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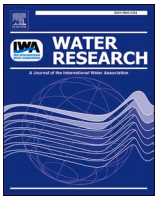

Glycerol conversion by aerobic granular sludge

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ABSTRACT

Glycerol is abundantly present in wastewater from industries such as biodiesel production facilities. Glycerol is also a potential carbon source for microbes that are involved in wastewater nutrient removal processes. The conversion of glycerol in biological phosphorus removal of aerobic granular sludge processes has not been explored to date. The current study describes glycerol utilization by aerobic granular sludge and enhanced biological phosphorus removal (EBPR). Robust granules with good phosphorus removal capabilities were formed in an aerobic granular sludge sequencing batch reactor fed with glycerol. The interaction between the fermentative conversion of glycerol and product uptake by polyphosphate accumulating organisms (PAO) was studied using stoichiometric and microbial community analysis. Metagenomic, metaproteomic and microscopic analysis identified a community dominated by *Actinobacteria* (*Tessaracoccus* and *Micropruina)* and a typical PAO known as *Ca.* Accumulibacter. Glycerol uptake facilitator (glpF) and glycerol kinase (glpK), two proteins involved in the transport of glycerol into the cellular metabolism, were only observed in the genome of the *Actinobacteria.* The anaerobic conversion appeared to be a combination of a substrate fermentation and product uptake-type reaction. Initially, glycerol fermentation led mainly to the production of 1,3-propanediol (1,3-PDO) which was not taken up under anaerobic conditions. Despite the aerobic conversion of 1,3-PDO stable granulation was observed. Over time, 1,3-PDO production decreased and complete anaerobic COD uptake was observed. The results demonstrate that glycerol-containing wastewater can effectively be treated by the aerobic granular sludge process and that fermentative and polyphosphate accumulating organisms can form a food chain in glycerol-based EBPR processes.

1. Introduction

Aerobic Granular Sludge (AGS) is a state-of-the-art biological wastewater treatment technology that performs simultaneous carbon, nitrogen, and phosphorus removal (de Kreuk et al., 2007, Pronk et al., 2015). Several factors distinguish AGS from conventional activated sludge systems. For instance, due to the compact structure and smooth surface of the granules, settling occurs rapidly and the redox gradient across the biofilm accommodates simultaneous conversions to take place within the granules (Bengtsson et al., 2018, de Bruin et al., 2004, de Kreuk and van Loosdrecht, 2006). These features reduce the treatment time significantly, enhance the solid-liquid separation and eliminate the need for multiple reactor compartments (Pronk et al., 2015). The formation of aerobic granules was first demonstrated in 1998 and applying certain environmental pressures was reported to be key in achieving granules (Heijnen and Van Loosdrecht, 1998). The underlying mechanisms for granulation and achieving a system with stable performance are extensively studied and described in the literature (Etterer and Wilderer, 2001); Yu (Liu et al., 2005, Morgenroth et al., 1997, Picioreanu et al., 1997, van Dijk et al., 2022, Winkler et al., 2013).

Several parameters are important for obtaining stable and smooth granules. Of prime importance is the selection of slow-growing microorganisms such as Polyphosphate Accumulating Organisms (PAO) and Glycogen Accumulating Organisms (GAO) that form dense smooth granules (de Kreuk and van Loosdrecht, 2004). When biodegradable organic carbon, usually Volatile Fatty Acids (VFA), is supplied to the system under anaerobic conditions, it is converted by certain microorganisms (PAOs for instance) into storage polymers known as Poly-Hydroxy-Alkanoates (PHA) (Oehmen et al., 2005). Microorganisms growing on storage polymers have a slower growth rate than

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heterotrophic bacteria growing on readily biodegradable organic carbon in the presence of an electron acceptor (van Loosdrecht et al., 1997). Generally, slow-growing microorganisms (such as methanogens, nitrifiers, anammox, PAOs, GAOs) form a more compact structure and smoother granular biomass than fast-growing microorganisms. In the subsequent aerobic phase, the stored organic carbon is oxidized to provide energy for the formation of biomass and replenishment of the intracellular polymer pool (Oehmen et al., 2007, Tarayre et al., 2016). Similarity in operational parameters such as anaerobic-aerobic cycles in both AGS and Enhanced Biological Phosphorus Removal (EBPR) is also a common link between the two system which selects organisms that are important for the successful application of these technologies.

EBPR is an environmentally sustainable and economically competitive technology (Bunce et al., 2018, Ketchum et al., 1987). The technology is based on the enrichment of the sludge with PAOs that can excessively accumulate phosphorus (P) in the form of poly-phosphate (poly-P) after which P removal is achieved through excess sludge discharge (Smolders et al., 1994). However, the stability of these systems depends on various factors such as the carbon source which act as the electron donor (Guerrero et al., 2011, Wei et al., 2014). In literature, an overwhelming amount of information exists on EBPR with VFAs mainly acetate and propionate as the carbon source (Bunce et al., 2018, Oehmen et al., 2007, Oehmen et al., 2005, Oehmen d et al., 2005, Pijuan et al., 2004, Pijuan et al., 2005, Smolders et al., 1994). However, when other sources of carbon such as alcohol or sugars are used, these systems manifest different behaviours. For instance, a period of adaptation is required by PAOs when ethanol is used (Puig et al., 2008) whereas methanol can be used via a syntrophic consortium between acetogens and PAOs (Tayà et al., 2013). In a study by (Begum and Batista, 2012), the authors reported EBPR deterioration due to the proliferation of GAOs over PAOs with glucose as substrate. In addition to traditional PAOs, the presence of fermentative microorganisms in dynamic feast-famine conditions that can perform fermentation and anaerobically accumulate storage compounds without cycling polyphosphate has been reported (Carucci et al., 1999, McIlroy et al., 2018). These fermentative GAOs (fGAO) play a potentially important role in EBPR systems since they can convert the substrate into PAO-utilizable product enabling PAO proliferation or they can compete for substrate uptake compromising the phosphorus removal efficiency of the system. Therefore, the carbon source in the wastewater plays a decisive role in the success of the EBPR process.

Glycerol is a compound that can be dominant in a variety of industrial wastewaters such as biodiesel and epoxy resin production (Pinto and De Araujo Mota, 2014). For instance, the production of every 10 kilograms of biodiesel generates about 1 kilogram of glycerol (Plácido and Capareda, 2016), and the wastewater generated from the epoxy resin production facilities contain a significant amount of glycerol. Due to its abundance and relatively low price, the use of glycerol has attracted the attention of different engineering communities for various applications (Lima et al., 2021). In wastewater treatment, glycerol has been reported to be a suitable source of carbon for the removal of nitrogen and phosphorus (Coats et al., 2015, Smyk and Ignatowicz, 2017, Yuan et al., 2010). However, the conversion of glycerol in AGS-EBPR processes or the formation of AGS using glycerol has not been studied in detail. The anaerobic conversion of glycerol by both mixed and pure microbial cultures has been extensively studied over the years ((Barbirato et al., 1995, Gupta et al., 2009); Katarzyna (Leja et al., 2011, Temudo et al., 2008)) and 1,3-propanediol (1,3-PDO) is the most common reported product among the products of glycerol fermentation in literature (Clomburg and Gonzalez, 2013, Moscoviz et al., 2016). Several studies have also reported the production of propionate from the fermentation of glycerol using mixed microbial cultures (Chen et al., 2016; (Yuan et al., 2010)). In EBPR systems, glycerol could directly be taken up by PAOs, but also potentially fermented to a variety of products that can subsequently be utilized by the PAOs or only be converted under aerobic conditions. The latter process could potentially harm the granulation process as fast aerobic growth would lead to less favourable sludge morphologies (Pronk et al., 2015). Given that 1,3-PDO is a more reduced compound than glycerol, microorganisms are required to generate more oxidized compounds (e.g. CO_2) to maintain their redox balance. Propionate synthesis from glycerol is both redox neutral (Table 1) and energy positive with 1 or more mol ATP produced per mol glycerol fermented ((Barbirato et al., 1997, Gonzalez-Garcia et al., 2017); Yin (Liu et al., 2011)) versus no ATP generated from 1,3-PDO production (Temudo et al., 2008).

$$Y_{ATP,1,3-PDO}\ Glycerol + H^+ + NADH \rightarrow 1,3\quad propanediol + H_2O \tag{1}$$

$$Y_{ATP,Propionate}\ Glycerol \rightarrow Propionate + H_2O + 1\ ATP \quad (Wood - Werkman\ cycle) \tag{2}$$

Information on the effect and conversion of glycerol in AGS and EBPR processes is lacking which is important for a wider application of the technology. Here, we study the potential of glycerol as an electron donor to promote aerobic granular sludge formation and evaluate the carbon and phosphorus removal of the system. We further elucidate the anaerobic-aerobic conversions by linking process stoichiometry to microbial community analysis.

