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Principles for stimulating concurrent selective microbial growth and polyhydroxyalkanoate (PHA) storage responses for enhanced PHA productivity

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ABSTRACT

Municipal wastewater treatment plants are a ubiquitous source of microbial biomass for PHA production. Technological feasibility of directly using municipal activated sludge (WAS) for a PHA accumulation bioprocess is demonstrated in the literature. However, PHA contents and yields may be lower due to a coexistence of PHA-storing and non-PHA-storing microorganisms in WAS. This work focused on metabolic principles for stimulating selective growth of PHA-storing microorganisms during a PHA accumulation bioprocess to enhance PHA productivity. Two model substrates, butyrate and acetate, were used to evaluate conditions and principles that may regulate this selective growth response. Conditions promoting selective growth of the PHA storers were consistently observed in the fed-batch PHA production process fed with butyrate. Productivity was increased to 4 times more PHA produced over 48 h, wherein PHA contents and average yields were improved from 0.39 gPHA/gVSS and 0.25 $gCOD_{PHA}$/gCOD to 0.61 gPHA/gVSS and 0.47 $gCOD_{PHA}$/gCOD, respectively. Respiration monitoring and mass balances, with metabolic modelling, suggest that expected underlying differences in ATP yields between two tested substrates are the main mechanistic drivers of the observed selective growth. This study proposed that if conditions are created such that ATP is produced in sufficient excess of the demands for PHA storage and cell maintenance, then those PHA storers will further grow concurrently. The selective microbial growth allows extra conversion of substrates into PHA. These principles, concerning the metabolic basis of the PHA production pathway, provide a foundation that can be applied to a range of substrates or substrate mixtures for enhanced PHA accumulation.

1. Introduction

Polyhydroxyalkanoates (PHAs), a class of naturally occurring polyesters, are recognized as alternative biobased biodegradable engineering materials with thermoplastic-like properties [1–3]. PHAs are anticipated with broad potential for engineering into applications that are typically facilitated by fossil plastics today [4]. A challenge is to establish economically viable and environmentally sustainable supply chains of PHAs for the plastics and chemical industries. Consequently, much research effort has been focused on production methods to increase the technology readiness, from laboratory to demonstration scales [5–9]. One route is in the use of municipal and/or industrial wastes and residual streams for production feedstocks [10–13]. Effective PHA production methods include open bioprocesses that inherently select for, and/or exploit the activity of, naturally occurring PHA producing bacteria. Advancing a broadly applicable microbial community-based PHA production approach is the focus for the present investigation [14,15]. Broadly applicable methods can help to facilitate development and growth of large scale PHA supply chain networks that can satisfy market needs.

Direct accumulation is one of the open mixed microbial culture (MMC) approaches to produce PHA [16]. This approach considers that

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significant amounts of bacterial biomass are already being produced by municipalities and industries worldwide as an integral part of biological wastewater treatment processes used for environmental protection. This biomass (activated sludge) often already contains many species of microorganisms that store PHA. It has been reported that a PHA storage response may enhance the robustness of microbial survival to environmental stresses in dynamic environments, such as shifts in temperature, oxygen levels, or exogenous substrate availability [17,18]. In direct accumulation, surplus (waste) activated sludge is transferred directly to a 24–48 h fed-batch bioprocess, where it is supplied with fermented waste streams as a feedstock. This approach stimulates and exploits the extant PHA accumulation potential of the biomass to reach a maximum possible PHA content [19]. It has been observed that when activated sludge is disposed from conditions of endogenous respirations to a sudden substrate influx, the response will be dominated by metabolism for PHA production [20]. This initial response allows for the PHA-storing microorganisms in waste activated sludge (WAS) to selectively compete due to a relatively higher substrate uptake rate and the captured substrate as stored intracellular PHA granules [21]. Technological feasibility and robustness have been demonstrated over a decade of testing from pilot to demonstration scales for producing commercial quality PHAs by direct accumulation using municipal WAS and various volatile fatty acid rich feedstocks [22,23].

Developments to directly use municipal WAS in the supply chain for PHA production are ongoing [24,25]. Unlike the typical mixed microbial PHA production approach that is purposefully enriched with PHA-storing phenotypes [21], WAS presents a more complex and diverse biomass. The complexity is mainly due to a coexistence of PHA storing and flanking (i.e. non-PHA storing) microorganisms. One bottleneck is that the fraction of PHA-storing microorganisms in WAS varies between different activated sludge sources. A lower fraction of PHA-storers means a reduced capacity within the biomass for PHA storage, resulting in lower levels (0.2 to 0.4 gPHA/gVSS) of PHA produced during direct accumulation [20]. Furthermore, in open culture systems with diverse MMC like activated sludge, any general activity and growth of the flanking microorganisms will reduce PHA production yields. However, if PHA storing microorganisms were to grow competitively within an open mixed culture, while still maintaining a significant PHA storage response, then the PHA productivity of a fed-batch accumulation process would become enhanced. Enhancement will be due to the selective growth that increases the PHA-storing biomass fraction (enrichment) concurrently during a batch accumulation process. This selective growth leads to an extended capacity of production in the same batch time, and results in more biomass with stored PHA (higher productivity). The combined responses of PHA storage with growth require that the fed substrate must simultaneously support both PHA production activity alongside the selective microbial growth of the PHA-storing phenotype. To contrast, the classical enrichment–accumulation approach works by successive feast-famine cycles. Selective growth (enrichment) of the PHA-storing phenotype requires repeated consumption of stored intracellular PHA as the sole carbon source, after each successive cycle of feast, under famine conditions [26–28].

Observations of concurrent growth and storage are made in the research literature [29,30]. Studies indicate that essential nutrient loading (i.e. nitrogen and phosphorus) or presence of selected metal elements (e.g. iron and calcium) during PHA accumulation can be important to support selective growth of the PHA-storing microorganisms [31–34]. However, these findings were primarily based on stable enrichment cultures dominated by PHA storing phenotypes and have not been linked to underlying mechanisms. Thus, the metabolic basis defining the conditions necessary for concurrent selective growth of PHA-storing microorganisms during PHA accumulation in a range of MMCs, such as WAS, remains unexplored.

Estévez-Alonso et al. found that calcium addition together with acetate created conditions for reproducible selective growth of PHA-storing microorganisms [32]. From those experiments, it was proposed that calcium complexation with acetate could affect cellular energy requirements of PHA-storers, resulting in a reduced ATP demand for PHA production. The interpreted reduction in ATP demand coincided with the consistently observed concurrent (selective) growth of PHA-storing microorganisms, which gave marked improvements in PHA production performance. It was of interest to test these principles in general, and independent of the previous observations specifically associated with calcium and acetate.

Building on the previous study, It was hypothesized that lowered ATP demands for PHA synthesis could enable a surplus ATP production. This surplus ATP can facilitate selective growth alongside the concurrent ongoing PHA storage activity. If such ATP surplus conditions can be generally achieved with common substrates, substrate mixtures, or practical bioprocess operating conditions, then enhanced direct PHA accumulation with WAS can be widely exploited for industrial scale supply chains.

Substrate type can influence the ATP balances in a completely different way from the observations of Estévez-Alonso et al. due to the calcium addition. The major products from acidogenic fermentation were considered in this work. Pathways for PHA synthesis from different short-chain organic acids have been reported in the literature [35–37]. Using acetate as a reference substrate, many other commonly produced fermentation acids exhibit lower ATP requirements for PHA synthesis (Table 1). The conversion of acetate to PHA further requires NADH, whereas substrates like butyrate, valerate and lactate generate NADH during the process. NADH serves as a key electron carrier in cellular metabolism, and its oxidation can drive ATP production. The ATP generated from this NADH oxidation can supplement the ATP supply for PHA synthesis. The right substrate, or substrate mixtures, should therefore result in PHA synthesis pathways that can be energy-neutral or would even result in a net energy gain.

Acetate and butyrate are two dominant products from the primary fermentation of organic streams. Their relative composition is known to be influenced by fermentation process conditions (e.g., solids retention time, pH) [38–41]. In previous studies, butyrate was reported to be preferred over acetate for PHA production with high theoretical product yield (0.94 Cmol/Cmol) in stable enrichment cultures that are dominated by PHA-storing phenotypes [42]. However, the mechanisms driving this preference have not been explored with respect to NADH/ATP generation in the PHA production pathway. They were also not considered with respect to ATP balances and conditions of enabling concurrent selective growth of PHA-storing phenotype within a more complex and diverse MMC system like municipal WAS.

In the present work, butyrate versus acetate were therefore selected as model substrates for comparing respiration, mass balances, and outcomes of selective growth during a fed-batch direct accumulation process using municipal WAS. These two substrates exhibit distinct ATP yields in their PHA production pathways as shown in Table 1. These differences make them ideal substrates to compare and test these principles of ATP balances for stimulating concurrent selective growth in diverse MMCs. Butyrate conversion to PHA has reduced ATP demand and generates NADH due to its more reduced redox state. Thus, butyrate

Table 1
ATP requirements and net NADH production for the PHA synthesis pathway of four selected major products from acidogenic fermentation.

Substrate type	ATP requirements for PHA synthesis per Cmol substrate	Net NADH production per Cmol substrate in conversion pathway*	ATP produced from the NADH production
Acetate	1	−0.25	−0.5
Butyrate	0.5	0.25	0.5
Valerate	0.4	0.2	0.4
Lactate	2/3	0.5	1

* The efficiency of the oxidative phosphorylation was assumed to be 2. A negative value in this column means NADH is required in the PHA synthesis pathway.

was used as a positive experimental control substrate. Acetate was used as the negative control substrate. It has increased ATP and NADH demand in the PHA conversion pathway, serving as a benchmark for PHA accumulation performances. The input biomass for fed-batch accumulations was not explicitly enriched for PHA-storing microorganisms as is most typically undertaken for MMC PHA production [14]. Instead, the WAS was used directly. This experimental approach allowed for the study of concurrent microbial growth and for the selectivity of this growth within diverse WAS microbial communities.

