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Master of Science Thesis

Glucose conversion by aerobic granular sludge

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Abstract: Aerobic granular sludge (AGS) is an innovative biotechnology extensively applied for treating municipal wastewater, and it can potentially treat sugar industry wastewater. Glucose is a prevalent substrate in sugar industry wastewater; nevertheless, the effect of glucose on AGS systems remains unexplored. In this study, an AGS reactor using glucose as the sole carbon source was operated in anaerobic-aerobic cycles. The system maintained a solids retention time (SRT) of 10 days, resulting in good granulation and enhanced biological phosphorus removal (EBPR) performance. The glucose fed was rapidly taken up within 10 minutes, with a portion stored as intracellular polymers such as glycogen and poly-hydroxy-alkanoates (PHAs), while another portion underwent anaerobic fermentation to lactate and formate. The carbon balance was not completely closed, with 16% of the carbon speculated to be utilized for the production of an unidentified polymer. The microbial community consisted of diverse organisms, with *Micropruina* identified as the most abundant genera and *Ca*. Accumulibacter (a typical type of PAOs) as the second most abundant genera based on metagenomic analysis. A batch test was conducted by adding an excess of glucose, lactate, and formate, revealing that lactate was the probable substrate utilized by PAOs. Additionally, *Micropruina* was hypothesized to be involved in glucose consumption, glycogen storage, and lactate production. *Micropruina* and *Ca*. Accumulibacter collaborate in utilizing glucose, providing them with a significant competitive advantage within the system. Due to their slow growth rate, these bacteria play a crucial role in achieving favorable granulation when supplied with glucose. Promoting the growth of these organisms can be a valuable strategy in engineering applications.

Keywords: Aerobic granular sludge (AGS), Glucose, Phosphate accumulating organisms (PAOs), Enhanced biological phosphorus removal (EBPR), Carbon balance

Content

1 Introduction

Aerobic granular sludge (AGS) is an innovative and efficient wastewater biotechnology capable of simultaneous removal of carbon, nitrogen, and phosphorus (de Kreuk et al., 2005). Consequently, AGS technology has found widespread application in municipal wastewater treatment (Pronk et al., 2015). Whereas municipal wastewater contains a diverse array of organic substrates, industrial wastewater often comprises distinct and specific organic compounds. Wastewater from sugar industry may contain specific sugars such as glucose (Rajesh Banu et al., 2020), and AGS technology is potentially suitable for its treatment.

The primary advantage of AGS lies in its ability to achieve efficient settling with a smaller spatial requirement compared to activated sludge, accomplished through the formation of stable and dense granules (de Kreuk et al., 2007). The selection of slow-growing bacteria, particularly phosphate accumulating organisms (PAOs), is considered crucial for the formation of dense and smooth granules (van Loosdrecht & de Kreuk, 2004), representing a typical type of bacteria in wastewater treatment. PAOs are responsible for enhanced biological phosphorus removal (EBPR) processes that were operated in anaerobic-aerobic cycles (Mino et al., 1998). During the anaerobic phase, PAOs acquire energy through hydrolyzing polyphosphate into phosphate, which is then utilized to uptake biodegradable substrates like volatile fatty acids (VFAs). The assimilated VFAs are used for synthesizing poly-hydroxy-alkanoates (PHAs) as energy storage compounds. In the subsequent aerobic phase, PHAs are utilized with oxygen for growth, while phosphate is assimilated to form polyphosphate, which remains available for the subsequent cycle. The operation of anaerobic-aerobic cycles provides a competitive advantage to PAOs, thereby enhancing EBPR and granulation.

To apply AGS technology for treating sugar wastewater, it's important to understand how the AGS system functions when fed with glucose. However, the specific impact of glucose on the granulation process and treatment performance of AGS remains unknown. Since EBPR and AGS operate in similar ways, studying the effect of glucose on EBPR can provide insights into its effect on the AGS system. Previous studies on glucose-fed EBPR systems have yielded conflicting results, with some demonstrating successful EBPR with glucose as the sole carbon source (Jeon & Park, 2000; Wang et al., 2002; Zengin et al., 2010; Ziliani et al., 2023) while others indicating instability or deterioration of EBPR under glucose feeding (Begum & Batista, 2012; Wang et al., 2001).

Given the perplexing outcomes regarding the impact of glucose and its potential industrial application, this study focuses on investigating a lab-scale AGS system that is fed with glucose as the sole carbon source. The study extensively examines the effects of glucose on the granulation process and EBPR performance, while also analyzing and discussing the conversion of glucose and its impact on the microbial community.

2 Methods

2.1 Setup and operation

A double-wall bubble column reactor was used to conduct experiments in the mode of Sequencing Batch Reactor (SBR). The reactor had a working volume of 2.8 liters with an internal diameter of 5.6 cm and a height of 160 cm. At the end of each cycle, 1.5 liters of effluent were withdrawn, leaving behind the remaining volume in the reactor for the next cycle. This accounted for a volumetric exchange ratio of 54%. The reactor had a constant temperature as room temperature which was regulated at 20°C. The pH was controlled at 7.0 ± 0.1 by adding either 0.5 mol/L NaOH or 0.5 mol/L HCl. Dissolved Oxygen (DO) level was controlled at 0% and 50% saturation during the anaerobic and aerobic phase respectively, which was achieved by adjusting the influent flow of nitrogen gas and filtered air. The reactor was initially inoculated with full-scale aerobic granular sludge obtained from municipal wastewater treatment located in Utrecht, the Netherlands and lab-scale aerobic granular sludge adapted to propionate and performing EBPR. The overall experimental setup is comparable to the one used by (Elahinik et al., 2022).

The influent was 1,500 mL consisting of 1,200 mL of demineralized water, 150 mL of medium A, and 150 mL of medium B. Medium A contained 20.83 mM glucose ($C_6H_{12}O_6$), 3.6 mM $MgSO_4 \cdot 7H_2O$, and 4.7 mM KCl. Medium B contained 41.1 mM NH_4Cl, 1.95 mM K_2HPO_4, 1.98 mM KH_2PO_4, 0.6 mM Allythiourea (ATU) to inhibit nitrification, and 10 mL/L of trace element solution. The trace element solution contained 4.99 g/L $FeSO_4 \cdot 7H_2O$, 2.2 g/L $ZnSO_4 \cdot 7H_2O$, 7.33 g/L $CaCl_2 \cdot 2H_2O$, 4.32 g/L $MnSO_4 \cdot H_2O$, 2.18 g/L $Na_2MoO_4 \cdot 2H_2O$, 1.57 g/L $CuSO_4 \cdot 5H_2O$, 1.61 g/L $CoCl_2 \cdot 6H_2O$, and 50 g/L EDTA. The combination of these feed streams resulted in an influent concentration of 400 mg/L COD, 57.6 mg/L NH_4^+-N, and 12.2 mg/L PO_4^{3-}-P.

