17	2.29	1.40	0.78	2.62	1.96	44.2	3.6
		±0.2						±0.1	± 0.3
	R3	1.83	3.97	1.06	0.55	1.51	4.49	48.5	10.2
		±0.2						±1.0	±0.2
	R4	2.45	2.97	1.29	0.67	2.02	2.77	48.3	4.4
		±0.1						±0.1	±0.5
3	R1	1.08	6.73	2.20	1.95	0.89	3.06	15.5	3.2
		±0.3						±1.2	±0.5
	R2	1.56	4.65	1.68	1.23	1.29	3.32	27.0	5.5
		±0.4						±0.5	±0.1
	R3	1.08	6.73	1.44	1.15	0.89	5.60	20.4	11.3
		±0.5						±0.7	±0.1
	R4	1.93	3.77	1.07	0.65	1.59	4.20	39.2	3.1
		±0.1						±0.1	±0.2

feast phases and cycle durations.

Biomass concentration was influenced by both feed nutrient concentration and nutrient-decoupling strategy. Simultaneous provision of carbon and nutrients favored biomass formation, with R1 maintaining the highest mixed liquor suspended solids (MLSS) concentration at all stages, whereas R4, which received neither nitrogen nor phosphorus during the feast phase, maintained the lowest (Table 2). Between Stages 2 and 3, biomass concentrations increased in R1, R2, and R3 as nutrient availability increased, whereas the PHB-containing biomass concentration in R4 decreased despite the higher nutrient dose. This suggests that biomass formation supported by mobilization of previously stored PHB was less efficient than direct growth on acetate when nutrients were simultaneously available [25]. Across the reactors, feast-phase phosphate availability was associated with higher biomass concentrations than feast-phase ammonium availability at an equivalent total nutrient dose. Furthermore, the overall decline in biomass from Stage 1 to Stage 2, despite increased ammonium but reduced phosphate in the feed, indicates that phosphate exerted the stronger constraint on biomass growth under these conditions.

Nutrient decoupling also increased PHB content at the end of the feast phase (PHB_{efst}), although the magnitude of this effect depended on feed nutrient concentration. R1, in which neither nitrogen nor phosphorus became strongly limiting during the feast phase, consistently exhibited the lowest PHB_{efst} . In contrast, ammonium decoupling in R2 and phosphate decoupling in R3 produced similar PHB_{efst} values of 44–49 wt%, demonstrating that limitation of either nutrient during carbon uptake promoted intracellular storage rather than direct biomass growth. Increasing the feed nutrient concentration reduced the average PHB_{efst} across the four reactors from 43.4 ± 3.7 wt% in Stage 2– 24.5 ± 5.8 wt% in Stage 3. Nevertheless, R4, in which both nitrogen and phosphorus were withheld during the feast phase, maintained a PHB_{efst} of 39.2 wt% in Stage 3, substantially above the stage average. Thus, complete nutrient decoupling preserved strong PHB storage even as overall nutrient availability increased.

PHB content at the end of the famine phase (PHB_{efam}) was instead closely associated with the timing of ammonium supply. In Stage 1, R2 and R4, which received ammonium during the famine phase, reduced PHB_{efam} to 2.3–2.9 wt%, whereas R1 and R3 retained substantially higher concentrations of 20.4 and 33.8 wt%, respectively. This difference reflects the role of nitrogen availability in enabling anabolic

growth during the famine phase. Stored PHB can be mobilized to acetyl-CoA and subsequently oxidized through central metabolism to provide carbon and energy for biomass synthesis [26]. When ammonium was supplied during the famine phase, this stored carbon could therefore be efficiently consumed for growth, whereas the absence of ammonium restricted anabolic demand and promoted PHB carryover into the subsequent cycle. The consistently higher PHB-free biomass concentration in R2 than in R3 across all stages further supports the importance of famine-phase nitrogen availability for converting stored PHB into new biomass.

3.2. Acetate uptake kinetics reveal a trade-off between PHB productivity and yield

Cycle analyses were conducted after each reactor had reached stable feast length, MLSS concentration, and PHB content, at approximately Days 100, 250, and 400 for Stages 1, 2, and 3, respectively. The resulting specific substrate uptake and PHB storage rates and stoichiometric yields are summarized in Table 3. The corresponding cycle profiles of acetate, PHB, ammonium, phosphate, and dissolved oxygen are shown in Fig. S1–S3. All carbon-based rates and yields are expressed on a Cmol basis, whereas ammonium and phosphate uptake rates are expressed on an elemental N and P basis, respectively.

