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Calcium enhances polyhydroxyalkanoate production and promotes selective growth of the polyhydroxyalkanoate-storing biomass in municipal activated sludge

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ABSTRACT

Activated sludge from municipal wastewater treatment processes can be used directly for the production of biodegradable polyesters from the family of polyhydroxyalkanoates (PHAs). However, municipal activated sludge typically cannot accumulate PHAs to very high levels and often low yields of polymer produced on substrate are observed. In the present work, it was found that the presence of calcium promotes selective growth and enrichment of the PHA-storing biomass fraction and significantly improved both PHA contents and yields. Calcium addition resulted in PHA contents of 0.60 ± 0.03 gPHA/gVSS and average PHA yields on substrate of 0.49 ± 0.03 $gCOD_{PHA}/gCOD_{HAc}$ compared to 0.35 ± 0.01 gPHA/gVSS and 0.19 ± 0.01 $gCOD_{PHA}/gCOD_{HAc}$ without calcium addition. After 48 h, three times more PHA was produced compared to control experiments without calcium addition. Higher PHA content and selective biomass production is proposed to be a consequence of calcium dependent increased levels of passive acetate uptake. Such more efficient substrate uptake could be related to a formation of calcium acetate complexes. Findings lead to bioprocess methods to stimulate a short-term selective growth of PHA-storing microorganisms and this enables improvements to the techno-economic feasibility for municipal waste activated sludge to become a generic resource for industrial scale PHA production.

1. Introduction

Microbial community engineering aims to produce energy carriers and chemicals from waste streams using microbial communities (Kleerebezem et al., 2015). Microbial community engineering relies on the use of ecological selection principles for the enrichment of microbial communities with a desired functionality. An example of the use of microbial community engineering principles is the production of biopolymers such as polyhydroxyalkonoates (PHAs) (Kourmentza et al., 2017; Sabapathy et al., 2020; Estévez-Alonso et al., 2021b). PHAs are naturally occurring polyesters that are produced intracellularly by bacteria to balance their growth while dealing with dynamic conditions, such as changes in substrate availability (Van Loosdrecht et al., 1997; Reis et al., 2003). In microbial community-based PHA production, PHA-storing bacteria are first enriched in a so-called *selection* reactor by applying intermittent presence and absence of carbon sources (volatile fatty acids) and/or uncoupling of organic carbon and growth nutrients supplies, namely nitrogen and phosphorus (Lemos et al., 2006; Lorini et al., 2020). Subsequently, the surplus biomass produced in the selection reactor is exploited in an *accumulation* reactor to maximize intracellular PHA content of the PHA-storing biomass (Sabapathy et al., 2020; Kourmentza et al., 2017). The maximum intracellular PHA content is constrained by the fraction of PHA-storing bacteria and the specific storage capacity of the microbial strains present in the microbial community. For instance, with enrichment cultures, where the whole microbial community can produce PHA, intracellular PHA contents of up to 0.9 gPHA/gVSS have been achieved (Johnson et al., 2009a; Jiang et al., 2011a). Notwithstanding many species of bacteria exhibit only moderate maximum storage capacity in the range from 0.5 to 0.8 gPHA/gVSS (Estévez-Alonso et al., 2021b; Sabapathy et al., 2020; Kourmentza et al., 2017). When selected microbial communities have a lower fraction of PHA-storing bacteria and therefore not all bacteria are able to produce PHAs, lower average PHA contents are observed, even if the PHA-storing bacteria that are present can accumulate up to 0.9 gPHA/gVSS (Marang et al., 2014; Crognale et al., 2019a).

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An example of a microbial community that exhibits a lower fraction of PHA-storing bacteria with moderate storage capacity is municipal activated sludge (Bengtsson et al., 2017; Pei et al., 2022a). The attraction to use of waste municipal activate sludge for PHA production is limited, in general, due to lower PHA contents in combination with lower PHA yields on substrate. Average PHA contents and yields on substrate that have been typically reported for municipal activated sludge are not higher than 0.5 gPHA/gVSS and 0.5 $gCOD_{PHA}/gCOD_{HAc}$ (Bengtsson et al., 2017; Kourmentza et al., 2017). These values are significantly lower than those that have been obtained with specifically enriched biomass. It has been recently shown that the PHA-storing bacteria already present in municipal activated sludge systems can accumulate up to 0.6 gPHA/gVSS (Pei et al., 2022a). Thus, if strategies are developed to consistently realize such high PHA contents with municipal activated sludge, it could broaden the generic potential for biopolymer supply chains. Municipal waste activated sludge could become an ubiquitous readily available biomass source for industrial scale microbial community based PHA production.

Strategies to reach higher PHA contents with municipal activated sludge are expected to rely on methods that increase the fraction of PHA-storing bacteria in the biomass (Pei et al., 2022a). An increase in the fraction of PHA-storing bacteria will consequently result in a process that reaches the biomass maximum PHA accumulation potential. It has been hypothesized that the fraction of PHA-storing bacteria can be increased directly in the PHA accumulation process if conditions for robustly promoting selective growth of the PHA-storing biomass can be identified and applied (Pei et al., 2022a). During cell division, daughter cells contain half of the cellular PHA content of the mother cells (Pfeiffer and Jendrossek, 2012). Consequently, more PHA can be formed in parallel to cell division. In this way, PHA production and biomass growth may occur concurrently and, theoretically given the right conditions, indefinitely. A challenge is to find those conditions where these two processes can co-occur, with selective growth of PHA storing bacteria only. In pure cultures, and highly enriched open cultures, examples for simultaneous growth and PHA accumulation have already been reported (Mulders et al., 2020; Cavaillé et al., 2016; Valentino et al., 2015; Grousseau et al., 2013). However, it is unknown if a similar strategy could be applied to diverse microbial communities that have not been specifically selected for PHA accumulation. For activated sludge systems, Cavaillé et al. (2013) found that different degrees of phosphorus limitation promoted simultaneous growth and accumulation of PHA. Nonetheless, this strategy required a strict phosphorus level control and resulted in lower PHA yields on substrate compared to enrichment cultures.

The present work stemmed from unexpected observations made during the search for selective conditions promoting for simultaneous growth and PHA accumulation. This work concerns an investigation of effects of calcium during PHA accumulation with municipal activated sludge. At lab-scale, volatile fatty acid solutions are often used to mimic acidic fermented feedstocks. Such feedstocks are to be used for industrial scale PHA production (Bengtsson et al., 2017). Sodium or potassium hydroxide are normally added to these feedstock solutions for pH control. In preliminary experiments, when calcium hydroxide was used instead to increase the pH of an acetic acid feedstock, it was observed that biomass growth and PHA production were positively affected. The addition of calcium in the feedstock solution induced changes that apparently created favourable conditions in the reactor for selective biomass growth concurrent to PHA production. However, since calcium addition could have influenced several water quality factors, the underlying causes for observed concurrent biomass growth with PHA production were not clear. The aim of this study was to identify the mechanism(s) behind the observed increase in PHA production and biomass growth due to calcium presence.

2. Materials and methods

2.1. Experimental set-up

PHA accumulation tests were performed over 24 and/or 48 h in a 1 L double-jacketed glass bioreactor, as previously described in Estévez-Alonso et al. (2022). Reactor temperature was 25 ±0.1°C, pH was monitored, and air was supplied at 1 L/min. In some experiments, the pH was controlled to 8.0 ± 0.1 with 1 M NaOH solution, as indicated in Table 1.