2. Materials and Methods

2.1. Experimental setup and reactor operation

The experiments were conducted in a bubble column reactor and operated in a Sequencing Batch Reactor (SBR) configuration. The working volume of the reactor was 2.8 L with an internal diameter of 5.6 cm and a total height of 90 cm. After effluent withdrawal, 1.3 L remained in the reactor after each cycle representing a volumetric exchange ratio of 54%. The pH was controlled at 7.1 ± 0.1 by either doing 1M NaOH or 1M HCl. The Dissolved Oxygen (DO) was controlled at 0% and 50% (3.5 mg/L) saturation during the anaerobic and aerobic phases, respectively, by a controlled mixture of nitrogen gas and air. The temperature was not controlled. The room temperature was controlled at 20 °C. The reactor was seeded with aerobic granular sludge from a pilot-scale municipal wastewater treatment reactor performing EBPR located in Harnaschpolder, the Netherlands.

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 35.7 mM glycerol, 3.6 mM $MgSO_4.7H_2O$, 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 $FeSO_4.7H_2O$, 2.2 g/L $Zn.SO_4.7H_2O$, 7.33 g/L $CaCl_2.2H_2O$, 4.32 g/L $MnSO_4.H_2O$, 2.18 g/L $Na_2MoO_4.2H_2O$, 1.57 g/L $CuSO_4.5H_2O$, 1.61 g/L $CoCl_2.6H_2O$ 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 of PO_4^3-P.

The reactor cycles consisted of 5 min of nitrogen sparging to ensure an anaerobic condition before feeding followed by 5 min of feeding, 60 min of nitrogen sparging, 120 min of aeration, 5 min of settling, and 5 min of effluent withdrawal. The off-gas was recirculated with a flow of 5 L/min to keep a steady DO concentration.

Table 1
List of reactions showing complete oxidation of each compound to CO_2. ƙ represents mole electrons per mole substrate.

Compounds	Reactions	ƙ
Glycerol	$C_3H_8O_3 + 3H_2O \rightarrow 3CO_2 + 14H^+ + 14e^-$	14
Propionate	$C_3H_6O_2 + 4H_2O \rightarrow 3CO_2 + 14H^+ + 14e^-$	14
1,3-Propanediol	$C_3H_8O_2 + 4H_2O \rightarrow 3CO_2 + 16H^+ + 16e^-$	16

2.2. Analytical methods

Concentrations of phosphate and ammonium 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 0.0015M phosphoric acid as eluent.

2.3. PHA and Glycogen determination

Before both glycogen and PHA analysis, the biomass sample from the reactor was fixed with the addition of 4% w/v paraformaldehyde, washed with demineralized water, freeze-dried, and pottered into a fine powder. The extraction and measurement of PHA were performed according to the protocol described by (Riis and Mai, 1988)) with minor adjustments. To summarize, the PHA in the freeze-dried biomass (30 mg) was hydrolysed and esterified in an HCL, Propanol, and Dichlorethane solution with a ratio of 1:4:5 v/v/v for 3h (Poly--Hydroxy-Butyrate & Poly-Hydroxy-Valerate) and 20h (Poly-Hydroxy-2-Methyl-Valerate) at 100°C with frequent manual vortexing. 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 propyl esters in the organic phase were then filtered and analysed by Gas Chromatography (6890N, Agilent, USA). Benzoic acid (50 μL) was used as an internal standard. Quantification of PHB, PHV, and PH2MV was done using commercial 3-hydroxybutyrate, R-3-hydroxyvalerate, and 2-hydroxyhexanoate as standards (Sigma-Aldrich, USA).

The glycogen content of the biomass was extracted according to the method described by (Smolders et al., 1994) but with the addition of 0.9 M HCl to the sample and heating it for 5 h at 100 °C with frequent manual vortexing. The digestate was then filtered using 0.45 μm Millipore filters and the poly-glucose was analysed using an HPLC.

2.4. Batch experiments

A batch test was performed by addition of excess glycerol (14 mM) and prolonging the anaerobic phase to 29 hours to determine the fermentation product. The propionate batch test was performed with a similar concentration in a normal cycle as described above. The pH was controlled at 7.1 ± 0.1 by either dosing 1M NaOH or 1M HCl. The Dissolved Oxygen (DO) was controlled at 0% and 50% saturation during the anaerobic and aerobic phases, respectively, by a controlled mixture of nitrogen gas and air. The temperature was not controlled. The room temperature was controlled at 20 °C. Liquid and biomass samples were collected for bulk liquid analysis and PHA and glycogen content determination.

2.5. Biomass measurements

Granules were taken from the reactor and washed with demineralized water to wash off the impurities and salts. The granules were then placed in an oven at 105 °C for 24 hours to determine the Total Suspended Solids (TSS). The dried biomass was then incinerated at 550 °C for 3 hours to determine the ash content and Volatile Suspended Solids (VSS).

2.6. Microscopy

A stereo zoom microscope (M205 FA, Leica Microsystems, Germany) was used to capture the images of the granules equipped with Qwin image analysis software (V3.5.1, Leica Microsystems, Germany).

Safranin and crystal violet were used to stain Gram-positive and -negative bacteria, respectively. Iodine was added to fix the safranin to the peptidoglycan molecules of Gram-positive bacteria. Microscopic images were captured with Axio Imager M2 (Zeiss, Germany) equipped with ZEN (blue edition) software.

Biomass samples were handled and stained for FISH according to the protocol described by (Bassin et al., 2011). A mixture of probes EUB338, EUB338-II, and EUB338-III (EUBmix) was used to stain all bacteria (Amann et al., 1990, Daims et al., 1999). To visualize PAOs, probe PAO651 was used (Crocetti et al., 2000). Probe Actino658 was used to visualize Actinobacteria (Kong et al., 2005). List of FISH probes tested are available in the supplementary material. The images were captured with an epifluorescence microscope equipped with filter set Cy3 (ET545/25x ET605/70m T565LPXR), Cy5 (ET640/30x ET690/50m T660LPXR), and FITC (ET470/40x ET525/50m T495LPXR) (Axio Imager M2, Zeiss, Germany).