Each operation cycle takes 200 mins and consists of: nitrogen gas mixing (5 mins) to ensure anaerobic condition in feeding phase; feeding (5 mins); anaerobic phase (60 mins) that mixed with nitrogen gas of 5 L/min; aerobic phase (120 mins) that mixed with recirculated air of 2.5 L/min; settling (5 mins); effluent discharge (5 mins). The reactor was operated at solids retention time (SRT) of 10 days.

2.2 Chemical analyses

Liquid samples taken from the bioreactor were all filtered by 0.22 µm filters before chemical analyses. Phosphate (PO_4^{3-}-P) and ammonia (NH_4^+-N) concentration were measured using a Gallery Discrete Analyzer (Thermo Fisher Scientific, USA). Chemical oxygen demand (COD) was measured using Hach Lange Kits (LCK614) with a spectrophotometer cuvette system (DR2800, Hach Lange, USA) Total organic carbon (TOC) was measured using a TOC-L CSH system (Shimadzu, Japan). Carbohydrates and volatile fatty acids (VFAs) were measured using an HPLC system (Vanquish, Thermo Fisher Scientific, USA) equipped with an RI and UV detector, Aminex HPX-87H column (BioRad, USA) using 1.5mM phosphoric acid as eluent. The off-gas of the reactor was measured using a Gas Chromatography - Mass Spectrometer system (Prima BT, Thermo Fisher Scientific, USA).

2.3 Biomass measurements

A mixed liquid sample was taken from the reactor at the end of aerobic phase and washed with demineralized water to get off the impurities. Then the sample was placed on an aluminum cup and in an oven at 105℃ for 24 hours to determine the Total Suspended Solids (TSS) of the reactor. The dried sample after 105℃ will be put in a furnace at 550℃ for 3 hours to determine the ash content and Volatile Suspended Solids (VSS). The difference of the sample weight before and after putting in an oven will be used for calculation. For determining TSS and VSS of the effluent, the samples were taken at the effluent phase. Then they were filtered through 1μm glass fiber filters (Pall Corporation, USA) to catch all the suspended solids, the rest steps were the same as the mixed liquid samples.

Freeze-dried biomass was used for PHA, glycogen and other biomass measurements. A biomass sample was taken from the reactor and fixed with 4% w/v paraformaldehyde, then washed by demineralized water at least twice, frozen in a -80℃ freezer, freeze-dried in a vacuum freeze dryer and pottered into powder to prepare the freeze-dried biomass. The PHA measurement protocol was adjusted from (Oehmen et al., 2005; Riis & Mai, 1988). About 30 mg of the powder freeze-dried biomass was mixed with 1.5mL of H_2SO_4 in methanol (3% v/v) and 1.5 mL of chloroform in a glass tube. Then the tube was heated at 100℃ with frequent manual vortexing for 20 hours for hydrolysis and esterification. After cooling to room temperature, 3 mL of ultrapure water was added to the sample, vortexed, and centrifuged to separate the two phases. The formed methyl esters in the organic phase were then filtered and analyzed by Gas Chromatography (6890N, Agilent, USA). Benzoic acid was used as an internal standard and added together with the freeze-dried biomass. Quantification of PHB and PHV was done using commercial Poly (3-hydroxybutyric acid-co-3-hydroxyvaleric acid) with 8 mol% PHV content (Sigma-Aldrich, USA). The GC system used was equipped with OPTIMA column (60 m length × 0.25 mm I.D. × 0.25 μm film). The system was operated with a split injection ratio of 1:30 and helium as the carrier gas (1.0 mL/min). The flame ionization detector (FID) unit was operated at 250℃ with an injection port temperature of 230℃. The oven temperature was programed to start with 120℃ for 4 mins, increased at 30℃/min to 180℃, maintained at 180℃ for 6 mins, then increased to 240℃ at 40℃/min and held for 16 mins.

Glycogen was extracted and measured according to the method described by (Smolders et al., 1994) with minor adjustments. About 5 mg of the powder freeze-dried biomass was mixed with 5 mL of 0.9M HCl and heated at 100℃ with frequent manual vortexing for 5 hours. After cooling to room temperature, the mixed liquid was filtered using 0.22 μm Millipore filters and neutralized with the same volume of 0.9M NaOH solution. Finally, the glucose in the sample was determined using the same HPLC system and used for calculating glycogen amount.

2.4 Batch test

A batch test was performed by operating 4 bottles in the anaerobic phase with different substrates together. N_2 gas was used for mixing biomass and maintaining anaerobic condition. Three bottles were run for 1 hour and fed with 3.15 mM lactate, 3.98 mM formate, and 11.7 mM glucose, respectively. The

fourth bottle was fed with 12.8 mM glucose and operated for 3 hours. Only the carbon source was controlled differently as mentioned, other compounds in the media were controlled at the same concentration as in the reactor. Biomass was taken from the end of aerobic phase in a normal reactor cycle, and 20 mL biomass was added in each bottle with a total volume of 250 mL. The pH was intermittently controlled at 7.0 ± 0.3 by manually adding either 0.5 M NaOH or 0.5 M HCl. All bottles had a constant temperature as room temperature which was regulated at 20℃. Liquid and biomass samples were collected for bulk liquid analysis and PHA and glycogen content determination.

2.5 Microscopy and FISH analyses

A stereo zoom microscope (M205 FA, Leica Microsystems, Germany) coupled with Qwin image analysis software (V3.5.1, Leica Microsystems, Germany) was utilized for capturing images of the granules. Microscopic images were captured using Axio Imager M2 (Zeiss, Germany) with ZEN (blue edition) software. Biomass samples were prepared and stained for FISH following the protocol outlined in (Bassin et al., 2011). Similar probes were employed as described in (Elahinik et al., 2022). A combination of probes EUB338, EUB338-II, and EUB338-III (EUBmix) was used to stain all bacteria, as reported in (Amann et al., 1990; Daims et al., 1999). To visualize PAOs, probe PAO651 was utilized, as outlined in (Crocetti et al., 2000). For further identification of the PAO genus *Candidatus* Accumulibacter, probes Accumulibacter type I and Accumulibacter type II were employed (Petriglieri et al., 2022). Epifluorescence microscope images were captured using filter sets Cy3 (ET545/25x ET605/70m T565LPXR), Cy5 (ET640/30x ET690/50m T660LPXR), and FITC (ET470/40x ET525/50m T495LPXR) with Axio Imager M2 (Zeiss, Germany).

2.6 Metagenomics

The granule biomass obtained from the reactor was crushed and subsequently utilized for DNA extraction. DNA extraction from the biomass was carried out using the DNeasy PowerSoil Pro Kit (Qiagen, Germany) following the manufacturer's provided protocol. The concentration of DNA was measured using a Qubit fluorometer (ThermoFisher Scientific, USA). Metagenome sequencing and subsequent processing of raw data, including read assembly and gene identification, were conducted by Novogene (Novogene Co., China).