The specific acetate uptake rate (q_{Ac}) increased with both feed nutrient concentration and nutrient availability during the feast phase, ranging from 0.11 to 0.25 Cmol/Cmol $\cdot$ h in Stage 1–0.47–0.71 Cmol/Cmol $\cdot$ h in Stage 3. The feast-phase specific PHB storage rate (q_{PHB}) increased concurrently, from 0.04 to 0.08 Cmol/Cmol $\cdot$ h in Stage 1–0.10–0.21 Cmol/Cmol $\cdot$ h in Stage 3, with the highest rates generally observed in the ammonium-decoupled R2 and fully decoupled R4. The feast storage yield, defined as $q_{\text{PHB}}/q_{\text{Ac}}$, ranged from 0.15 to 0.63 Cmol/Cmol, consistent with values reported for acetate-fed mixed microbial cultures and remaining below the theoretical maximum of approximately 0.6–0.67 Cmol/Cmol. The highest storage yields were obtained in R2 (0.56–0.63) and R4 (0.37–0.41), whereas R1 and R3 exhibited lower values. These results indicate that withholding ammonium during the feast phase directed a greater fraction of assimilated acetate toward PHB storage than withholding phosphate alone.

Despite the parallel increase in q_{Ac} and q_{PHB} , volumetric PHB productivity and PHB yield showed contrasting relationships with

Table 3
Kinetic and stoichiometric parameters of the enriched biomass in the four reactors (R1–R4) across the three operation stages.

Stages	Stage 1				Stage 2				Stage 3			
Reactor	R1	R2	R3	R4	R1	R2	R3	R4	R1	R2	R3	R4
Strategy	Control	N-dec	P-dec	NP-dec	Control	N-dec	P-dec	NP-dec	Control	N-dec	P-dec	NP-dec
MLSS productivity (mgMLSS/L $\cdot$ h)	53.1	30.5	53.5	28.5	109.6	44.2	39.2	57.9	212.9	129.3	158.6	63.5
$r_{\text{PHB}_{\text{fst}}}$ (mgPHB/L $\cdot$ h)	8.66	5.20	11.9	3.66	13.0	6.76	7.88	10.8	15.8	15.9	13.2	9.93
$r_{\text{PHB}_{\text{fam}}}$ (mgPHB/L $\cdot$ h)	N/A ^a	0.09	1.51	0.21	N/A ^a	0.13	0.96	0.21	N/A ^a	0.59	1.36	0.17
q_{Acetate} (Cmol/Cmol $\cdot$ h)	0.16	0.11	0.25	0.19	0.31	0.21	0.31	0.32	0.48	0.47	0.79	0.57
$q_{\text{PHB}_{\text{deg}}}$ (Cmol/Cmol $\cdot$ h)	0.04	0.07	0.08	0.05	0.05	0.11	0.08	0.20	0.11	0.20	0.12	0.22
$q_{\text{NH}_4\text{fst}}$ (Nmmol/Cmol $\cdot$ h)	3.63	N/A ^b	6.90	N/A ^b	6.54	N/A ^b	16.7	3.23	8.89	19.3	16.4	14.6
$q_{\text{PO}_4\text{feast}}$ (Pmmol/Cmol $\cdot$ h)	0.03	0.01	0.18	0.01	0.06	0.10	0.26	0.23	0.16	0.55	0.69	0.19
$q_{\text{NH}_4\text{fam}}$ (Nmmol/Cmol $\cdot$ h)	N/A ^b	1.11	N/A ^b	3.76	0.27	1.18	N/A ^b	1.54	1.13	1.54	4.83	2.90
$q_{\text{PO}_4\text{fam}}$ (Pmmol/Cmol $\cdot$ h)	0.01	0.01	0.02	0.02	0.02	N/A ^b	0.01	0.03	0.03	N/A ^b	0.06	0.06
YPHB/Acetate (PHBCmol/AcCmol)	0.20	0.19	0.25	0.11	0.16	0.16	0.18	0.18	0.10	0.14	0.09	0.11

^a No sludge discharge or feeding in the famine phase of the control reactor (R1).

^b Nutrient not supplied in that phase (decoupled); rate not defined.

acetate uptake kinetics. Across the twelve steady-state conditions, PHB productivity increased with q_{Ac} ($R^2 = 0.36$), whereas PHB yield decreased as q_{Ac} increased ($R^2 = 0.46$) (Fig. 3). The positive relationship with productivity can be explained by two effects. First, because the F/F ratio was fixed at 0.20, faster acetate uptake shortened the feast phase and consequently the total cycle duration, allowing more cycles and therefore more PHB production per unit time [27]. Second, increasing feed nutrient concentration generally increased biomass concentration, thereby increasing the amount of PHB that could be stored volumetrically even when the fraction of acetate converted to PHB declined.

The negative relationship between q_{Ac} and PHB yield indicates a progressive loss of storage selectivity at higher uptake rates. Under conditions with greater feast-phase nutrient availability, rapid acetate uptake occurred while sufficient nitrogen and/or phosphorus remained available to support direct biomass synthesis. A larger fraction of the assimilated carbon was therefore directed toward growth and associated energy metabolism rather than intracellular PHB storage [25,28]. In contrast, when ammonium was withheld during the feast phase, carbon uptake occurred under a stronger growth limitation, and a greater proportion of acetate was channeled into PHB. This distinction is also reflected in the MLSS productivity of the control R1, which reached 213 mg MLSS/L·h in Stage 3, consistent with simultaneous carbon and nutrient availability favoring biomass formation rather than storage.