2.2. Sludge source and feedstock

Activated sludge from Bath WWTP (The Netherlands) was used as the principal PHA-storing biomass for the PHA accumulation tests. The activated sludge samples were taken after gravity belt thickening and had a total solids concentration of 55 gTS/L. Validation experiments with calcium hydroxide were also performed with activated sludge from four other different Dutch WWTPs (Leeuwarden, Beverwijk, Almere and Winsum). These validation experiments were compared to outcomes from a parallel study (Pei et al., 2022a). For these other experiments, activated sludge samples were taken directly from the main aerobic process volume. The mixed liquor suspended solids were concentrated by gravity settling for 30–60 min with decanting, and had a total solids concentration of 6 to 12 gTS/L. The concentrated mixed liquor was delivered on the same day to Wetsus (Leeuwarden, The Netherlands) and stored at 4°C for further analysis.

The accumulation feedstock, with nutrients ratio 100:1 (COD:N by weight), was prepared with tap water as follows: 50 g/L acetic acid and 1.91 g/L NH_4Cl. Phosphate levels were changed by the addition of KH_2PO_4, as described in Table 1. The feedstock was adjusted to pH 4.5 with 20 $gCa(OH)_2$, 15.5 $gMg(OH)_2$, 30 gKOH or 21.5 gNaOH, as described in Table 1. Side experiments were performed with the supplementary addition of $CaCl_2$ and KCl to the feedstock.

2.3. PHA accumulation experiments

For each assay, the concentrated activated sludge samples were diluted with tap water to nominally 2–3 gVSS/L and allylthiourea (50 mg/L) was added. The mixed liquor was brought to 25°C and conditioned with constant aeration overnight to establish a baseline of endogenous microbial activity. Subsequently, an automated acclimation was performed. Acclimation comprised three feast and famine cycles as previously reported (Morgan-Sagastume et al., 2017). Feast conditions were generated with a pulse input to reach a maximum substrate level of 150 mgCOD/L and the duration of the feast was determined from changes in respiration based on dissolved oxygen concentration trends. The famine period was dynamically adjusted to be three times longer than each respective feast time. In total, the acclimation period had a duration of 4–5 h. The trends in respiration were used to determine the oxygen mass transfer coefficient (k_La).

After the third acclimation famine period, the accumulation test was started automatically. Accumulation was driven with the same feast influent pulses and control logic, but now without any famine period between pulses. Pulse inputs were controlled from on-line monitoring of dissolved oxygen according to Valentino et al. (2015).

2.4. Analytical methods

The PHA accumulation process was monitored by online data logging (DO, pH and temperature), liquid and solids analyses. Grab samples were used for liquid and solids analyses, biomass PHA content and $CaCO_3$ determination. Suspended solids were separated from the mixed liquor by centrifugation (3250 rcf at 4°C for 20 min). The supernatant after membrane filtration (0.45 μm pore size filters) was stored at −20°C pending liquid analyses. Acetic acid concentration was determined

Table 1
Overview of the accumulation tests and feedstock solutions used.

Experiment	Feedstock solution			Reactor		
	Counterion	Salt	Phosphate	pH control	Duration	N° of tests
–	–	–	mgP/L	pH 8 ± 0.1	h	–
1	Ca^{2+}	–	25	No	48	7
2	Ca^{2+}	KCl	25	No	24	3
3	Ca^{2+}	–	250	No	24	2
4	Ca^{2+}	–	25	Yes	24	1
5	K^{+}	–	25	No	48	7
6	K^{+}	$CaCl_2$	25	No	24	4
7	K^{+}	–	2.5	No	24	2
8	K^{+}	–	25	Yes	24	1
9	Na^{+}	–	25	No	48	1
10	Mg^{2+}	–	25	No	48	1

by ultra-high pressure liquid chromatography and ammonium, nitrite, nitrate, and phosphate concentrations were determined by ion chromatography, as previously reported (Estévez-Alonso et al., 2021a). The harvested biomass pellet dry weight and ash contents were estimated based on standard methods and referenced to the sample volume for total and volatile suspended solids (TSS and VSS), respectively.

The aliquot for PHA determination was directly acidified to pH 2 with 37% HCl. After thorough mixing for 5 min, suspended solids were collected (3250 rcf at 4°C for 20 min). The biomass pellet was retained and dried at 105°C. Dried pellets were ground and analysed by thermogravimetric analysis as described previously (Chan et al., 2017). Similarly, the aliquot for $CaCO_3$ determination was directly centrifuged (3250 rcf at 4°C for 20 min) and the biomass pellet was retained and dried at 105°C. Dried pellets were ground and analysed by thermogravimetric analysis. 5 mg of ground sub-sample were introduced to the furnace at 80°C and heated to 105°C (10 °C /min) under nitrogen atmosphere. After drying at 105°C for 10 min, the sample was heated to 550°C (10 °C/min), under nitrogen atmosphere. At 550°C, the atmosphere was switched to air and temperature was held for 30 min. After 30 min at 550°C, the sample was heated to 900°C. The weight loss trends in air were used to determine inorganic content and the calcium carbonate fraction with respect to the biomass dried total and volatile solids.

2.5. Microscopy analysis

Mixed liquor sludge samples were taken at selected time points during the PHA accumulation process. The mixed liquor was fixed with formaldehyde to a final concentration of 3.7% and preserved in a solution with ratio 1:1 of 1x PBS and pure ethanol. The fixed samples were 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 with confocal laser scanning microscope with statistics from fields of view, as previously described (Pei et al., 2022b). Respective overlaid stained images were acquired with a 16-bit depth in separate channels and analysed by Fiji Image J (ImageJ2, Ver 1.52P) software. An average biomass volume-to-volume ratio of polymer to non PHA-storing biomass (v/v) was estimated (Pei et al., 2022b). This ratio was used for the determination of the fraction of PHA-storing biomass in the microbial community.

2.6. Microbial community structure

Fresh samples were collected at selected time points from the accumulation reactor and stored at –20 °C until DNA extraction. Before extraction, the samples were washed with 1x PBS solution and sonicated for 30 s. DNA was extracted from 0.5 mL mixed sample using a FastDNA® SPIN kit for soil (MPBio, USA) according to manufacturer instructions. Extracted DNA samples were purified with the kit DNA Clean & Concentrator™-5 ZYMO Research according to manufacturer instructions. DNA purity and concentrations were measured by a NanoDrop® spectrophotometer (Thermo Fisher Scientific, Germany) and a Quantus™ Fluorometer (Promega Corporation, USA), respectively. Purified DNA samples were normalized to 20 ng/μl for library preparation and 16s rRNA gene amplicon sequencing on an Illumina MiSeq at MrDNA (TX, USA). Libraries for bacteria were constructed using primers 515F (Parada et al., 2016) and 926R (Quince et al., 2011). Using QIIME2 v2019.10 (Bolyen et al., 2019) quality filtering and sequence data processing was applied. Sequences were demultiplexed with cutadapt (Martin, 2011). Trimming was performed after the first two bases and at lengths 190 and 200 bp for forward and reverse reads, respectively. DADA2 was used for error correction, amplicon sequence variant (ASV) inference and dereplication using default parameters, except that maxEE values were set to 4 (Callahan et al., 2016). Taxonomic assignment was performed with a naïve Bayesian classifier (Bokulich et al., 2018) trained on the curated MiDAS4 database tailored to wastewater microbial communities (Dueholm et al., 2022). The feature table and taxonomic information along with metadata were imported in R v4.0.3 using qiime2R (Bisanz, 2018) into phyloseq (McMurdie and Holmes, 2013). A reproducible workflow including all QIIME2 scripts and a R markdown document is available on Github (https://github.com/pietervanveelen/AEST_PHA_accumulation). The raw sequence data are deposited in the European Nucleotide Archive under project accession number PRJEB56263.