3 Results

3.1 Reactor operation

The inoculum consisted of a mixture of full-scale granules and lab-scale granules adapted to propionate and performing EBPR. Subsequent to the inoculation and commencement of the operation, the full-scale granules rapidly disintegrated into flocculated biomass, causing a continuous decrease in

the volume of the granular sludge bed. Following a few weeks, the biomass acclimated, resulting in a gradual color transition of the granules from white to brown. Eventually, a stable settling sludge bed was achieved (Figure 3-1). Analysis of stereoscopic images revealed that the granule size primarily ranged from 0.3 to 1 mm, with minimal suspended sludge observed. In conclusion, glucose feeding facilitated successful granulation.

After day 40, the TSS concentration of the reactor exhibited reduced fluctuations. The reactor attained a pseudo-steady state on day 80, characterized by a stable TSS concentration averaging 5.7 g/L (Figure 3-2). In addition, the VSS concentration exhibited a very similar trend to TSS, and the VSS/TSS ratio was very stable at 80%. The effluent TSS could reflect how much biomass was lost when discharging in the effluent phase, and thus how clean the effluent was. The average effluent TSS concentration was 0.089 g/L, representing a daily loss of 1/17th of the total biomass.

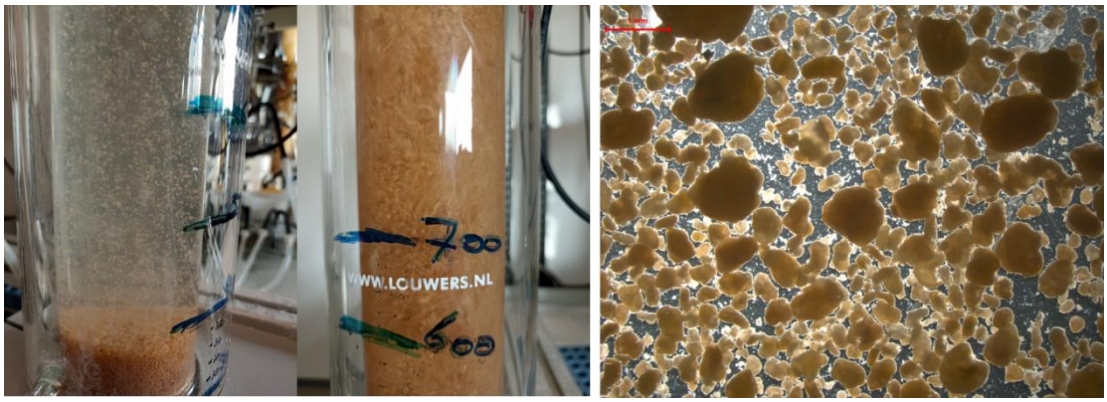

Figure 3-1 Settling granular sludge bed of the bioreactor and stereo zoom image of granules (Day 88)

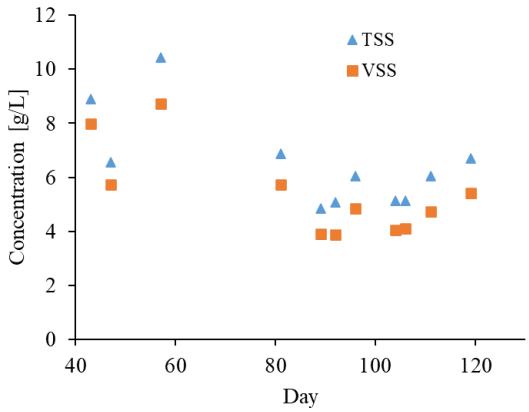

Figure 3-2 TSS and VSS of the reactor changed over time (between day 40 and 120)

3.2 Chemical conversion in reactor cycles

The chemical conversion experiment was conducted after the reactor reached the pseudo-steady state. Figure 3-3 illustrates the strong EBPR performance of the reactor, with a P release of 54.7 mg PO_4-P/L during the anaerobic phase, followed by subsequent P uptake during the aerobic phase. The ratio of

anaerobic P release to the C source fed was 0.27 mol-P/mol-C. For the kinetics, the maximum P release rate reached 21.8 mg PO_4-P/g VSS/h, while the maximum P uptake rate was 12.0 mg PO_4-P/g VSS/h. T These findings indicate the presence of abundant PAOs in the microbial community and their substantial utilization of the available substrate. Since nitrification was inhibited in the system, the decline in NH_4^+ concentration was attributed to its utilization by microorganisms as a nitrogen source, reflecting microbial growth. From Figure 3-3, the majority of NH_4^+ reduction (76%) occurred during the aerobic phase, indicating significant microbial growth in this phase. This growth pattern aligns with the behavior of PAOs, who store substrate as carbon and energy source during the anaerobic phase and utilize them for growth in the subsequent aerobic phase.

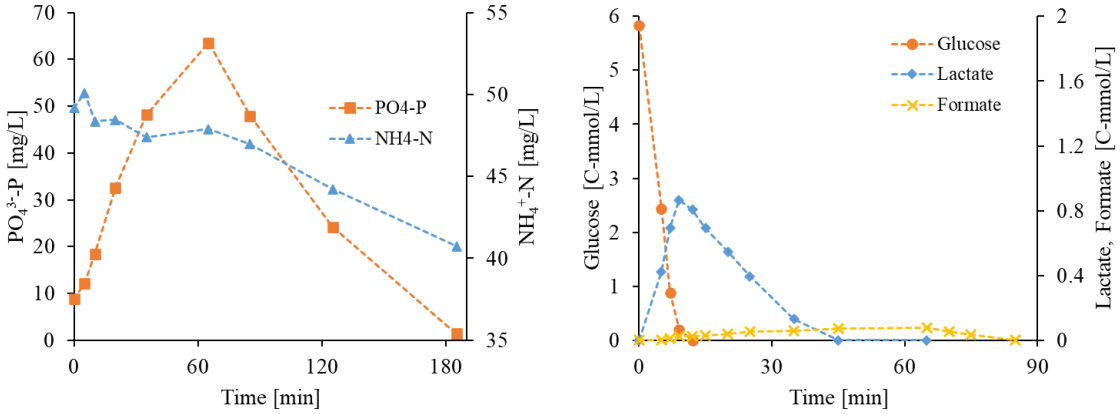

Figure 3-3 Chemical conversion in a normal cycle: (left) PO_4^{3-} and NH_4^+ bulk liquid concentration change; (right) glucose, lactate, and formate bulk liquid concentration change

Glucose uptake occurred rapidly within the initial 10 minutes of each cycle, reaching a maximum uptake rate of 341 mg glucose/g VSS/h. At the same time, lactate and formate were produced as fermentation products. Lactate reached its maximum concentration around 10 mins, after which it was completely consumed before the end of the anaerobic phase. In contrast, formate production was gradual throughout the entire anaerobic phase and was subsequently consumed at the beginning of the aerobic phase. These findings suggest that glucose consumption involves multiple intricate steps and likely involves the participation of at least two abundant organisms within the microbial community.

