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# Impact of aerobic availability of readily biodegradable COD on morphological stability of aerobic granular sludge

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## ABSTRACT

Operational disturbances in aerobic granular sludge (AGS) systems can result in aerobic availability of readily biodegradable COD (rbCOD). Different from activated sludge, morphological consequences on the short and long term are not well described in literature. This study investigated the effect of incomplete anaerobic uptake of acetate on the morphological and process stability of AGS using a lab-scale reactor. A fraction of the total acetate load was dosed aerobically, which was increased stepwise while monitoring granular morphology. A good granular morphology and an SVI of 40 ml/g were obtained during initial enrichment and maintained for  $\leq 20\%$  aerobic acetate load dosed at 4 mg COD/g VSS/h. Biological phosphorus removal efficiency was initially unaffected, but the aerobic acetate dosage rate did decrease the aerobic phosphate uptake rate. This led to loss of phosphorus removal for  $>20\%$  aerobic acetate load dosed at 8 mg COD/g VSS/h over the course of 12 days. Subsequently, significant outgrowth formed on the granular surfaces and developed over time into finger-like structures. Under these high aerobic acetate loads the SVI increased to 80 ml/g and resulted in significant biomass washout due to deteriorating settling properties of the sludge. The sludge settleability and biological phosphorus removal recovered 10 days after aerobic feeding of acetate was stopped. Aerobic presence of rbCOD can be tolerated if mostly anaerobic acetate uptake is maintained, thereby ensuring stable granular morphology and good settleability. The high enrichment of phosphate accumulating organisms in the granular sludge through bottom-feeding and selective wasting of flocs makes aerobic granular sludge resilient to morphological deterioration in aerobic presence of rbCOD.

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## 1. Introduction

Aerobic granular sludge (AGS) is becoming a well-established technology for wastewater treatment due to the compactness, energy savings and good effluent quality (Bengtsson et al., 2018; Derlon et al., 2016; Pronk et al., 2017a). The current commercial technology (Nereda<sup>®</sup>, a trademark owned by Royal HaskoningDHV) is based on sequentially operated batch reactors (SBRs) (Giesen et al., 2013). The influent is fed from the bottom of the reactor resulting in uptake of readily biodegradable chemical oxygen demand (rbCOD) and conversion to storage polymers in the microbial cells under anaerobic conditions. The storage polymers are oxidised in the subsequent aeration phase where they are used for microbial growth and nutrient removal. This process design selects for well-settling, granular sludge, which is further enhanced

by subsequent selective discharge of the worst-settling, flocculent sludge fraction (Beun et al., 2002; De Kreuk and Van Loosdrecht, 2004; Liu et al., 2005).

There has been a significant amount of research orientated towards the long-term morphological stability of AGS in SBRs. An increasing body of literature has been developed on AGS formed on various organic substrates and a variety of operational conditions. The results obtained on granular stability have led to the general view that long-term stability of AGS is very variable, and dependant on the applied process conditions (Corsino et al., 2017; De Kreuk and Van Loosdrecht, 2004; Franca et al., 2018; Kent et al., 2018; Liu and Liu, 2006). A limitation of many studies is their empirical nature; different process conditions are evaluated but the underlying mechanisms explaining the observations are often not provided or even studied. Specific conclusions can therefore be difficult to extrapolate to a more general context. It is the authors' opinion that this clouds the current understanding of the principles governing the formation and stability of AGS in general. More-

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over, this potentially hampers implementation and development of alternative technologies for acquiring granular sludge in full-scale municipal wastewater treatment.