2.7. Microbial community analysis by metagenomics and metaproteomics

DNA from fresh biomass was extracted using the DNeasy PowerSoil Pro Kit (Qiagen, Germany) according to the protocol from the manufacturer. DNA concentration was determined with Qubit fluorometer (ThermoFisher Scientific, USA). Metagenome sequencing and raw data processing (reads assembly and identification of genes) were performed by Novogene (Novogene Co., China). Protein extraction and shotgun proteomic analysis were performed according to (Kleikamp et al., 2022). Briefly, approx. 250 ng of the proteolytic digest was analysed using a nano-liquid-chromatography system consisting of an EASY nano-LC 1200 equipped with an Acclaim PepMap RSLC RP C18 separation column and a QE plus Orbitrap mass spectrometer (ThermoFisher Scientific, Germany). The Orbitrap was operating in data-dependent acquisition (DDA) mode, where the top 10 signals were isolated at a window of 2.0 m/z and fragmented using an NCE of 28. Fragments were acquired at 17 K resolution with a max IT of 75 ms and an AGC target of 2e5. Unassigned, singly and >6 times charged mass peaks were excluded from fragmentation. The analysis of the mass spectrometric raw data was performed by database searching using the metagenomics constructed reference database (or alternatively a focused database containing only Uniprot genomes of the 3 main genera) and PEAKS X (Bioinformatics Solutions Inc., Canada). Thereby, the search allowed for 20 ppm parent ion and 0.02 m/z fragment ion mass error, 2 missed cleavages, carbamidomethylation as fixed, and methionine oxidation and N/Q deamidation as variable modifications. Peptide-spectrum matches were filtered for a 1% False Discovery Rate (FDR) and protein identifications with 2 or more unique peptides were considered significant. Annotation of functions of the proteins identified using the metagenomics reference sequence database was performed using the KEGG database using GhostKoala: www.kegg.jp/ghostkoala.

2.8. ATP & NADH balances

To evaluate the substrate distribution between the fermentative organisms and the PAO community, ATP and NADH balances were made for each organism. The balance was made based on the assumption 1- that glycerol is anaerobically converted to propionate and glycogen and that the produced propionate is subsequently taken up by the PAO community to synthesize PHA (verified via mass balances) and 2- the majority of the community was dominated by PAOs (using FISH and proteomics) indicating minor contribution to substrate distribution by other potential communities (see discussions for further elaborations). The parameters used in the calculations to estimate propionate production are listed through equations 3 – 6 derived from Principles of biochemistry (Nelson et al., 2000). Note that the degree of reduction of glycerol and propionate are the same and thus, the NADH balance is a conserved moiety in this reaction.

$$-Glycerol - ATP + DHAP + NADH \quad (3)$$

$$-2\ DHAP - ATP + \ Glycogen \quad (4)$$

$$-DHAP + Pyruvate + 2\ ATP + NADH \quad (5)$$

$$-Pyruvate - 2\ NADH + Propionate \quad (6)$$

The ATP and NADH balance over the PAOs was performed according to the model describing the anaerobic metabolism of propionate by PAOs in EBPR systems by (Oehmen et al., 2005). In short, PAOs derive the required energy for propionate uptake and PHA synthesis through poly-P and glycogen hydrolysis. The latter process also generates reducing equivalents, i.e. NADH, required for the maintenance of redox balance within the cell.

3. Results

3.1. Reactor operation

Granular sludge from a pilot municipal Wastewater Treatment Plant (WWTP) in the Netherlands performing EBPR was used to inoculate the reactor. After a few weeks of reactor operation and the formation of new biomass, the sludge colour changed from dark brown to light brown/orange. The settling time was step-wise decreased from 20 to 5 minutes within 4 weeks of operation. With the development of granular sludge, the effluent became clearer and the flocculent biomass disappeared from the system. Once granular biomass formation stabilized (from day 65 onwards), a Solids Retention Time (SRT) of 12 days was maintained by manual sludge removal and solids washout via the effluent. After the reactor reached a pseudo-steady state, an average Sludge Volume Index (SVI_5) of 33.4 mL/g was obtained from the sludge settled bed in the reactor after 5 minutes of settling. The TSS and VSS concentration in the reactor stabilized at an average of 7.3 and 5.5 g/L (i.e. 24% ash content), respectively.

Once a pseudo-steady-state was achieved (day 126 onwards) an average P-release of 64.3 mg/L was obtained with a specific P-release and -uptake rate of 10.9 and 16.3 mgP/gVSS/h, respectively. Pseudo-steady-state was defined on the basis of stable conversion of substrates and phosphate throughout the cycles. The anaerobic glycerol uptake rate was 2 C-mmol/gVSS/h (76 mgCOD/gVSS/h). The anaerobic P-release to complete carbon uptake (P:C) ratio during a typical cycle was 0.23 P-mol/C-mol with an aerobic ammonium consumption rate of 0.8 mgN/gVSS/h for biomass growth (no nitrification).

Fig. 2 shows the concentrations of 1,3-PDO and orthophosphate in the bulk liquid at the end of the anaerobic phase versus the percentage of rbCOD (representing glycerol) removed anaerobically. Coupled with increased anaerobic glycerol removal and the reduction of 1,3-PDO production, P release was increased. Initially, the leftover COD from the anaerobic phase was oxidized in the subsequent aerobic phase. The aerobic conversion caused floc formation that was washed out once the settling time decreased to 5 minutes. As the anaerobic production of 1,3-PDO decreased, a sludge bed with smooth granules was formed (Fig. 1). Anaerobic 1,3-PDO formation decreased over time, indicating formation of other fermentation products (such as propionate). The formation of a different fermentation product was also reflected in increasing P release, likely due to uptake of formed propionate by PAOs.

3.2. Chemical transformations in a typical cycle

Fig. 3 shows the anaerobic-aerobic chemical transformations during a cycle. In the anaerobic phase, glycerol uptake, P release, PHA accumulation, and minor glycogen degradation occurred concurrently. In the aerobic phase, PHA oxidation, P uptake, and glycogen replenishment were observed.

The ratio of glycogen degraded and PHA accumulated to glycerol consumed in the anaerobic phase was 0.27 $C\text{-}mol_{glycogen}/C\text{-}mol_{glycerol}$ and 0.97 $C\text{-}mol_{PHA}/C\text{-}mol_{glycerol}$. The average distribution of the PHA content in the biomass at the end of the anaerobic phase was 54% PHV and 46% PH2MV. No PHB was detected. The mass balance performed over the anaerobic glycerol uptake, PHA production, and glycogen degradation closed with a recovery of 77% and 81% for carbon and

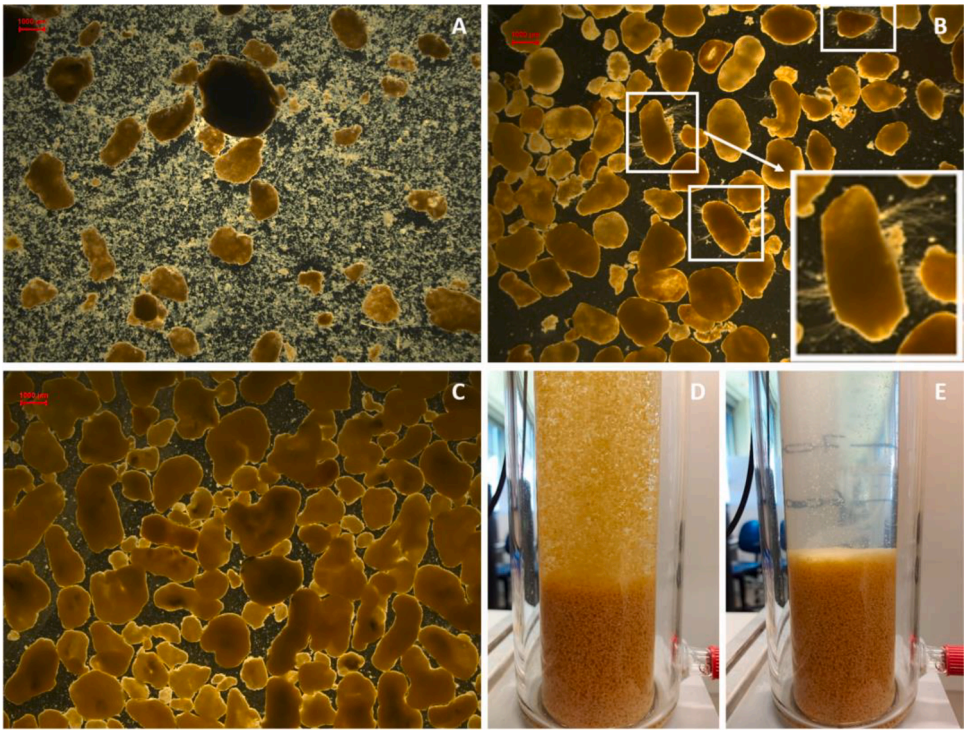

Fig. 1. Stereoscopic overview of the aerobic granular sludge grown on glycerol. Picture (A) was taken during the initial days of reactor operation, (B) day 37, (C) end of the experimental period day 226, (D & E) shows biomass settling and the settled bed day 226, respectively. The pictures show the washout of the flocculent biomass and the formation of dense smooth granules over time. Scale bar equals 1000 μM.

electron (COD), respectively. The incomplete carbon and electron recovery was assumed to be due to CO_2 losses and anaerobic growth on the stored glycogen. The anaerobic growth was calculated using the theoretical glycogen production of 0.5 $mol_{glycogen}/mol_{glycerol}$ based on ATP calculations and the anaerobic biomass yield of 0.2 gCOD/gCOD for *Propionibacterium* (Sykes, 1975). See discussion for the explanation of anaerobic glycogen production and growth.