For PAOs metabolism, intracellular polymers like PHA and glycogen are used to support EBPR. In the system, a typical behavior of PHA was observed, with an increase during the anaerobic phase and subsequent consumption during the aerobic phase to replenish for the next cycle (Figure 3-4). I PHV was identified as the predominant type of PHA produced, with an anaerobic accumulation of 7.6 mg PHV/g TSS biomass. Conversely, the expected glycogen behavior, characterized by a decrease in the anaerobic phase followed by an increase in the aerobic phase, was not observed. Instead, the glycogen concentration showed a rapid initial increase, followed by a continuous decrease during both the anaerobic and aerobic phases (except for an outlier at 35 mins), and finally returning to nearly the same level by the end of the cycle.

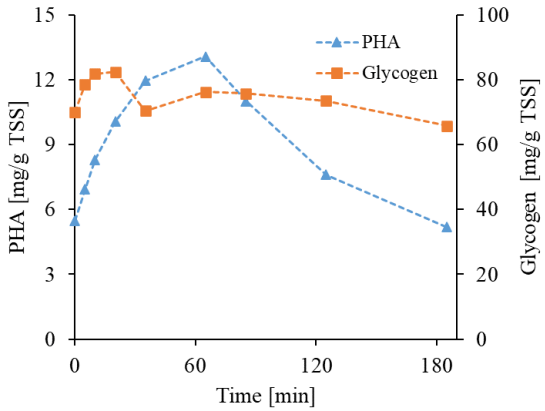

Figure 3-4 PHA and glycogen in biomass change in a normal cycle

Based on the chemical analyses in a normal cycle, glucose could be fermented to intermediate products and stored as intracellular polymers. However, when examining the carbon balance during the anaerobic phase of a normal cycle (see Appendix), it became apparent that the total carbon balance was only 48.2%, indicating a significant portion of the fed carbon was unaccounted for. These unexplained carbon losses were not attributed to known fermentation products or intracellular polymers. Furthermore, the electron balance (see Appendix) also exhibited a significant deficit (53.2% coverage). The contribution of different chemicals to the carbon and electron balance was similar, as their oxidation states were comparable.

3.3 Finding the missing carbon

To ensure the comprehensive coverage of the carbon and electron balance, two additional measurements were conducted: the bulk liquid test and the off-gas measurement. The purpose of the bulk liquid test was to determine if any significant chemical components were missing from the HPLC measurement of the bulk liquid. This was achieved by measuring the changes in total organic carbon (TOC) and chemical oxygen demand (COD) to assess the overall carbon and electron changes in the bulk liquid. Subsequently, the identified products from the HPLC analysis (glucose, lactate, and formate) were compared against the total carbon and electron changes. The results indicated that 95.5% of the carbon and 93.3% of the electrons were accounted for by the identified chemicals, thus confirming the completeness of the HPLC measurement of the bulk liquid.

The off-gas measurement was employed in the anaerobic phase to quantify the production of H_2, CH_4, CO_2 and other gases. The levels of H_2 and CH_4 remained stable during the 1-hour anaerobic phase, while CO_2 concentration was initially high but gradually decreased until the end of the anaerobic phase (see Appendix). Table 3-1 provides the average concentration and contribution of these gases to the overall balance. The off-gas measurement accounted for an additional 23% of the carbon and 7% of the electrons, but there was still a fraction of carbon and electrons that remained unexplained in the balance.

Table 3-1 The concentration of H_2, CH_4 and CO_2 and their contribution to carbon and electron balance

	Average concentration [ppm]	Carbon coverage	Electron coverage
H_2	39	/	0.6%
CH_4	112	3.2%	6.4%
CO_2	697	19.9%	/
Total	/	23.1%	7.0%

3.4 Batch test

To gain insight into the fermentation processes occurring in the reactor, a batch test using different substrates was conducted. It was observed that the biomass fed with excess glucose will underwent a rapid color change, transitioning from its original brown to a distinct yellow (Figure 3-5). In contrast, the biomass fed with lactate and formate retained its brown color, similar to that observed in the reactor. The pH of both glucose-fed bottles required frequent adjustments through the addition of NaOH, which can be attributed to the formation of lactic acid leading to a decrease in pH. For the bottle running for 3h, the pH didn't need adjustments after 2.5 hours, when lactate production ceased. In contrast, the bottles fed with lactate and formate maintained stable pH levels without the need for adjustments.

Figure 3-5 Setup of the batch test with 4 bottles (from left to right: lactate dosing; formate dosing; glucose dosing; glucose dosing with 3-hour anaerobic time)

The chemical conversion in the glucose-fed (1h) bottle is illustrated in Figure 3-6. Glucose exhibited a consistently stable consumption rate of 336 mg glucose/g VSS/h (average between 11 and 61 mins). Notably, significant and continuous phosphorus (P) release was observed during the 1-hour period; however, the P/C ratio was only 0.018 mol P/mol C. The NH_4^+ concentration remained stable throughout the 1-hour duration, indicating a negligible anaerobic biomass growth. The batch test also demonstrated the production of fermentation products (lactate and formate) observed in the reactor. Moreover, acetate and propionate were also generated. Since acetate and propionate are more easily used by PAOs, their presence may not be as prominent in a normal cycle with limited glucose. Overall, the fermentation

processes observed in the batch test closely resembled those in the reactor.

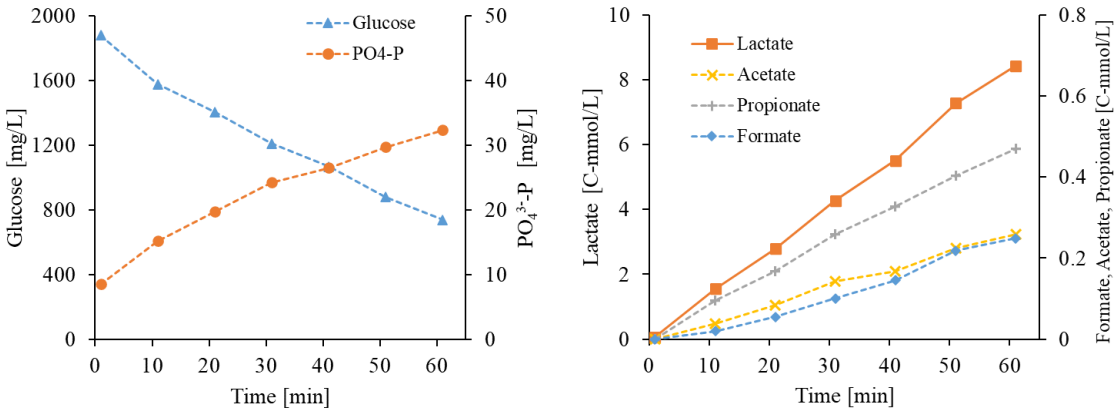

Figure 3-6 Chemical conversion in the glucose-fed (1h) bottle: (left) glucose and PO_4^{3-} change; (right) lactate, formate, acetate, and propionate change

