Aerobic granular sludge phosphate removal using glucose

Elahinik, Ali; Li, Linghang; Pabst, Martin; Abbas, Ben; Xevgenos, Dimitrios; van Loosdrecht, Mark C.M.; Pronk, Mario

DOI
10.1016/j.watres.2023.120776

Publication date
2023

Document Version
Final published version

Published in
Water Research

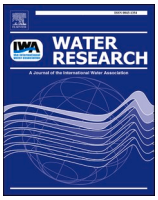
Citation (APA)
Elahinik, A., Li, L., Pabst, M., Abbas, B., Xevgenos, D., van Loosdrecht, M. C. M., & Pronk, M. (2023). Aerobic granular sludge phosphate removal using glucose. *Water Research*, *247*, Article 120776. https://doi.org/10.1016/j.watres.2023.120776

Water Research 247 (2023) 120776

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres



Aerobic granular sludge phosphate removal using glucose

Ali Elahinik [a,*], Linghang Li [a], Martin Pabst [a], Ben Abbas [a], Dimitrios Xevgenos [a], Mark C.M. van Loosdrecht [a], Mario Pronk [a,b]

[a] Department of Biotechnology, Delft University of Technology, van der Maasweg 9, Delft 2629HZ, the Netherlands
[b] Royal HaskoningDHV, Laan 1914 no 35, Amersfoort 3800AL, the Netherlands

ARTICLE INFO

Keywords:
Aerobic granular sludge
Enhanced biological phosphorus removal
Proteomics
Fermentative GAO
Ca. Accumulibacter
Micropruina

ABSTRACT

Enhanced biological phosphate removal and aerobic sludge granulation are commonly studied with fatty acids as substrate. Fermentative substrates such as glucose have received limited attention. In this work, glucose conversion by aerobic granular sludge and its impact on phosphate removal was studied. Long-term stable phosphate removal and successful granulation were achieved. Glucose was rapidly taken up (273 mg/gVSS/h) at the start of the anaerobic phase, while phosphate was released during the full anaerobic phase. Some lactate was produced during glucose consumption, which was anaerobically consumed once glucose was depleted. The phosphate release appeared to be directly proportional to the uptake of lactate. The ratio of phosphorus released to glucose carbon taken up over the full anaerobic phase was 0.25 Pmol/Cmol. Along with glucose and lactate uptake in the anaerobic phase, poly-hydroxy-alkanoates and glycogen storage were observed. There was a linear correlation between glucose consumption and lactate formation. While lactate accounted for approximately 89 % of the observed products in the bulk liquid, minor quantities of formate (5 %), propionate (4 %), and acetate (3 %) were also detected (mass fraction). Formate was not consumed anaerobically. Quantitative fluorescence *in-situ* hybridization (qFISH) revealed that polyphosphate accumulating organisms (PAO) accounted for 61 ± 15 % of the total biovolume. Metagenome evaluation of the biomass indicated a high abundance of *Micropruina* and *Ca.* Accumulibacter in the system, which was in accordance with the microscopic observations and the protein mass fraction from metaproteome analysis. Anaerobic conversions were evaluated based on theoretical ATP balances to provide the substrate distribution amongst the dominant genera. This research shows that aerobic granular sludge technology can be applied to glucose-containing effluents and that glucose is a suitable substrate for achieving phosphate removal. The results also show that for fermentable substrates a microbial community consisting of fermentative organisms and PAO develop.

1. Introduction

Aerobic Granular Sludge (AGS) technology is a biological wastewater treatment technique that offers significant advantages over conventional activated sludge systems, including a smaller footprint and improved energy efficiency (Bengtsson et al., 2018; de Bruin et al., 2004; de Kreuk et al., 2005). To ensure the formation of AGS and maintain stable reactor performance, various parameters have been identified as crucial (van Dijk et al., 2022). Amongst these parameters, the type of substrate plays a key role. A suitable substrate for granulation is one that is taken up and stored under anaerobic conditions by the microorganisms, and subsequently oxidized during an aerobic phase to provide energy for microbial growth processes (Pronk et al., 2015). This scenario selects for slow-growing organisms which are associated with the formation of smooth and stable granules (de Kreuk and van Loosdrecht, 2004). Substrates that do not undergo anaerobic sequestration are utilized aerobically by fast-growing heterotrophic organisms causing floc formation or filamentous growth and negatively influencing stable granulation (van Loosdrecht et al., 1997). Organisms that grow on storage polymers such as polyhydroxy-butyrate have a lower growth rate ($< 2\ d^{-1}$; Beun et al., 2002) than normal heterotrophs directly growing on the available substrate (e.g. *E. Coli* $< 2\ h^{-1}$; Buchanan and Klawitter, 1992).

Enhanced biological phosphorus removal (EBPR) is an efficient and sustainable technique for phosphate removal from water bodies (Ketchum et al., 1987). A common link between the AGS and EBPR process is the operational parameter, i.e. the induction of cyclical anaerobic-aerobic phases, which selects for a slow-growing organism

* Corresponding author.
E-mail address: a.elahinik@tudelft.nl (A. Elahinik).

https://doi.org/10.1016/j.watres.2023.120776
Received 3 August 2023; Received in revised form 25 September 2023; Accepted 21 October 2023
Available online 23 October 2023

known as polyphosphate accumulating organism (PAO). When acetate or propionate is the available substrate, PAOs tend to dominate the system, ensuring compact granule formation and effective phosphate removal (Oehmen et al., 2005; Pronk et al., 2015). Acetate and propionate can also be utilized by glycogen accumulation organisms (GAO) that can compete with PAOs, negatively affecting the EBPR efficiency. Despite their metabolic flexibility, PAOs are unable to directly utilize substrates such as certain sugars (Elahinik et al., 2022). Presence of substrates that cannot be directly sequestered into storage polymers in the anaerobic phase, either result in aerobic oxidation which is unfavourable for both the AGS and EBPR process or are fermented by fermentative organisms. The fermentation products can often be sequestered by PAO and similar organisms such as GAOs. The fermentative organisms often also store a fraction of the substrate and are denoted as "G-bacteria" (Cech and Hartman, 1993) or fermentative GAO (fGAO) (McIlroy et al., 2018). They are observed in wastewater treatment plants performing EBPR around the world but understanding their role has had limited attention (Seviour et al., 2000). It is important to study these organisms as they can compete for substrate, decreasing EBPR efficiency, or form non-granular and fragile biofilms, impacting solid-liquid separation.

Glucose, a substrate found in polymers in municipal wastewater (e.g. cellulose and starch) and in the effluent of various industries, such as food and sugar production facilities, has been marginally studied concerning granule formation. Regarding EBPR studies, glucose has been used in combination with fatty acids (e.g. glucose and acetate) in the past which makes it difficult to distinguish the effect of glucose independently (Gebremariam et al., 2012; Ziliani et al., 2023). In a study by Jeon & Park (2000), the long-term effect of glucose as the sole carbon source on the EBPR process using an activated sludge system was evaluated. The authors reported a probable synthesis of a lactate polymer which has not been reported in non-modified microorganisms in the literature. Therefore, in this research, an AGS reactor operating in sequencing batch mode was studied with glucose as the sole substrate to evaluate its potential for granulation and study the microbial community in relation to process stoichiometry and EBPR efficiency.