The aim of this study was to investigate whether reduced ATP demand, combined with net NADH generation in the conversion of substrates to PHA, correlates with concurrent selective growth response of the PHA-storing fraction in WAS. Furthermore, the study sought to determine if such selective growth significantly enhances PHA productivity in a direct accumulation bioprocess. Outcomes of replicate butyrate direct accumulations were studied with respect to parallel accumulations performed with acetate. Two distinct sources of municipal WAS were evaluated with replicate experiments for relative effects of the substrates on process mass and ATP balances, microbial community developments, and the PHA accumulation performance indicators.

2. Material and methods

2.1. Experimental set-up

PHA accumulation tests were conducted over a 48-h period in double-jacketed glass fed-batch reactors with working volume of 1 L. Reactors were maintained with constant airflow (1 L/min, MV-302, Bronkhorst, Germany), temperature (25 ± 0.1 °C), and mixing (150 rpm with standard three-bladed turbine - R60, CAT Scientific, Germany). Dissolved oxygen (DO) and pH were logged (COS81D and CPS11D, Endress & Hausser, The Netherlands) at 0.1 Hz via a 4-channel transmitter (LiquilineCM444, Endress &Hausser, The Netherlands). Substrate was dosed by diaphragm pumps (Stepdos 10, KNF, The Netherlands) actuated by PLC (Logo! 8 and Logo! TDE, Siemens, Germany). Dose volumes were measured by logged steps of weight loss (0.1 Hz) of the stock substrate solution (PCE-BT 2000, PCE Instruments, The Netherlands).

2.2. Activated sludge sources and substrate

Municipal WAS came from two different types of Dutch WWTPs, Bath and Almere (Table 2). Two replicate batches of WAS were delivered, being thickened waste sludge samples (nominally 55 gTS/L) from Bath WWTP, and grab samples of mixed liquor from the aerobic stage from Almere WWTP (nominally 8 gTS/L after 30 min gravity settling and decanting). WAS grab samples were delivered directly on the same day by courier to Wetsus (Leeuwarden, the Netherlands) and stored refrigerated (4 °C) upon arrival for no more than 2 weeks pending accumulation experiments.

The accumulation feedstock was either 46.8 g/L acetic acid or 27.6 g/L butyric acid (Acetic acid glacial 100 % and butyric acid ≥99 %, Merck, Germany) to target 50 gCOD/L. The COD:N:P ratio (by weight) was trimmed to 100:1:0.05 by adding 1.91 g/L NH_4Cl and 110 mg/L KH_2PO_4. pH was brought to 4.5 with KOH addition.

Table 2
Details of selected municipal WWTPs supplying WAS grab samples.

WWTP	Capacity (kPE*)	Process	P-removal	Primary settling
Bath	536	AO	Chemical	Yes
Almere	329	A^2O	Biological	No

* kPE: 1000 person equivalents; AO: anaerobic-oxic; A^2O: anaerobic-anoxic-oxic.

2.3. Feed-on-demand PHA accumulation experiments

The experiments were conducted in fed-batch mode without any volume withdrawals except for sampling. An activated sludge solids grab sample was taken from the refrigerator and diluted with tap water to reach a MLVSS concentration of nominally 2–3 gVSS/L with added allylthiourea (50 mg/L). The mixed liquor that was then aerated overnight at 25 °C and established in this time a stable dissolved oxygen (DO) concentration indicating a steady baseline level of endogenous respiration. PHA production was conducted under fully aerobic conditions, resulting exclusively in polyhydroxybutyrate (PHB), from either acetate or butyrate as sole substrates [43].

A direct accumulation process started with a standardized conditioning step that has been referred to as an acclimation [44,45]. Conditioning in this context aimed to stimulate the physiological state of the activated sludge biomass and ensure that all biomass samples underwent the same conditions after cold storage and before initiating PHA accumulation. Notwithstanding, conditioning did not incur any significant amount of active growth (no enrichment) nor any significant net PHA accumulation due to relatively small amounts of substrate used and the timing of its addition. The conditioning process was an automated sequence of three pulse-feed inputs giving a maximum of 150 mgCOD/L per pulse under constant air flow rate for aeration. For each conditioning pulse, increase and decrease in respiration levels were monitored by changes in DO concentration to estimate the time for substrate uptake. The next conditioning pulse was given with a delay of 3 times the estimated substrate uptake time. The total conditioning time ranged from 5 to 6 h depending on the activated sludge source. After conditioning, accumulation was started by automatic control. A typical DO profile for the feed-on-demand PHA accumulation experiment including the conditioning step is provided in Fig. S1 (Supporting Information 1). Accumulation was driven with the same respiration-based control logic for pulse-fed input of substrate reaching a maximum substrate concentration of 150 mgCOD/L but without any delay between successive feed pulses. Accumulation sustained a high average respiration rate based on these small “just-in-time” supplied substrate pulses [46]. Feed-on-demand pulse input timing was triggered with on-line monitoring of DO according to the previously established control strategy [19].

2.4. Analytical methods

Online signal logging (DO, pH and temperature) was made alongside mixed liquor grab samples (2 × 15 mL) that were taken at selected times. Samples were processed immediately after sampling. 15 mL were directed to basic water quality analyses. Suspended solids were separated by centrifugation (5250 ×*g* and 20 °C for 15 min). The supernatant was then filtered (0.45 μm) pending liquid analyses. Ammonium, nitrite, nitrate, phosphate, acetic acid, and butyric acid concentrations were determined by ion chromatography (Metrohm Compact IC Flex 930, Metrohm, Switzerland). Soluble chemical oxygen demand (sCOD) was determined by Hach-Lange (Germany) LCK514 kits. The harvested biomass pellet dry weight and its ash content were measured by standard methods [47] and referenced to the sample volume for estimating total and volatile suspended solids (TSS and VSS) concentrations, respectively.

15 mL were used for PHA determination. The sample was acidified directly to pH 2 with 3 M HCl. The acidified suspended solids were then mixed thoroughly before solids separation by centrifuge (5250 ×*g* and 20 °C for 15 min). The pellet was retained, dried at 105 °C overnight, and then ground by mortar and pestle. Average biomass PHA content was estimated by TGA, as previously described [48]. This method has been validated to be accurate and comparable to independently performed digestion, extraction, and gas chromatography determination, regardless of PHA type [49]. In each measurement sequence a positive control biomass sample with known PHA content was measured, giving a precision for the present work of 39 ± 0.5 % gPHA/gVS (n = 8). The

active biomass (X_a) was defined as the non-PHA fraction of the VSS. X_a elemental composition (C,H,O,N) was assessed (Flash*Smart* CHNS/O Analyzer, Thermo Fisher Scientific, USA) with mass balance correction based on the VSS PHA content.

For microscopy, 1 mL mixed liquor grab samples were taken directly from the reactor and fixed with formaldehyde at a final concentration of 3.7 %. The fixed samples were preserved in 1:1 of 1× PBS and cold ethanol and stored at −20 °C until further processing. Fixed samples were stained with BODIPY 493/503® (Thermo Fisher Scientific, MA, USA) in combination with Sypro™ Red (Thermo Fisher Scientific, MA, USA) and were examined by confocal laser scanning microscopy (LSM 880, Carl Zeiss, Germany) with image analyses as previously described [50]. Images with illuminated respective stains were acquired at 16-bit depth in separate channels, and processed (ImageJ2, Ver 1.52P). An average biomass volume-to-volume ratio for the PHA to non PHA-storing biomass (*v*/v) and the average PHA content in the PHA-storing biomass fraction were estimated as previously described [51].

For the microbial community analysis, fresh samples were collected at 0 h, 24 h and 48 h, preserved with added cold ethanol at 1:1 (v/v) and stored at −20 °C. DNA was extracted from 0.5 mL of the preserved sample using a FastDNA® SPIN kit for soil (MPBio, USA) according to manufacturer instructions. Extracted DNA samples were diluted 10 times for PCR. Library construction for 16S rRNA gene amplicon sequencing (MrDNA Molecular Research LP, TX, United States), and sequence data processing were performed as previously described [32], but using QIIME2 version 2023.2 [52]. Taxonomic classification was assigned with SILVA version 138.1 as reference. A reproducible workflow, including all QIIME2 scripts and an R markdown document, is available on Github (https://github.com/pietervanveelen/Xing_PHA_direct_accumulation). The raw sequence data have been deposited in the European Nucleotide Archive under project accession number PRJEB75957.

2.5. Data analysis

For the data analysis, all measured parameters were corrected for effects of sample withdrawal and feedstock addition with liquid and mass balance considerations.

2.5.1. Respiration monitoring

The DO recovery phase from the three conditioning pulses was used to estimate the process oxygen mass transfer rate k_La [min^{-1}] based on the oxygen mass balance:

$$\frac{dC_L}{dt} = k_La\,(C^* - C_L) - r_{Oexog} - r_{Oendog} \quad (1)$$

where C_L is the DO concentration [mg/L]; C* is the DO saturation concentration [mg/L] determined from temperature-dependent empirical equation [53], and was defined as the value in equilibrium with the concentration in the bulk gas phase at 25 °C; r_{Oexog} is the oxygen uptake rate [mgO_2/min] due to exogeneous substrate addition; r_{Oendog} is the background biomass respiration level established after aerating the activated sludge overnight [mgO_2/min]. Nonlinear regression analysis of the measured DO was used to calibrate the system k_La for each experiment:

$$C_L = C_r^* - \left(C_r^* - C_L\right)e^{-k_La\bullet t} \quad (2)$$

where C_r^* is the observed reference DO saturation level after overnight aeration and is equal to$C^* - \frac{r_{Oendog}}{k_La}$. The average k_La from the three conditioning re-aeration trends was applied for respiration rate monitoring given a constant aeration air flow rate maintained throughout each experiment (provided in Table S2, supporting information 3).