The effect of the presence of both rbCOD and oxygen as electron acceptor is a prime example of such confusion. Strategies to prevent and mitigate bulking sludge in conventional activated sludge processes have primarily focussed on the impact of substrate concentrations or floc loading rates (F/M ratio) on floc morphology (Martins et al., 2004b). The obtained insights initially led to the first investigations into AGS under full aerobic conditions (Beun et al., 1999; Heijnen and Van Loosdrecht, 1998; Morgenroth et al., 1997). In general, a dense and smooth biofilm requires substrate uptake limited by the maximum biomass specific uptake rate, while a transport-limited substrate uptake yields less dense and irregular biofilms (Picioreanu et al., 2000; van Loosdrecht et al., 1997a). In the case of biological nutrient removal using activated sludge, design has focussed traditionally on maximizing rbCOD gradients in the activated sludge reactors (Chudoba, 1985; Chudoba et al., 1985). This is implemented by designing plug-flow reactors or adding so-called aerobic selector tanks (Chudoba et al., 1973). In these configurations, rbCOD is first converted into storage polymers such as polyhydroxyalkanoates (PHAs) (van Loosdrecht et al., 1997b). There are two important conditions to minimise the sludge volume index (SVI). Firstly, a sufficiently high dissolved oxygen concentration ( $>2\text{ mg/l}$ ) is required, in case of aerobic conversion of rbCOD, to prevent transport-limitation for oxygen during formation of PHA (Martins et al., 2003a). Second, the ratio of the rbCOD sludge loading rate in the selector over the maximum biomass specific uptake rate ( $q_S/q_S^{\text{max}}$ ) should be close to unity for a good sludge volume index ( $\text{SVI}_{30} < 100\text{ ml/g}$ ) (Martins et al., 2003b).

AGS can be cultivated with an aerobic feeding strategy (i.e., employing the same completely oxidative storage of rbCOD to PHA), but this requires a relatively high shear rate, a high selective pressure on settling speed, and a sufficiently high dissolved oxygen concentration to obtain a granular morphology (Arrojo et al., 2004; Beun et al., 2000; Morgenroth et al., 1997; Mosquera-Corral et al., 2005; Schwarzenbeck et al., 2004; Tay et al., 2001). With the increase of granule size over time, the interior of these aerobically fed granules gets deprived of substrates and decays, resulting in granular instability. Long-term stability is in general not achieved in aerobically fed granular sludge processes (Beun et al., 2000; Morgenroth et al., 1997).

Design of anaerobic and anoxic selectors, to obtain well-settling sludge, has proven less problematic than for aerobic selectors. The general observation for anaerobic selectors is that complete uptake of rbCOD in the anaerobic contact zone yields a well-settling, dense flocculent sludge morphology, independent of the hydrodynamic conditions in the anaerobic selector (Martins et al., 2004a; Wanner et al., 1987). The removal of all rbCOD during the anaerobic stage of the process into PHA was shown to be a critical design criterion to form granular sludge in aerobic processes for nutrient removal as well (De Kreuk and Van Loosdrecht, 2004). Different from aerobic feeding, long-term stability of granular sludge morphology was obtained using a process with anaerobic feeding/aerobic growth (De Kreuk et al., 2007, 2005).

The sensitivity of anaerobically fed AGS reactors to the presence of rbCOD in the aeration phase has not been studied. This is an important aspect for design and operation, and knowledge on this sensitivity aids in the further development of AGS processes. Not only rbCOD bypassing the anaerobic stage, but also aerobic hydrolysis of slowly biodegradable COD makes rbCOD available in the aerated phase (Pronk et al., 2015a). Both can result in transport-limited substrate uptake rates of either oxygen or rbCOD, and potentially lead to deterioration of sludge morphology and settleabil-

ity. If there is some tolerance for the aerobic presence of rbCOD, it would ease the control and design of AGS processes.

The effect of aerobic presence of rbCOD on the morphology of activated sludge and granular sludge, respectively, could vary due to the intrinsic differences in process configurations. In both cases, phosphate accumulating organisms can significantly compete for substrate uptake with other heterotrophs under aerobic conditions due to the ability to sequester substrates under aerobic conditions (Pijuan et al., 2005). For a completely mixed activated sludge, a fraction of 20% of the rbCOD-load dosed aerobically was reported to have no negative effect on the SVI of a lab-scale system enriched for enhanced biological phosphorus removal (EBPR) (Martins et al., 2004a). Aerobic granular sludge processes might react differently than flocculent sludge systems due to the difference in feeding and the need to selectively waste flocculent sludge.

In this work, the effect of aerobic presence of rbCOD on the morphological stability of AGS was investigated. The first aim was to determine the fraction of rbCOD that can be consumed aerobically while maintaining well-settling AGS. In case of deterioration, the second aim was to clarify via which mechanism(s) it would occur. A lab-scale AGS system with acetate as carbon source was used to investigate the impact on granular morphology and overall sludge settling when a fraction of acetate is dosed under aerobic conditions. Acetate was aerobically dosed at a rate that ensured a negligible residual concentration (i.e., to force transport-limited substrate uptake rates). Changes in sludge morphology and nutrient removal were monitored over time.