2. Material and methods

2.1. Experimental setup and reactor operation

A bubble column reactor with a working volume of 2.8 L, an internal diameter of 5.6 cm and a total height of 90 cm was operated in sequencing batch reactor (SBR) mode. After 1.5 L effluent withdrawal at the end of each cycle, a volumetric exchange ratio of 54 % was maintained. The pH was controlled at 7.0 ± 0.1 by automatic dosing of 0.5 M NaOH or HCl. The Dissolved Oxygen (DO) concentration was controlled at 0 % and 50 % (3.5 mg/L) saturation during the anaerobic and aerobic phase, respectively, by a mixture of nitrogen gas and air. The off-gas was recirculated with a flow of 5 L/min to maintain the DO concentration. The temperature of the reactor was not directly controlled but the room temperature was controlled at 20 °C. The reactor was inoculated with a mixture of aerobic granular sludge from a full-scale municipal wastewater treatment reactor performing EBPR located in Harnaschpolder, the Netherlands and glycerol-adapted sludge performing EBPR from previous experiments. Each cycle consisted of 5 min of nitrogen sparging to ensure anaerobic condition before feeding followed by 5 min of feeding, 60 min of nitrogen sparging (anaerobic phase), 120 min of aeration (aerobic phase), 5 min of settling, and 5 min of effluent withdrawal, equivalent to a total duration of 200 min. The sludge retention time (SRT) was controlled at 10 days via manual sludge removal. The SRT was calculated based on the solids in effluent and solids manually removed.

The synthetic influent fed at the beginning of each cycle with a total volume of 1500 mL consisted of 1200 mL of demineralized water, 150 mL of nutrient medium and 150 mL of carbon medium. Carbon medium contained 20.8 mM glucose, 3.6 mM $MgSO_4{\cdot}7H_2O$, and 4.7 mM KCl. The nutrient medium 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 $Zn.SO_4{\cdot}7H_2O$, 7.33 g/L $CaCl_2{\cdot}2H_2O$, 4.32 g/L $MnSO_4.H_2O$, 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 sources resulted in a final influent concentration of 400 mg/L COD, 58 mg/L NH_4^+-N, and 12 mg/L of PO_4^{3-}-P.

Batch experiments were performed in 250 mL Schott bottles, sparged with nitrogen to ensure an anaerobic environment, and controlled pH at 7 ± 0.1 by manual dosing of 0.5 M HCl or NaOH. Biomass at the end of the aerobic phase (end of the cycle) was taken from the reactor and used as inoculum (20 mL) for each test.

2.2. Analytical methods

Concentrations of PO_4^{3-}-P and NH_4^+-N were measured using a Gallery Discrete Analyser (ThermoFisher Scientific, USA). Chemical Oxygen Demand was measured with a spectrophotometer cuvette system (DR2800, Hach Lange, USA). Volatile fatty acids and sugars were measured using an HPLC (Vanquish, ThermoFisher Scientific, USA) equipped with an RI and UV detector, Aminex HPX-87H column (Bio-Rad, USA) using 0.0015 M phosphoric acid as eluent.

2.3. PHA and glycogen determination

Biomass samples from the reactor were fixed with 4 % w/v paraformaldehyde, washed with demineralized water, freeze-dried overnight, and crushed into fine powder. For extraction, approximately 30 mg of the powered biomass was hydrolysed and esterified in a 3 % sulphuric acid, methanol, and chloroform solution for 24 h at 100 °C with frequent manual vortex. For phase separation, 3 mL of ultrapure water was added to the samples and the formed esters in the organic phase were then filtered and analysed by a GC (6890 N, Agilent, USA). Quantifications of PHB, PH2MV and PHV were done using commercial 3-hydroxybutyrate, 2-hydroxyhexanoate and a synthetic copolymer of (*R*)−3-hydroxybutyrate-(*R*)−3-hydroxyvalerate (Sigma-Aldrich, USA) as standards, respectively. Benzoic acid was also added to the samples as an internal standard.

2.4. Biomass measurements

To determine the Total Suspended Solids (TSS) in the reactor, granules at the end of the cycle were taken and filtered through 0.45 μm glass fibre filters and placed in an oven at 105 °C for 24 h. The dried biomass sample was then incinerated at 550 °C for 3 h to determine the ash content. The Volatile Suspended Solids (VSS) were then calculated based on the difference between TSS and the ash content.

2.5. Microscopy and fish

To observe and capture the morphology of the granules, a stereo zoom microscope (M205 FA, Leica Microsystems, Germany) equipped with Qwin image analysis software (V3.5.1, Leica Microsystems, Germany) was used. For Fluorescence *in-situ* Hybridization (FISH), the handling and staining of the biomass samples were done using the protocol described by Bassin et al. (2011). The images were captured with an epifluorescence microscope equipped with filter set Cy3 (ET545/25x ET605/70 m T565LPXR), Cy5 (ET640/30x ET690/50 m T660LPXR), and FITC (ET470/40x ET525/50 m T495LPXR) (Axio Imager M2, Zeiss, Germany). Quantitative FISH (qFISH) was done as a percentage of total biovolume which was stained with a general probe (EUBmix) and a specific probe (PAO651) (Daims et al., 1999). Quantification was done over 9 representative pictures using daime software (DOME, Vienna, Austria) (Daims et al., 2006).

2.6. Metagenomics and metaproteomics

The DNA from the biomass samples was extracted using the DNeasy PowerSoil Pro-Kit (Qiagen, Germany) following the manufacturer's protocol. Metagenome sequencing and raw data processing were conducted by Novogene (Novogene Co., China). For protein extraction and metaproteome analysis, the methodology described by Kleikamp et al. (2022) was followed. Similar to (Elahinik et al., 2022), the functional proteins were annotated using the KEGG and EggNOG databases, and the metaproteome raw data was analysed using the metagenome-constructed dataset. Pathway reconstruction with expressed proteins was performed with GhostKOALA.

2.7. Substrate distribution considerations

To calculate substrate distribution amongst the dominant genera, ATP balances over metabolic pathways were made assuming glucose conversion in the anaerobic phase followed a three-step process; 1- uptake of glucose and storage as glycogen, 2- fermentation of glycogen/glucose to lactate by fGAOs, and 3- lactate conversion into PHA by PAOs. To assess the energy balance within the fGAOs, it was assumed that the ATP needed for glucose uptake and storage as glycogen is provided through lactate fermentation *(1)* (Jeon and Park, 2000). The ATP generated from lactate production can be 1 or 2 mol depending on whether the reaction follows the Entner–Doudoroff (ED) or the Embden-Meyerhof-Parnas (EMP) pathway, respectively (Mino et al., 1994). Assuming the EMP pathway, (supported by the expression of proteins involved in the EMP pathway) (Table 1), this balance suggests that half of the glucose is utilized for glycogen synthesis, while the other half is converted into lactate.

$$-Glucose - 2\ ATP + Glycogen \quad (1)$$

$$-Glucose + 2\ ATP + 2\ Lactate \quad (2)$$

The ATP balance for the subsequent uptake of lactate and storage as PHA by PAOs was done according to the model described by Mino et al. (1994). The model takes into account the ATP needed for transport (1 $mol_{ATP}/mol_{substrate}$) and acetyl-CoA formation (1.5 $mol_{ATP}/mol_{lactate}$), supplied through poly-P hydrolysis. According to models describing the anaerobic metabolism of PAOs, the ATP needed for substrate uptake and PHA synthesis is generated through poly-P hydrolysis, and glycogen conversion is primarily used for NADH generation (Smolders et al., 1994). When lactate is utilized, the cells can maintain a redox balance through simultaneous conversion of pyruvate into acetyl-CoA and propionyl-CoA as shown in Eq. (3) (Mino et al., 1994). The produced NADH is then used in the condensation of acetyl-CoA and propionyl-CoA into PHA.

$$-2\ Pyruvate - 1\ ATP + \ Propionyl\_CoA + Acetyl\_CoA \ + CO_2 + NADH \quad (3)$$

3. Results

3.1. Reactor operation

A combination of full-scale and laboratory-scale aerobic granular sludge performing EBPR was used as inoculum. During the experimental period, the reactor performance was monitored on a daily basis via online pH, dissolved oxygen, and acid/base dosing measurements. Additionally, several cycle measurements were performed when the reactor reached a steady state to ensure reactor stability and that the presented figures are reliable and representative. The reactor reached a pseudo-steady state in terms of stable conversions within 4 weeks and in terms of granular sludge formation after about 2 months of reactor operation. The granules exhibited a smooth and stable structure, devoid of filamentous growth (Fig. 1) with excellent settling properties indicated by an average sludge volume index (SVI_5) of 24 mL/g. Stereoscopic imaging revealed a granule size range of 0.3 to 1 mm. The sludge

Table 1
Key metabolic pathways and the relevant detected expressed proteins by organisms with 75 % of the total protein mass. Enzymes typically involved in EMP (*) and ED (**) pathways.