Previous studies have reported different relationships between acetate uptake kinetics and PHB performance depending on the limiting nutrient and the severity of nutrient limitation. Under phosphorus limitation, faster acetate uptake was associated with increases in both PHB yield and content [16], whereas under moderate nitrogen limitation, an increase in storage yield was accompanied by a decrease in PHB content [8]. In a nitrogen-decoupled system operated at different OLRs, a slight increase in PHB content was reported without a corresponding improvement in yield [15]. In addition, the reduced PHB yield and productivity at low substrate uptake rates were attributed partly to operation at an F/F ratio above 0.25 [5]. In the present study, maintaining the F/F ratio at 0.20 removed variation in feast fraction as a major operational confounder and revealed a consistent trade-off across the tested conditions: faster acetate uptake increased volumetric PHB productivity but reduced the fraction of substrate carbon recovered as PHB.

3.3. Nutrient decoupling redirects carbon between PHB storage, biomass growth, and respiration

Partitioning feast-phase acetate carbon among PHB storage, biomass formation, and respiration explains why reactors with similar end-of-feast PHB contents (PHB_{efst}) reached those levels through different metabolic routes (Fig. 4). This partitioning follows the feast–famine

metabolic framework for mixed cultures, in which acetate assimilated during the feast phase is simultaneously routed to PHB storage, biomass growth, and energy generation, with the balance among these routes shifting according to the degree of nutrient limitation [29]. Ammonium decoupling produced the strongest diversion of acetate carbon toward PHB storage. In R2, 56–63% of feast-phase acetate carbon was stored as PHB, while only a negligible fraction was incorporated into biomass. In contrast, the control R1 stored only 18–23% of acetate carbon as PHB and allocated a comparable fraction, 18–24%, to biomass formation, with the remaining carbon predominantly respired.

The phosphate-decoupled R3 displayed a distinctly different pattern. Only 15–25% of feast-phase acetate carbon was converted to PHB, similar to or even lower than the fraction observed in R1, despite R3 exhibiting one of the highest PHB_{efst} values. This apparent discrepancy indicates that PHB content alone does not represent the amount of PHB newly stored within a cycle. In R3, ammonium was available during the feast phase but absent during the famine phase, allowing direct growth during acetate uptake while restricting subsequent utilization of stored PHB for biomass formation. Consequently, residual PHB was carried over between cycles and contributed to the high PHB_{efst} measured in the following feast phase. R2, by contrast, achieved a similarly high PHB_{efst} primarily through direct feast-phase storage under ammonium limitation. Carbon partitioning therefore distinguishes true within-cycle storage from accumulation caused by incomplete PHB consumption between cycles and demonstrates that feast-phase ammonium limitation was more effective than phosphate limitation in directing newly assimilated acetate toward PHB. The different responses to nitrogen and phosphate limitation likely reflect their distinct metabolic roles. Nitrogen limitation directly restricts protein synthesis and biomass growth, causing acetate-derived acetyl-CoA and reducing equivalents to be redirected toward PHB synthesis as a carbon and electron sink [25,28]. Phosphate limitation acts more indirectly through ATP, nucleic acid, and phospholipid synthesis, and may be buffered by intracellular polyphosphate reserves [30]. The availability of ammonium during the feast phase in R3 allowed growth to proceed in parallel with acetate uptake, thereby reducing the immediate diversion of carbon toward PHB storage.

Famine-phase carbon partitioning was similarly governed by ammonium availability. In R1 and R3, where ammonium was absent during the famine phase, the relatively small fraction of PHB that was degraded was used predominantly for respiration, with negligible biomass formation. This limited anabolic utilization allowed a substantial fraction of stored PHB to remain and be carried into the subsequent cycle. In contrast, in the ammonium-decoupled R2 and R4, 31–81% of the carbon released from PHB degradation was incorporated into biomass. Thus, supplying ammonium during the famine phase enabled stored PHB to support biomass synthesis rather than being retained across cycles.