In the other glucose-fed bottle, which operated for 3 hours, glucose consumption patterns are depicted in Figure 3-7. Glucose consumption displayed an initial rapid and continuous rate during the first 2 hours, reaching depletion around the 2.5-hour mark. The consumption rate averaged 292 mg/g VSS/h (average from 11 to 91 mins), similar to the rate observed in the 1-hour bottle. Changes in NH_4^+ and PO_4^{3-} mirrored those observed in the 1-hour bottle, with NH_4^+ remaining constant throughout the period and continuous release of PO_4^{3-} at a P/C ratio of 0.023 mol P/mol C. Furthermore, the fermentation production of formate, acetate, and propionate exhibited high similarities. The only distinction was the cessation of lactate production during the last 30 minutes (Figure 3-7) , which coincided with the depletion of glucose, strongly indicating a direct relationship between lactate production and glucose consumption.

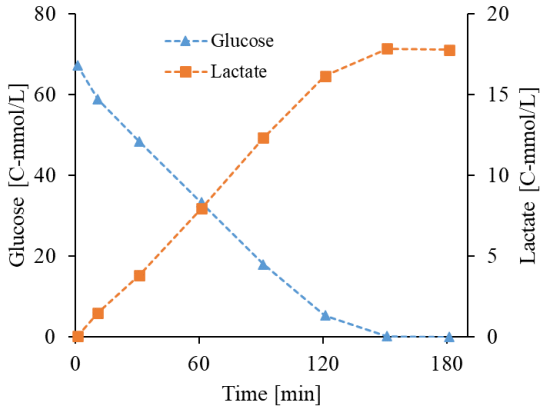

Figure 3-7 Glucose and Lactate change in the glucose-fed (3h) bottle

The lactate-fed bottle and the formate-fed bottle exhibited continuous phosphorus (P) release throughout the experiment. The lactate-fed bottle demonstrated a higher release rate of 12.4 mg P/g VSS/h, whereas the formate-fed bottle had a rate of 8.1 mg P/g VSS/h. Lactate consumption was slow but consistent, with a rate of 13.7 mg lactate/g VSS/h (see Appendix), while formate concentration remained unchanged.

Both the glucose-fed and lactate-fed bottles showed significant accumulation of PHA after 1 hour, with PHV being the predominant type of PHA produced. The glucose-fed bottle exhibited a PHA accumulation of 8.34 mg/g VSS, while the lactate-fed bottle had an accumulation of 6.96 mg/g VSS. These values were close to the 7.60 mg/g VSS observed in a normal cycle, suggesting potential PHA saturation for PAOs in the system. In contrast, PHA production in the formate-fed bottle was minimal.

Significant glycogen accumulation (118.6 mg glycogen/g TSS) was observed in the glucose-fed bottles, while both the lactate-fed and formate-fed bottles showed limited glycogen consumption. Consequently, the glucose-fed bottle achieved 68.4% carbon coverage in total without off-gas measurement, representing a 20% increase compared to normal cycles. Considering a similar percentage of carbon coverage in the off-gas analysis, the overall carbon balance is nearly closed.

3.5 Microbial Community

A microscopic image was captured on day 96 to illustrate the microbial community composition. The image revealed the presence of multiple abundant species, suggesting that the energy derived from glucose conversion was utilized by various organisms (Figure 3-8). When comparing the granules to the suspended solids in the effluent, the organisms' types appeared to be similar, but the granules exhibited a higher prevalence of large cells (see Appendix).

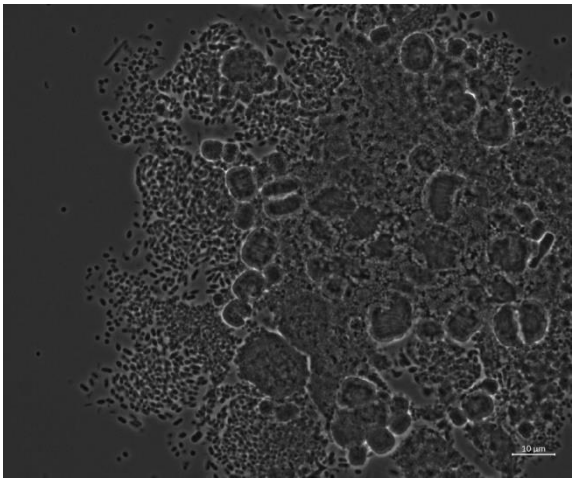

Figure 3-8 Microscopic picture of the granular biomass on Day 96 (Scale bar equals 10 µm)

To obtain more conclusive information about the microbial community, fluorescence in situ hybridization (FISH) analysis was conducted. As depicted in Figure 3-9, the red color indicated the presence of PAOs, which were found to be abundant throughout the community. Further characterization of PAOs revealed a higher prevalence of *Ca*. Accumulibacter type I, while *Ca*. Accumulibacter type II was minimally detected. Overall, the FISH results verified the existence of PAOs, corroborating the observations of chemical conversion in the reactor. However, numerous unstained cells remained in the sample, necessitating further investigation.

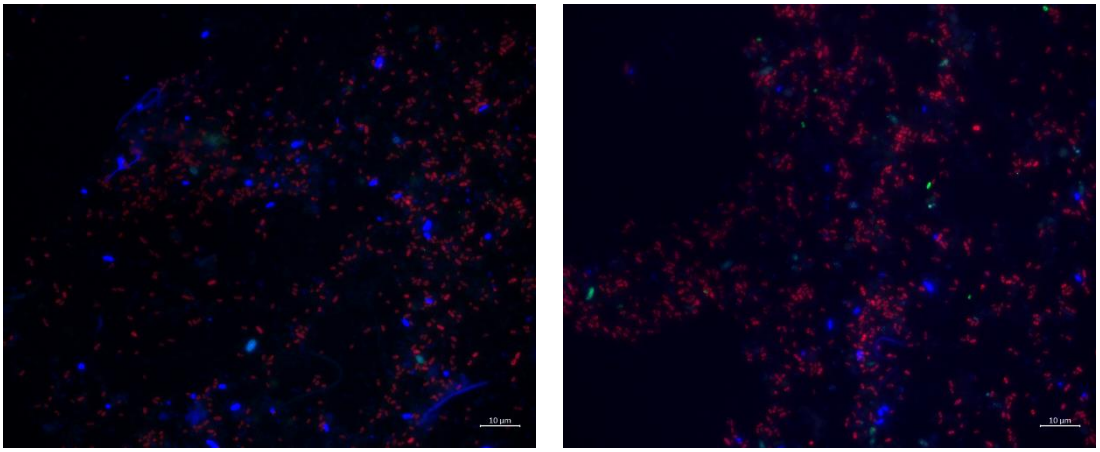

Figure 3-9 FISH picture to determine microbial community: Picture (left) determine PAOs (Color representation – blue: EUB(mix); red: PAOs651); Picture (right) determine *Ca*.Accumulibacter type (Color representation – blue: EUB(mix); red: *Ca*.Accumulibacter I; green: *Ca*.Accumulibacter II)

The metagenomics analysis identified four genera that exhibited significant abundance, collectively accounting for 21.7% of the total genes in the community. The relative abundance of these four genera is illustrated in Figure 3-10. The presence of abundant *Ca*. Accumulibacter confirmed the findings of the FISH analysis. *Micropruina* emerged as the most prevalent genus in the community, which likely explains the inability to detect these cells using the employed FISH probes.