Trends for the average oxygen consumption yield on substrate (Y_{os}, mgO_2/mgCOD) for each successive pulse were estimated based on the integrated respiration rate and the measured mass of substrate added for each pulse. Y_{os} represents the fraction of substrate oxidized by oxygen to generate energy required for metabolic activities:

$$Y_{os} = \frac{\int_{t_o}^{t_d} r_{Oexog}\,dt}{V_f \bullet C_f} \quad (3)$$

where t_o is the time of pulse input; t_d is the time of substrate depletion. t_d was defined from the DO trend by the maximum value of dC_L/dt in the downswing of respiration for every pulse event base on Eq. (1) [46]; V_f is the pulse feed dose volume monitored gravimetrically; C_f is the COD concentration of the feed solution [mgCOD/mL].

2.5.2. Kinetics of PHA accumulation

PHB has a COD content of 1.67 gCOD/gPHB. Biomass PHB content was estimated by TGA (gPHA/gVSS) on grab samples in time. The asymptotic trend of PHB content (f_{PHA}) was empirically represented by least-squares regression to the function describing first order accumulation kinetics, as previously described [54]:

$$f_{PHA} = A_0 + A_1\left(1 - e^{-\frac{t}{\tau}}\right) \quad (4)$$

where A_0 is the initial PHA content, A_1 the maximum PHA content, and τ is the first order time constant for PHA accumulation to an estimated plateau value.

2.5.3. Kinetics of active biomass growth, substrate consumption and yields

Quadratic or cubic polynomials could empirically follow the observed smooth trends of measured parameters in time. Polynomial constants were estimated by linear regression analyses in time for measured total biomass production (X_v, gVSS) and substrate consumption (S_s, gCOD):

$$\begin{aligned} \hat{y} &= ax^2 + bx + c, & \hat{y}' &= 2ax + b \\ \hat{y} &= ax^3 + bx^2 + cx + d, & \hat{y}' &= 3ax^2 + 2bx + c \end{aligned} \quad (5)$$

where, a, b, c and d are empirical constants of best fit and ŷ is the predicted parameter value (y) as a function of time used for the mass balances, i.e., VSS production and COD consumption. The function first derivative (ŷ') gave trends of estimated observed rates in time. X_a was estimated as a continuous function of time based on Eqs. (4) and (5):

$$X_a = X_V \bullet (1 - f_{PHA}) \quad (6)$$

Trends of instantaneous yields ($Y_{Xa,\,i}$, $gCOD_{Xa}$/gCOD and $Y_{PHA,i}$, $gCOD_{PHA}$/gCOD) were in this way determined from the respective ratios of the first derivatives of the trend lines defined using eqs. (4) to (6).

2.5.4. Modelling metabolism and reaction rates

The applied process model of metabolic pathways and reaction rates (Fig. 1) was based on two previous works [37,55]. Acetate (HAc) as a sole carbon source is converted to acetyl-CoA after active transport (r_1). Acetyl-CoA is channelled, to the tricarboxylic acid cycle (TCA) for growth (r_8) and NADH production (r_4), and converted to PHB (r_6). Two acetyl-CoA units condense to produce acetoacetyl-CoA, which is reduced to hydroxybutyryl-CoA at the expense of NADH. In contrast, for butyrate (HBu) active transport into cells, hydroxybutyryl-CoA can be directly produced with simultaneous NADH generation (r_2). Available hydroxybutyryl-CoA can be directed to PHB synthesis or else it can be partially decomposed to the intermediate acetyl-CoA (r_3) for reducing equivalents necessary for energy demands, and X_a production.

The following conversion rates were estimated for each experiment: specific oxygen uptake rate (SOUR), specific substrate uptake rate (SSUR), and PHB production rate. An ATP balance was constructed from the set of assumed metabolic processes (where, r_1 is the specific acetate uptake rate (CmolAc/CmolX$_a$/min); r2 is the specific butyrate uptake

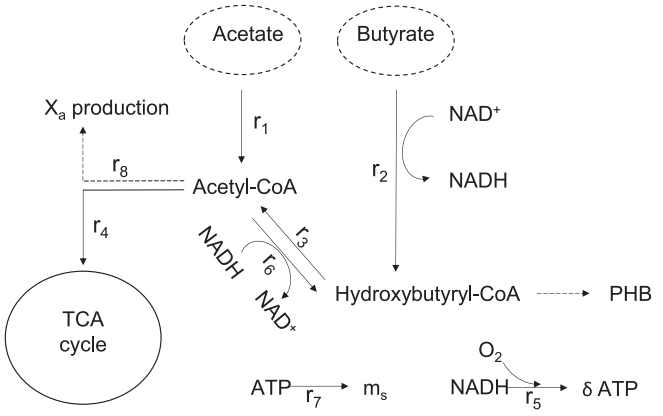

Fig. 1. Schematic representation of PHA production from butyrate and acetate. Reaction rates r_1 to r_8 are listed in Table 3.

Table 3
Internal reactions considered for PHA production from butyrate and acetate (all in C-mol units). The efficiency of the oxidative phosphorylation (δ) was assumed to be 2.0; α_x represents an ATP demand per CmolX$_a$ produced.

Reaction		Stoichiometry
r1	Acetate uptake, activation	1HAc + 1ATP ➔ 1AcCoA
r2	Butyrate uptake	1HBu + 0.5ATP ➔ 1PHB + 0.25NADH$_2$
r3	PHB consumption	1PHB + 0.25ATP➔ 1AcCoA + 0.25NADH$_2$
r4	Catabolism	1AcCoA ➔ 1CO$_2$ + 2NADH$_2$
r5	Oxid. Phosphorylation	NADH$_2$ + 0.5O$_2$ ➔ H$_2$O + δATP
r6	PHB production	1 AcCoA +0.25 NADH$_2$ ➔ 1 PHB
r7	Maintenance	ATP ➔ m$_s$
r8	Anabolism	α_xATP ➔ X$_a$

rate (CmolBu/CmolX$_a$/min); r_3 is the difference between r_2 and the PHB production rate (CmolPHB/CmolX$_a$/min). The difference between estimated rates of ATP production and PHB-synthesis ATP consumption define a surplus in ATP production. This surplus ATP during the PHA accumulation process is theoretically available for processes of cell maintenance metabolic activity as well as supporting an energy demand for X$_a$ production (r_7 and α_x r_8).

$$\text{For acetate}: -r_1 - \alpha_x r_8 - r_7 + \delta r_5 = 0 \quad (7)$$

$$\text{For butyrate}: -0.5r_2 - 0.25r_3 - \alpha_x r_8 - r_7 + \delta r_5 = 0 \quad (8)$$

2.5.5. Microbial community analysis

Microbial taxon count data were filtered to retain bacterial amplicon sequence variants (ASVs) with >0.05 % of the total sequence abundance, retaining 2107 out of 4846 ASVs (98.6 % of sequences). Rarefaction curves for all samples were saturated with sequencing depth averaging (range) to 61,666 sequences per sample (42061–82,447). Therefore, microbial diversity was estimated based on unrarefied data, summarized at the genus level, using R packages *phyloseq* [56]. Microbial community dynamics among time points 0, 24 and 48 h of the accumulations were evaluated, and compared between Bath and Almere WWTP sludge fed with either acetate or butyrate. Principal coordinate analysis (PCoA) using the ecological Bray–Curtis dissimilarity index was performed using vegan R package [57]. A heatmap for each combination of activated sludge in time with respect to feed was generated to visualize relative abundance trends of genera consistently observed between replicate experiments, and specific for the volatile fatty acid substrate fed, or specific for the activated sludge source.

3. Results

PHA accumulation tests were run with butyrate or acetate as sole substrates in a set of experiments using two distinct types of activated sludge as the starting biomass. PHA production performances were evaluated with mass balances from biomass and water quality analyses. Differences and changes between and within the activated sludge microbial communities were assessed by selective staining with confocal microscopy and with 16S rRNA gene sequencing analyses.

3.1. PHA accumulation performance

An example of observed PHA accumulation trends over 48 h with butyrate versus acetate is given in Fig. 2. Measured data were fitted to trends by least squares regression analysis with Eqs. (4) to (6) to empirically represent continuous respective developments in time for making mass balances and rate estimations over the accumulation period. Higher substrate COD consumption rates were observed with butyrate feed (Fig. 2A). These increased feed consumption rates were associated with higher PHA content and mass of PHA produced (Fig. 2B and C). After 48 h, 5.1 g PHA (8.5 g COD$_{PHA}$) were produced in the butyrate-fed reactor, compared to only 1.4 g PHA (2.0 g COD$_{PHA}$) in the acetate-fed reactor.