Pathway	Protein	KEGG Symbol	Betaproteobacteria	Actinobacteria
Glycogen synthesis	Phosphoglucomutase	*pgm*	+	+
	Glucose-1-phosphate adenylyltransferase	*glgC*	+	+
	Glycogen synthase	*glgA*	+	–
	1,4-alphaglucan branching enyzme	*glgB*	+	+
	Glycogen phosphorylase	*glgP*	+	+
	Glycogen debranching enzyme	*TreX*	+	+
PHA synthesis	D-lactate dehydrogenase	*dld*	+	+
	Starch synthase	*glgE*	–	+
	PHA synthase	*phaC*	+	–
	PHA polymerase	*phaE*	–	–
	Acetyl-CoA transferase	*phaA*	–	–
	Acetoacetyl-CoA reductase	*phbB*	+	–
	Propionyl-CoA synthetase	*prpE*	+	–
	Propionyl-CoA carboxylase alpha	*pccA*	+	–
	Propionyl-CoA carboxylase beta	*pccB*	–	–
	Acetyl-CoA synthetase	*acs*	+	+
	Methylmalonyl-CoA mutase	*mut*	–	+
Polyphosphate metabolism	Polyphosphate glucokinase	*ppgk*	–	–
	Phosphoglycerate	*pgk*	+	+
	Polyphosphate kinase	*ppk*	+	+
	Polyphosphate AMP phosphotransferase	*pap*	+	–
	Phosphate acetyl transferase	*pta*	+	+
	Exopolyphosphatase	*ppx*	+	–
Glucose metabolism	Phosphotransferase system	*pts*	–	+
	6-phosphofructokinase*	*pfk*	+	+
	Fructose-1,6-bisphosphatase*	*fbp*	+	+
	Triosephosphate isomerase*	*tpi*	+	+
	Glyceraldehyde 3-phosphate dehydrogenase*	*gapA*	+	+
	glucose-6-phosphate 1-dehydrogenase**	zwf	–	–
	6-phosphogluconate dehydrogenase**	gnd	–	–
	Glucokinase	*glk*	–	–

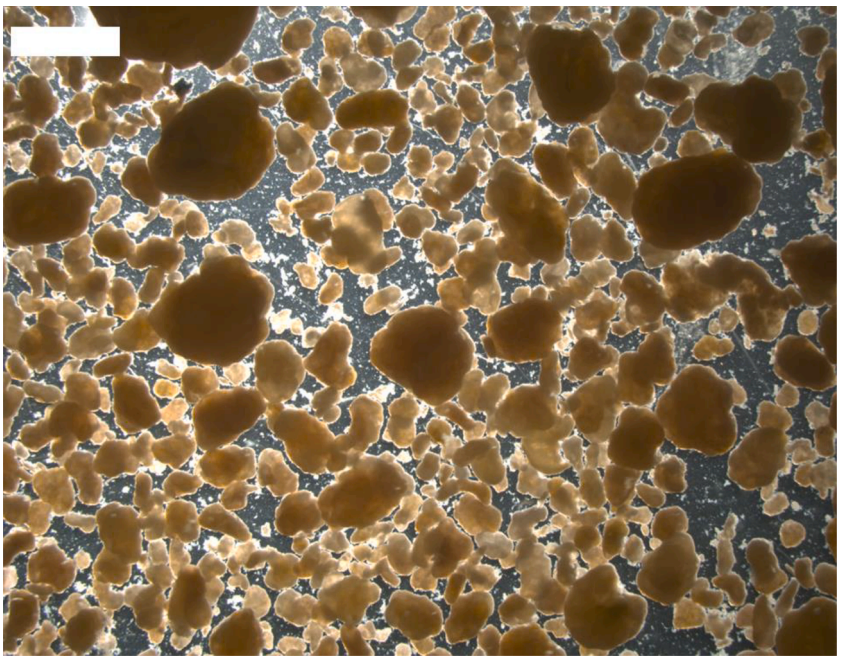


Fig. 1. Stereoscopic image of aerobic granular sludge fed with glucose after 88 days of reactor operation. The scale bar is equal to 1 mm.

bed displayed a light brown-orange colour, which was darker compared to granules fed with acetate. The TSS in the reactor averaged around 5.7 g/L, with a VSS to TSS ratio of approximately 80 %. The effluent was relatively clear, with an average TSS concentration of approximately 90 mg/L.

During stable conditions, the anaerobic conversions were a combination of substrate (glucose) fermentation and product (lactate) uptake type reaction. As shown in Fig. 2, glucose was rapidly taken up (273 mg/gVSS/h) and phosphate was released (15.5 mgP/gVSS/h) in the anaerobic phase. Simultaneously, a minor amount of lactate was produced alongside glucose consumption. The lactate was also anaerobically consumed once glucose was depleted. The phosphate release appeared to be directly proportional to the uptake of lactate when glucose was no longer available. The calculated ratio of phosphorus released to lactate carbon uptake was 0.55 Pmol/Cmol based on the ATP balance (half of glucose converted to lactate). When lactate was depleted, the rate of phosphate release also decelerated. The secondary phosphate release can be attributed to cellular maintenance processes and had a rate of 2.3 mgP/gVSS/h. In the aerobic phase, phosphate was seemingly taken up in two stages expressed by the primary (9.3 mgP/gVSS/h) and the secondary (3.9 mgP/gVSS/h) phosphate uptake rates. The observed delay in phosphate uptake within the initial five minutes into the aerobic phase was maybe related to the aerobic utilization of formate that was produced in the anaerobic phase (formate formation described in Section 3.2). The aerobic specific ammonium consumption rate was 0.93 mgN/gVSS/h for biomass growth since nitrification was inhibited due to ATU addition.

The ratio of phosphorus released to glucose carbon taken up in the anaerobic phase was 0.25 Pmol/Cmol. Along with glucose and lactate uptake in the anaerobic phase, PHA and glycogen storage was observed

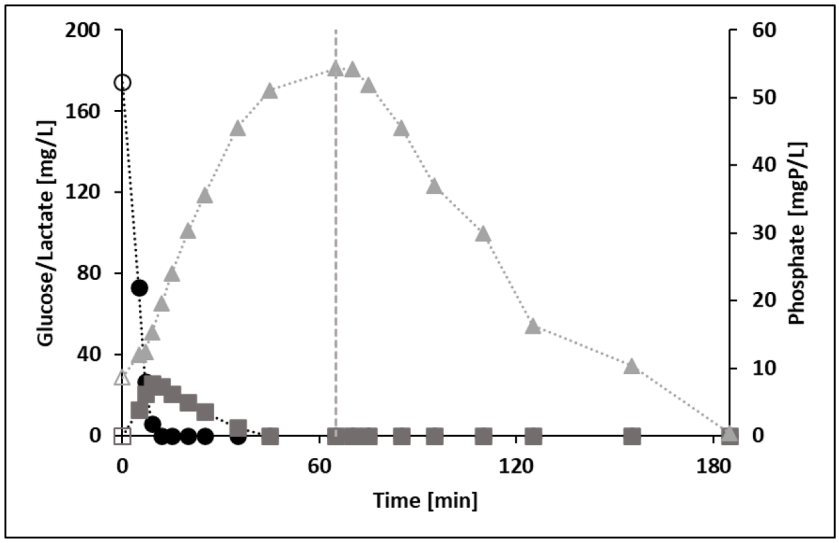


Fig. 2. Overview of a representative cycle during steady state condition. Glucose (circles), lactate (squares), and phosphate (triangles) transformations are shown. The first hollow markers show the calculated concentrations based on dilution and influent concentration. The dashed-vertical line shows the switch between the anaerobic and aerobic phases.