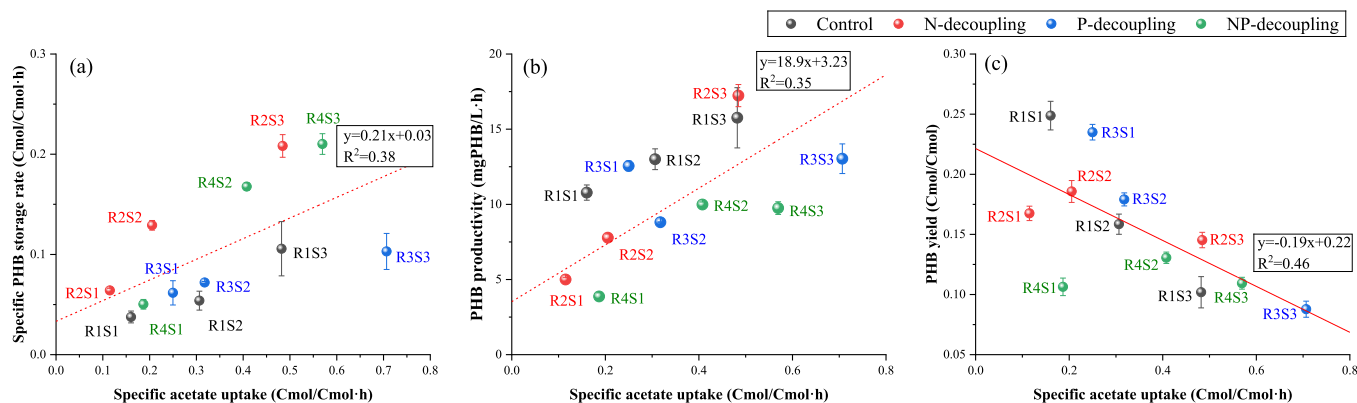


Fig. 3. Relationships between the specific acetate uptake rate and (a) specific PHB storage rate, (b) PHB productivity, and (c) PHB yield in the four reactors across three operational stages (RxSy: reactor x, stage y; R1, control; R2, N-decoupling; R3, P-decoupling; R4, NP-decoupling).

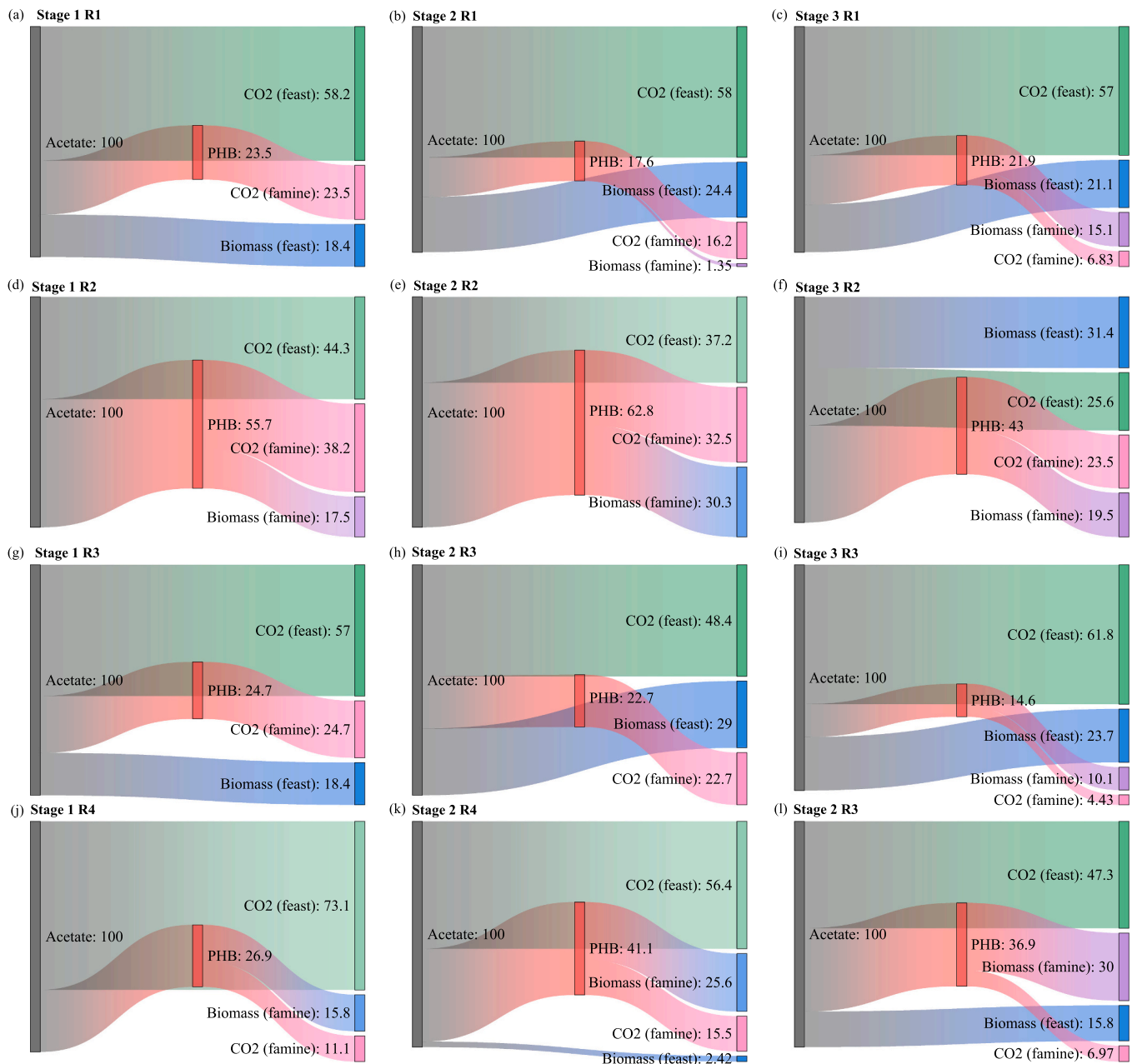


Fig. 4. Carbon partitioning into PHB storage, biomass growth, and respiration during feast and famine phases in the four reactors across three operational stages (a–l). R1, control; R2, N-decoupling; R3, P-decoupling; R4, NP-decoupling.