3.3. Batch experiments

To identify the products formed from glycerol fermentation in the anaerobic phase, a batch test with excess glycerol (14 mmol/L) and an extended anaerobic period of 23 hours was conducted. Once glycerol was completely removed, the formation of mainly propionate (74% molar basis) along with minor amounts of lactate, formate, acetate, valerate, iso-butyrate, and 1,3- PDO was observed. The mass balance performed over the entire anaerobic phase with glycerol uptake, product formation, PHA production, and glycogen degradation closed with a recovery of 98% and 106% for carbon and electron balance, respectively. The distribution of the PHA at the end of the anaerobic phase was 9% PHB, 53% PHV, and 38% PH2MV. Further information regarding the batch experiment can be found in the supplementary material.

To compare the results obtained from the conversion of glycerol, a batch test with propionate (similar initial concentration) was conducted (Fig. 4). Similar to the glycerol reactor, propionate uptake, P release, PHA accumulation, and glycogen degradation occurred in the anaerobic phase. In the aerobic phase, PHA oxidation, P uptake, and glycogen replenishment were observed. The anaerobic balance performed over propionate uptake, PHA production, and glycogen degradation closed with a recovery of 87% and 92% for carbon and electron (COD) balance. The incomplete carbon and electron recovery was assumed to be due to CO_2 losses and possibly anaerobic growth. The distribution of the PHA content in the biomass at the end of the anaerobic phase was 60% PHV and 40% PH2MV with no PHB detection.

3.4. ATP & NADH balances

The ATP & NADH balances were made over the anaerobic phase to estimate substrate distribution among the fGAOs and the PAOs. We assumed a two-step process, fermentation of glycerol to propionate and glycogen by fGAOs and propionate uptake by PAOs. First, an ATP balance was made over the fGAOs by assuming that the ATP generated via propionate formation from glycerol is used for substrate uptake and possibly storage processes (Table 3). Second, the amount of glycogen hydrolyzed by the PAOs was estimated based on the NADH required for the synthesis of PHA produced in the anaerobic phase (Table 4). Third, by taking the theoretical propionate yield and the estimated hydrolyzed glycogen into account, an ATP balance was made over the PAOs (Table 5).

3.5. Microbial community

Biomass samples from the reactor were frequently observed under the microscope to track the evolution of the microbial community during the experimental period. Visually, two morphologically distinct types of microorganisms were observed (Fig. 5). Gram staining was performed as the first identification technique and for FISH protocol adjustment. Tetrad-shaped microorganisms were identified as Gram-positive and the microorganisms growing in pairs were Gram-negative. The presence of storage compounds (dark spots) was only observed in the latter morphotype (Fig. 5A).

Gram staining showed a sparse presence of the tetrad-shaped microorganisms (Fig. 5D). FISH analysis of the biomass indicated the dominant presence of PAOs along with tetrad-shaped microorganisms in the sludge sample (Fig. 6). The presence of both Gram-positive and –negative made hybridization difficult. Thus, to visualize both types of microorganisms in one sample, the protocol to hybridize the cells was adjusted and several strategies were tested. To prevent Gram-negative cells disintegration and ensuring hybridization of Gram-positive cells, permeabilization was not performed instead the hybridization duration was increased to 3 days. Other probes have also been tested to visualize the tetrad-shaped microorganisms but no signal was observed.

To determine the composition of the microbial community a whole metagenome sequencing was performed. Genome information of the functional bins has been submitted to the NCBI database under the accession number PRJNA891235. Among all identified taxonomies (data not shown – see supplementary material) 2 of the most abundant genera matched microscopic observations; namely *Tessaracoccus* and *Ca.* Accumulibacter (Fig. 5). Subsequently, metaproteomics was performed that provided the expressed pathways. These furthermore enabled to track the biochemical conversions of the respective genera. First, the metaproteome raw data were analysed using the complete metagenome-constructed sequence database. The 3 most abundant genera, based on spectral counts (and with 2 or >2 unique peptides per protein) were *Ca.* Accumulibacter, *Tessaracoccus,* and *Micropruina* with approx. 55%, 7%, and 5%, respectively. However, other >30% were extremely low abundant taxa with only a few protein matches and very low protein sequence coverage. Therefore, the database searching was repeated with (Uniprot) genomes from only the 3 most abundant genera. This (as expected) increased the coverage towards those taxa significantly. Considering this result, the fact that most other taxonomies showed only a very small number of significant protein matches as well as the microscopy data, we hypothesised that 90% of the protein biomass may originate from the 3 main microorganisms. Among this protein mass, over 90% of the expressed proteins are associated with *Ca.* Accumulibacter. The remaining fraction was approximately 9% and 1% for *Tessaracoccus* and *Micropruina* (collectively referred to as fGAOs), respectively. The key anaerobic functioning metabolic pathways such as substrate uptake, fermentation, PHA synthesis, and polyphosphate metabolism in the genome of *Ca.* Accumulibacter, *Tessaracoccus,* and *Micropruina* were analysed based on the expressed proteins involved in the reactions summarized in Table 6. Central metabolic pathways such as the TCA cycle, glycolysis, and gluconeogenesis were not tracked as they are shared by the majority of microorganisms.

4. Discussions

Overall, the experimental results have indicated that glycerol can be a good substrate for biological phosphorus removal and that good granulation can be achieved. The observed microbial community is more complex when compared to traditional studied laboratory EBPR communities with acetate or propionate as substrate.

4.1. Anaerobic conversions

As shown in Fig. 2, the system achieved a pseudo-steady-state after 4 months of operation which was likely due to a small competitive difference between different fermentative organisms leading to 1,3-PDO production in the initial months. The conversion seemed to be a combination of a substrate fermentation and product uptake type of reaction (Fig. 7). In stable conditions, the main fermentation product was likely propionate since PHV & PH2MV were the synthesized storage polymers that corresponded to the uptake of propionate (Oehmen et al., 2005). These results are also in agreement with similar studies that used glycerol as the carbon source for EBPR (Yang et al., 2018, Yuan et al., 2010) where propionate was also identified as the main glycerol fermentation product. Additionally, two batch tests indicated that propionate was likely the main intermediate product. First, in a batch test where an excess amount of glycerol was fed to the system (to saturate the product-utilizing organisms, such as the PAOs) propionate accumulation was observed. Unlike a normal cycle, a minor amount of PHB formation (0.3 C-mmol/gTSS) was observed towards the end of the anaerobic