Accumulations were performed with the same relative levels of nutrient supply, specifically a COD:N:P ratio of 100:1:0.05 (by weight). No buildup of soluble ammonium, nor phosphate, concentrations were observed in the liquid phase for either case during accumulations. Measured X$_a$ elemental compositions are provided in Table S1 (Supporting information 2). There was no measurable change in the X$_a$ elemental composition for either acetate-fed or butyrate-fed reactors. X$_a$ was estimated to be $CH_{1.87}O_{0.51}N_{0.15}$ giving 1.45 gCOD/gX$_a$. According to the dry weight measurements, the observed net active growth was low for acetate-feed (Fig. 2D), and only 0.2 g X$_a$ (0.3 g COD$_{Xa}$) was produced. In contrast, butyrate-feed promoted that active biomass almost doubled in mixed liquor VSS in the same 48 h accumulation time from 2.2 g X$_a$ (3.2 g COD$_{Xa}$) to 4.2 g X$_a$ (6.1 g COD$_{Xa}$). The active growth estimated from N uptake and measured N content of the active biomass showed a similar trend to the dry weight measurements (Fig. 2D). Reproducibly more significant X$_a$ production for butyrate was observed compared to acetate accumulations, regardless of the sludge source ($X_{a,\ t=48}/X_{a,\ t=0}$ of 2.0 ± 0.3 for butyrate versus 1.3 ± 0.1 for acetate).

A summary of results for the full set of accumulation experiments are provided in Table 4. Butyrate-feed consistently resulted in a greater extent of X$_a$ production. About four times higher volumetric PHA productivity was achieved. Average yields of both PHA and active biomass over 48 h were significantly increased. Biomass PHA contents nearly doubled compared to accumulations using acetate for either Bath or Almere activated sludge (Fig. S2, supporting information 3). With Bath WAS, a maximum biomass PHA content of 0.61 gPHA/gVSS was obtained from feeding butyrate compared to 0.39 gPHA/gVSS with acetate. For Almere WAS, 0.35 gPHA/gVSS was obtained with butyrate versus 0.22 gPHA/gVSS for acetate.

Representative images from staining and confocal microscopy are shown in Fig. 3. For Bath WAS and at 30 h with a stable biomass PHA content, it was estimated that 65 % ± 9 % (*v*/v) of the cell volume was occupied by PHA using butyrate as compared to 44 % ± 3 % (v/v) for acetate.

3.2. Kinetics of PHA storage and biomass production

Instantaneous yields on substrate for PHA and active biomass production were calculated from the estimated trends from the measured dry weights (Fig. 4A). With acetate, PHA yield decreased progressively as the biomass asymptotically reached an interpreted steady maximum PHA content. Active biomass production progressed over the interval from 5 to 20 h, but ultimately ongoing substrate consumption was diverted away from any further net PHA or active biomass production.

With butyrate, PHA yield on substrate decreased during the first 5 h as PHA content similarly approached a maximum level asymptotically.

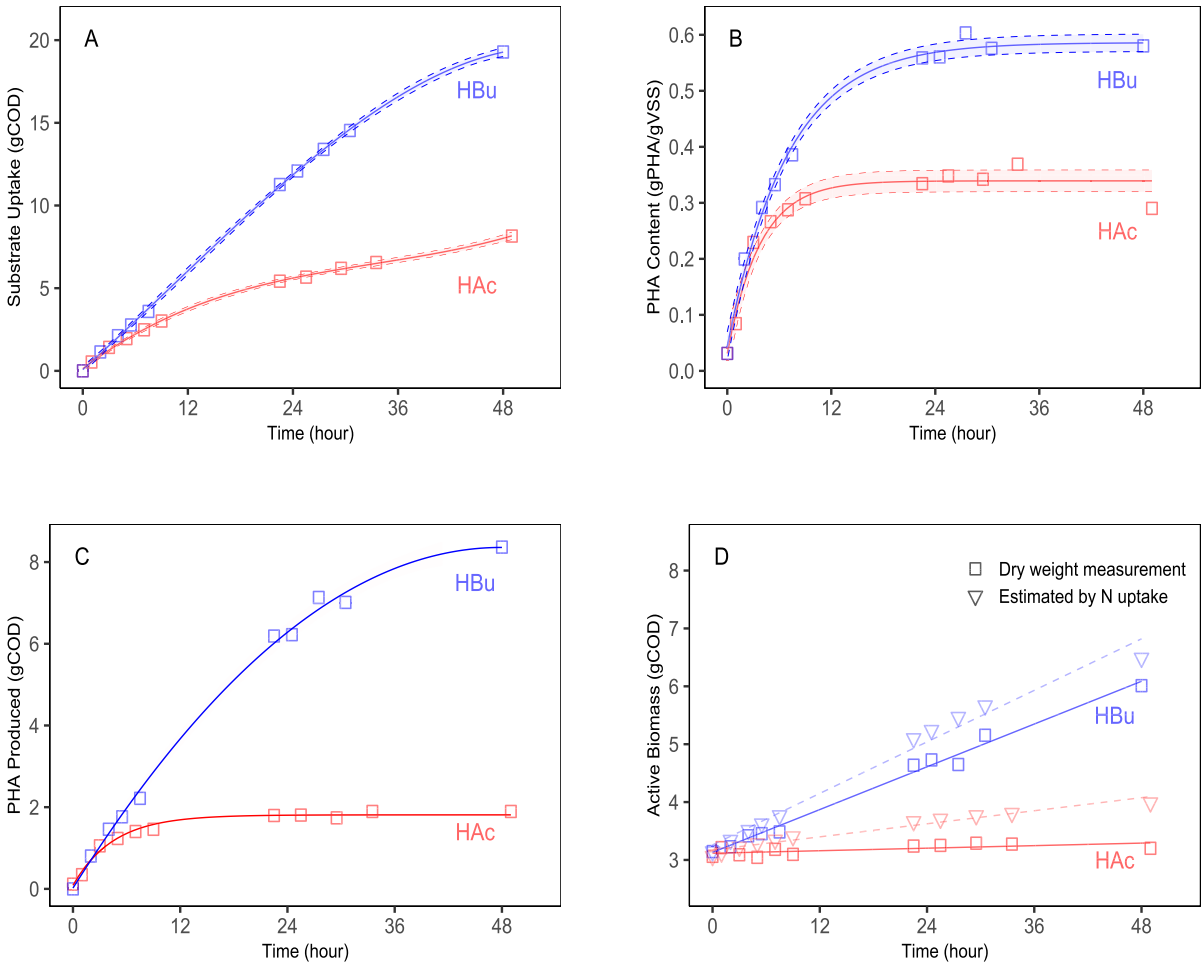

Fig. 2. Typical trends for (A) soluble COD consumption, (B) biomass PHA content, (C) PHA produced and (D) active biomass estimated by both dry weight and N uptake measurements during accumulation tests with WAS from Bath WWTP. Dashed lines show the trend 95 % confidence intervals from least squares regression analysis of the measurement values.

However, PHA yield on butyrate remained sustained at around 0.6 $gCOD_{PHB}/gCOD_{HBu}$ alongside substrate utilization for active biomass production (≈ 0.15 $gCOD_{Xa}/gCOD_{HBu}$). PHA to active biomass ratio increased over time with both acetate and butyrate feed (Fig. 4B). Despite the higher level of active growth in butyrate-fed case, trends in mass balances suggest that the PHA accumulation process remained selective for the PHA-storing phenotype (i.e. $gPHA/gX_a$ developments). PHA mass increased to 1.3 times that of the active biomass after 30 h in the butyrate-fed case.

3.3. Metabolic responses to butyrate versus acetate in municipal WAS

The metabolic responses of the Bath and Almere WWTP WAS to butyrate and acetate were evaluated based on oxygen mass balances for each successive substrate input pulse (Fig. 5A). In replicate experiments, no substantial active growth was observed during the initial 2-h period. PHA storage response was the dominant metabolic response in this initial period with negligible X_a yield (Fig. 4A). Over the initial 2-h with WAS from Bath WWTP, Y_{os} for butyrate was 0.17 ± 0.01 $gO_2/gCOD$, compared to 0.26 ± 0.01 $gO_2/gCOD$ for acetate. Almere WWTP activated sludge gave comparable results with 0.16 ± 0.02 $gO_2/gCOD$ for butyrate and 0.27 ± 0.01 $gO_2/gCOD$ for acetate.

Y_{os} increased progressively over the course of the accumulations and this increase was relatively more substantial for acetate reaching 0.69 $gO_2/gCOD$ (Fig. 5A). In contrast with butyrate, Y_{os} increased at a slower rate to reach only 0.39 $gO_2/gCOD$ by 48 h.

Oxygen (OUR) and substrate (SUR) uptake rates were estimated from the timing of each successive triggered pulse of substrate input, and from oxygen and substrate mass balances. X_a was estimated as a function of time (Eq. (6)) to obtain the specific uptake rates, SSUR and SOUR (Fig. 5B and C). Unpaired *t*-tests indicated no significant difference between SOUR values for butyrate and acetate feed during the course of parallel accumulations, regardless of which sludge source was used (CI = 95 %, Table S2, supporting information 3). Bath WAS exhibited overall higher average SOUR of 0.57 ± 0.08 $mgO_2/gX_a/min$ (n = 232) compared to 0.29 ± 0.11 $mgO_2/gX_a/min$ (n = 166) for Almere WAS. At the same time, significantly higher SSUR was observed for butyrate (Table S2, supporting information 3). For butyrate, the lower Y_{os} was due to a higher SSUR. Over 48 h, SOUR remained relatively constant compared to SSUR. SSUR decreased significantly, and more rapidly for acetate-feed, over time from their initial levels.