2.7. Data analysis

All measured parameters were corrected for effects of sample withdrawal and feedstock addition from liquid and mass balance considerations (Johnson et al., 2009b). The biomass PHA content was expressed as mass fraction of the volatile suspended solids (gPHA/gVSS). Active biomass (X_a) was estimated as the total VSS minus PHA mass. Active biomass was assumed to be represented as $CH_{1.8}O_{0.5}N_{0.2}$ (Roels, 1980). The trend for change in PHA content (f_{PHA}) was represented by least squares regression to the empirical function:

$$f_{PHA} = A_0 + A_1\left(1 - e^{-kt}\right) \quad (1)$$

where A_0, A_1 and k are constants that allow for estimation of rates as a function of time and comparison between performances of different activated sludge samples (Bengtsson et al., 2017). Initial and average specific production/consumption rates and PHA yields on substrate were estimated for different times. The average PHA yields on substrate are reported on a COD-basis and calculated from the amount of PHA (1.67 gCOD/gPHB) produced and substrate (1.07 gCOD/gHAc) added as a function of time. Average specific production and consumption

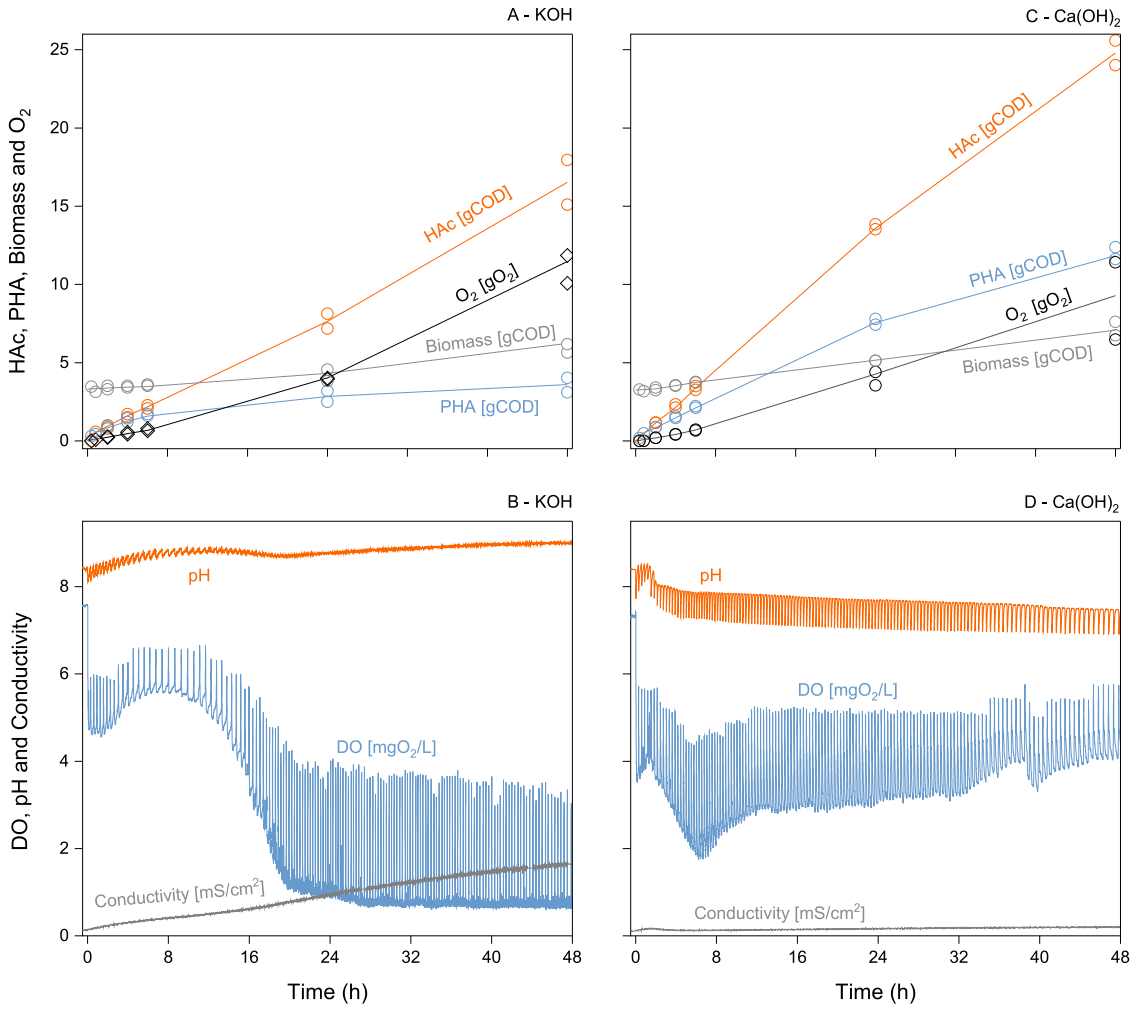

Fig. 1. Trends of dissolved oxygen, pH, conductivity (Figure C & D), acetate consumption, oxygen consumption, PHA production, and biomass production (Figure A & B) during PHA accumulation tests with either KOH or $Ca(OH)_2$ added to the substrate and activated sludge from Bath WWTP. For the top graphs A and B, measured values at selected times are connected simply by straight lines to help in readability.

rates were calculated based on the cumulative amounts of acetic acid, PHA, biomass and oxygen consumed with respect to the estimated active biomass levels ($gCOD/gX_a/h$).

The average PHA content in the PHA-storing biomass fraction was calculated as follows:

$$\text{Average } f_{PHA} \text{ in PHA-storing fraction} = \frac{f_{PHA}}{f_{PHA} + DE \cdot X_a} \quad (2)$$

where DE is the volume to volume ratio of polymer to non PHA-storing biomass by the end of the accumulation process obtained from staining and microscopy image statistical analyses (Section 2.5).

Microbial community analysis was based on an average of 71245 sequences per sample (range = 21463–149580), which were then filtered to retain only abundant bacterial taxa (ASVs), each representing greater than 0.05% of the total sequence abundance (i.e. retaining > 99% of sequences, 2398 ASVs). A dataset comprising timepoints 0, 24 and 48 h was rarefied (26538 reads per sample, 100 iterations) to account for unequal sampling. Microbial community composition dynamics were visualized using principal coordinate analysis (PCoA) on the ecological Bray–Curtis dissimilarity matrix using *vegan* (Oksanen et al., 2020). A permutational multivariate analysis of variance (PERMANOVA) was applied to test the effects of feedstock and operation time on microbial biomass composition. The contributions of the 15 most dominant bacteria were depicted in the PCoA. ANOVA was used to evaluate these effects on Shannon diversity.