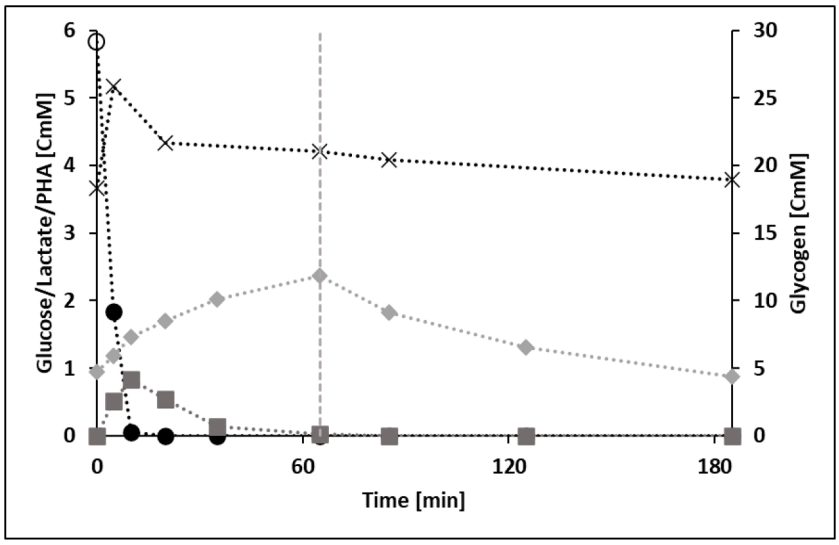


Fig. 3. Glucose (circles), lactate (squares), PHA (diamonds), and glycogen (crosses) profile during the complete cycle. The first hollow marker representing glucose shows the calculated concentration based on dilution and influent concentration. The dashed-vertical line shows the switch between the anaerobic and aerobic phases.

(Fig. 3). The electron balance distribution performed over the anaerobic phase shown in Fig. 3 closed with a recovery of 94 % (molar basis). This glucose-COD was recovered as storage of glycogen (46 %) and PHA (29 %), as well as growth (18 %) estimated based on anaerobic ammonium consumption of 0.7 mgN/gVSS. A minor amount of formate was anaerobically produced ($\leq$ 1 %) which was oxidized aerobically.

3.2. Batch tests

To gain further insights into the conversions occurring within the reactor during the anaerobic phase, a series of batch tests were conducted. The first batch test involved separate bottles containing formate and lactate, where biomass from the reactor (collected at the end of the cycle) was used to assess their anaerobic utilization (Fig. 4). Nitrogen gas was continuously sparged to ensure an anaerobic environment in the bottles. As anticipated, formate demonstrated no anaerobic utilization, aligning with the observations made in the reactor. Lactate was anaerobically utilized at a rate of 13.7 mg/gVSS/h and PHV formation was observed. No glycogen accumulation was observed. Electron balancing performed over anaerobic lactate uptake, PHA accumulation, and biomass growth closed with a recovery of 108 %. With both substrates P-release was observed, with the assumption that P-release in the presence of formate was attributed to maintenance processes and the higher P-release in the presence of lactate was due to lactate consumption. The P-release rate with lactate was 11.6 mgP/gVSS/h with a P/C ratio of 0.45 Pmol/Cmol.

To identify the product formation during the anaerobic phase fed with glucose a batch test with a high glucose addition (10 times more than in the regular operation) was performed. A linear correlation between glucose consumption and lactate formation was observed (Fig. 5). Lactate accounted for approximately 89 % of the products in the bulk liquid (by mass fraction) with minor quantities of formate (5 %), propionate (4 %), and acetate (3 %). The rates of glucose uptake and lactate production were determined to be 310 and 83 mg/gVSS/h, respectively. Note that the intrinsic lactate production rate was likely to be higher than the observed rate due to simultaneous consumption. During the test, glycogen accumulation (21.3 mg/gVSS) and PHA production (6.7 mg/gVSS) were also observed (as expected).

3.3. Microbial community

Biomass samples at the end of the experimental period were taken from the reactor for microscopic and meta-omics analysis. Phase contrast images showed that distinct microbial communities with diverse morphologies were present (Fig. 6.A). A representative FISH picture of the biomass is shown in Fig. 6.B. and qFISH results revealed that the PAOs accounted for 61 $\pm$ 15 % of the total biovolume.

Extracted DNA and proteins of the biomass were analysed and the genome information of the functional bins was submitted to the NCBI database under the accession number PRJNA1020750. The metaproteome was analysed using the metagenome-constructed database. The relative abundance of the top 7 genera accounting for 21 % and 34

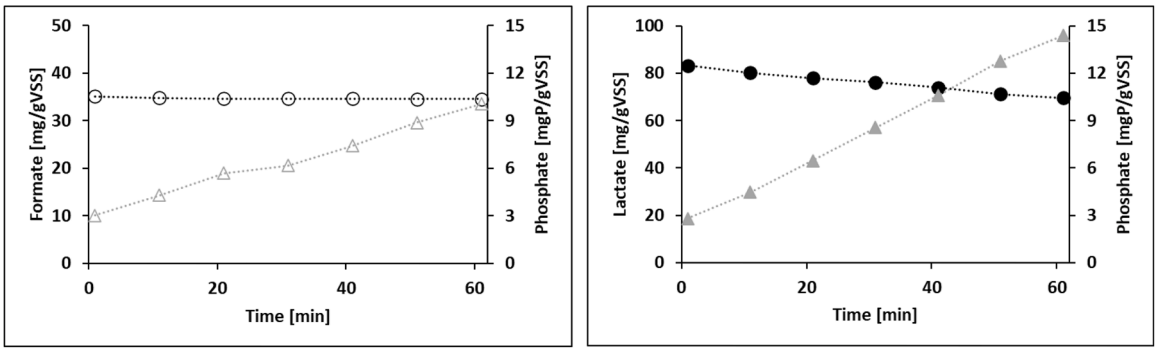


Fig. 4. Lactate (left) and formate (right) batch tests showing substrate (circles) and phosphate (triangles) anaerobic transformations.

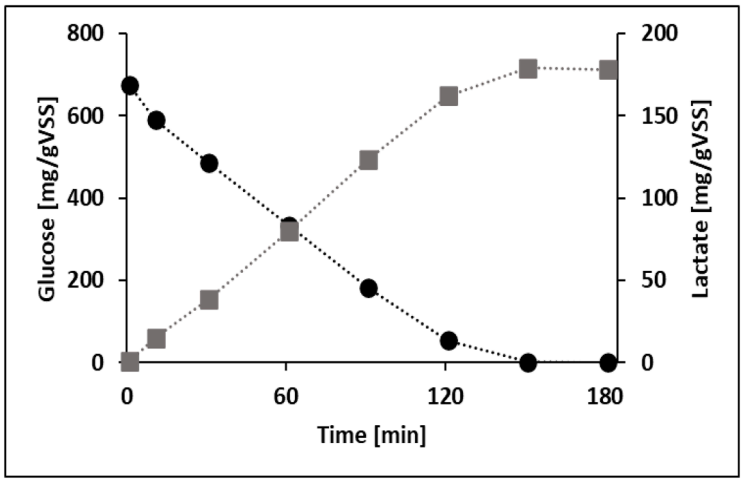


Fig. 5. Anaerobic glucose consumption (circles) and lactate production (squares) as a function of time.