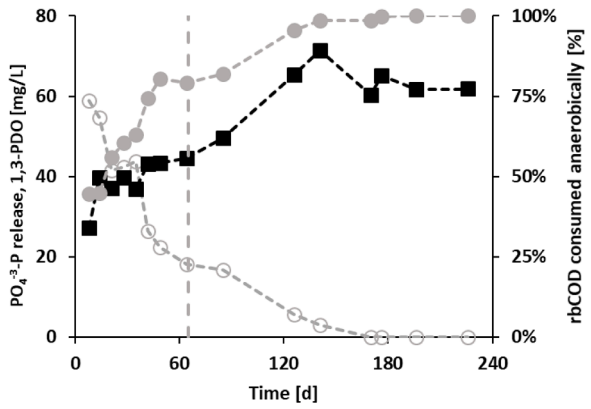

Fig. 2. Concentrations of 1,3-PDO (hollow circles), percentage of rbCOD removed (closed circles), and orthophosphate (squares) at the end of the anaerobic phase over time. rbCOD represents glycerol in COD equivalents. The dashed vertical line indicates the start of SRT control.

phase which might have been the result of redox imbalances. PHB is formed from the condensation of 2 acetyl-CoA molecules which can be derived from acetate. Part of the glycerol in the batch test was converted into acetate (7% mol fraction), thus PHB formation can be expected. However, in a normal cycle with lower glycerol concentration less acetate is produced which can explain absence of PHB detection. Similarly, a small amount of PHB was also observed at the end of the anaerobic phase in a batch test with excess propionate (data not shown – see supplementary material). This observation indicates that the duration of the anaerobic phase and/or the initial concentration of carbon substrate may affect PHA distribution. Second, the direct uptake of propionate in a separate batch test (Fig. 4) supports the argument that the microbial community was able to take up propionate as substrate.

The fraction of glycerol ending up as propionate was estimated based on an ATP balance over the fGAOs. By inferring the balance, half of the consumed glycerol is directly converted into propionate (0.5 $mol_{propionate}/mol_{glycerol}$) similar to the yield reported by (Guerrero et al., 2012). The other half is assumed to be used for the accumulation of storage compounds such as glycogen. Both *Tessaracoccus* and *Micropruina* are facultative anaerobes that can metabolize glycerol and accumulate glycogen (Maszenan et al., 1999). Therefore, due to this potential occurrence of simultaneous accumulation and consumption of glycogen in the biomass, the hydrolysed glycogen by the PAO was estimated based on the amount of PHA synthesized, assuming that PHA was only synthesised by the PAOs. The estimated hydrolysed glycogen (3.3 C-mmol) and the measured value (3.2 C-mmol) did not differ significantly which supports the arguments for both simultaneous accumulation and consumption of glycogen during the anaerobic period and PHA synthesis by the PAOs. Using the estimated amount of hydrolysed glycogen by the PAOs and the theoretical propionate yield, an ATP balance was made over the PAOs with 101% recovery. As shown in Table 5, the PAOs produced most of their ATP requirement through poly-P hydrolysis, manifesting a typical PAO metabolism. The amount of ATP required for VFA-CoA activation and transport of substrate into the cell was calculated based on PHA measurements. The amount of ATP produced was calculated using the total P-release (assuming that P-release was only due to PAO activity) and glycogen hydrolysis. Estimation of substrate utilization between PAOs and fGAOs from the

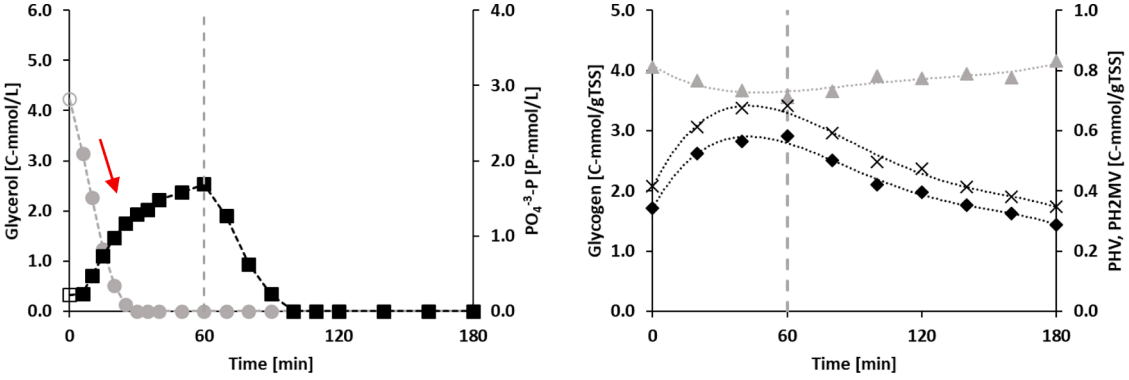

Fig. 3. Left - Glycerol (circles), PO_4^{3-}-P (squares) and right- PHV (crosses), PH_2MV (diamonds), and glycogen (triangles) profiles during a typical cycle. The dashed vertical line indicates the switch between the anaerobic and aerobic phases. The first hollow point in the graph (left) is calculated based on dilution and influent concentration. The arrow indicates the start of secondary P release which occurs after complete glycerol uptake.

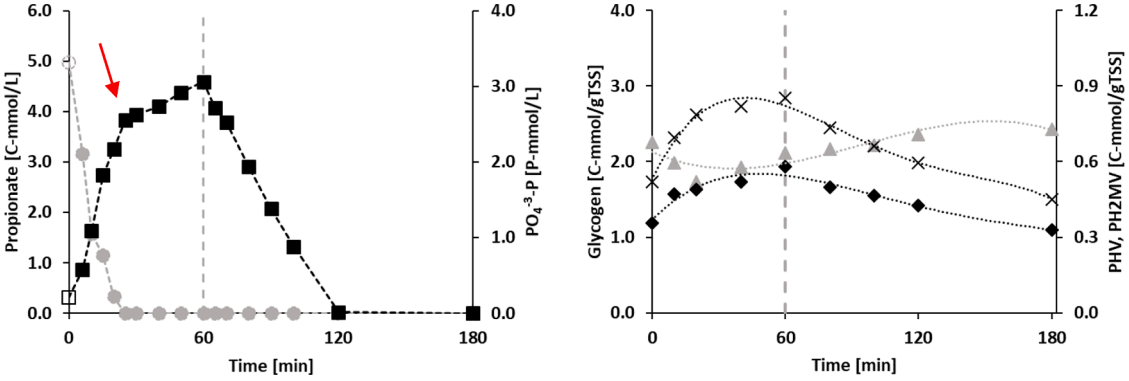

Fig. 4. Left - Propionate (circles), PO4-3-P (squares), PHV (crosses), PH2MV (diamonds), and glycogen (triangles) profiles during the batch test. The dashed vertical line indicates the switch between the anaerobic and aerobic phases. The first hollow point in the graph (left) is calculated based on dilution and influent concentration. The arrow indicates the start of secondary P release which occurs after complete glycerol uptake.