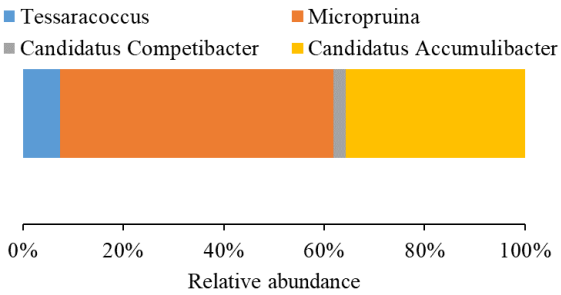

Figure 3-10 Genomic relative abundance of the abundant genera in the microbial community

4 Discussion

4.1 EBPR behavior comparison

To gain further insights into the EBPR performance, the findings of this study were compared with those of previous studies. The system in this study exhibited a comparable P/C ratio and P uptake rate to the glucose batch in the EBPR system, as shown in Table 4-1. The main difference was that our system had a higher anaerobic P max release rate, which can be explained by having more active fermenters. Overall, these findings suggest that the system performance and the role of PAOs in our study were comparable to those observed in previous studies. By comparing both EBPR system fed with acetate and AGS system fed with glycerol, the EBPR performance of our system could be considered significant and

thus indicated good PAOs activity.

Table 4-1 Comparison of EBPR performance in this study with previous studies

	Glucose batch (floc)	**Glucose reactor (granular)**	Acetate batch (floc)	Glycerol reactor (granular)
Sole carbon source	Glucose	**Glucose**	Acetate	Glycerol
P/C ratio [mol-P/mol-C]	0.26	**0.27**	0.52	0.23
P release rate(max) [mg P/g VSS/h]	9.8	**21.8**	19.1	10.9
P uptake rate(max) [mg P/g VSS/h]	10.4	**12.0**	12.5	16.3
Reference	(Ziliani et al., 2023)	**This study**	(Ziliani et al., 2023)	(Elahinik et al., 2022)

According to (Zengin et al., 2010), PHV accounted for the majority (77%) of total PHA production, aligning with our findings where PHV was the predominant PHA. (Jeon & Park, 2000) reported a significant increase in glycogen content after feeding, followed by its predominant consumption during the anaerobic phase, while it remained constant during the aerobic phase. A similar glycogen trend was observed in our study, which diverges from the typical glycogen behavior exhibited by PAOs. Therefore, it is plausible that an alternative glycogen behavior exists.

An explanation for the observed glycogen changes is as follows: The significant increase in glycogen after feeding can be attributed to glycogen-accumulating organisms (GAOs) utilizing glucose to synthesize glycogen. Subsequently, the decrease in glycogen may be due to PAOs utilizing glycogen as a reducing power in their metabolic processes, while GAOs employ glycogen to produce lactate for energy generation. During the aerobic phase, PAOs produce glycogen, whereas GAOs utilize glycogen for growth. Consequently, in the anaerobic phase, a decrease in glycogen content corresponds to our findings and the results reported by (Jeon & Park, 2000). In the aerobic phase, if the rate of glycogen consumption by GAOs is similar to the rate of glycogen production by PAOs, the glycogen level will remain stable, as demonstrated by (Jeon & Park, 2000). However, if the rate of GAOs consuming glycogen exceeds the rate of PAOs producing glycogen, the glycogen level will continue to decrease, as observed in our study.

4.2 Substrate used by PAOs

The anaerobic metabolism of PAOs involves the hydrolysis of polyphosphate to generate energy for substrate uptake. In the acetate-fed PAOs system, hydrolysis occurred continuously, leading to a gradual increase in the bulk P concentration during acetate uptake. Once acetate was depleted, the P release ceased (Smolders et al., 1994). However, in the normal cycle of this study, glucose uptake occurred rapidly within the first 10 minutes, while P release continued throughout the 1-hour anaerobic phase. This observation suggests that glucose is unlikely to be the substrate directly utilized by PAOs. There was a paper claiming

that glucose could be putatively used by PAOs (Ziliani et al., 2023). However, their reactor was fed with a mixture of glucose and acetate (1:1), rather than glucose alone. Moreover, the absence of analyses for general sugars or organic acids in both normal reactor cycles and batch tests resulted in a lack of information regarding fermentation processes. Additionally, the presence of 50% PAOs protein abundance in proteomics did not establish the high enrichment of PAOs in the community. Consequently, based on these limitations, the conclusion drawn in the aforementioned study regarding the putative use of glucose by PAOs is contradicted by the findings of this study.

In a normal reactor cycle, lactate was anaerobically produced and consumed, suggesting its potential as a substrate for PAOs. A comparison of the P release profile and lactate change profile (Figure 3-3) indicates a correlation between these two processes. During the initial 35 minutes, when lactate was present with significant amount, a high P release rate was observed, whereas between 35 and 65 minutes, when lactate was minimal, the P release rate decreased. Comparison of the P release rates showed that the lactate-fed bottle exhibited the highest rate (12.4 mg/g VSS/h), which closely resembled the maximum P release rate observed in a normal cycle (12.0 mg/g VSS/h). These findings strongly suggest a connection between lactate and P release. Additionally, the high PHA accumulation observed in the lactate-fed bottle, similar to that in a normal cycle, can be attributed to the direct synthesis of PHA by PAOs using lactate. Collectively, these relationships provide evidence supporting lactate as a potential substrate for PAOs in this system.

There was also a possibility that lactate was first fermented to other chemicals then used by PAOs. In the glucose-fed bottle, propionate and acetate were produced and they are both good substrates for PAOs. However, in the lactate-fed bottle, there was no intermediate product when excess lactate was fed. If acetate and propionate were fermented from lactate, then they should be detected in the lactate-fed bottle as well. The possibility of lactate being fermented to other substrate for PAOs can not been seen from this study. Formate was not consumed at all in anaerobic phases, so it was excluded from being a substrate for PAOs.