Taken together, the carbon balance shows that the timing of ammonium supply controlled both stages of the PHB cycle. Withholding ammonium during the feast phase promoted the conversion of incoming acetate to PHB, whereas supplying ammonium during the famine phase promoted the conversion of stored PHB to biomass. This dual control reflects the dependence of PHB-driven growth on nitrogen availability. In the absence of ammonium, stored PHB may be oxidized for maintenance, but its conversion into new biomass is constrained by limited nitrogen assimilation and biosynthetic cofactor supply [26]. Ammonium phase allocation therefore emerged as the dominant operational lever governing carbon partitioning among storage, growth, and respiration in this system.

3.4. Nutrient-decoupled enrichment shapes fed-batch PHB storage capacity

The maximum PHB storage capacity of each enriched community was evaluated in fed-batch accumulation tests using both synthetic medium and fermented waste activated sludge (WAS). All values represent the means of three analytical replicates (Fig. 5).

The initial PHB content, measured after overnight aeration to minimize residual intracellular polymer, decreased with increasing feed nitrogen concentration across the stages, from 6.7 wt% in Stage 1–4.8 wt% in Stage 2 and 4.0 wt% in Stage 3 for the synthetic-medium tests. No consistent relationship was observed with phosphorus concentration.

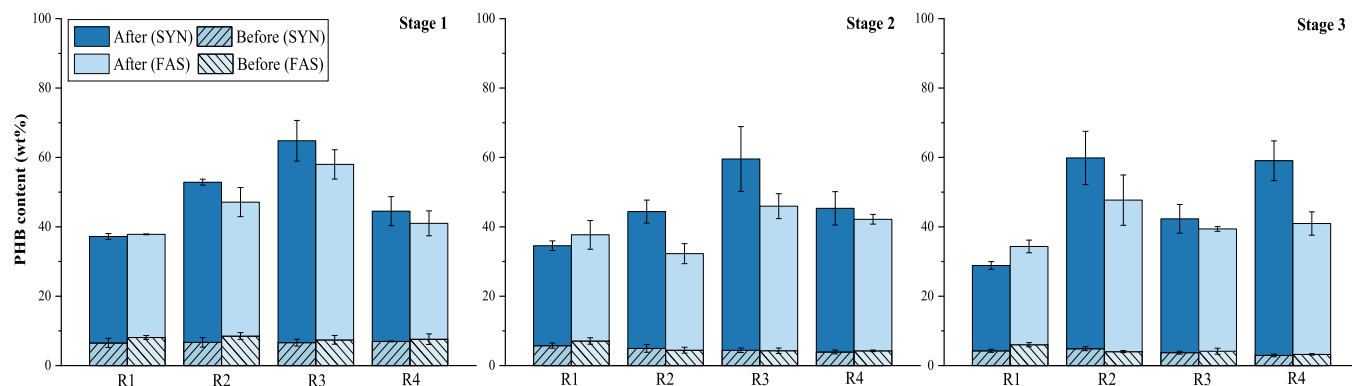


Fig. 5. PHB content before and after fed-batch accumulation using synthetic wastewater (SYN) and fermented waste activated sludge (FAS) in the four reactors across three operational stages. R1, control; R2, N-decoupling; R3, P-decoupling; R4, NP-decoupling.

The lower initial PHB content at higher nitrogen availability is consistent with more extensive mobilization of stored PHB during the famine phase when nitrogen limitation was alleviated.

Communities enriched under feast-phase nutrient limitation generally exhibited the greatest PHB storage capacity. On synthetic medium, R3 reached the highest PHB contents in Stages 1 and 2, at 58.2 ± 5.9 and 55.1 ± 9.3 wt%, respectively, exceeding the other reactors by 17–38%. R3 experienced phosphate limitation during the feast phase together with ammonium limitation during the famine phase, indicating that this enrichment regime selected for a community with a high subsequent storage capacity. In Stage 3, the ammonium-decoupled R2 and fully decoupled R4 reached the highest PHB contents, at 55.0 ± 7.7 and 56.1 ± 5.7 wt%, respectively. The strong performance of the ammonium-decoupled communities is consistent with previous reports that feast-phase ammonium limitation enriches microorganisms with high PHA storage capacity [5,31]. The similarly high capacity of R3 further indicates that phosphate decoupling can also impose an effective selection pressure for PHB-storing communities, an aspect that has received comparatively little attention.

Accumulation on fermented WAS generally resulted in lower PHB contents than accumulation on synthetic medium, consistent with previous comparisons between synthetic substrates and fermented waste streams [32,33]. The largest decrease was observed for R2 in Stage 2, where PHB content declined from 39.4 wt% on synthetic medium to 27.8 wt% on fermented WAS. This loss of performance was likely related to residual ammonium in the fermented WAS. Because only phosphate was removed before the accumulation test, R2, which had been enriched under an ammonium-free feast phase, was exposed to ammonium during fed-batch accumulation. The resulting change in nutrient regime reduced the selective advantage for storage that characterized its enrichment condition. This result highlights the importance of matching the nutrient environment during accumulation to that under which the biomass was enriched.