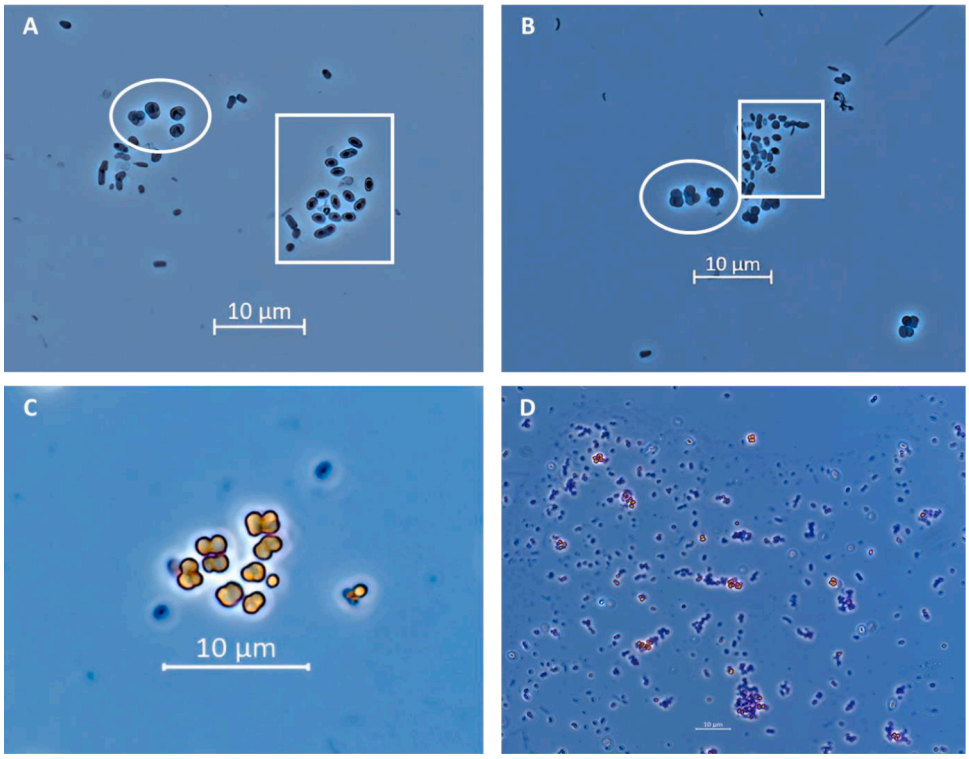

Fig. 5. Microscopic image of glycerol-cultivated biomass sample taken with 1000x magnification. Picture (A & B) cocci-shaped microorganisms arranged in tetrads (circles), rod-shaped cocci arranged in pairs or single cells (squares), (C) close-up of Gram-positive cells stained with Safranin, (D) Gram-positive cells (orange) and Gram-negative cells (violet).

fermentation of anaerobically stored glycogen is further discussed below.

4.2. Propionate vs glycerol as substrate

As shown in Table 2, the P:C ratio increased from 0.23 to 0.47 P-mol/C-mol (51%) when propionate was used as the substrate which is likely due to the direct uptake of propionate by the PAOs. The PHA yields, however, only increased from 0.97 C-mol/C-mol$_{glycerol}$ to 1.13 C-mol/C-mol$_{propionate}$ (14%) which is likely the result of higher glycogen degradation. The extra P-release during the batch experiment could be explained by the "uncoupling effect" of higher propionate concentration in the bulk liquid than the propionate generated from the fermentation of glycerol in a normal cycle. The increased propionate concentration likely induces a shift in the energy metabolism of PAOs also known as the overflow metabolism which leads to higher substrate uptake rates that is reflected in higher poly-P hydrolysis (Verhagen et al., 2020). The extra ATP generation was also observed when a balance was made over the PAOs during the propionate batch experiment with an excess of 49%, corresponding well to the increased P:C ratio (data not shown). Moreover, during cycle measurements, a considerable "secondary" P-release with a rate of 4.6 mgP/gVSS/h was observed after complete glycerol uptake. A secondary P-release was also observed during the batch test with propionate at a similar rate of 4.8 mgP/gVSS/h. In literature, the reported secondary P-release rates vary between 0.6 – 3.5 mgP/gVSS/h (Oehmen et al., 2005, Puig et al., 2008, Smolders et al., 1995) and seem to be related to the maintenance needs of the microorganisms. The higher rate observed in this study is an indication for the uptake of an extra substrate by the PAOs after all carbon substrate has been sequestered. We postulate that fGAOs have initially stored glycogen, which was fermented after the depletion of glycerol, with the released energy used for growth. Thus, in the anaerobic phase, a fraction of stored glycogen is fermented into products and used by PAOs which can be calculated using the secondary P-release. Combining the propionate yield with the fraction of glycogen fermented into PAO-utilizable products indicated that 90% of the substrate was available for PAOs for the production of PHA while the remaining fraction was used by the fGAOs for biomass synthesis. However, the anaerobic biomass formation by fGAOs is based on our postulation and thus, requires further investigations.

4.3. Glycerol-driven EBPR

In the majority of EBPR studies, simple substrates such as acetate and propionate have been used as carbon sources. Using VFAs as substrate results in the selection of one group of microorganisms performing carbon uptake and storage with the energy and reducing power derived from poly-P and glycogen hydrolysis, respectively. Such an approach is based on a simple system that is convenient for metabolic studies but it fails to fully describe more complicated EBPR narratives. In EBPR studies where a different substrate such as glycerol is used, the fermentative organisms are traditionally thought to anaerobically generate the acetate and propionate for the PAO population. This consortium of fermentative organisms and substrate hoarders might also be a feature shared by similar EBPR systems where substrates other than glycerol are used. For example, (Tayà et al., 2013) reported a successful methanol-driven EBPR that was achieved by a syntrophic consortium of methanol fermenters and PAOs. In our study, a glycerol-driven EBPR system was achieved with a microbial consortium of glycerol fermenters and PAOs.

Over time, P-release and -uptake improved as the anaerobic COD removal increased which could be due to a shift in the microbial population and the formation of a stable granular sludge bed with VFA-producing microorganisms. Incomplete anaerobic COD uptake in the initial days of reactor operation was likely because a larger fraction of glycerol was fermented into 1,3-PDO that was not taken up by the PAOs (Fig. 2). The produced 1,3-PDO was oxidized immediately in the aerobic phase which lead to some filamentous growth (Fig. 1B). Generally, rapid

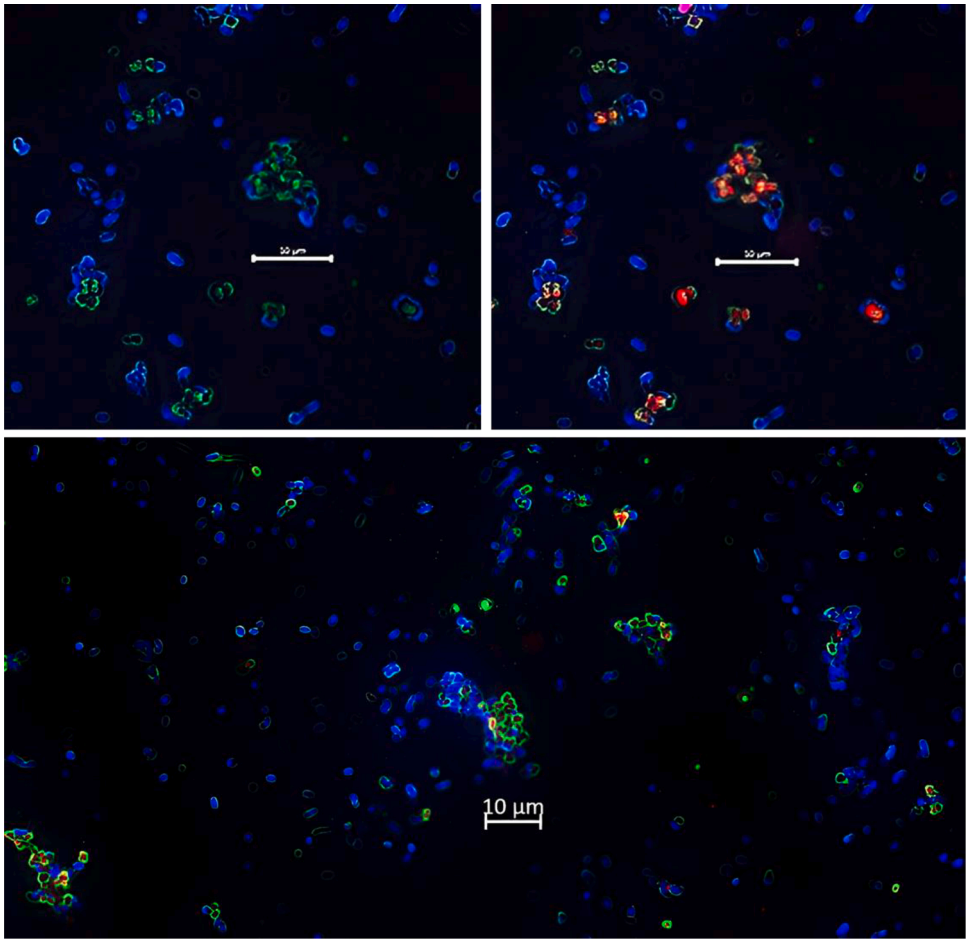

Fig. 6. FISH images of glycerol-cultivated biomass sample taken with 630x magnification. Probes EUBmix was used to stain all bacteria (fluos-green), PAO651 to stain PAOs (Cy3-blue), and Actino658 to stain Tetrad-shaped cells (Cy5-red). Both images were overlaid with phase-contrast pictures to show that all cells were stained. Scale bar equals 10 µm.