3. Results

3.1. Preliminary results giving rise to the work described in this paper

In preliminary tests with municipal activated sludge for PHA production, calcium hydroxide was used to increase the pH of an acetic acid feedstock solution. In these tests, a distinctly different biomass response was observed compared to experiments where KOH was used (Fig. 1). This response was also observed in replicate experiments with four other types of municipal activated sludge, as provided in the SI. The addition of calcium hydroxide in the feedstock solution induced changes in the reactor conditions that affected the calcium and salts concentrations, phosphate availability and pH changes over the accumulation. Calcium carbonate precipitation was observed already in the first two hours of the accumulation, and therefore the concentration of soluble calcium in the mixed liquor did not increase above 500 $mgCa^{2+}/L$ over the time of accumulation. After 48 h, 50 to 55% of the total dry solids in the reactor could be identified as calcium carbonate. Calcium balances showed that the calcium added to the reactor could be found back mainly as calcium carbonate or dissolved calcium. The precipitation of calcium carbonate induced a slow, but constant decrease of pH from an initial value between 8.2 and 8.4 to a final value between 7.0 and 7.5 after 48 h of accumulation. Similarly,

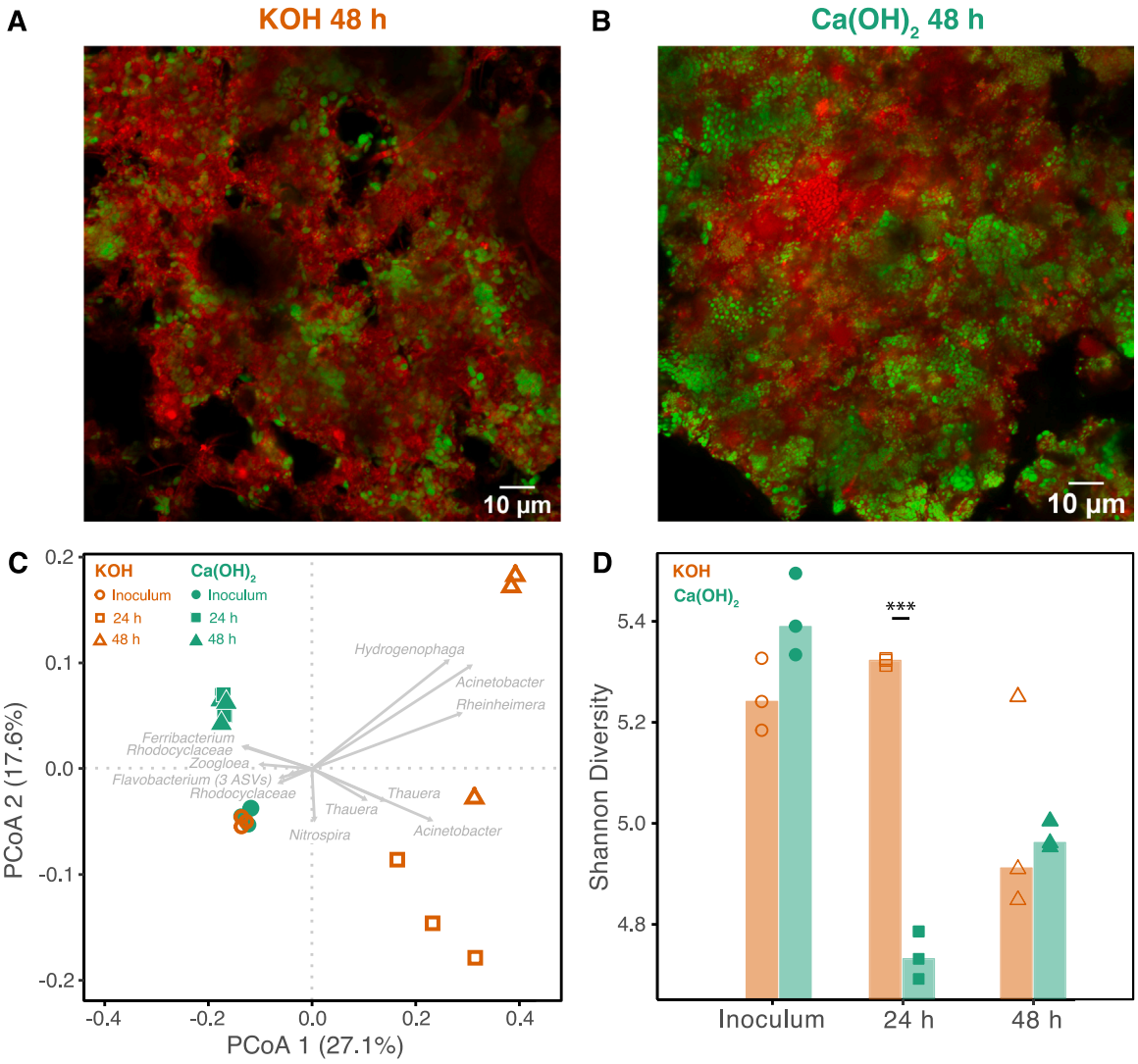

Fig. 2. Representative microscopy images of the PHA accumulations with (A) KOH and (B) $Ca(OH)_2$ at 48 h. Red staining depicts the typical floc morphology and green stain shows the distribution of PHA granules in the biomass. (C) Shifts in microbial community in experiments with $Ca(OH)_2$ and KOH feedstocks. Principal coordinate analysis from experiments in triplicate shows predictable shifts in microbial community composition with $Ca(OH)_2$ feedstock. These shifts were driven by known PHA-storing bacteria, while different and less predictable community compositions developed with KOH feedstock. (D) Evolution of the Shannon diversity in experiments with $Ca(OH)_2$ and KOH feedstocks. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

phosphate concentrations were below the detection limit from the start of the accumulation.

To elucidate the cause for the biomass response due to calcium added to the feedstock, deepened investigations were performed with activated sludge from Bath WWTP. PHA accumulation tests were performed with $Ca(OH)_2$, $Mg(OH)_2$, KOH and NaOH to evaluate if the observed biomass response was specific to calcium. PHA accumulations tests were also performed to evaluate the potential for influences on concurrent selective biomass growth with PHA production caused by salinity, phosphate concentration, and pH control.

3.2. PHA accumulations with $Ca(OH)_2$ and KOH

A comparison of 48 h PHA accumulations tests with $Ca(OH)_2$ or KOH is provided in Fig. 1. In the presence of calcium, a higher substrate consumption was observed. This increased consumption was associated with much higher PHA production, 11.5–12.4 $gCOD_{PHA}$ compared to only 3.1–4.0 $gCOD_{PHA}$ in KOH tests. Maximum biomass PHA contents in the range of 0.56–0.64 gPHA/gVSS were obtained with the addition of $Ca(OH)_2$ compared to only 0.35–37 gPHA/gVSS for KOH cases. Similarly, the average PHA yield on substrate obtained at 48 h was higher for $Ca(OH)_2$, 0.44–0.52 $gCOD_{PHA}/gCOD_{HAc}$, compared to 0.18–0.21 $gCOD_{PHA}/gCOD_{HAc}$ for KOH. Lower average oxygen yields on substrate were also observed for $Ca(OH)_2$, 0.29–0.46 $gO_2/gCOD_{HAc}$, compared to 0.65–0.69 $gO_2/gCOD_{HAc}$ for KOH (Fig. 3). COD mass balances closed well for $Ca(OH)_2$ and KOH tests, 105 ± 6% and 102 ± 5%, respectively.