## 2. Materials and methods

### 2.1. Experimental set-up and operation

A lab-scale reactor with a working volume of 2.7 L and aspect ratio of 5 was operated as a sequential batch reactor with a volume exchange ratio of 0.55. The bioreactor was operated continuously in 3-hour cycles for 192 days. The cycle first consisted of an anaerobic phase where synthetic wastewater was fed through the bottom of a settled sludge bed (AN feeding, maximum duration of 60 min). A short mixing phase was then applied prior to enabling DO-control to homogenize the bulk-liquid (AE mix, 4 min). Next, a mixed aerobic phase with dosage of acetate to mimic the continued availability of rbCOD was followed by a mixed aeration phase without additional dosage (AE feeding and AE reaction, combined minimum duration of both phases was 106 min). A settling period (settling, 5 min) was then followed by the discharge of effluent and unsettled sludge (discharge, 5 min). The duration of the respective anaerobic and aerobic (carbon source only) feeding phases were set according to the anaerobic/aerobic acetate distribution of the current stage (Table 1), while maintaining a constant total cycle duration. The total organic loading rate ( $1.63\text{ g COD L}^{-1}\text{ d}^{-1}$ ) was kept constant over the course of the study. AGS previously enriched at the same conditions as described in this section was used as inoculum (conditions listed in Table 1, 0% aerobic rbCOD load).

A synthetic wastewater of 1.5 L per cycle was used as anaerobic feed and consisted of 1.2 L of deionized water together with 150 mL carbon source (medium A) and 150 mL nitrogen and phosphorous source (medium B). Medium A contained 63 mM  $\text{NaCH}_3\text{COO}\cdot 3\text{H}_2\text{O}$ , 3.6 mM  $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ , and 4.7 mM KCl. Medium B contained 42.8 mM  $\text{NH}_4\text{Cl}$ , 4.2 mM  $\text{K}_2\text{HPO}_4$ , 2.1 mM  $\text{KH}_2\text{PO}_4$ , and 10 mL  $\text{L}^{-1}$  trace elements solution (Vishniac and Santer, 1957), but using 2.2 mg/L  $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$  instead of 22 mg/L (Pronk et al., 2017b, 2015b). The combination of medium A, medium B, and tap water led to a synthetic wastewater composition of 366 mg COD  $\text{L}^{-1}$ , 60 mg  $\text{NH}_4^+\text{-N L}^{-1}$ , and 9.3 mg  $\text{PO}_4^{3-}\text{-P L}^{-1}$ . The synthetic wastewater was fed at a superficial liquid velocity of  $0.6\text{ m h}^{-1}$ . The aerobic acetate load was dosed using a 10x more diluted so-

**Table 1**

Operational parameters of reactor operation during stepwise increase of aerobically dosed acetate load fraction. For each condition, the total cycle time was kept constant at 3 h. AN = anaerobic, AE = aerobic, VSS = volatile suspended solids.

Aerobic acetate load (%)	Specific aerobic acetate dosage rate (mg COD/ g VSS/h)	Duration (days)	Operating phase duration (min)					
			AN feeding	AE mix	AE feeding	AE reaction	Settling	Discharge
0 <sup>a</sup>	0	27	60	4	–	106	5	5
2	4.6	15	58.8	4	12	95.2	5	5
5	3.6	9	57	4	30	79	5	5
10	3.5	33	54	4	60	52	5	5
15	4.0	5	51	4	90	25	5	5
20	4.4	23	48	4	118	–	5	5
25	7.8–13.2 <sup>b</sup>	24	45	4	75	46	5	5
35	11.1	29	39	4	106	21	5	5
0 <sup>c</sup>	0	27	60	4	–	106	5	5

<sup>a</sup>) Reference period <sup>b</sup>) The MLVSS concentration in the reactor remained stable for two weeks after increasing the aerobic acetate load to 25%, but then decreased due to an increase in washout of biomass from deteriorating settling properties. The specific aerobic acetate dosage rate therefore increased as the MLVSS concentration in the reactor decreased. <sup>c</sup>) Recovery period.