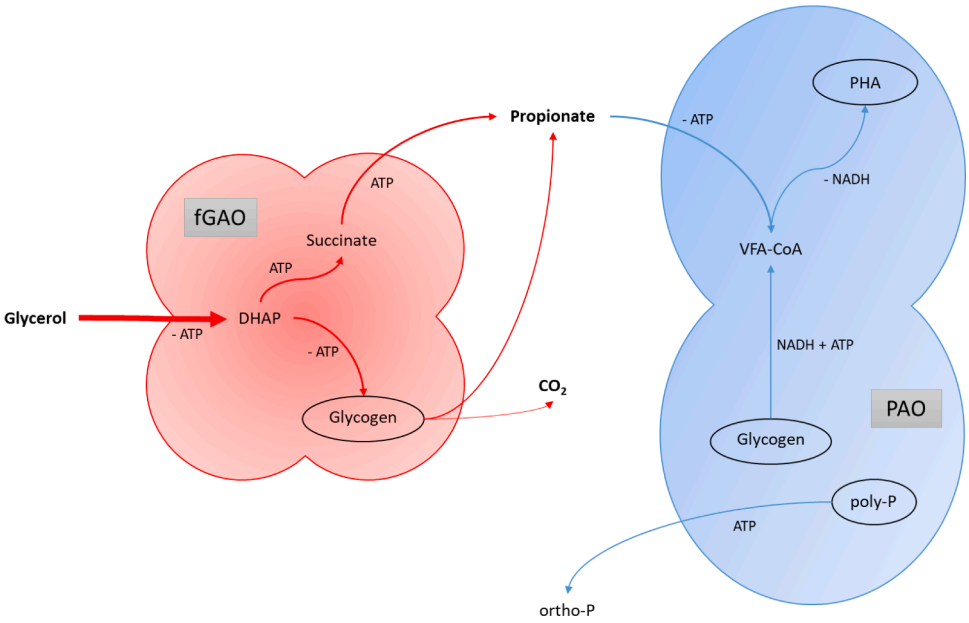

Fig. 7. Graphical representation of anaerobic glycerol conversion by aerobic granular sludge. DHAP: dihydroxyacetone phosphate, PHA: polyhydroxyalkanoates, Poly-P: polyphosphate, VFA-CoA: the combination of acetyl and propionyl-CoA.

Table 2
Comparison of anaerobic stoichiometries with glycerol and propionate as substrate. P_{rel}: Phosphorus release, PHA_{pro}: PHA production, C_{up}: Carbon uptake, Gly_{deg}: Glycogen degradation

Conversions	Glycerol	Glycerol	Propionate	Propionate	Units
	This study	(Guerrero et al., 2012)	This study	(Oehmen et al., 2005)	
P_{rel}/C_{up}	0.23	0.20	0.47	0.42	P-mol/C-mol
PHA_{pro}/C_{up}	0.97	0.31	1.13	1.23	C-mol/C-mol
PHB_{pro}/C_{up}	0.00	0.08	0.00	0.04	C-mol/C-mol
PHV_{pro}/C_{up}	0.53	0.14	0.68	0.55	C-mol/C-mol
$PH2MV_{pro}/C_{up}$	0.44	0.09	0.45	0.65	C-mol/C-mol
Gly_{deg}/C_{up}	0.27	0.25	0.30	0.33	C-mol/C-mol

Table 3
ATP balance over the fermentative organisms.

Fermentative organisms				
ATP required	ATP produced	ATP balance	Estimated	
Glycerol uptake and storage mol/C-mol	Propionate synthesis mol/C-mol	%	Propionate yield mol/mol	Glycogen Yield
-0.33	0.33	100	0.5	0.5

Table 4
NADH balance over the PAOs.

NADH required		PAO NADH produced	NADH balance	Estimated
PHV mol/C-mol	PH2MV mol/C-mol	Glycogen hydrolysed mol/C-mol	%	Glycogen hydrolysed C-mmol
-1.31	-0.88	2.13	97	3.28

Table 5
ATP balance over the PAOs. VFA-CoA is the combination of acetyl- and propionyl-CoA.

ATP required		PAO ATP produced	NADH balance	ATP balance
VFA-CoA activation mol/C-mol	Transport mol/C-mol	Poly-P hydrolysis mol/C-mol	Glycogen hydrolysis mol/C-mol	%
-0.33	-0.11	0.35	0.09	101

substrate uptake in the presence of an electron acceptor (e.g. oxygen) could lead to filamentous growth on the outer fraction of granules (Beun et al., 1999). The slow replacement of the 1,3-PDO fermentative bacteria by propionate fermentative bacteria indicates that the competitive difference between the both is small. It seemed that lowering the SRT control to 12 days after day 65 helped to outcompete the 1,3-PDO fermentation process. This SRT control could be a potentially practical way to select for good conditions in full-scale reactors where glycerol is an important substrate for EBPR processes.

4.4. Substrate storing fermentative anaerobes

Many microorganisms are capable of accumulating internal storage compounds (van Loosdrecht et al., 1997). The storage in aerobic feast-famine processes and in anaerobic-aerobic processes have been well described. Anaerobic feast-famine conditions and substrate storage got only limited attention in the literature. For instance, (Shimada et al., 2007) showed the accumulation and degradation of trehalose by a mixed microbial culture growing in a sequencing batch reactor converting glucose into methane. Recently, in a similar study where sucrose-rich synthetic wastewater was used, anaerobic accumulation of glycogen and glycogen-like compounds was reported (Ni et al., 2015). In this study, we observed the fermentation of glycerol and accumulation of glycogen at the end of the anaerobic phase presumably by the fGAOs. The anaerobic uptake of glycerol could lead to the accumulation of storage compounds (not PHA) by *Tessaracoccus* (Maszenan et al., 1999). The degree of this accumulation can partly be explained by the mechanism by which microorganisms derive their energy from the fermentation process. For instance, several catabolic pathways exist in the conversion of glycerol into propionate. A common pathway found predominantly in *propionibacterium* is the Wood-Werkman cycle which involves a transcarboxylase transferring a carboxyl group from methylmalonyl-CoA to pyruvate to generate propionyl-CoA (Gonzalez-Garcia et al., 2017). As shown in Table 6, the transcarboxylase enzymes are also expressed in the genome of *Tessaracoccus*. This cycle conserves an ATP which is otherwise lost to the fixation of CO_2 to oxaloacetate and thus the energy can be used for growth and storage processes. Likely half of the glycerol is fermented to propionate while the other half is stored as glycogen or poly-glucose. When glycerol is depleted, the fGAOs start to grow fermentative on the stored compound, resulting in the production of VFAs such as propionate in the bulk liquid that is subsequently taken up by the PAOs. In line with the accumulation of propionate observed in a batch experiment after glycerol depletion, the formation of propionate from glucose fermentation using granular sludge was also reported by (Shimada et al., 2007). Thus, it is likely that the stored glycogen was also fermented into propionate.