Nevertheless, communities exhibiting high storage capacity on synthetic medium generally retained comparatively high storage capacity on fermented WAS. R3, for example, showed the highest PHB accumulation under both substrates, suggesting that the storage phenotype developed during enrichment remained at least partly transferable to a more complex waste-derived feedstock [34]. Small amounts of poly (hydroxybutyrate-co-hydroxyvalerate) (PHBV) were also detected in R3 and R4 during accumulation on fermented WAS but not on the acetate-only synthetic medium. This is consistent with the presence of odd-carbon volatile fatty acids, particularly propionate and valerate, which together accounted for 38% of the WAS COD and can provide precursors for hydroxyvalerate formation [35]. Comparable PHBV formation, with hydroxyvalerate fractions tuned by the odd-carbon acid content of fermented residues, has recently been reported for

mixed-culture systems fed agri-food fermentates, where feedstock pre-treatment likewise governed storage performance [36]. Notably, PHBV formation occurred only in R3 and R4, the two communities enriched with restricted phosphate availability during the feast phase. Although this pattern suggests that feast-phase phosphate limitation may have favored organisms capable of incorporating odd-carbon substrates into PHBV, the available data are insufficient to establish a direct causal relationship, and no consistent association with the famine-phase nutrient regime was observed.

Phosphate decoupling appears particularly practical for PHB production from real waste streams. Fermented wastewater and WAS-derived feedstocks often retain substantial ammonium, which is difficult to remove selectively [33], whereas phosphate can be removed by simple precipitation [3]. A phosphate-limited feast phase can therefore be established more directly than a nitrogen-limited feast phase, which would generally require additional ammonium-removal pretreatment [37]. This advantage was reflected in the fed-batch tests, where the phosphate-decoupled culture (R3) showed the highest PHB storage capacity in Stages 1 and 2 with both synthetic medium and fermented WAS. The maximum contents obtained here (55–58 wt%) are within the range recently reported for uncoupled-feeding enrichments, where combining uncoupled feeding with microaerophilic selection reached up to 82 wt% [27], suggesting that oxygen management could further raise the storage capacity of nutrient-decoupled systems. The trade-off was slower reactor stabilization, as phosphate limitation extended the time required to reach steady state, consistent with previous observations under phosphate-limited enrichment [9]. Thus, phosphate dosing timing provides a practical control handle for balancing PHB storage capacity and start-up speed. Process control at larger scale could rely on the DO signal, which indicated acetate depletion in the SBRs and guided carbon addition during the fed-batch tests. This feed-on-demand strategy requires only standard DO instrumentation and is therefore readily scalable. Key remaining constraints include feedstock variability, nutrient imbalance requiring pretreatment, and downstream polymer recovery, consistent with recent pilot-scale assessments [37].

Overall, PHB storage capacity was shaped by both the accumulation substrate and the enrichment nutrient regime. Fermented WAS reduced PHB accumulation when its nutrient balance differed from the enrichment conditions, indicating that nutrient-to-carbon adjustment of waste-derived feedstocks is needed to fully exploit the enriched storage phenotype.

3.5. Nutrient decoupling shapes microbial community structure and PHB-associated phenotypes

Sequencing identified 324 genera, with the 20 most abundant genera accounting for approximately 86% of total reads on average. Changes in