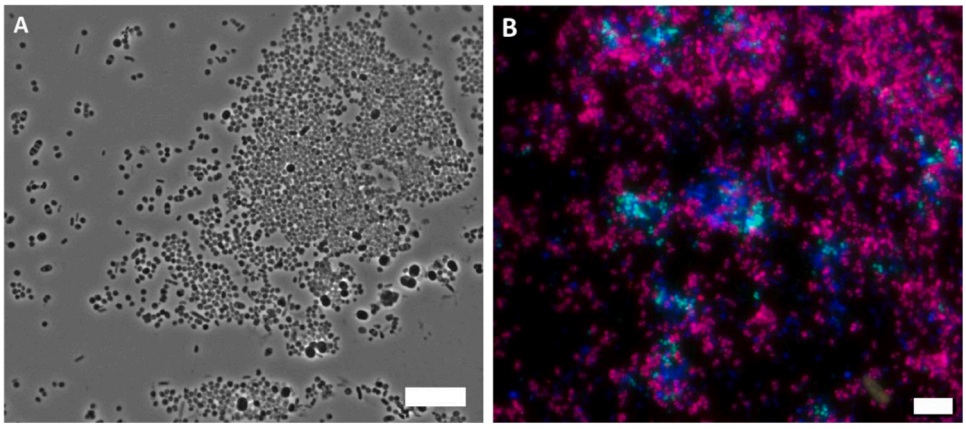


Fig. 6. Phase contrast and FISH picture of the microbial population. The scale bar is equal to 10 μm. Blue represents all microorganisms stained with probe EUBmix, magenta shows the PAO population stained with probe PAO651, and cyan shows the cluster stained with probe Tess644.

% coverage according to metagenomics and metaproteome analysis, respectively, is shown in Fig. 7. The "other" excluded fraction accounted for extremely low abundant taxa with few protein matches and low protein sequence coverage. The area of the peptide per protein was summed to calculate protein mass and estimate the relative abundance. Based on protein mass fraction and metagenome contigs, *Ca.* Accumulibacter and *Micropruina* were identified as the dominant genera amongst all of the identified taxa which corresponded to microscopic

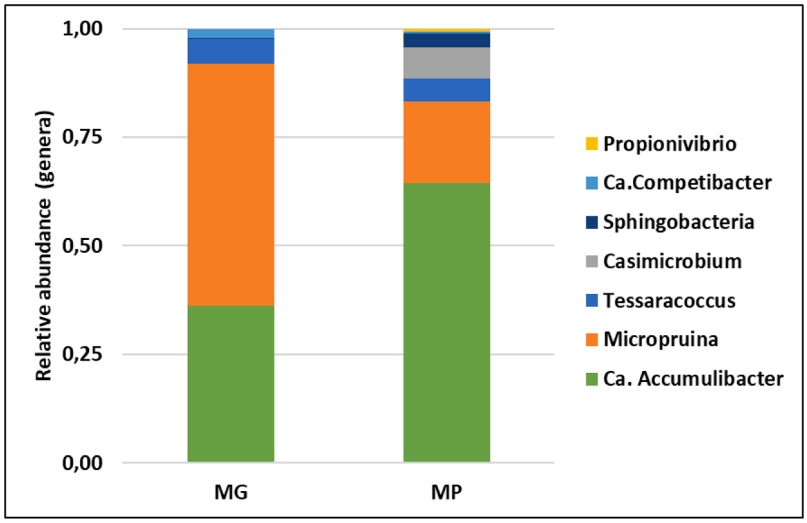


Fig. 7. Relative abundance of the top genera according to metagenome (MG) contigs and metaproteome (MP) analysis using protein mass fraction.

observations (Fig. 6). Based on class distribution, *Actinobacteria* and *Betaproteobacteria* accounted for 19 and 43 % of all of the protein mass, respectively. Moreover, the metaproteome analysis provided the expressed proteins that were used to investigate the anaerobic conversions and associate the reactions to the respective class (Table 1). Since *Ca*. Accumulibacter and *Micropruina* were the respective dominant members of the *Betaproteobacteria* and *Actinobacteria* classes, and the protein sequence coverage was higher in class distribution (as expected), the expressed proteins are shown as a function of class rather than genus.

3.4. ATP balances

To further evaluate the anaerobic conversions and estimate substrate distribution amongst PAOs and fGAOs, an ATP calculation was performed based on equations in Section 2.7. By combining the existing information from literature, our experimental results, and the genetic potential of *Micropruina* (fGAOs) and *Ca*. Accumulibacter (PAOs), glucose conversion in the anaerobic phase was assumed to follow a three-step process (Fig. 8); 1- uptake of glucose and storage as glycogen, 2- fermentation of glycogen (and glucose) to lactate by fGAOs, and 3- lactate sequestration into PHA by PAOs. According to the energy balance within fGAOs, half of the glucose is utilized for glycogen synthesis, while the other half is converted into lactate (Table 2). When comparing theoretical estimates to measured values, it was found that both PHA and glycogen synthesis aligned closely with the theoretical estimates. The amount of glycogen synthesized was determined to be 0.5 Cmmol/Cmmol, which closely matched the measured value of 0.46 Cmmol/Cmmol. Additionally, by extrapolating the theoretical lactate yield, the estimated PHA yield was calculated to be 0.5 Cmmol/Cmmol, which also exhibited a strong correlation with the measured value of 0.47 Cmmol/Cmmol. Finally, an ATP balance was performed for the PAOs and considered the energy required for lactate transport and PHA synthesis was supplied only through polyphosphate hydrolysis (96 % recovery, Table 3).

Table 2
Theoretical ATP balance performed over the fermentative organisms assuming ATP required for glycogen synthesis is replenished by lactate fermentation.

fGAO				
ATP required	**ATP produced**	**ATP balance**	**Theoretical**	
Glycogen synthesis mol/C-mol	Lactate synthesis mol/C-mol	%	Glycogen yield mol/mol	Lactate yield mol/mol
−0,33	0,33	100	0,50	0,50

Table 3
ATP balance over the PAOs.

PAO			
ATP required		**ATP produced**	**ATP balance**
VFA-CoA activation mol/C-mol	Lactate transport mol/C-mol	Poly-P hydrolysis mol/C-mol	%
0,12	0,17	0,28	96

4. Discussion

In summary, the experimental findings suggest that glucose is a suitable substrate for the EBPR process and that it can facilitate the formation of stable granules. The anaerobic conversions seemed to be a biotransformation cascade; substrate fermentation (and storage) and product uptake reaction by different microbial communities. Microbial evaluation of the biomass indicated a dominant presence of *Ca*. Accumulibacter and *Micropruina* in the system were mainly responsible for metabolizing glucose.

4.1. Anaerobic conversions and substrate distribution

During the anaerobic phase, several biochemical reactions took place simultaneously. Glucose was rapidly taken up within the first 10 min

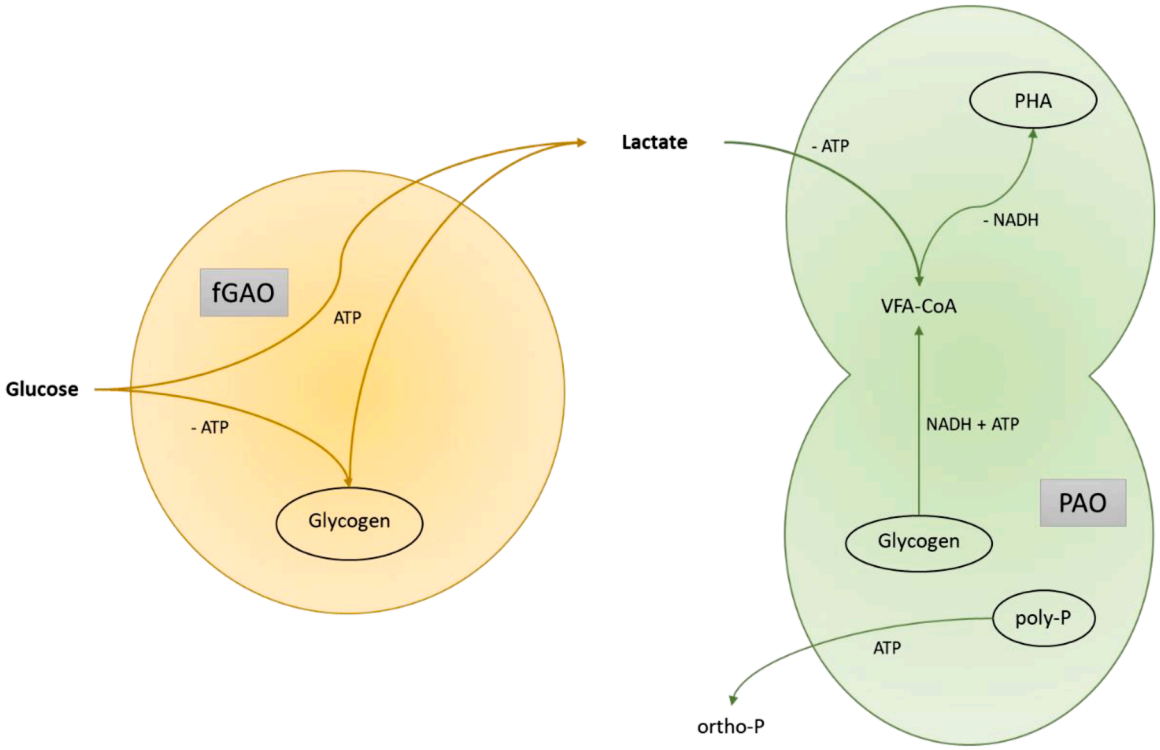


Fig. 8. Conceptual overview of microbial interaction between fGAOs and PAOs utilizing glucose in the anaerobic phase of AGS-EBPR process.