Previous studies on EBPR fed with glucose have also observed the production of lactate, suggesting its role as a substrate for PAOs (Jeon & Park, 2000; Zengin et al., 2010). The metabolic pathway proposed by (Jeon & Park, 2000) indicated that lactate can undergo conversion to Acetyl-CoA and Propionyl-CoA , enabling PHA synthesis. The exclusive synthesis of PHV in this study can be attributed to the fact that one HV monomer is formed by combining Acetyl-CoA and Propionyl-CoA. Additionally, if lactate is utilized by PAOs, the synthesis of PHV would not require additional reducing power. Consequently, it is likely that glycogen is not involved in anaerobic metabolism of PAOs, although the explanation regarding glycogen can still be valid when excluding the effect of PAOs.

Based on the PHA concentration comparison, lactate might not be the only substrate used by PAOs. The batch result showed that acetate and propionate could be produced when glucose was consumed, and they are very likely to be better substrates for PAOs. Consequently, a hypothesis was formulated suggesting that acetate and propionate, in addition to lactate, can be utilized for PHA synthesis by PAOs, resulting in higher PHA accumulation. This observation can explain the lower PHA concentration in the lactate batch bottle compared to both the normal cycle and glucose batch bottle, as the latter two contained

additional substrates beyond lactate. The glucose batch bottle, with its abundant glucose content, had a higher potential for acetate and propionate production, which PAOs could utilize for increased PHA synthesis. Consequently, the glucose batch bottle exhibited the highest PHA concentration among the three scenarios. In a normal cycle with a lower glucose amount, the specific PHA biomass concentration fell between that of the lactate batch bottle and the glucose batch bottle.

4.3 Closing carbon balance

The carbon balance issue was well addressed in the results section. The common method of measuring bulk liquid and intracellular polymer accounted for only approximately 50% of the carbon in a normal reactor cycle. To delve deeper into the carbon balance, it is important to consider the three forms in which the supplied glucose can be converted: liquid, gas, and solids. The bulk liquid test has ruled out the possibility of glucose being converted into substances present in the bulk liquid. The off-gas measurement accounted for an additional 20% of carbon coverage, yet there remained a 30% deficit in the carbon balance.

Regarding the solid form, one potential pathway involves biomass growth. The synthesis of new cells requires nitrogen source, which is supplied in the form of NH_4^+ in the medium. Because nitrification is inhibited in the system, NH_4^+ decrease can reflect how much biomass is synthesized. In a normal reactor cycle, there was 2.2 mg NH_4-N/L decrease, reflecting 13.5% of the total carbon fed. Despite measuring intracellular polymers such as PHA and glycogen, these two measurements do not account for all polymers including extracellular polymers. The remaining 16% of the unaccounted carbon is presumed to be an unknown polymer, so the overall carbon balance and conversion can be shown in Figure 4-1.

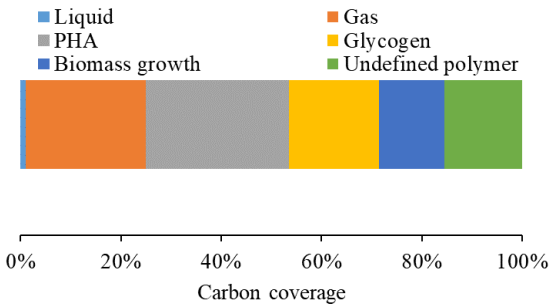

Figure 4-1 Carbon balance of the glucose anaerobic conversion in a normal reactor cycle

This unknown polymer is speculated to be converted from glycogen or glucose, but the conversion percentage is limited. Therefore, when excess glucose was supplied in the batch test, the absolute amount of unknown polymer conversion probably wouldn't increase much. As a result, the contribution of unknown polymer production to the carbon balance would be considerably diminished, while glycogen accumulation would be notably elevated. This aligns with the batch test result, which demonstrated a substantial increase in glycogen accumulation, covering an additional 20% of the carbon. Additionally, the study by (Jeon & Park, 2000) proposed the existence of a lactate polymer that could potentially be

converted from glycogen, which is consistent with our findings and analysis.

4.4 Roles in the microbial community

Substrate uptake involves a critical step of rapid glucose assimilation and glycogen storage; however, the specific species responsible for this process remains unclear. Given that this step offers the greatest advantage to harness energy from the supplied substrate, it is highly probable that the most prevalent genus, *Micropruina*, carries out this function. By comparing the conversion shown in this study, it can be deduced that the species responsible for this step is *Micropruina glycogenica* (Shintani et al., 2000). From the pure culture study of (Shintani et al., 2000), it was demonstrated that *Micropruina glycogenica* is capable of assimilating glucose and converting it into glycogen under anaerobic conditions. The substantial increase in glycogen content aligns closely with the findings of our study. Its coccoid shape and average diameter of 1 μm align with the morphology of the most prevalent cells depicted in the microscopic image (Figure 3-8).

In establishing a connection between the microbial community and chemical conversion, *Micropruina glycogenica* and *Ca*. Accumulibacter play pivotal roles in glucose utilization. Glucose supplied will initially be rapidly assimilated by *Micropruina* and stored as glycogen. A portion of the glycogen will then undergo fermentation to lactate for energy. The released lactate will serve as a substrate for *Ca*. Accumulibacter and be used for synthesizing PHA. These intracellular polymers stored under anaerobic conditions will be used for growth during aerobic conditions. Since there is minimal substrate available for other organisms during the aerobic phase, *Micropruina glycogenica* and *Ca*. Accumulibacter have the competitive advantage to dominate the microbial community. Furthermore, in terms of energy distribution, approximately 34% of the electrons derived from the substrate are allocated to PHA, primarily utilized by *Ca*. Accumulibacter. Therefore, in the long term, 34% of the substrate energy will be used for *Ca*. Accumulibacter growth. The metagenomics analysis revealing a relative abundance of 36% for *Ca*. Accumulibacter further corroborates this analysis from an alternative perspective.

Based on the principle of granulation, slow-growing bacteria is the key to obtain granulation (van Loosdrecht & de Kreuk, 2004). *Micropruina glycogenica* is demonstrated to be slow-growing bacteria (Shintani et al., 2000), which elucidates its ability to promote granulation in the glucose-fed system. Considering that *Ca*. Accumulibacter is also slow-growing bacteria (Mino et al., 1998), promoting the growth of both *Micropruina glycogenica* and *Ca*. Accumulibacter, as well as and their collaboration, represents a crucial strategy in engineering AGS systems fed with glucose.

5 Conclusion

- ✧ Good granulation and significant EBPR performance were obtained in the AGS system using glucose as the sole carbon source.