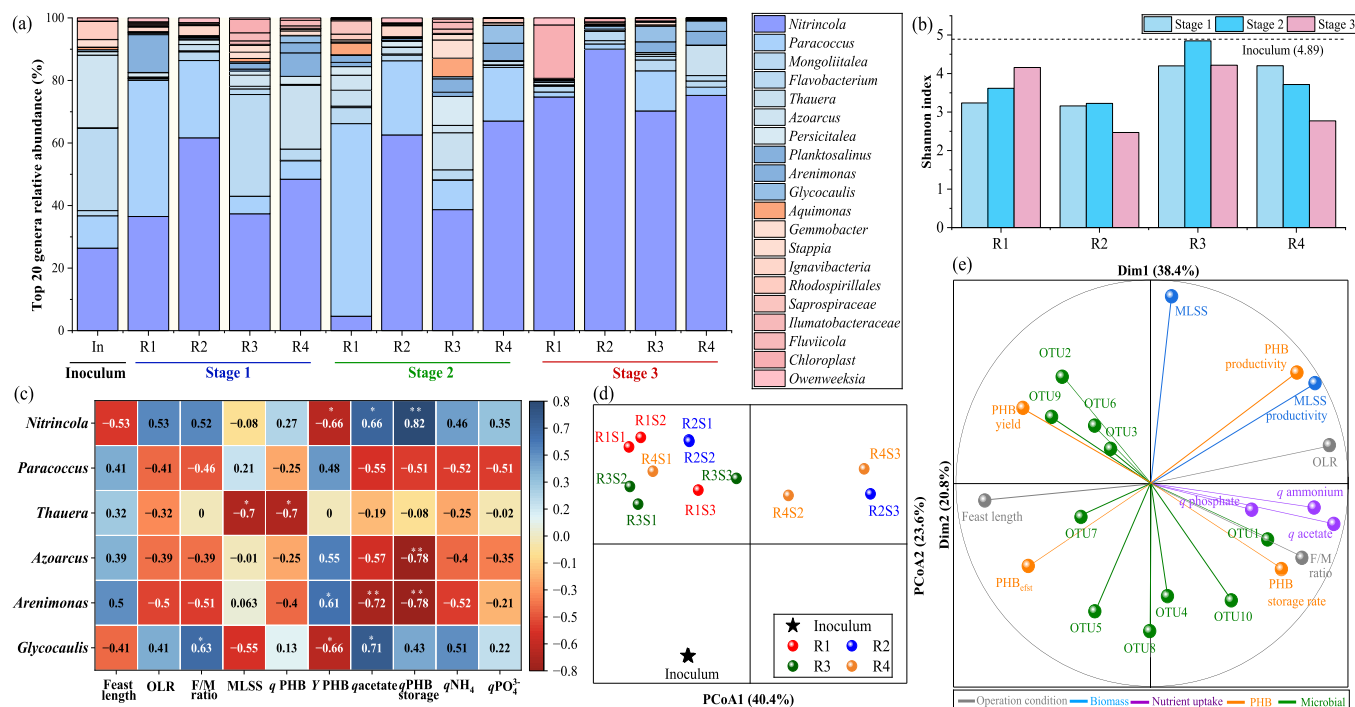


Fig. 6. Microbial community composition, diversity, and associations with operational parameters and PHB production: (a) relative abundance of the top 20 genera, (b) Shannon diversity index, (c) Spearman correlations between genera and process parameters, (d) Bray–Curtis principal coordinate analysis (PCoA), and (e) multiple factor analysis (MFA). R1, control; R2, N-decoupling; R3, P-decoupling; R4, NP-decoupling.

alpha diversity and community composition, together with associations between dominant genera and operational and PHB-production variables, are shown in Fig. 6.

The inoculum was more diverse than most enriched communities, with an ACE richness of 263 and a Shannon index of 4.9, and the subsequent decline in richness indicated selective enrichment under the imposed operating conditions. Because the inoculum itself originated from a PHB-enriched culture, the reduction would likely have been greater if conventional wastewater-treatment sludge had been used as the starting community. For comparison, Wang et al. (2016) reported an ACE of 1218 and a Shannon index of 5.37 for aerobic activated sludge, compared with 263 and 4.9, respectively, for the inoculum in WWTP [38]. Among the reactors, the phosphate-decoupled R3 exhibited the highest richness in Stages 1 and 2, with ACE values of 269 and 299. From Stage 1 to Stage 2, increasing nutrient availability increased richness in all reactors, although the increase was smallest in the ammonium-decoupled R2, suggesting that feast-phase nutrient decoupling maintained a strong selective pressure even as the total nutrient dose increased.

A different pattern emerged in Stage 3, where the further increase in nutrient concentration was accompanied by a pronounced decline in diversity. Relative to Stage 2, the mean ACE across the four reactors decreased by 42% and Faith's phylogenetic diversity by 33%, with the largest reductions observed in R4 (52% and 47%, respectively). This decline coincided with substantial shortening of the feast phase and a decrease in mean SRT from 1.9 to 1.2 d. Short SRTs can restrict the retention of slower-growing populations and reduce opportunities for interspecies interactions, thereby decreasing community diversity [39]. The observed richness patterns therefore appear to reflect the combined effects of feast-phase nutrient availability and biomass retention rather than nutrient concentration alone.

Bray–Curtis principal coordinate analysis further demonstrated that both enrichment and nutrient-placement strategy shaped community composition. The inoculum was clearly separated from all enriched communities, confirming substantial restructuring during reactor operation. Within the enriched communities, reactors tended to separate

according to the phase-specific availability of nitrogen and phosphorus. In Stage 1, communities receiving phosphate during the feast phase (R1 and R2) differed from those in which phosphate was withheld until the famine phase (R3 and R4), with R3 showing particularly distinct composition. As operation progressed, communities exposed to similar nutrient-placement regimes became more similar, whereas R1 in Stage 3, which experienced simultaneous feast-phase availability of both nitrogen and phosphorus, became increasingly distinct from the nutrient-decoupled reactors. These results extend previous observations that COD:N ratio, OLR, and pH influence PHA-enriching communities [39, 40] by showing that the timing of nutrient availability within the feast–famine cycle is also associated with community assembly.