Biomass growth was slightly higher in $Ca(OH)_2$ assays, 3.4–4.3 $gCOD_X$, compared to 2.6–3.3 $gCOD_X$ in KOH tests. In $Ca(OH)_2$ accumulations, biomass growth was observed already from the beginning of the accumulation. In KOH experiments, biomass growth only started after 4-6 h. From microscope staining, a higher fraction of PHA-storing biomass was observed at 48 h in $Ca(OH)_2$ compared to KOH, as illustrated in Fig. 2. The fraction of PHA-storing biomass at 48 h was 0.76–0.80 (v/v) compared to 0.29–0.51 (v/v) in experiments with KOH. Despite different values in the fraction of PHA-storing biomass, the derived average PHA content within just the PHA-storing fraction of the biomass was estimated to be similar in both cases, 0.63 and 0.58 gPHA/gVSS. 16s rRNA gene amplicon sequencing analysis demonstrated that the addition of $Ca(OH)_2$ versus KOH to the feedstock promoted differential developments of the microbial community composition, as shown in Fig. 2 (PERMANOVA; interaction timepoint:feedstock: F2, 17 = 8.4, P = 0.002; Fig. 2C). Addition of

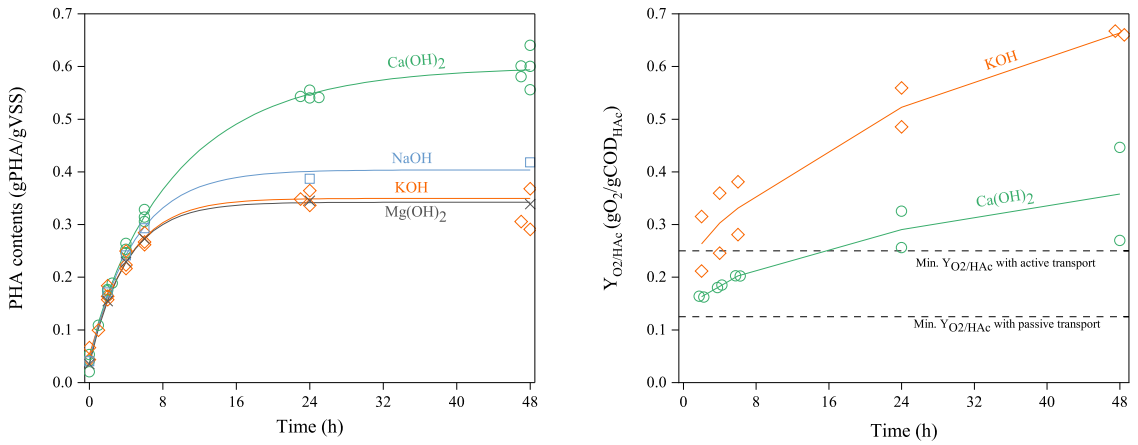

Fig. 3. PHA content evolution for PHA accumulations with KOH, NaOH, $Mg(OH)_2$ and $Ca(OH)_2$ (left) and evolution of average oxygen yields for PHA accumulations with KOH and $Ca(OH)_2$ (right).

$Ca(OH)_2$ caused a rapid drop in diversity within 24 h with a shift in biomass composition that remained stable up to 48 h. This $Ca(OH)_2$ induced shift was associated with *Ferribacterium*, *Zoogloea* and other dominant *Rhodocyclaceae* genera. Conversely, addition of KOH induced a different kind of shift with retained microbial composition up to 24 h followed by a later decrease in diversity between 24 and 48 h corresponding to when most growth took place. Shifts induced by KOH addition were associated with *Acinetobacter*, *Thauera*, *Hydrogenophaga* and other dominant genera that, in contrast to $Ca(OH)_2$, continued to change in composition between 24 and 48 h.

3.3. PHA accumulations with $Mg(OH)_2$ and NaOH

To investigate if the observed effect was calcium ion, and/or cation valence specific, additional PHA accumulation tests were performed with Mg^{2+} and Na^+ as the hydroxide counterion. $Mg(OH)_2$ and NaOH were used to raise the feedstock pH to 4.5 instead. The addition of $Mg(OH)_2$ and NaOH resulted in similar pH profiles as in experiments with KOH. Also, the process performance in terms of PHA production was very similar the KOH experiments, as observed in Fig. 3. These results pointed to a specific effect related to, or created directly by, the presence of calcium cations during the accumulation process.

3.4. Increasing calcium concentrations in KOH experiments

To further evaluate the effect of calcium addition, accumulation experiments with KOH and extra $CaCl_2$ amounts were performed. With increasing calcium concentration, the PHA production and biomass growth increased, as observed in Fig. 4. The test with KOH and 30 $gCaCl_2/L$ had the same dissolved calcium concentration as the feedstock with $Ca(OH)_2$ and replicated the observations of tests with only $Ca(OH)_2$. Calcium carbonate precipitation occurred from the beginning of the experiment and similar trends of pH were observed. Despite these similarities in reactor profile, the PHA production performance was lower than in experiments with $Ca(OH)_2$, but still significantly higher than in experiments with only KOH. An identifiable key distinction between tests with added calcium salts, and experiments with only $Ca(OH)_2$ was the much higher increase in salinity, due to the addition of $CaCl_2$ to the feedstock.

3.5. Increasing salinity levels in $Ca(OH)_2$ experiments

The addition of $CaCl_2$ to experiments with KOH in the feedstock resulted in higher salts concentrations in the reactor compared to experiments with $Ca(OH)_2$. To evaluate the influence of salinity in $Ca(OH)_2$ experiments, control experiments with $Ca(OH)_2$ and different KCl concentrations were performed. Similar trends were observed in all experiments, independent of the concentration of KCl in the feedstock. Calcium carbonate precipitation resulted in a progressive decrease in pH to around 7.0–7.5. Despite similarities to experiments with $Ca(OH)_2$, higher concentrations of KCl reduced the PHA production performance, as observed in Fig. 4. Experiments with $Ca(OH)_2$ and 40 gKCl/L were analogous to experiments with KOH and 30 $gCaCl_2/L$, and similar results were obtained, as it was expected.

3.6. Phosphate excess and limitation in accumulations with $Ca(OH)_2$ and KOH

In experiments with $Ca(OH)_2$, phosphate concentrations were not detectable (< 0.33 mgP/L) in the reactor after 2-3 h of accumulation. The addition of $Ca(OH)_2$ in the feedstock is anticipated to promote the precipitation of calcium phosphate, making the conditions phosphate limiting during most of the PHA accumulation time, notwithstanding observed biomass growth. To evaluate for an influence of phosphate limitation, control experiments were performed to evaluate KOH with phosphate limitation, as well as experiments with $Ca(OH)_2$ with phosphate excess. In these cases, no systematic differences were observed due to the imposed phosphate limitation or phosphate excess, respectively.

3.7. pH control in PHA accumulations with $Ca(OH)_2$ and KOH

The addition of $Ca(OH)_2$ in the feedstock resulted in a distinctly different pH profile during accumulations compared to KOH experiments. This difference was interpreted to be mainly due to precipitation of calcium carbonate. To allow for a more direct comparison between KOH and $Ca(OH)_2$ tests, PHA accumulation tests with pH controlled to 8 were performed, as depicted in Fig. 4. pH control did not have an observable influence in the KOH experiments. However in the $Ca(OH)_2$ tests with pH control, lower PHA contents, PHA yields on substrate, and total PHA and biomass mass increase were observed. pH control was performed with additions from a 1 M NaOH stock solution, and 0.87 g of sodium were added to the reactor over 24 h.