lution of medium A (medium C). Medium C contained 6.3 mM NaCH<sub>3</sub>COO·3H<sub>2</sub>O, 0.36 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, and 0.47 mM KCl, resulting in a concentration 366 mg COD L<sup>-1</sup>. All media were dosed using peristaltic pumps and only flow rate and feeding duration were changed according to experimental stage (see Table 1). Media compositions remained the same throughout the study. The temperature was controlled at 20 ± 1 °C through the double-jacketed reactor wall using a water bath with thermostat. The pH was controlled during aeration at 7.0 ± 0.1 by dosage of either a 1 M solution of hydrochloric acid or a 1 M sodium hydroxide solution. During aeration, a recirculation gas flow was maintained at 6 l min<sup>-1</sup> (superficial gas velocity of 4.2 cm s<sup>-1</sup>). A dissolved oxygen (DO) concentration of 2 mg/l was maintained during the aeration phase via addition of compressed air or dinitrogen gas using mass flow controllers. The average sludge retention time (SRT) was controlled at 10 days by manual removal of biomass at the end of the aeration phase on a two day basis (once per 16 cycles).

## 2.2. Operational conditions during stepwise increase of aerobically dosed rbCOD fraction

To force transport-limited uptake of acetate during the stepwise increase of the aerobically dosed acetate load, the acetate sludge-loading rate should be significantly lower than the maximum aerobic biomass specific uptake rate. Under these conditions, there is the maximal risk of obtaining bulking sludge and open porous biofilms (Martins et al., 2003b; Picoreanu et al., 2000). The maximum aerobic uptake rate was determined during the reference period as 49 mg COD/g VSS/h. The aerobic acetate loading rate was initially set to 1/10 of this maximum rate (5.8 mg COD/h), thereby ensuring a transport-limited substrate uptake rate. The aerobic acetate feeding rate was increased to 1/5 of the maximal substrate uptake rate (11.6 mg COD/h) as the aerobic acetate load was increased from 20% to 25% of the total load to maintain a constant total cycle time. The load distribution was achieved by altering the duration of acetate dosage in the anaerobic phase and aerated phase (Table 1). The reactor was operated at each aerobic acetate load fraction for at least one SRT prior to transitioning to the next step (Table 1). A longer time period was applied when a clear morphological change was detected within one SRT.

## 2.3. Analysis of reactor performance

Samples were taken during aeration and filtered through a 0.45 µm PVDF filter (Millipore). NH<sub>4</sub><sup>+</sup>-N, NO<sub>x</sub>-N, and PO<sub>4</sub><sup>3-</sup>-P concentrations were measured by using a Thermo Fisher Gallery Dis-

crete analyzer (Thermo Fisher Scientific, Waltham, USA). The concentration of acetate was determined by high-performance liquid chromatography (HPLC) with an Aminex HPX-87H column from Biorad, coupled to an UV detector, using 0.01 M phosphoric acid as eluent. Mixed liquor suspended solids (MLSS) and volatile suspended solids (MLVSS) concentrations in the reactor were determined according to the standard methods ("2540 SOLIDS," 2017). The sludge volume after 10 min of settling and effluent discharge (SV<sub>10</sub>) was determined in between cycles in-situ. The SRT was calculated by dividing the average amount of VSS in the reactor over the sample period by the sum of the VSS in the effluent from selective wasting and manual, mixed wasting of sludge (averaged per week). The amount sludge wasted manually was adjusted to maintain the desired average SRT.

## 2.4. Imaging of sludge morphology

The morphological features of the sludge were assessed from both the reactor and effluent prior to changing fraction of acetate dosed aerobically at each experimental stage or when a clear morphological change was observed. Sludge was collected from the reactor at the end of the aeration phase and from the effluent directly after discharge. A mixed sample from either source was transferred to a glass petri dish and examined by the means of an Olympus reverse microscope coupled with a Leica Digital Camera, together with its software QWin Pro (version 3.1.)

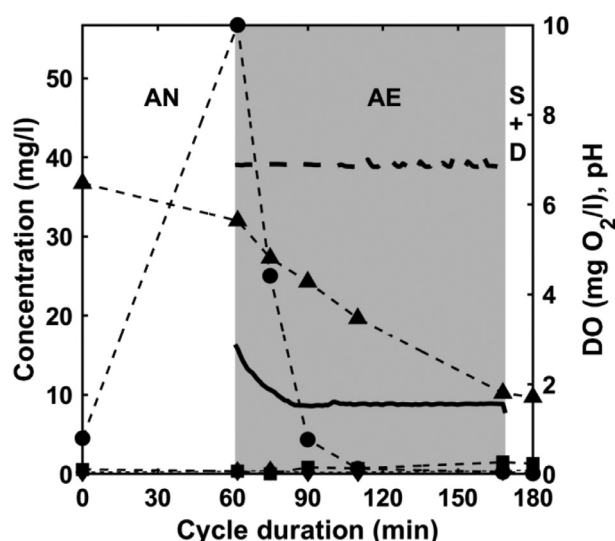
## 2.5. Maximum specific acetate uptake rate under aerobic conditions