Nitrincola became the dominant genus across the reactors after enrichment, increasing from an initial relative abundance of 27%. Its abundance was particularly high in R2 and R4, which received ammonium during the famine phase, compared with R1 and R3, where ammonium was unavailable during famine, especially in Stages 1 and 2. *Nitrincola* has been described as a halotolerant denitrifying genus whose abundance can respond to COD:N conditions [41], and PHA-related genes, including *phaC*- and *phaP*-like genes, have been reported in members of this genus [42]. Its enrichment in reactors exhibiting strong PHB-storage performance therefore suggests a potential contribution to the storage phenotype, although genus-level abundance alone cannot establish its functional role. *Paracoccus*, which represented approximately 10% of the inoculum, increased to 44–62% in R1 during Stages 1 and 2. A comparable enrichment of *Paracoccus*, accompanied by an increase in maximum PHA content to about 60 wt%, was reported for a mixed culture selected on complex saline wastewater [43], supporting its role as a storing organism under nutrient-replete feast conditions. This is consistent with the known ability of *Paracoccus* spp. to accumulate PHB even when nutrients remain available during carbon uptake [10]. Its abundance subsequently declined in Stage 3 as *Nitrincola* became more dominant. *Mongoliitalea* reached 33% relative abundance in R3 during Stage 1, coinciding with high PHB_{est}. Because this genus has rarely been associated with PHB accumulation, its possible contribution to the storage phenotype warrants further investigation.

The correlation analysis further linked community shifts with reactor performance and separated the dominant genera into two functional patterns (Fig. 6). *Nitrincola* was positively correlated with the specific acetate uptake rate ($\rho = 0.66$, $p < 0.05$) and showed the strongest positive correlation with the specific PHB storage rate ($\rho = 0.82$, $p < 0.01$), but was negatively correlated with PHB yield ($\rho = -0.66$, $p < 0.05$). This pattern places *Nitrincola* in a fast-uptake, high-storage-rate guild that supports volumetric productivity but lowers yield. *Glycocalyx* showed a similar pattern, with a positive correlation with acetate uptake ($\rho = 0.71$, $p < 0.05$). In contrast, *Arenimonas* was positively correlated with PHB yield ($\rho = 0.61$, $p < 0.05$) and negatively correlated with both acetate uptake ($\rho = -0.71$, $p < 0.01$) and PHB storage rate ($\rho = -0.78$, $p < 0.01$). *Azoarcus* followed the same direction, suggesting a slow-uptake, high-yield guild. *Thauera* was negatively correlated with biomass concentration and PHB productivity ($\rho = -0.70$, $p < 0.05$ for both).

These results indicate that the productivity–yield trade-off identified in Section 3.2 was associated not only with kinetic shifts but also with changes in community structure. Operating conditions that accelerated acetate uptake, including higher feed nutrient concentration and famine-phase ammonium supply, coincided with a gradual shift from the high-yield guild toward the high-rate guild, with corresponding changes in bulk kinetic parameters. The ability of *Nitrincola* to occupy this high-rate niche is consistent with *phaC1*- and *phaP*-like genes reported in its family [42] and with its enrichment at elevated COD:N ratios [44]. Under the fixed F/F ratio, feast length, OLR, and acetate uptake rate were mutually coupled, while biomass concentration and volumetric PHB productivity were also operationally linked. Therefore, these correlations should be interpreted as associations rather than independent causal effects, and they do not by themselves resolve whether the community shifts were the cause or the consequence of the corresponding kinetic conditions. Nevertheless, they suggest that nutrient-decoupling strategy selects microbial communities with different kinetic traits, which in turn affects the achievable balance between PHB productivity and yield.

4. Conclusion

This study clarified how nutrient decoupling strategy and feed concentration govern PHB production in single-stage feast–famine SBRs operated at a fixed F/F ratio. Ammonium availability primarily controlled feast length and, when supplied during the famine phase, supported biomass growth using stored PHB. In contrast, feast-phase phosphate availability shortened the time required to reach steady state and promoted higher biomass accumulation. Carbon balance analysis distinguished active feast-phase PHB storage under ammonium limitation from cross-cycle PHB carry-over under phosphate limitation, demonstrating that feast-phase nitrogen limitation directed the largest fraction of acetate carbon toward PHB even when similar PHB contents were ultimately achieved. PHB productivity increased with the specific acetate uptake rate, whereas PHB yield decreased, revealing a clear productivity–yield trade-off that was accompanied by shifts in microbial community composition. *Nitrincola* was associated with high acetate uptake and PHB storage rates, while other genera showed distinct relationships with yield and nutrient conditions. Cultures enriched under nutrient-limited feast conditions also exhibited the highest fed-batch PHB storage capacity and largely retained this capacity when fermented WAS was used as the substrate, with combined nutrient limitation additionally promoting PHBV formation. Overall, these findings show that the timing and type of nutrient limitation regulate carbon allocation, biomass growth, and microbial selection in different ways, providing a mechanistic basis for selecting nutrient-decoupling strategies for wastewater-based PHB production. Further work should examine feast-phase phosphate limitation in greater detail and investigate the PHB-storage potential of less-studied genera such as *Mongoliitalea*.

CRedit authorship contribution statement

Seongbong Heo: Writing – review & editing, Writing – original draft, Validation, Data curation, Conceptualization. **Yongqiang Liu:** Supervision, Conceptualization. **Young Mo Kim:** Writing – review & editing, Validation.

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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.dwt.2026.101946.

Data Availability

Data will be made available on request.

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