4.5. Microbial community

A metabolic pathway for glycerol conversion was hypothesised based on the metaproteome data obtained for the microbial community (Fig. 8). Although *Ca.* Accumulibacter possesses a diverse metabolic potential, these microorganisms seem to lack the enzymatic machinery required for uptake and conversion of glycerol (Table 6). Glycerol uptake facilitator (glpF) and glycerol kinase (glpK), two proteins involved in the transport of glycerol into cellular metabolism (Voegele et al., 1993), were observed in the genome of *Tessaracoccus* and *Micropruina.* Both of these organisms belong to the *propionibactericeae* family which is one of the suborders of the class *Actinobacteria.* As the name suggests, the production of propionic acid is the hallmark of *propionibacteria* (Stackebrandt et al., 2006) and illustrated in a study by (Barbirato et al., 1997) where propionate production from glycerol fermentation by different strains of *Propionibacterium* was reported. Interestingly, these microorganisms have been also commonly observed in activated sludge samples with phosphorus removal capabilities around the world (Nielsen et al., 2019). Genomic analysis of *Tessaracoccus* and *Micropruina* showed that these organisms lack PHA synthase (phaC), a key enzyme encoding for PHA synthesis (Zher Neoh et al., 2022). In an earlier study by (Maszenan et al., 1999) absence of poly-P and PHA granules in *Tessaracoccus* was also reported as these compounds were not observed when stained by Methylene blue and Nile blue, respectively. However, staining methods are prone to error due to false positive results and provide limited information about the potential of enzymatic machinery of the

Table 6
Key metabolic pathways and the expressed proteins involved in the reaction by organisms with over 90% of the protein mass in the reactor.

Pathway	Protein	Symbol		Ca. Accumulibacter	Tessaracoccus	Micropruina
Glycerol uptake	Glycerol kinase	*glpK*	-	+		+
	Glycerol uptake facilitator	*glpF*	-	+		+
	Glycerol dehydrogenase	*glpD*	-	+		+
Fermentation	Pyruvate kinase	*pyk*	+	+		+
	Alcohol dehydrogenase	*adh*	+	-		-
	Pyruvate decarboxylase	*pdc*	+	-		-
	D-lactate dehydrogenase	*dld*	+	-		-
Storage polymers	PHA synthase	*phaC*	+	-		-
	PHA polymerase	*phaE*	+	-		-
	Glucokinase	*glk*	+	-		-
	Starch synthase	*glgE*	+	+		+
Polyphosphate metabolism	Polyphosphate glucokinase	*ppgk*	-	+		-
	Polyphosphate kinase	*ppk*	+	+		+
	Polyphosphate AMP phosphotransferase	*pap*	+	-		-
VFA transporter	Propionyl-CoA synthetase	*prpE*	+	-		-
	Propionyl-CoA carboxylase	*pccB*	+	+		-
	Propionyl-CoA transferase	*pct*	+	-		-
	Acetyl-CoA synthetase	*acs*	+	-		+
	Acetyl-CoA carboxylase	*accC/B*	+	+		-
	Phosphate acetyl transferase	*pta*	+	-		-
Trans-carboxylation	Methylmalonyl-CoA mutase	*mut*	+	+		+
	Methylmalonyl-CoA epimerase	*mcee*	-	+		-
	Methylmalonyl-CoA carboxyltransferase	*YgfG*	-	+		-

organisms. Here, through proteomics, we have investigated the presence or absence of storage compounds by searching for the existence of key enzymatic reactions in each organism. *Tessaracoccus* have the capacity to produce poly-glucose, but PHA production proteins were not identified. Although poly-P granules were not observed in *Tessaracoccus* and *Micropruina* they seem to possess some of the enzymes involved in poly-P metabolism. Indicated by the presence of polyphosphate kinase (ppk), these microorganisms can likely accumulate poly-P but are not able to utilize it for energy generation since AMP phosphotransferase (pap) is absent. Further study on the capabilities of these fGAOs in the future could elucidate their full potential.

4.6. Practical implications

- Stable granulation by PAO-dominated microbial community can be achieved by steering glycerol fermentation to propionate over 1,3-Propanediol. Potentially the SRT can be a control parameter.
- The dominance of polyphosphate accumulating organisms in the culture indicates that the extracellular matrix is likely similar to those produced using municipal wastewater indicating that glycerol-based sludge might also be used to extract the Kaumera biopolymers.

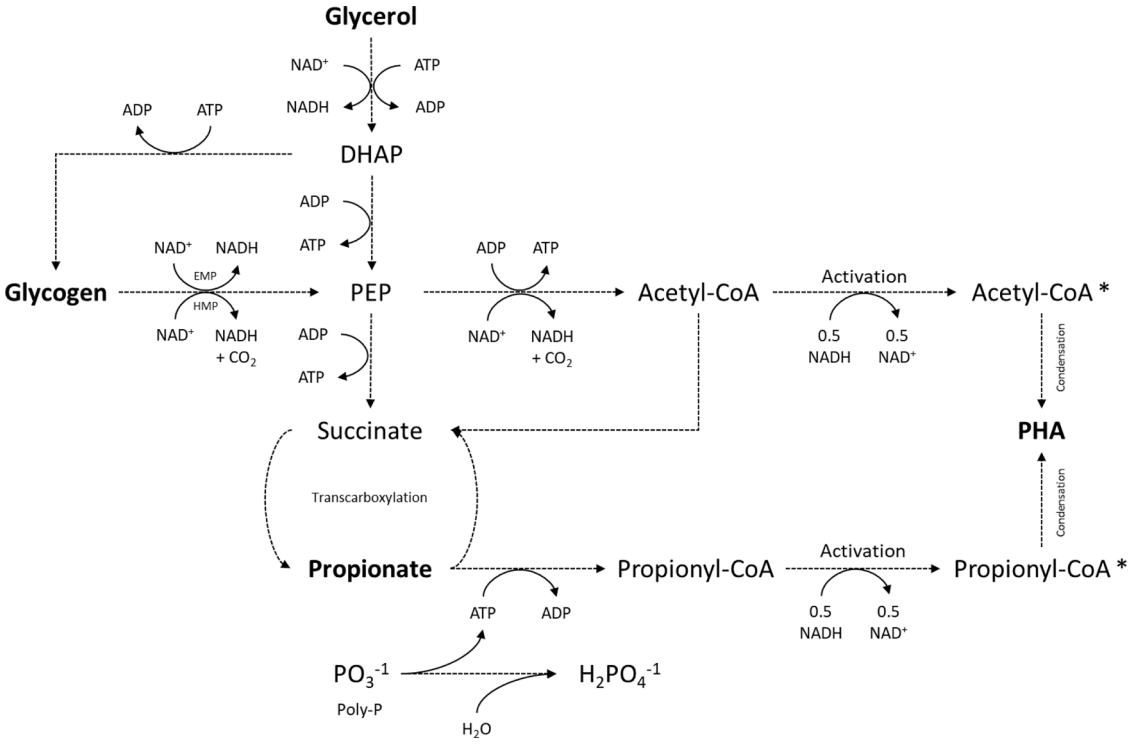

Fig. 8. Overview of proposed anaerobic glycerol conversion pathway by aerobic granular sludge. DHAP: Dihydroxyacetone phosphate, PEP: Phosphoenol pyruvate, PHA: Poly-hydroxy-alkanoates. Conversion of glycerol into propionate follows the Wood-Werkman cycle.

- Glycerol as a relatively cheap carbon source could be dosed to wastewater treatment plants to overcome carbon deficiency.

5. Conclusion

- Successful and stable granulation with glycerol as the sole carbon source was shown.
- Glycerol-driven EBPR was achieved by a microbial consortium of glycerol fermenters and PAOs.
- Lack of *glpF, glpD,* and *glpK* in the genome of *Ca.* Accumulibacter implied that these organisms are likely not able to directly utilize glycerol.
- Over 90% of the expressed proteins in glycerol-based aerobic granular sludge belonged to *Ca.* Accumulibacter.

Declaration of Competing Interest

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

Data Availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2022.119340.

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