and glycogen concentration increased within the same time frame (Fig. 3). In a separate batch with 10 times higher glucose concentration, the direct correlation between glucose conversion and lactate production was verified (Fig. 4). In this test, the glucose uptake rate was similar as in the regular reactor operation. The batch experiments also indicated that P-release was associated with the uptake of lactate rather than the uptake of glucose. Based on these observations and the analysis of the microbial community, we hypothesized that the anaerobic conversion followed a biotransformation cascade, which was similar to the findings of Jeon & Park. (2000) where glucose was used as the sole electron donor in an EBPR system. However, Jeon & Park. (2000) proposed a probable metabolic pathway which included a "lactate polymer" synthesis based on an undefined peak in their ^{13}C NMR spectra. Despite multiple attempts, we were unable to detect any lactate-based storage compounds from the biomass in this study. To the best of the author's knowledge, lactate-based biopolymers are currently only produced through genetically modified organisms (Taguchi et al., 2008). Moreover, since glucose can be used for extracellular polymer formation (Fuhrer et al., 2005), the undefined NMR peak spectra could potentially be products like gluconate that are extracellularly produced and constitute the extracellular polymeric substance (EPS).

We hypothesize that glucose was initially assimilated and stored as glycogen by the fGAOs, with the subsequent conversion into lactate. While it remains unclear whether simultaneous glycogen storage and lactate production occurred, it is still a plausible scenario. Subsequently, the generated lactate served as a substrate for PAOs, contributing to the production of PHA. To validate our hypothesis, we compared the theoretical estimates and the measured values. The measured P/C ratio during a batch test with lactate as the only substrate was 0.45 Pmol/Cmol (Fig. 4). This value is comparable to the estimated value of 0.55 Pmol/Cmol during a normal cycle which was calculated with the assumption that half of the glucose was converted into lactate and that the lactate was only utilized by PAOs. In literature, a lactate yield of 0.65 g/g_{sugar} was reported (Tsapekos et al., 2020), which is slightly higher than the estimated lactate yield here but that is likely attributed to the utilization of a pure culture. Regarding the overall P/C ratio, the ratio of 0.25 $Pmol/Cmol_{glucose}$ is similar to the ratio of 0.24 Pmol/Cmol reported by Ziliani et al. (2023) using glucose in a batch test. The P/C ratios obtained with sugar substrates are generally lower $\approx$ 0.25 Pmol/Cmol (Elahinik et al., 2022; Guerrero et al., 2012; Ziliani et al., 2023) compared to the ratios obtained using VFAs $\approx$ 0.5 Pmol/Cmol (Oehmen et al., 2005; Smolders et al., 1994). This difference is likely due to partial consumption of the substrate by other organisms (e.g. fermentative population) when sugars are used as substrate. The partial consumption of substrate decreases the available food for PAOs which results in lower P/C ratios. According to the theoretical lactate yield (0.5 $mol_{lactate}/mol_{glucose}$) and the assumption of polyphosphate hydrolysis by PAOs only (supported by the absence of *pap* in *Actinobacteria*), the ATP balance over PAOs closed with 96 % recovery (Table 3). Moreover, the balance suggested that the energy required for the uptake of lactate was only provided via polyphosphate hydrolysis and that additional glycogen cleavage was likely not occurring.

Regarding the PHA distribution, PHV was the only detected polymer similar to the findings of Jeon & Park. (2000). Detection of PHV is an indication of acetyl- and propionyl-CoA generation, two precursors of PHV synthesis (Oehmen et al., 2005). In lactate metabolism, a proposed mechanism suggests the simultaneous oxidation and reduction of pyruvate, leading to the production of acetyl-CoA and propionyl-CoA (Mino et al., 1994). Given that low amounts of acetate and propionate were also detected during the batch test conducted with excess glucose, it is reasonable to anticipate such reactions took place. Furthermore, the NADH produced via the acetyl-CoA pathway can be consumed in the methylmalonyl-CoA pathway (a.k.a. Wood-Werkman cycle) generating propionyl-CoA, maintaining the redox balance within the cell.

4.2. Proteomic insights

The microbial community analysis showed that the two predominant genera in the system were *Ca*. Accumulibacter and *Micropruina,* which are referred to as PAOs and fGAOs, respectively. In an EBPR study by Jeon & Park (2000) where glucose was used as the sole electron donor, the authors hypothesized that EBPR was likely achieved by two groups of microorganisms despite any microbial community analysis; lactic acid-producing organisms and PAOs. In this study, the dominant presence of PAOs (*Ca*. Accumulibacter) and lactic acid producers (*Micropruina* a.k.a. fGAOs) were observed which confirms their hypothesis. Both of these organisms have been observed in EBPR performing WWTPs around the world and information related to their metabolism is available in the literature (Hesselmann et al., 1999; McIlroy et al., 2018). Of particular relevance to this study is the genus *Micropruina*, as they can metabolise glucose, supported by the activity of the phosphotransferase system (*pts*) and their described physiological characteristics in literature (Shintani et al., 2000). *Micropruina* belongs to the *Actinobacteria* class and thrives particularly in carbohydrate-rich environments (Seviour et al., 2000). They are Gram-positive facultative anaerobes that can perform fermentation and glycogen storage (Shintani et al., 2000). By using the combination of FISH and Raman spectroscopy, McIlroy et al. (2018) reported the observation of glycogen in *Micropruina* but neither PHA nor polyphosphate. In this study, the absence of PHA synthesizing proteins in these organisms confirmed that these organisms are unable to produce PHA polymers. Regarding polyphosphate metabolism, the expression of *ppk* enzyme indicates polyphosphate accumulation ability but the absence of *pap* suggests that *Micropruina* do not rely on energy generation from polyphosphate hydrolysis which is in agreement with previous findings (Elahinik et al., 2022). Detection of lactate dehydrogenase and expression of PHA synthesizing proteins in *Ca*. Accumulibacter suggests that these organisms are indeed able to metabolize lactate anaerobically and likely produce PHA using lactate. Moreover, as shown in Table 1, glycolysis in both organisms followed the EMP pathway which is also the commonly reported pathway in EBPR studies (Guedes da Silva et al., 2020; Martín et al., 2006; Ziliani et al., 2023).