3.4. Microbial community structure analysis

Diversity of the two activated sludge bacterial communities and accumulation population dynamics were evaluated by 16S rRNA gene sequencing. Compositional changes were significant during the accumulations (time point: R^2 = 14 %, pseudo-$F_{2,23}$ = 3.4, P < 0.001) (Fig. S4, supporting information 4), and independent of the distinct differences in the composition between Bath and Almere WAS (R^2 = 40 %, pseudo-$F_{1,23}$ = 4.4, P = 0.002). The substrate type fed influenced how and how much compositions changed during PHA accumulations (R^2 =

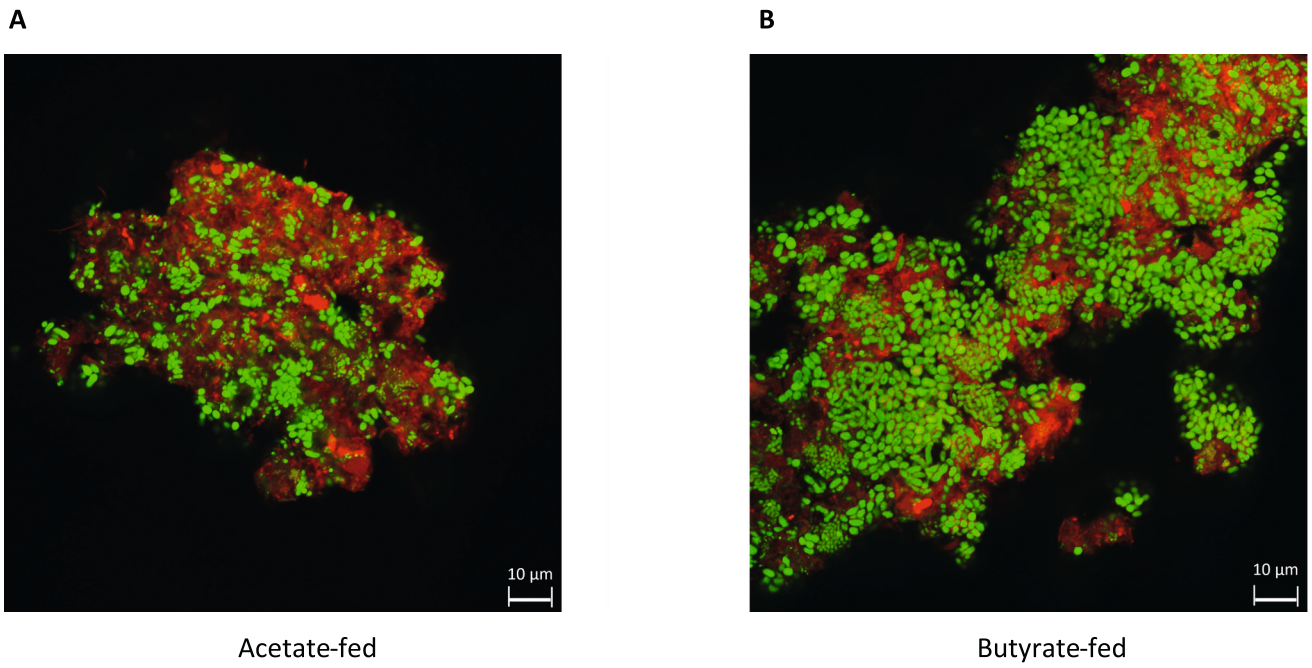

Fig. 3. Representative images of stained activated sludge flocs in PHA accumulations with (**A**) acetate and (**B**) butyrate at 30 h. Red (protein) staining depicts the floc morphology and green (PHA) staining reveals the distribution of PHA granules in the flocs.

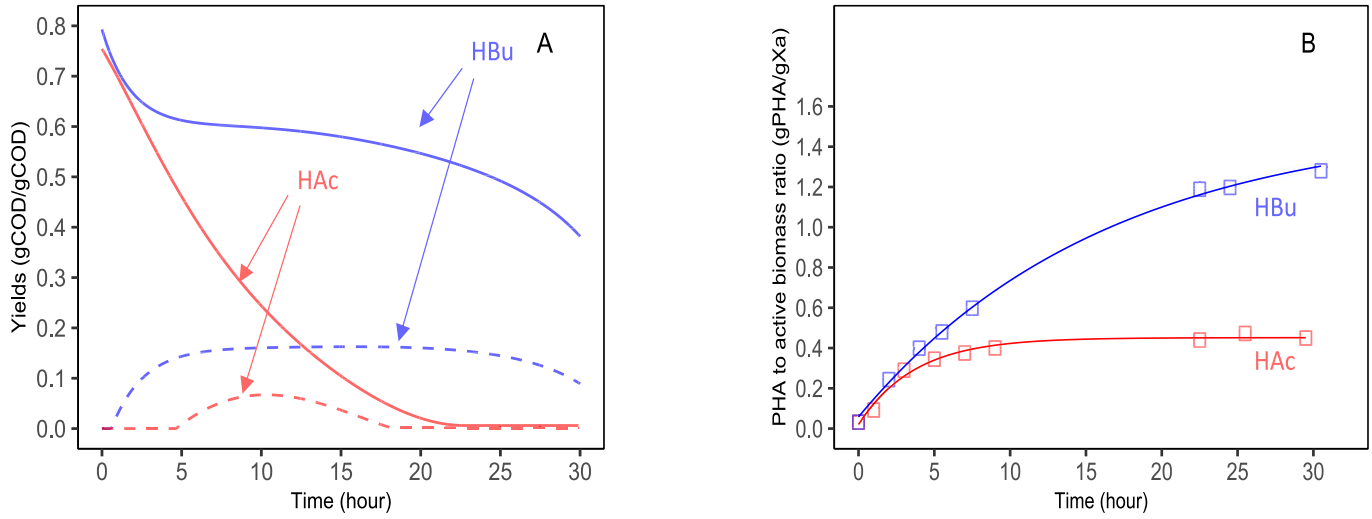

Fig. 4. Performance of PHA accumulation tests with the same biomass from Bath WWTP using acetate (HAc) or butyrate (HBu) as substrate. (**A**): Instantaneous PHA yields (solid lines) and active biomass yields (dashed lines) on substrate. (**B**): Trends of PHA to active biomass ratio.

9 %, pseudo-$F_{1,23} = 20.4$, $P < 0.001$). With acetate feed, changes in compositions were characterized by apparent enrichment of specific genera (Fig. 6), which translated into a significant reduction of Shannon diversity after 48 h (Fig. S5, supporting information 4). For Bath WAS, the enrichment was most pronounced for *Flavobacterium* in both replicates, and on average from 11 % to 27 % (Fig. 6, Fig. S6 in supporting information 4). For Almere activated sludge, the shift in microbial communities was mainly attributable to a relative increase of *Acinetobacter*. In contrast, butyrate fed accumulations exhibited relatively more stable compositions (Fig. S4, supporting information 4). While minor increases of up to 3 % were detectable (e.g., *Zoogloea, Dechloromonas*), no compositional change reflecting an enrichment of WAS bacterial genera >3 % was detected when fed with butyrate (Fig. 6), despite the fact that total active biomass (X_a) levels almost doubled in all these cases.

4. Discussion

4.1. Enhanced PHA production is promoted by concurrent selective growth

Active growth with ongoing PHA production in all butyrate cases led to more biomass produced with a higher average PHA content (Table 4). Replicate experiments with Bath and Almere WWTP waste activated sludge showed that higher average PHA yields were repeatedly associated with higher active biomass yields for butyrate-feed compared to acetate-feed (Table 4). A consistent minor offset was observed, with N uptake measurements estimating more active growth compared to suspended solids mass balances (Fig. 2D). This measured difference and the observed foaming in the reactor suggests some degree of unavoidable biomass segregation in the foam scum from the MLVSS measured in the

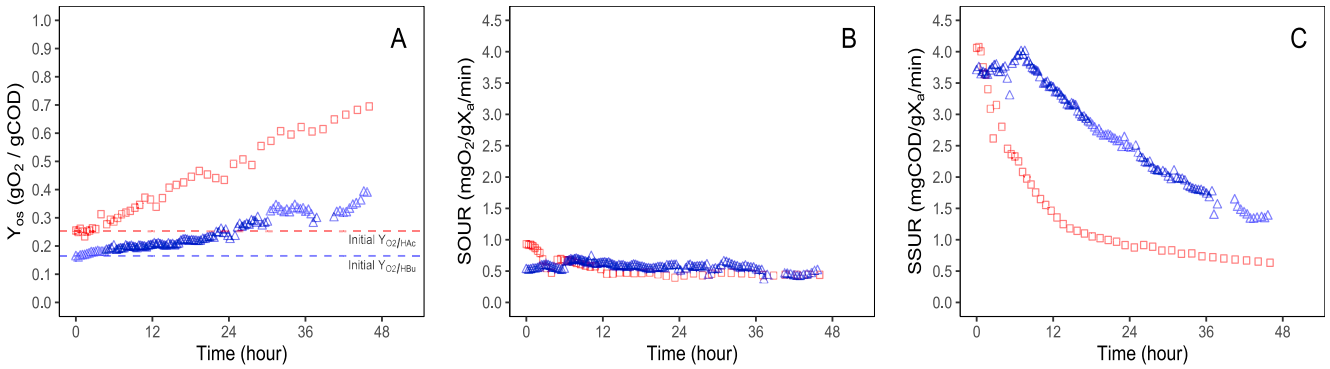

Fig. 5. Evolution of the substrate input pulse-to-pulse derived (**A**) oxygen yield on substrate, (**B**) SOUR and (**C**) SSUR for each respective feed dose in time with acetate-fed (□) or butyrate-fed (Δ) reactors over 48-h of direct PHA accumulation using WAS from Bath WWTP.