4. Discussion

The combination of tests performed in this work lead to the conclusion that the presence of calcium is beneficial for selective growth of PHA-storing bacteria and PHA accumulation in waste activated sludge. Calcium presence resulted in PHA contents of up to 0.6 gPHA/gVSS, higher than previously reported. These results broaden the potential to exploit municipal waste activated sludge as a generic resource for

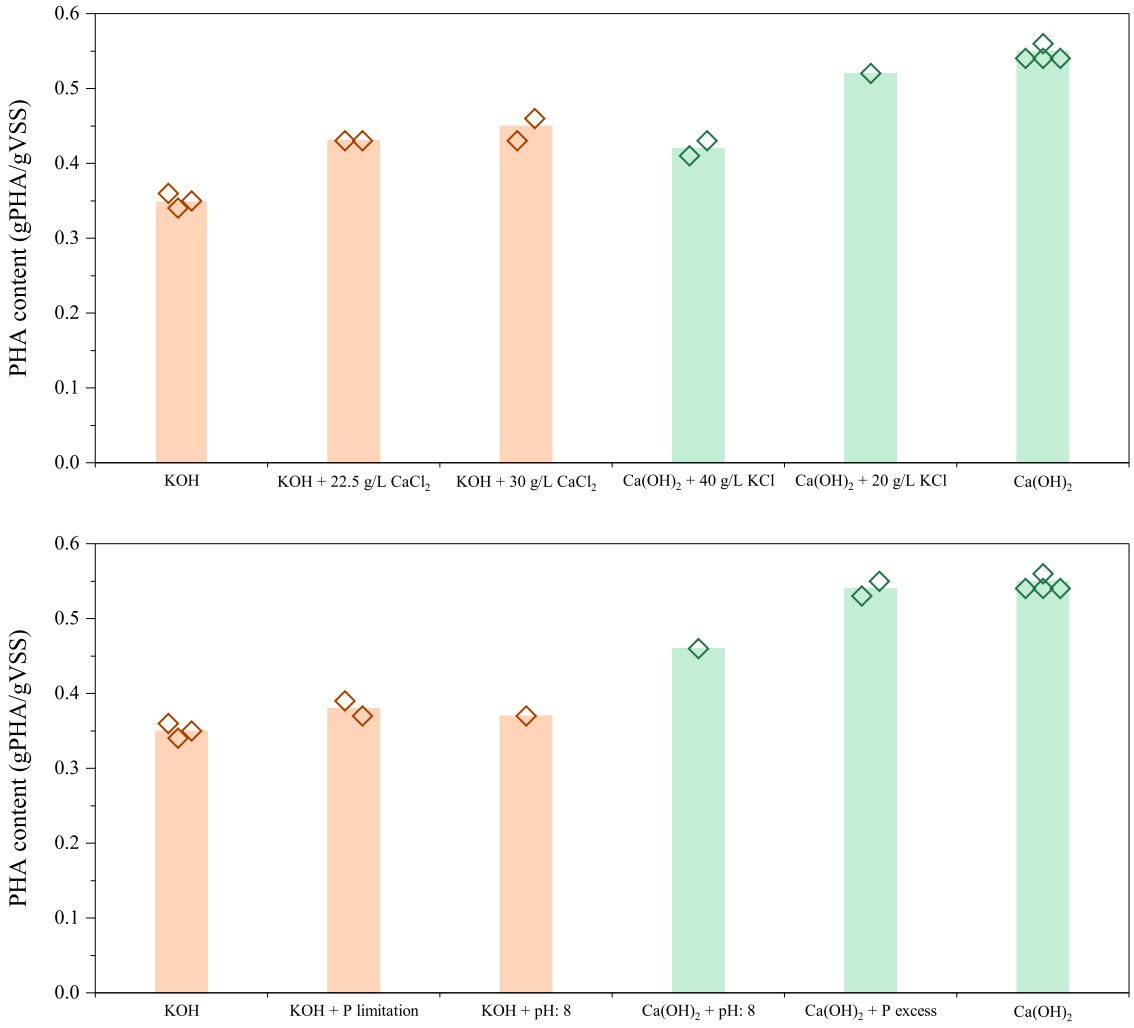

Fig. 4. Maximum PHA contents in KOH tests with increasing calcium concentrations, $Ca(OH)_2$ tests with increasing salinity levels, $Ca(OH)_2$ and KOH tests with phosphate excess (250 mgP/L) and limitation (2.5 mgP/L), and $Ca(OH)_2$ and KOH tests with pH controlled to 8.

industrial scale PHA production. With reference to the literature and by process of elimination results were examined to identify causal mechanisms for the observed influence of calcium. One explanation that could not be ruled out was the potential for formation of calcium-acetate complexes that can be more efficiently transported into the cell compared to the native acetate ion. Development of the consideration leading to this hypothesis follows.

4.1. Bacterial growth and PHA production are affected by the presence of calcium

In this work, PHA production and selective biomass growth were positively affected by the presence of calcium during accumulation. Calcium was provided due to the addition of $Ca(OH)_2$ to the feedstock used for PHA accumulation. The reproducibility of the increase in PHA production rates and yields was verified with more than ten PHA accumulations with $Ca(OH)_2$ and KOH, and with activated sludge samples obtained from different WWTPs. The increase resulted only when $Ca(OH)_2$ was added to the feedstock and could not be mimicked by the addition of KOH, NaOH or $Mg(OH)_2$, as observed in Fig. 3. Calcium caused precipitation of mineral salts that lowered reactor phosphate concentrations and promoted a decrease in pH. These conditions were replicated by the addition of $CaCl_2$ in experiments with KOH, as observed in Fig. 4. However, added $CaCl_2$ also resulted in a higher salinity. A negative effect of increased salinity was verified by the addition of KCl in the feedstock of $Ca(OH)_2$ tests, as observed in Fig. 4. Even though a higher salinity did negatively influence the PHA production performance, a salt effect alone could not explain observed differences between $Ca(OH)_2$ and KOH experiments. A higher pH in $Ca(OH)_2$ experiments (8 ± 0.1) also resulted in lower PHA production rates. The addition of sodium due to pH control was not high enough to explain the decrease in performance as a consequence of salinity. This outcome suggests that the observed influence that calcium may exert is pH dependent. In the same line of reasoning from control experiments, phosphate limitation due to mineral precipitation in cases with $Ca(OH)_2$ was not found to be a cause for improved PHA production. The combination of tests and control experiments lead to the interpretation that it was the presence of calcium, directly and not the associated changes in environment created by the addition of calcium, that resulted to stimulate selective bacterial growth with concurrent PHA production right from the start of accumulation.

4.2. Calcium addition affects the cellular energy requirements and may promote passive acetate uptake

Calcium ions are known to play different roles in eukaryotic cells (Clapham, 2007). However, the role of calcium in prokaryotic cells remains unclear and research is limited (Domínguez et al., 2015). High calcium concentrations are expected to negatively affect cell activity, due to creating a high osmotic pressure. However, calcium

has also been reported to benefit the stability of cell flocs and granules (Gagliano et al., 2020). More related to the current work, calcium has been reported to provoke metabolic shifts in polyphosphate accumulating microorganisms, to be involved in the formation of PHA granules, and in ion co-transport processes in the animal colon and in plant leaflets (Zhang et al., 2015; Trinidad et al., 1996, 1999; Borchert, 1986; Wolever et al., 1995).