4.3. Influence of carbohydrates on the microbiology of EBPR systems

When substrates such as acetate or propionate are used, a highly enriched monoculture is usually achieved that performs anaerobic substrate uptake and PHA synthesis. Alternatively, when non-VFA substrates such as glycerol or glucose are used, a consortium of microorganisms is enriched that performs a biotransformation cascade. The cascade entails the fermentation of substrate into fermentation products by fermentative organisms that use the obtained energy for substrate storage. The fermentation products are subsequently taken up and stored by PAO or GAO-like organisms. During the cascade, storage of glycogen or glycogen-like materials by fermenters can also take place, hence the name "G-bacteria" which was proposed by Cech & Hartman (1993) for these microorganisms. In a minireview by Seviour et al. (2000), the physiology of this group of organisms and why they thrive in certain environments is thoroughly discussed. In short, systems that operate under anaerobic-aerobic conditions with carbohydrates in the influent, allow the proliferation of fGAOs, which also corresponds to the observations in this study. The storage strategy gives these organisms a competitive advantage through uncoupling substrate uptake and growth (Rombouts et al., 2020; Shimada et al., 2007). They can maximize their substrate uptake rate during the feast period and grow in famine conditions using their storage pool. In this study, the observations suggest that a part of the stored glycogen was fermented anaerobically (producing lactate) and likely the other part was utilized aerobically.

4.4. Practical implications

- The application of AGS technology is suitable for the treatment of wastewater with high sugar content since stable granulation can be achieved.
- The dominance of fGAOs may be unfavourable for an efficient EBPR system, however, their complete absence can be detrimental to the overall EBPR process, particularly when sugars like glucose or glycerol are used as the sole electron donor. These organisms compete for substrate but also produce PAO-utilizable products. However, finding ways to provide a selective advantage towards pure fermentative organisms that, unlike fGAOs, do not store substrate might further optimize phosphate removal.
- In situations of carbon scarcity, a potential remedy is to introduce external carbon sources to mitigate the risk of EBPR failures. Glucose can serve as a viable substitute for an external carbon source, considering its cost, availability, and that it is first fermented into PAO-utilizable products.
- In the case of biopolymer recovery, the abundant presence of organisms other than the typical PAOs in the sludge can impact the properties of the extracted Kaumera biopolymer (de Bruin et al., 2022; Tomás-Martínez et al., 2023).

5. Conclusion

- Long-term stable reactor performance and successful granulation were shown with glucose as the sole carbon source in an AGS system.
- Phosphate removal was achieved through a collaboration between fGAOs (*Micropruina)* and PAOs (*Ca*. Accumulibacter).
- Lactate was the main product of glucose conversion in the anaerobic phase.
- Absence of PHA synthesizing enzymes in *Micropruina* indicates that these organisms are likely unable to produce PHA which confirms previous findings in the literature.
- Detection of lactate dehydrogenase and expression of PHA synthesizing proteins in *Ca*. Accumulibacter suggests that these organisms are able to metabolize lactate anaerobically and likely produce PHA using lactate.
- Lack of PTS protein components in *Ca*. Accumulibacter suggests that these organisms are not able to directly utilize glucose.

6. Declaration of AI-assisted technologies in scientific writing

During the preparation of this work, the author used ChatGPT 3.5 in order to improve the structure and readability of some sentences. After using this tool, the author reviewed and edited the content as needed and takes full responsibility for the content of the publication.

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.

Acknowledgments

This project was funded by WATER-MINING which is a research and innovation project that develops energy-efficient technologies for treating municipal and industrial wastewater, whilst promoting resource recovery. WATER-MINING (Grant Agreement No. 869474) is partially funded by the European Union Horizon 2020 research and innovation programme. For further information regarding the project, the reader can refer to (Petrik et al., 2022).

References

Bassin J.P., Pronk M., Muyzer G., Kleerebezem R., Dezotti M., & van Loosdrecht M.C.M. (2011). Effect of elevated salt concentrations on the aerobic granular sludge process: linking microbial activity with microbial community structure. Appl. Environ. Microbiol., 77(22), 7942–7953. 10.1128/AEM.05016-11.

Bengtsson, S., de Blois, M., Wilén, B.M., Gustavsson, D., 2018. Treatment of municipal wastewater with aerobic granular sludge. Crit. Rev. Environ. Sci. Technol. 48 (2), 119–166. https://doi.org/10.1080/10643389.2018.1439653.

Beun, J.J., Dircks, K., Van Loosdrecht, M.C.M., Heijnen, J.J., 2002. Poly-β-hydroxybutyrate metabolism in dynamically fed mixed microbial cultures. Water Res. 36 (5), 1167–1180. https://doi.org/10.1016/S0043-1354(01)00317-7.

Buchanan, R.L., Klawitter, L.A., 1992. The effect of incubation temperature, initial pH, and sodium chloride on the growth kinetics of Escherichia coli O157:H7. Food Microbiol. 9 (3), 185–196. https://doi.org/10.1016/0740-0020(92)80046-7.

Cech, J.S., Hartman, P., 1993. Competition between polyphosphate and polysaccharide accumulating bacteria in enhanced biological phosphate removal systems. Water Res. 27 (7), 1219–1225. https://doi.org/10.1016/0043-1354(93)90014-9.

Daims, H., Brühl, A., Amann, R., Schleifer, K.H., Wagner, M., 1999. The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. 22 (3), 434–444. https://doi.org/10.1016/S0723-2020(99)80053-8.

Daims, H., Lücker, S., Wagner, M., 2006. daime, a novel image analysis program for microbial ecology and biofilm research. Environ. Microbiol. 8 (2), 200–213. https://doi.org/10.1111/J.1462-2920.2005.00880.X.

de Bruin, L.M.M., de Kreuk, M.K., van der Roest, H.F.R., Uijterlinde, C., van Loosdrecht, M.C.M., 2004. Aerobic granular sludge technology: an alternative to activated sludge. Water Sci. Technol. 49 (11–12), 1–7. https://doi.org/10.2166/wst.2004.0790.

de Bruin, S., Vasquez-Cardenas, D., Sarbu, S.M., Meysman, F.J.R., Sousa, D.Z., van Loosdrecht, M.C.M., Lin, Y., 2022. Sulfated glycosaminoglycan-like polymers are present in an acidophilic biofilm from a sulfidic cave. Sci. Total Environ. 829, 154472 https://doi.org/10.1016/J.SCITOTENV.2022.154472.

de Kreuk, M.K., Pronk, M., van Loosdrecht, M.C.M., 2005. Formation of aerobic granules and conversion processes in an aerobic granular sludge reactor at moderate and low temperatures. Water Res. 39 (18), 4476–4484. https://doi.org/10.1016/j.watres.2005.08.031.

de Kreuk, M.K., van Loosdrecht, M.C.M., 2004. Selection of slow growing organisms as a means for improving aerobic granular sludge stability. Water Sci. Technol. 49 (11–12), 9–17. https://doi.org/10.2166/wst.2004.0792.

Elahinik, A., Haarsma, M., Abbas, B., Pabst, M., Xevgenos, D., van Loosdrecht, M.C.M., Pronk, M., 2022. Glycerol conversion by aerobic granular sludge. Water Res. 227, 119340 https://doi.org/10.1016/J.WATRES.2022.119340.

Fuhrer, T., Fischer, E., Sauer, U., 2005. Experimental identification and quantification of glucose metabolism in seven bacterial species. J. Bacteriol. 187 (5), 1581. https://doi.org/10.1128/JB.187.5.1581-1590.2005.

Gebremariam, S.Y., Beutel, M.W., Christian, D., Hess, T.F., 2012. Effects of glucose on the performance of enhanced biological phosphorus removal activated sludge enriched with acetate. Bioresour. Technol. 121, 19–24. https://doi.org/10.1016/J.BIORTECH.2012.06.086.

Guedes da Silva, L., Olavarria Gamez, K., Castro Gomes, J., Akkermans, K., Welles, L., Abbas, B., van Loosdrecht, M.C.M., Wahl, S.A., 2020. Revealing the metabolic flexibility of "candidatus Accumulibacter phosphatis" through redox cofactor analysis and metabolic network modeling. Appl. Environ. Microbiol. (24), 86. https://doi.org/10.1128/AEM.00808-20.

Guerrero, J., Tayà, C., Guisasola, A., Baeza, J.A., 2012. Glycerol as a sole carbon source for enhanced biological phosphorus removal. Water Res. 46 (9), 2983–2991. https://doi.org/10.1016/j.watres.2012.02.043.