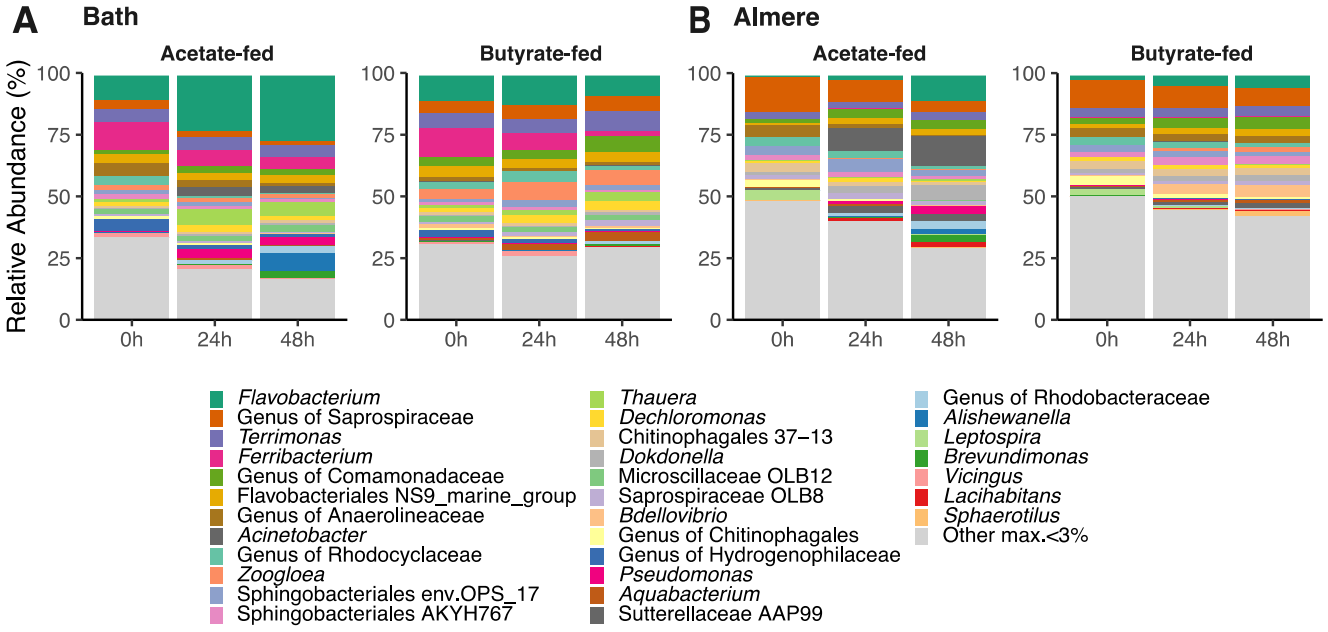

Fig. 6. Development of genus relative abundance during PHA accumulation tests conducted with activated sludge from Bath WWTP (A) and Almere WWTP (B). Each condition was performed in duplicate and average values are presented.

broth. However, even with respectively similar minor amounts of uncontrolled biomass loss from the reactors over 48 h, reproducibly more significant absolute amounts of X_a production were observed for butyrate compared to acetate accumulations based on either suspended solids or nitrogen mass balances. Despite an onset of significant active growth, the PHA to active biomass ratio increased over time (Fig. 4B), indicating that the PHA storage response still remained a dominant activity that was not overtaken by the active biomass production. Thus, observed higher PHA contents in butyrate cases suggest that the active growth must have been, at least to some degree, selective to the PHA storing phenotype. The selective nature for the active growth was also supported by staining and confocal microscopy (Fig. 3). Significantly more of the floc bio-volume became occupied by PHA when using butyrate during these direct accumulations. Simultaneous biomass growth and storage has been previously reported to take place, and it benefited overall PHA production without observed risk that an onset of a generic growth response would overtake the extant PHA storage activity [32,34].

Concurrent growth of PHA accumulating bacteria also affected the dynamics of the direct accumulation process. Developments of instantaneous PHA yield revealed different trends after about 4 h of accumulation for butyrate versus acetate (Fig. 4A). For butyrate, selective growth of PHA-storing phenotypes enabled ongoing substrate utilization for PHA production due to newly formed storing capacity. Thus, during butyrate accumulations, PHA production rates could be maintained relatively stable and relatively high (Fig. 2C).

In contrast, with acetate, PHA yield decreased progressively as the biomass asymptotically reached a plateau maximum level of PHA content (Fig. 4A). Ultimately, PHA production became negligible already after 10 h. Some substrate contributed to the active biomass production (5 to 20 h) but increasing amounts of substrate consumed did not contribute to further net production of PHA and/or active biomass. This outcome fits with trends of independently determined feed-pulse average Y_{os} derived from logged DO and oxygen mass balances (Fig. 5A). By 48 h, a majority of the acetate COD (≈ 70 %) provided with each fed pulse was respired to carbon dioxide. Such a high Y_{os} suggested in time that acetate COD became largely spilled in an apparent futile cycle once the biomass became saturated with PHA. Organic substrate was spilled to carbon dioxide in non-growth and non-storage associated ATP production, indicating an influence of increased maintenance demands and/or some form of stress response. From a process efficiency perspective, prolonged acetate accumulations led to wasted substrate with diminishing productivity in time. Notwithstanding, the biomass PHA content was nevertheless still remarkably sustained. Efficient

methods of direct accumulation using municipal activated sludge require methods of automated process termination as substrate may become less effectively utilized over the process time depending on the substrate or substrate mixture. This work suggests that online assessment of oxygen consumption yield on substrate could be a useful process control parameter to estimate and monitor, in real time. Triggering the accumulation termination time by actively detecting the onset of substrate wastage due to maintenance activity or futile cycling can be one way to achieve the most optimal outcome in scaled up industrial practice. Energy (aeration) and substrate utilization must be spent as effectively as possible per ton of PHA.

4.2. ATP overflow leads to combined storage and growth response in WAS

PHA production has become interpreted as an "overflow" mechanism in which PHA is produced only when the substrate is not all needed or cannot be used directly for the growth and maintenance purposes [37]. However, this interpretation may not fully capture the observations from recent enrichment culture studies. Johnson et al. observed that a highly enriched MMC exhibited a dominant PHA storage response with negligible growth in a feast phase after a period of famine even when both acetate and nutrients were present in excess [58]. In the present study, new insights for considering PHA accumulation and microbial growth in WAS system are proposed. When municipal activated sludge is transferred to a direct PHA accumulation bioprocess from a state of famine, the environment can be favourable for a dominant PHA storage response. Simultaneous growth during PHA storage requires a sufficient ATP overflow from PHA storage. This ATP overflow was defined, for the present work, as the ATP production rate minus the ATP consumption rates due to PHA synthesis and cell maintenance.

4.2.1. Storage response can dominate the initial phase of direct accumulation

Results from the replicate experiments with both Bath and Almere WWTP waste activated sludge suggested an initial dominant PHA storage metabolic response with negligible X_a yield, independent of substrate, butyrate or acetate (Fig. 4A). Negligible net active growth response during the initial hours can be due to the combination of limiting supplied growth nutrients combined with the method of pulse feeding and biomass needs for adaption away from its immediate environmental history of famine [59]. It has been reported that PHA storage kinetics of substrate uptake is one mechanism by which the PHA storing phenotype within a biomass has competitive advantage when conditions shift suddenly from famine to feast. [21,60] Maximum theoretical yields for PHB have been previously estimated to be 0.74 gCOD/gCOD (0.66 Cmol/Cmol) on acetate [61], and 0.85 gCOD/gCOD (0.94 Cmol/Cmol) on butyrate [62]. Initial accumulation yields estimated in the present work did fit well to these expected respective reported theoretical values (Fig. 4A). These initial yields suggest that, at the accumulation start, metabolic activity was dominated by PHA storage. In the present work the biomass was not explicitly enriched for PHA-storing microorganisms. WAS was used directly after a conditioning step that gave a wake-up call [44].

4.2.2. Lower ATP demand with net NADH production for butyrate

The amount of oxygen consumed during respiration is directly proportional to the amount of ATP produced through oxidative phosphorylation (Fig. 1). Thus, average Y_{os} of each feed pulse was estimated for evaluating differences in ATP demands during the accumulations and between the two substrates. It can be concluded from these results that distinct WWTP processes gave surplus activated sludge with consistently similar initial storage responses to butyrate and acetate and with lower Y_{os} for butyrate (Fig. 5A). The analysis from pulse to pulse of input feed reveals an interpreted selective response from the PHA-storing phenotype (initially) with lower ATP demands (lower Y_{os}) for PHA synthesis using butyrate versus acetate. Decrease in ATP demands may be mainly attributed to the apparent difference in the energy expenditure for active substrate transport into cells (Table 3, Reactions 1 and 2). NADH is also produced in the metabolic pathway for butyrate conversion, as butyrate has a higher degree of reduction than the product PHA monomer. This NADH production allows PHA synthesis to generate the electron carriers required for oxidative phosphorylation, thus minimizing the need to run the TCA cycle. ATP can be produced through the reoxidation of the generated NADH. Sufficient ATP surplus, with given essential nutrient availability, can allow for the PHA-storing phenotype to grow concurrently and competitively in a mixed culture while still maintaining a relatively dominant storage response.

4.2.3. Respiration rate (SOUR) as the limiting factor of the metabolic processes

All direct accumulation experiments were conducted under fully aerobic conditions (Fig. S1, supporting information 1). The substrate type, either butyrate or acetate, did not have substantial influence on SOUR levels during accumulations (Fig. 5B). SOUR for butyrate or acetate feeds were not significantly different and this was in contrast to the disparity in trends and levels in the SSUR (Fig. 5C). Marang et al. studied metabolic differences for an enrichment culture with acetate versus butyrate and similarly found a constant SOUR regardless of substrate type [42]. A constant SOUR indicated for a fixed rate of electron transfer through the electron transport chain (ETC) and the subsequent reoxidation of electron carriers (e.g., NADH) by oxygen. In the present work, this rate is estimated to have been about 2.0 mol e^-/CmolX_a/d for Bath activated sludge and 1.1 mol e^-/CmolX_a/d for Almere sludge. Assuming that all ATP production is based on reoxidation of reduced electron carriers in the ETC [63], a fixed ATP production rate is interpreted for both feed cases. Heijnen proposed that cells may have become limited in energy production capacity over evolutionary time [64]. The restricted membrane space for embedding electron transport proteins may limit energy generation via electron transport phosphorylation, resulting in a constrained maximum ATP production rate. The estimated rate difference between Bath and Almere activated sludge are anticipated to be due to the quality of the X_a, given that Bath WAS has a higher initial degree of enrichment of PHA-storing phenotypes [20].