- ✧ Glucose was taken up rapidly, with a portion stored as intracellular polymers such as glycogen and PHAs and the remainder anaerobically fermented into lactate and formate.
- ✧ *Micropruina* was the predominant genus, followed by *Ca*. Accumulibacter, within the microbial community.
- ✧ *Micropruina glycogenica* was presumably to take up glucose and release lactate, then lactate was presumably to be the substrate for *Ca*. Accumulibacter to perform EBPR.
- ✧ The carbon and electron balance cannot be completely closed, with the missing portion speculated to be transformed into an unidentified polymer.
- ✧ The dominant organisms in the system are slow-growing bacteria, which account for the successful granulation and offer potential as an important strategy in engineering practice.

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Appendix

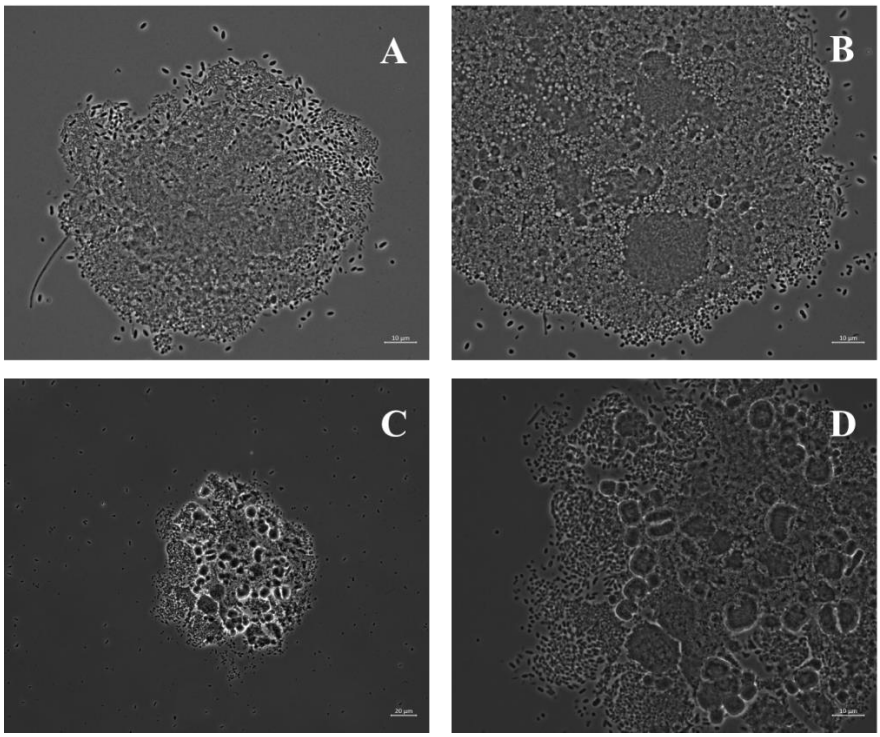

Figure 0-1 Microscopic picture of the biomass on Day 96 (A and B were from effluent suspend biomass; C and D were from granular sludge)

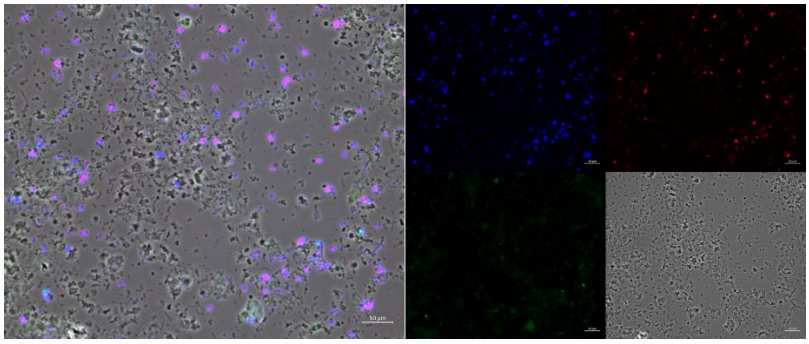

Figure 0-2 FISH picture to determine PAOs and *Ca*.Accumulibacter (Color representation – blue: PAOs651; red: *Ca*.Accumulibacter I; green: *Ca*.Accumulibacter II)

Table 0-1 Carbon balance in the anaerobic phase of normal reactor cycles (Unit for compounds: C-mmol/L)

Time range (min)	glucose	lactate	formate	Glycogen	PHA	Carbon coverage
0~65	-5.831	0.000	0.080	1.069	1.659	**48.2%**

Table 0-2 Electron balance in the anaerobic phase of normal reactor cycles (Unit for compounds: e-mmol/L)

Time range (min)	glucose	lactate	formate	Glycogen	PHA	Electron coverage
0~65	-23.33	0.00	0.16	4.28	7.96	**53.2%**

Table 0-3 Carbon balance in the glucose-fed bottle (1h) (Unit for compounds: C-mmol/L)

Time range (min)	glucose	lactate	formate	Acetate	Propionate	Glycogen	PHA	Carbon coverage
1~61	-38.0	8.38	0.248	0.260	0.470	15.066	1.570	**68.4%**

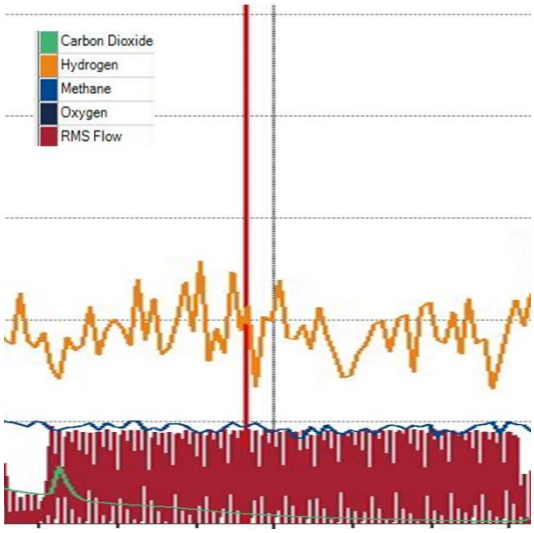

Figure 0-3 Off-gas measurement in the anaerobic phase of a normal cycle

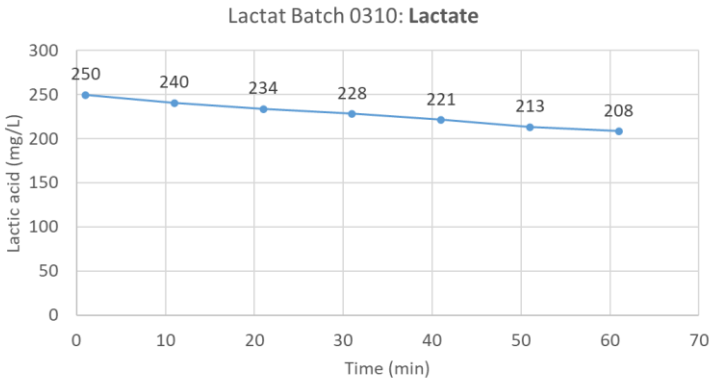

Figure 0-4 Lactate consumption in the lactate-fed bottle

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