Hesselmann, R.P.X., Werlen, C., Hahn, D., Van Der Meer, J.R., Zehnder, A.J.B., 1999. Enrichment, phylogenetic analysis and detection of a bacterium that performs enhanced biological phosphate removal in activated sludge. Syst. Appl. Microbiol. 22 (3), 454–465. https://doi.org/10.1016/S0723-2020(99)80055-1.

Jeon, C.O., Park, J.M., 2000. Enhanced biological phosphorus removal in a sequencing batch reactor supplied with glucose as a sole carbon source. Water Res. 34 (7), 2160–2170. https://doi.org/10.1016/S0043-1354(99)00383-8.

Ketchum, R.L., Irvine, R.L., Breyfogle, R.E., Manning, J.F., 1987. A comparison of biological and chemical phosphorus removals in continuous and sequencing batch reactors. J. Water Pollut. Control Fed. 59 (1), 13–18. https://www.jstor.org/stable/25043163?seq=1.

Kleikamp, H.B., Grouzdev, D.S., Schaasberg, P., Van Valderen, R., Van Der Zwaan, R., Van De Wijgaart, R., Lin, Y., Abbas, B., Pronk, M., Van Loosdrecht, M., Pabst, M., 2022. Comparative metaproteomics demonstrates different views on the complex granular sludge microbiome. bioRxiv (Cold Spring Harbor Laboratory). https://doi.org/10.1101/2022.03.07.483319.

Martín, H.G., Ivanova, N., Kunin, V., Warnecke, F., Barry, K.W., McHardy, A.C., Yeates, C., He, S., Salamov, A.A., Szeto, E., Dalin, E., Putnam, N.H., Shapiro, H.J., Pangilinan, J.L., Rigoutsos, I., Kyrpides, N.C., Blackall, L.L., McMahon, K.D., Hugenholtz, P., 2006. Metagenomic analysis of two enhanced biological phosphorus removal (EBPR) sludge communities. Nat. Biotechnol. 24 (10), 1263–1269. https://doi.org/10.1038/nbt1247.

McIlroy, S.J., Onetto, C.A., McIlroy, B., Herbst, F.A., Dueholm, M.S., Kirkegaard, R.H., Fernando, E., Karst, S.M., Nierychlo, M., Kristensen, J.M., Eales, K.L., Grbin, P.R.,

Wimmer, R., Nielsen, P.H., 2018. Genomic and *in situ* analyses reveal the *Micropruina* spp. as abundant fermentative glycogen accumulating organisms in enhanced biological phosphorus removal systems. Front. Microbiol. 9 (MAY), 1–12. https://doi.org/10.3389/fmicb.2018.01004.

van Loosdrecht, M.C.M., Pot, M.A., Heijnen, J.J., 1997. Importance of bacterial storage polymers in bioprocesses. Water Sci. Technol. 35 (1), 41–47. https://doi.org/10.1016/S0273-1223(96)00877-3.

Mino, T., Satoh, H., Matsuo, T., 1994. Metabolisms of different bacterial populations in enhanced biological phosphate removal processes. Water Sci. Technol. 29 (7), 67–70. https://doi.org/10.2166/WST.1994.0309.

Oehmen, A., Yuan, Z., Blackall, L.L., Keller, J., Oehmen, A., Yuan, Z., Blackall, L.L., Keller, J., 2005a. Comparison of acetate and propionate uptake by polyphosphate accumulating organisms and glycogen accumulating organisms. Biotechnol. Bioeng. 91 (2), 162–168. https://doi.org/10.1002/bit.20500.

Oehmen, A., Zeng, R.J., Yuan, Z., Keller, J., 2005b. Anaerobic metabolism of propionate by polyphosphate-accumulating organisms in enhanced biological phosphorus removal systems. Biotechnol. Bioeng. 91 (1), 43–53. https://doi.org/10.1002/bit.20480.

Petrik, L.F., Ngo, H.H., Varjani, S., Osseweijer, P., Xevgenos, D., van Loosdrecht, M., Smol, M., Yang, X., Mateo-Sagasta, J., 2022. From wastewater to resource. One Earth 5 (2), 122–125. https://doi.org/10.1016/j.oneear.2022.01.011.

Pronk, M., Abbas, B., Al-zuhairy, S.H.K., Kraan, R., Kleerebezem, R., van Loosdrecht, M. C.M., 2015. Effect and behaviour of different substrates in relation to the formation of aerobic granular sludge. Appl. Microbiol. Biotechnol. 99 (12), 5257–5268. https://doi.org/10.1007/s00253-014-6358-3.

Rombouts, J.L., Kranendonk, E.M.M., Regueira, A., Weissbrodt, D.G., Kleerebezem, R., Loosdrecht, M.C.M., 2020. Selecting for lactic acid producing and utilising bacteria in anaerobic enrichment cultures. Biotechnol. Bioeng. 117 (5), 1281–1293. https://doi.org/10.1002/bit.27301.

Seviour, R.J., Maszenan, A.M., Soddell, J.A., Tandoi, V., Patel, B.K.C., Kong, Y., Schumann, P., 2000. Minireview microbiology of the "G-bacteria" in activated sludge. Environ. Microbiol. 2 (6), 581–593. https://doi.org/10.1046/j.1462-2920.2000.00153.x.

Shimada, T., Zilles, J., Raskin, L., Morgenroth, E., 2007. Carbohydrate storage in anaerobic sequencing batch reactors. Water Res. 41 (20), 4721–4729. https://doi.org/10.1016/j.watres.2007.06.052.

Shintani, T., Liu, W.T., Hanada, S., Kamagata, Y., Miyaoka, S., Suzuki, T., Nakamura, K., 2000. *Micropruina* glycogenica gen. nov., sp. nov., a new Gram-positive glycogen-accumulating bacterium isolated from activated sludge. Int. J. Syst. Evol. Microbiol. 50 (1), 201–207. https://doi.org/10.1099/00207713-50-1-201/CITE/REFWORKS.

Smolders, G.J.F., Meij, J.V.D., Van Loosdrecht, M.C.M., Heijnen, J.J., 1994. Model of the anaerobic metabolism of the biological phosphorus removal process : stoichiometry and pH influence. Biotechnol. Bioeng. 43 (6), 461–470.

Taguchi, S., Yamada, M., Matsumoto, K., Tajima, K., Satoh, Y., Munekata, M., Ohno, K., Kohda, K., Shimamura, T., Kambe, H., Obata, S., 2008. A microbial factory for lactate-based polyesters using a lactate-polymerizing enzyme. Proc. Natl. Acad. Sci. U. S. A. 105 (45), 17323–17327. https://doi.org/10.1073/PNAS.0805653105.

Tomás-Martínez, S., Chen, L.M., Pabst, M., Weissbrodt, D.G., van Loosdrecht, M.C.M., Lin, Y., 2023. Enrichment and application of extracellular nonulosonic acids containing polymers of Accumulibacter. Appl. Microbiol. Biotechnol. 107 (2–3), 931–941. https://doi.org/10.1007/S00253-022-12326-X/FIGURES/5.

Tsapekos, P., Alvarado-Morales, M., Baladi, S., Bosma, E.F., Angelidaki, I., 2020. Fermentative production of lactic acid as a sustainable approach to valorize household bio-waste. Front. Sustain. 1, 538783 https://doi.org/10.3389/FRSUS.2020.00004/BIBTEX.

van Dijk, E.J.H., Haaksman, V.A., van Loosdrecht, M.C.M., Pronk, M., 2022. On the mechanisms for aerobic granulation - model based evaluation. Water Res. 216 (November 2021), 118365 https://doi.org/10.1016/j.watres.2022.118365.

Ziliani, A., Bovio-Winkler, P., Cabezas, A., Etchebehere, C., Garcia, H.A., López-Vázquez, C.M., Brdjanovic, D., van Loosdrecht, M.C.M., Rubio-Rincón, F.J., 2023. Putative metabolism of *Ca.* Accumulibacter via the utilization of glucose. Water Res. 229, 119446 https://doi.org/10.1016/J.WATRES.2022.119446.