4.2.4. Surplus ATP production supports concurrent growth alongside the storage response

To reveal metabolic principles that stimulate PHA-storing phenotypes to outcompete flanking populations in a complex MMC while storing PHA, mass balances were applied in combination with the known metabolism for determining and contrasting rates of ATP turnover in the microbial consortia. In the present investigation, it was important to understand how metabolic energy was distributed functionally in time over the course of the direct PHA accumulation bioprocess from batch to batch. Direct measurements of standing biomass intracellular ATP levels [65] would not be able to indicate for rates and fluxes because ATP utilization is coupled rapidly to its production. Therefore, the approach of an ATP rate balance that integrates to the cumulative ATP distribution (Fig. 7) was applied. It provided a means to ascertain any consequential impact of lowered ATP demands for PHA synthesis for butyrate versus acetate, given an relatively constant ATP production rate due to aerobic respiration.

The ATP balance uses the measured parameters, derived trends, mass balances, and the metabolic model (Table 3). ATP produced due to aerobic respiration, and consumed due to PHB production, was estimated (Fig. 7, Fig. S3 in supporting information 3). A pronounced higher ATP surplus is predicted with butyrate compared to PHA accumulation using acetate. When biomass PHA content was 95 % of its estimated maximum level (3τ hours), the surplus between ATP generation and ATP demand for PHA storage was about 2–2.5 times greater for butyrate versus acetate. This surplus ATP can become consumed due to biomass production, cellular maintenance, or hydrolysis in futile cycles [62]. Despite the same levels in supply of essential nutrients (N and P),

Table 4
Summary of results for replicate PHA accumulation tests with WAS from Bath and Almere WWTPs ($n = 2$ in all cases).*

Sludge source	Carbon source	Time constant (h)	Max. PHA content (gPHA/gVSS)	Xa produced at 48 h (gX_a)	PHA productivity at 48 h (gPHA/L/d)	$Y_{PHA/s}$ (gCOD/gCOD)	$Y_{X/s}$ (gCOD/gCOD)
Bath	HAc	4.7 ± 0.9	0.38 ± 0.01	0.35 ± 0.21	0.65 ± 0.05	0.25 ± 0.04	0.04 ± 0.01
	HBu	6.8 ± 0.2	0.58 ± 0.03	1.87 ± 0.15	2.46 ± 0.33	0.46 ± 0.01	0.16 ± 0.02
Almere	HAc	6.3 ± 0.6	0.21 ± 0.01	0.69 ± 0.03	0.31 ± 0.02	0.15 ± 0.02	0.05 ± 0.02
	HBu	17.8 ± 0.5	0.34 ± 0.01	1.34 ± 0.22	1.25 ± 0.16	0.29 ± 0.04	0.12 ± 0.03

* $Y_{PHA/S}$: Average yield of PHA produced on substrate feed at 48 h. $Y_{X/S}$: Average yield of net active biomass produced on substrate feed at 48 h. PHA Volumetric productivity is given as the average for a 48-h accumulation process. HAc = acetate, HBu = butyrate.

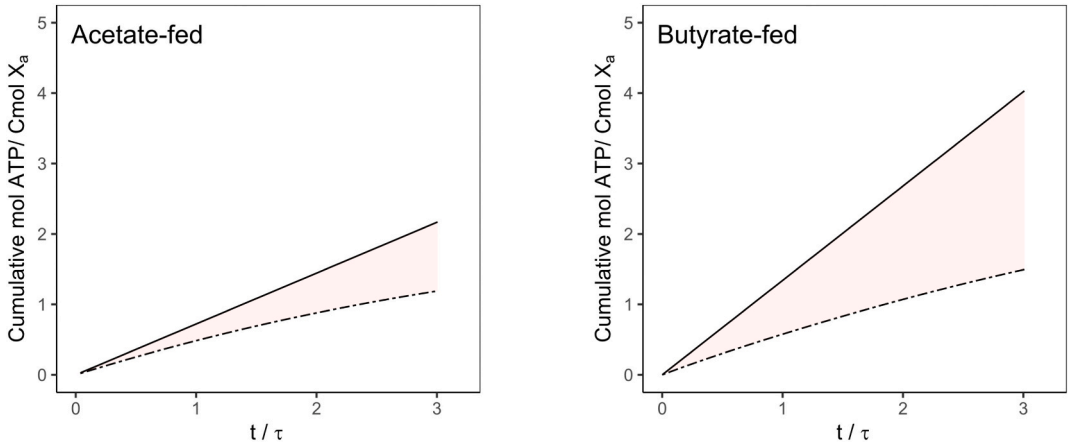

Fig. 7. Cumulative ATP generation (solid line) and ATP demand for PHB synthesis (dash line) over the normalized accumulation time with Bath WAS. Shaded area represents the total estimated amount of surplus ATP available for supporting maintenance activities and potentially also active growth.

microbial growth rates were found to be significantly different between butyrate and acetate feed cases (Fig. 2D). The greater surplus ATP production from butyrate supported higher levels of active growth. In contrast, the disparity between ATP production and ATP consumption with acetate only resulted in limited microbial growth. Additionally, excess ATP consumption in a potential futile cycle occurred with acetate feed once the biomass became saturated with PHA, as indicated by increasing oxygen consumption per unit of substrate (Fig. 5A). Necessity of surplus ATP is supported by previous research and interpretation based on ATP demand, where conditions associated with calcium similarly enabled significant selective growth using acetate [32].

4.3. A question of cell growth in size or in number

Measurements of the community analysis by 16sRNA did not reveal dominant trends in the population dynamics despite the significant observed increase in active biomass with the butyrate feed. This outcome was a conundrum, and it underlies a challenge of interpretation of generic microbial community sequencing data with certainty. For the butyrate feed, mass balances support that the measured active growth must have been due to selective growth of PHA-accumulators (Fig. 4B). Possible explanations are discussed below with respect to the growth strategies of PHA storers, along with their impacts on the outcomes of microbial community analysis and PHA storage capacity.

Observations of more active biomass (by mass) can be due to more individual daughter cells, but also due to the growth of larger individuals. Consider two scenarios of doubling mass of biomass by growth: a larger cell (growing in size from 1 μm to 1.4 μm) could have an advantage over the alternative of cell division into equally sized daughter cells (dividing into two 1 μm cells). The former scenario generates more total bio-volume available for PHA storage (Fig. 8). Thus, cell "expansion" might be an effective selective growth strategy compared to "division" in terms of providing a competitive advantage in resources, including nutrients, and access to substrate via PHA

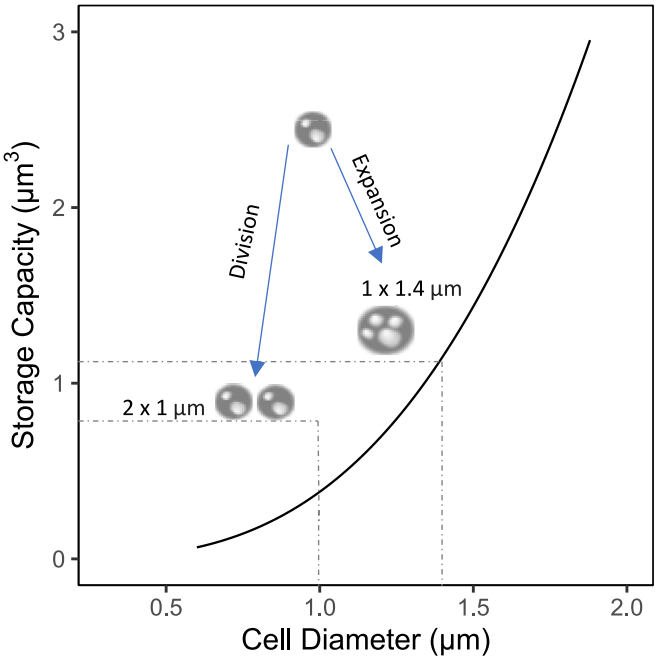

Fig. 8. Evaluation of cell storage capacity under different growth strategies. It is assumed the thickness of cell envelope is 50 nm, and the bacteria are simplified as being spherical. The amount of biomass for 2 cells is equal to the biomass of the 1 larger cell depicted.

production. An interpretation of increase in single-cell volume is supported by previous microscopy image analyses with selective staining for the same activated sludge. Pei et al. reported cell size increase of PHA storing microorganisms in activated sludge through selective fluorescent

staining with CLSM [50]. In those studies, the presence of PHA-storing microorganisms with adaptive stretchability has been documented.

Active biomass increase due to expanded cell size would not change microbial community compositions detected by the 16S RNA community analysis or quantitative PCR, because the increase in mass of individual cells storing PHA would not lead to multiplication of chromosomal DNA. The average PHA contents in just the PHA-storing biomass fraction were higher in the butyrate reactor compared to the acetate reactor. The estimated PHA accumulation capacity for PHA accumulating bacteria in the biomass increased from 0.55 ± 0.01 gPHA/gVSS (acetate fed) to 0.68 ± 0.04 gPHA/gVSS (butyrate fed). Larger cells have been reported to be capable of accommodating larger PHA granules, which may facilitate an easier downstream polymer recovery process [66]. Validation of the 'larger cell' interpretation and any associated effects on polymer properties (e.g. molecular weight) remains part of ongoing investigation. The extent of morphological changes is recommended to be confirmed by means of direct observation at the cellular level with ultra-high-resolution imaging by transmission electron microscopy.