In polyphosphate accumulating microorganisms, a direct effect of calcium was observed. An increasing calcium concentration resulted in a lower available phosphate concentration, due to calcium phosphate precipitation. Due to lower available phosphate, a metabolic shift was observed from polyphosphate to glycogen metabolism (Zhang et al., 2015). The use of glycogen slightly increased the production of PHA, with a higher content of hydroxyvalerate. In the present work, even though phosphate limitation was observed, the microbial community was not highly enriched in polyphosphate or glycogen accumulating microorganisms. This hypothesis cannot be extrapolated in general for heterotrophic bacteria and thus, it cannot explain the results obtained in $Ca(OH)_2$ tests.

Tian et al. (2019) have suggested that calcium has a structural role in the formation of PHA granules, that are also used as calcium storage units. Calcium was found to be the most abundant cation in PHA granules from *C. necator* H16 (up to 4 $mgCa^{2+}$/gPHB) and it was proposed that calcium ions play a role in bundling the carboxyl end-groups during initial stages of the polymerization. This would imply that a minimum concentration of calcium is required to efficiently drive PHA production. In the work of Tian et al. (2019), this minimum concentration was around 25 $mgCa^{2+}$/L. In the present work, the concentration of calcium was higher than 25 $mgCa^{2+}$/L in all cases, with and without calcium addition in the feedstock. Consequently, this hypothesis can also not explain the results obtained in $Ca(OH)_2$ tests.

Alternatively, calcium may play a role in the transport of acetate into the cell. Animal colon and plant research experiences have shown evidence for calcium and short chain volatile fatty acids co-transport (Trinidad et al., 1996, 1999; Borchert, 1986; Wolever et al., 1995). In these reports, two mechanisms were given as potential explanations. The first mechanism proposes that acetate is passively transported through the cell membrane in its undissociated form. Once the undissociated acetate enters the cytoplasm, it dissociates due to higher pH in the cytoplasm compared to in the colon. The liberated proton can be excreted from the cell in exchange for an extracellular calcium ion. However, this first mechanism seems unfeasible, as the undissociated acetate can be directly used for PHA production or growth and consequently no proton can be excreted. A similar mechanism to the one proposed above is normally observed in biological reactors for phosphorus removal operated at low pH values (Smolders et al., 1994), but still it cannot explain the results observed in this work. In the current work, the reactor pH is higher than the intracellular pH, and more energy needs to be used to overcome the electric potential difference across the cell membrane. The second mechanism proposes the formation of extracellular calcium acetate complexes that diffuse through the cell membrane. Once the calcium acetate complex enters the cell, it dissociates and the calcium ion is excreted from the cell in exchange for a proton that enters the cell through the ATP-ase system. This second mechanism can potentially explain the results of the present work. Calcium acetate complexes can be formed in aqueous solutions at pH values similar to those observed in the PHA accumulation tests (Hacht, 2008). The formation of calcium acetate complexes is affected by the soluble calcium concentration, which is also affected by the precipitation of calcium carbonate and pH. For instance, at alkaline pH, HCO_3^-/CO_3^{2-} equilibrium shifts towards CO_3^{2-} and calcium carbonate precipitation can occur at lower calcium concentrations. At lower calcium concentrations, the concentration of calcium acetate complexes may be too low to enable passive transport. This interpretation can explain why experiments with $Ca(OH)_2$ at pH 8 resulted in lower PHA and biomass production rates compared to experiments where pH decreased to values between 7.0 and 7.5.

If calcium acetate complexes are formed and diffuse over the cell membrane, lower energy requirements for acetate transport should also be observed. In the present work, initial oxygen yields on acetate in experiments with calcium were in the range 0.16–0.18 $gO_2/gCOD_{HAc}$. In previous metabolic models for PHA production at pH 7, the minimum yield of oxygen per g of acetate was 0.25 $gO_2/gCOD_{HAc}$ (van Aalst-van Leeuwen et al., 1997). In this model, energy is required in the production of PHA from acetate in two steps: 1) to actively transport the acetate through the cell membrane and 2) to convert acetate to acetyl-CoA. At pH 7, each of these steps would require 1 ATP per mol of acetate. If passive transport through the cell membrane is included in this model, the minimum oxygen yield on acetate would be 0.13 and not 0.25 $gO_2/gCOD_{HAc}$, as observed in Fig. 3. The initial oxygen yields observed in the current work suggest a potential contribution of passive acetate transport over the cell membrane.

Similarly, magnesium acetate complexes could be formed in $Mg(OH)_2$ experiments. However, even though Mg^{2+} was present in equimolar concentration as those of Ca^{2+} in $Ca(OH)_2$ experiments, it did not result to stimulate the effect of PHA accumulation with selective growth. In experiments with $Mg(OH)_2$, pH development and salinity were more similar to experiments with KOH rather than experiments with $Ca(OH)_2$. It remains unclear why or if magnesium would or should result in similar outcomes as calcium.

4.3. Simultaneous PHA production and selective biomass growth is promoted as consequence of the lower cellular energy requirements

As a consequence of the lower energy requirements, higher biomass and PHA production rates were observed. Biomass growth was identified to be the main driver to cause increased levels of PHA production. This growth response was selective towards the PHA-storing fraction of the biomass, as observed by increased degree of enrichment from the microscopy with staining and 16S rRNA gene analyses. At 48 h, 76%–80% of the population was able to store PHA as compared to only 29%–51% when KOH was used. Interestingly, the average PHA contents in just the PHA-storing biomass fraction of both reactors were estimated to be at similar levels, 0.58 and 0.63 gPHA/gVSS for KOH and $Ca(OH)_2$, respectively. In $Ca(OH)_2$ experiments, the sequencing data suggest that the biomass increase is at least partly due to the growth of *Ferribacterium* and *Zoogloea* species. The genus *Ferribacterium* is composed of strictly anaerobic chemo-organotrophs that oxidize organic acids with ferric iron, nitrate or fumarate as electron acceptor and it is associated with the production of PHB (Cummings et al., 1999). In WWTP Bath, iron is added to chemically removed phosphorus, and in the anoxic tank nitrate is present. These conditions likely explain the presence of this genus in the activated sludge from WWTP Bath, but they cannot explain why species of this genus (as identified) would have a competitive advantage in a fully aerated PHA accumulation reactor. For $Ca(OH)_2$ tests with four other activated sludge (see supplementary information), the growth response was associated with other well-known PHB producers from the genera *Pseudomonas*, *Zoogloea* and *Thauera* (Jiang et al., 2011b; Stouten et al., 2019; Queirós et al., 2015; Verlinden et al., 2007). Thus, certain species within the biomass seem to selectively benefit. Calcium may therefore not benefit all species of the PHA storing phenotype to the same extent. Further research should therefore focus on optimization of nutrients dosing and finding conditions that could potentially lead to the selective growth of superior PHA accumulators due to improved yields on substrate. Preliminary experiments with $Ca(OH)_2$ have indicated that a stricter nitrogen limitation resulted in similar PHA contents to KOH tests (data not shown) and this highlights the importance of biomass growth in the results that were obtained in the present work.