4.4. A question of the growth selectivity

Accumulations with relatively low levels of net active growth (acetate feed) did exhibit clear and reproducible population dynamics with dominant "winners", like *Flavobacterium* in the Bath case and *Acinetobacter* in the Almere case. *Flavobacterium* is common to activated sludge [67]. Previous studies reported this genus in PHA enrichment cultures [68,69]. However, the PHA storage capacity of *Flavobacterium* has not been evaluated in the research literature. From FISH and PHA staining performed on Bath sludge, this genus is expected to have only limited PHA accumulation capacity (unpublished results). Increase in *Acinetobacter* abundance was observed from Almere when fed with acetate. Pei et al. found that an EBPR waste activated sludge exhibited limited PHA accumulation potential [20], and the ability of *Acinetobacter* to accumulate PHA under aerobic conditions has not been demonstrated in the literature. Estévez-Alonso et al. similarly observed dominant trends for "winners" given acetate feed, with and without the effects of calcium [32]. Therefore, competition for acetate for the PHA storing phenotype may require inherent selective pressures in the bioprocess to avoid undue flanking growth. Early developments for GAO enriched biomass required specialized approaches towards expressing optimal accumulation potential [70]. Ways to maximize PHA content in PAO enriched activated sludge have not yet been studied to the same level of understanding.

The genera linked measured compositional changes with acetate (e.g., *Flavobacterium* and *Acinetobacter*) were not observed for butyrate feed (Fig. S5). The significantly higher SSUR and the associated (over) growth with butyrate may help to limit chances for competitive growth of flanking populations (Fig. 5C). It has been reported that upon addition of a substrate to an endogenously respiring activated sludge, a lag phase ensues before the biomass shifts up with a growth response towards its maximum possible rate. This transient response is anticipated to be due in part to intracellular transport and conversion processes [71]. Kinetics of up- and downshifts in biomass physiological state have been previously modelled [46,72]. "Just-in-time" feed-on-demand accumulation control based on respiration response creates conditions for the possible recurrence or prolongation of this transient response, as the next feed pulse is given only when the exogenous substrate provided from the previous pulse becomes depleted. Notably, due to the higher uptake rate for butyrate, significantly more feast pulse cycles were observed compared to acetate-feed over the course of the parallel run accumulations (99–120 cycles versus 48–53 cycles in 48 h). The average shorter pulse times for butyrate (with the same COD provided per pulse) meant a higher frequency transient, which may mitigate growth of flanking populations. Estévez-Alonso et al. reported that smaller but more frequent pulses in similarly run feed-on demand accumulations resulted in higher PHA accumulation potentials when using municipal waste activated sludge [45]. Deepened understanding of selectivity dynamics for growth during mixed culture PHA accumulation needs further development.

4.5. Outlook

The present study suggests that conditions supporting ATP-driven mechanisms for selective growth will enhance PHA productivity within short-term (24–48 h) with fed-batch accumulations that directly use waste activated sludge as input biomass. It is logical to therefore consider extending the accumulation process indefinitely. However, in these developments [32], for such fed-batch accumulation experiments that were run for up to 144 h, a loss of selectivity for PHA accumulation over time resulted with outcomes of lowered biomass PHA contents (unpublished results). The loss of productivity was interpreted to be due to the onset and development in the growth of flanking populations over the extended production time. Therefore, conditions of selectivity due to ATP-driven mechanisms in diverse mixed cultures cannot be expected to be indefinitely stable. Selectivity for growth is understood to result from a combination of factors, including—but not limited to—nutrient availability and the history of the biomass. For example, in direct accumulation, WAS starts under conditions transitioning from endogenous respiration to sudden substrate availability. Microorganisms, that are not initially competitive to quickly assimilate substrate by storing PHA, may just take longer to eventually engage competitively after a lag phase within such diverse microbial communities.

The potential for direct fed-batch accumulation using one initial source biomass batch for repeated production cycles, and/or in other wider applicable bioprocess scenarios that exploit these metabolic principles, even in pure culture systems, needs further investigation. This work sets the stage for general improvement in PHA accumulation methods. It motivates a wider testing of the proposed ATP-driven mechanisms to benefit productivity for PHA production including bioprocess methods using pure and/or enrichment mixed cultures.

Principles based on general metabolic energy requirements for selective growth during direct accumulation were nevertheless demonstrated to be reproducible with butyrate as the sole substrate. These principles provide a basis for an approach that can be extended to substrate mixtures as found in fermented organic waste based on the ATP and NADH yields for individual substrates reported in Table 1 and discussed in the literature [62,73,74]. The systematic impact of VFA composition on surplus ATP production remains to be elucidated. Future research should explore whether metabolic pathways can predict conversion efficiencies and surplus ATP-driven mechanisms for substrate mixtures. Additionally, it is of interest to understand if other bioprocess strategies (e.g. inducing conditions for promoting passive transport [32]) can similarly support surplus ATP production for concurrent selective growth with PHA storage.

Direct PHA accumulation with selective microbial growth is also expected to be applied to larger scale PHA production despite the experimental work was carried out with 1 L lab reactors in this work. Comparability of direct PHA accumulation performance was evaluated across multiple production scales—including a 200 L pilot and a 4 m^3 demonstration-scale bioreactor in the literature [23]. Production scale was not found to influence the accumulation performance outcomes, given all other factors being kept the same (i.e. substrate quality, total accumulation time, bioprocess control methods, etc.).

The present investigation has addressed methods for enhanced productivity in PHA *quantity* by stimulating selective growth in a direct accumulation bioprocess. PHA *quality* and its quality control are also critical for an industrial polymer production process. PHA quality relates to average monomer content, *co*-polymer blend distribution, and the molecular weight distribution [75]. Previous research shows repeatedly, for direct accumulation on waste activated sludge, that the average monomer content, and co-polymer blend composition are a function of

the feedstock composition [23,45]. Similarly, the applied bioprocess conditions (including attention to details of the downstream processing – i.e. biomass acidification and drying conditions) will deliver polymers with similarly high weight average molecular weight (order of 1000 kDa) and polydispersity index (≈ 2) [76]. Acetic and butyric acids produce PHB during direct accumulation with WAS [43]. The PHB will be formed in granules by a biological process of step chain elongation, with steps of chain initiation, propagation and termination [77,78]. The higher the probability for chain termination is, the lower the average molecular weight will be. In previous work, delay in dosing with feed-on-demand was interpreted to result in outcomes of reduced molecular weight [19]. On the other hand, feeding in excess has also led to slightly reduced but still statistically significantly lower molecular weights on average when using direct accumulation [45]. The interpreted ATP-driven mechanisms markedly enable to increase production productivity due to active growth. The combined growth and storage responses may influence intracellular carbon fluxes resulting in a change the PHA storage termination probability for polymerization. Therefore, any potential influence of concurrent growth on the molecular weight of PHA produced from WAS needs to be evaluated and confirmed through continued investigation.

Nutrient (N and P) availability relative to substrate supply is critical for promoting selective growth of PHA storing microorganisms while suppressing non-storing populations during direct accumulation. In this work, a consistent nutrient limitation (COD:N:P = 100:1:0.05 on mass basis) was applied to compare selective growth and PHA storage responses for butyrate and acetate feeds. The findings showed the robustness of the ATP-driven mechanism. However, the applied nutrient loadings were suboptimal to fully support growth response in butyrate fed systems. Nutrient demands should be balanced with the requirement for selective biomass growth. Therefore, control of nutrient loading and aeration level for optimal selective growth during direct accumulation of PHA with WAS is required. In future research and technology upscaling, bioprocess design and control must be developed to accommodate varying feedstock qualities, including organic carbon concentrations and relative nutrient contents [46,79].

5. Conclusion

In this work, the simultaneous growth and PHA storage responses of waste activated sludge microorganisms were observed and explained. This simultaneous growth of PHA-storing microorganisms requires surplus ATP supply in sufficient excess of demand for transport, maintenance and PHA storage. Butyrate is a substrate that has lowered ATP demand and generates net NADH when converted to PHA, and this NADH can be converted to ATP in surplus of what is needed for the butyrate conversion. The surplus ATP generated during butyrate conversion is subsequently used for microbial growth. The increased active biomass production allows for extra PHA accumulation in the municipal waste activated sludge. By stimulating combined growth and storage responses, volumetric PHA productivity was improved by four times, and PHA contents of up to 0.61 gPHA/gVSS were obtained. High PHA yields of nominally 0.6 $gCOD_{PHB}/gCOD_{HBu}$ can be maintained over 20 h of accumulation alongside this concurrent and selective microbial growth. This study establishes principles from which possibilities can be explored for enhanced PHA production in waste activated sludge.

CRediT authorship contribution statement

Yizhou Xing: Writing – original draft, Visualization, Investigation, Formal analysis, Conceptualization. **Ruizhe Pei:** Writing – review & editing, Formal analysis, Conceptualization. **Marta F.S. Cardoso:** Writing – review & editing, Investigation. **H. Pieter J. van Veelen:** Writing – review & editing, Visualization, Formal analysis. **Mark C.M. van Loosdrecht:** Writing – review & editing, Supervision, Conceptualization. **Robbert Kleerebezem:** Writing – review & editing, Supervision, Conceptualization. **Alan Werker:** Writing – original draft, Supervision, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cej.2025.165907.

Data availability

Data will be made available on request.

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