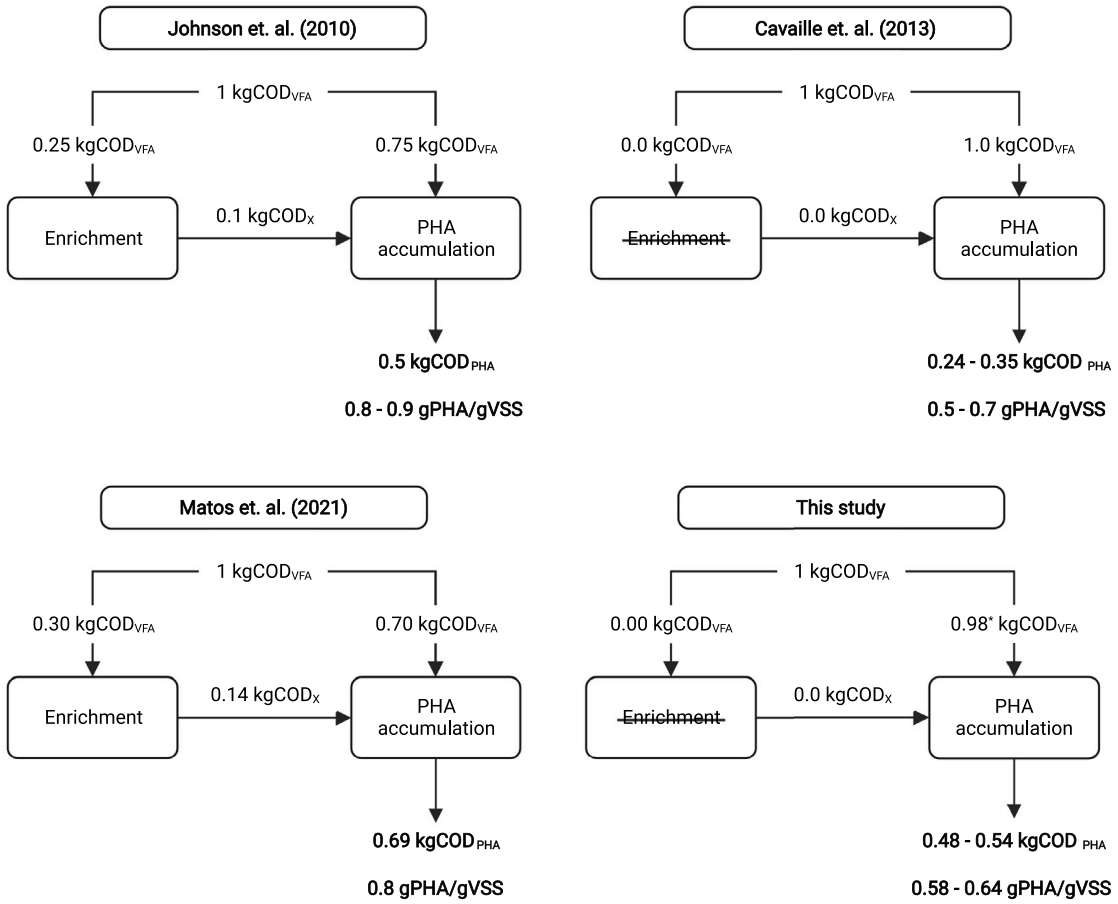

Fig. 5. Comparison between the different PHA production routes: left) enrichment accumulation and right) direct accumulation. For the enrichment accumulation route, data is from Johnson et al. (2009a) and Matos et al. (2021). For the direct accumulation route, data is from Cavaillé et al. (2013) and this study. (*) In this study, 0.02 kgCOD$_{VFA}$ was used in the acclimation step. This figure was created by *Biorender*.

4.4. Implications for microbial community-based PHA production

Even if the mechanism(s) from which calcium promotes the selective growth of the PHA-storing biomass fraction remains to be elucidated, the outcomes have significant implications. In $Ca(OH)_2$ experiments a three-fold increase in PHA mass was obtained, compared to KOH, NaOH, $Mg(OH)_2$ experiments. This increase in PHA production allowed to reach PHA contents of up to 0.64 gPHA/gVSS, which are in line with the highest PHA contents reported to date with municipal activated sludge (Kourmentza et al., 2017; Sabapathy et al., 2020; Estévez-Alonso et al., 2021b). These high PHA contents motivate to exploit municipal waste activated sludge as a generic resource for industrial scale PHA production (Fig. 5). Additionally, the principles are suggestive for PHA production process strategies that can be applied generically for any activated sludge, as was also observed in this work. Differences in the maximum PHA content to be achieved will naturally be dependent on the fraction of PHA-storing biomass at the start. A higher fraction of PHA-storing biomass in the activated sludge would likely result in a higher final PHA content. Apart from this, the results suggest that specific species of PHA storing bacteria may benefit from the effects of lower yields on substrate due to presence of calcium. Therefore, even certain enrichment cultures could produce more polymer with less substrate. It would be of specific interest to explore the effect of calcium expanded to more highly enriched cultures, such as a *Plasticicumulans acidivorans* dominated culture.

In the present work, an excess of calcium was added to the feedstock in the form of $Ca(OH)_2$. However, in practical scenarios with waste streams, this approach is not feasible. Alternatively, calcium may already be present in the waste stream or otherwise calcium salts can be added to optimal concentrations directly to the mixed liquor. It is critical to determine the minimum criteria of soluble calcium concentration and pH that are necessary to efficiently drive the kind of simultaneous growth and accumulation response observed in this work. For instance, in the $Ca(OH)_2$ experiments performed in the current work, the concentration of calcium in the liquid did not exceeded 500 mgCa^{2+}/L, due to calcium carbonate precipitation. If it is found that a high calcium concentration is necessarily required to drive simultaneous growth and accumulation response, strategies to separate the precipitated calcium carbonate from the organic solids will need to be further developed. In this work, calcium carbonate was removed by acidification with HCl. Post accumulation acidification is known to be beneficial for the polymer stability for its preservation before downstream processing (Bengtsson et al., 2017).

5. Conclusions

The presence of calcium resulted in PHA production with simultaneous selective growth of the PHA-storing biomass fraction in waste activated sludge. As a result, higher PHA contents up to 0.64 gPHA/gVSS and consistently higher PHA yields on substrate, 0.49–0.55 gCOD$_{PHA}$/gCOD$_{HAc}$, were obtained using a full-scale waste activated sludge and within a relatively short production cycle. This strategy opens a potential to apply these principles generically for any activated sludge, as long as there is already sufficient enrichment of the PHA-storing

phenotype in the activated sludge to enable for short-term enrichment with concurrent PHA storage.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A

This appendix illustrates the documented functionalities of main community members found in calcium and potassium reactors. This appendix is inspired by the previous work of Gerben Stouten and coworkers (Stouten et al., 2019). and based on the Midas Field Guide database (Dueholm et al., 2022).

- *Ferribacterium*. Strictly anaerobic chemo-organotrophs that oxidize organic acids with ferric iron, nitrate or fumarate as electron acceptor and it is associated with the production of PHB (Cummings et al., 1999)
- *Zoogloea*. Zoogloea is a well known PHA-storing genus associated with the production of PHB and is often found in colder environments (Stouten et al., 2019).
- *Acinetobacter*. Acinetobacter is a strictly aerobic heterotrophic bacteria associated with the production of polyphosphate and PHA (Saunders et al., 2016).
- *Thauera*: Denitrifying bacteria typically found in activated sludge systems capable of PHA production (Thomsen et al., 2007).
- *Hydrogenophaga*: Chemoorganotrophic and facultative chemolithoautotrophic bacteria capable of PHA production from organic acids (Crognale et al., 2019b).
- *Nitrospira*: Aerobic chemolithoautotrophic bacteria considered to be the most common nitrate oxidizing bacteria in wastewater treatment systems (Daims et al., 2001).
- *Flavobacterium*: Obligate aerobic bacteria, chemoorganictrophic typically found in activated sludge systems and lately found in some PHA enrichment reactors (Wang et al., 2017).

Appendix B. Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.watres.2022.119259.

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