During a cycle in the reference period, the DO-controller was turned off 10 min after the aeration phase had started at the DO-concentration set point of 2 mg/l. The outputs of the mass flow controllers for both compressed air and nitrogen gas were fixed to their last setting. The reactor was then pulsed with a sodium acetate solution to obtain a bulk concentration of 20 mg COD/l. The volumetric uptake rate was then determined by measuring the duration of the temporary drop in dissolved oxygen concentration due to the increased oxygen consumption rate. The reactor MLVSS concentration was used to calculate the biomass specific uptake rate (mg COD/g VSS/h).

## 2.6. Fluorescent in-situ hybridisation (FISH)

The handling, fixation and staining of FISH samples was performed as described in (Bassin et al., 2011). A mixture of PAO462, PAO651, and PAO846 probes (PAOmix) was used for visualizing polyphosphate accumulating organisms (PAO) (Crocetti et al.,





**Fig. 1.** Concentration profiles for a typical cycle (27 days after the start of the experiments) during the reference period with complete anaerobic acetate uptake for PO<sub>4</sub>-P (circles), NH<sub>4</sub>-N (triangles), NO<sub>2</sub>-N (diamonds) and NO<sub>3</sub>-N (squares) (left axis). pH (dashed line, controlled at 7) and DO (solid line) (right axis). Measurements were only meaningful in the mixed aeration phase. Alternating shading denotes phase changes within the cycle (AN: anaerobic phase; AE: aerobic phase; S + D: settling and discharge).

2000). A mixture of GAOQ431 and GAOQ989 probes (GAOmix) was used for visualizing glycogen accumulating organisms (GAO) (Crocetti et al., 2002). A mixture of EUB338, EUB338-II and EUB338-III probes was used for staining all bacteria (Amann et al., 1990; Daims et al., 1999). Images were taken with a Zeiss Axio-plan 2 epifluorescence microscope equipped with filter set 26 (bp 575e625/FT645/bp 660e710), 20 (bp 546/12/FT560/bp 575e640), 17 (bp 485/20/FT 510/bp 551e565) for Cy5, Cy3 and fluos respectively.

### 3. Results

#### 3.1. Reference reactor operation

The reactor was seeded with aerobic granular sludge formed at lab-scale in an earlier cultivation using the same synthetic wastewater composition and initial operational conditions (Table 1). Granulation and conversions were allowed to stabilize over the course of one month. Complete anaerobic acetate uptake and complete phosphorus removal were achieved during this period. The focus in this research was on stable granulation and conversions rather than the optimisation of effluent concentrations (i.e., total nitrogen concentration). Fig. 1 depicts the concentration profiles during one cycle prior to initiating the stepwise increase of the fraction of acetate dosed in the aerobic period. This period was used as a reference throughout the study.

The effluent concentration of phosphate during the steady-state operation was always very low (i.e. < 0.1 mg PO<sub>4</sub>-P/l). The maximum biomass specific rate for aerobic phosphate uptake was 20 mg P/g VSS/d. The presence of a strong PAO-community was also indicated using FISH-microscopy (supplementary materials). All the acetate was taken-up during the anaerobic feeding period. Nitrification was present, albeit not yet complete. The reactor fully granulated and sludge settleability was stable with low SVIs throughout the reference period (SVI<sub>10</sub> = 30–40 ml/g, Fig. 2A). Granules in the reactor had a smooth surface and were heterogeneous in size and shape, with an average diameter > 1 mm (Fig. 3A, stage I). Sludge wasted through selective discharge mainly con-

sisted of the smallest granule size fraction (Fig. 3B, stage I). The mixed liquor volatile suspended solids (MLVSS) concentration was  $5.2 \pm 0.6$  g VSS/l (Fig. 2B). The solids retention time (SRT) after start-up was controlled at 10 days.

#### 3.2. Effect of aerobic transport-limited acetate uptake rate on sludge morphology and settleability

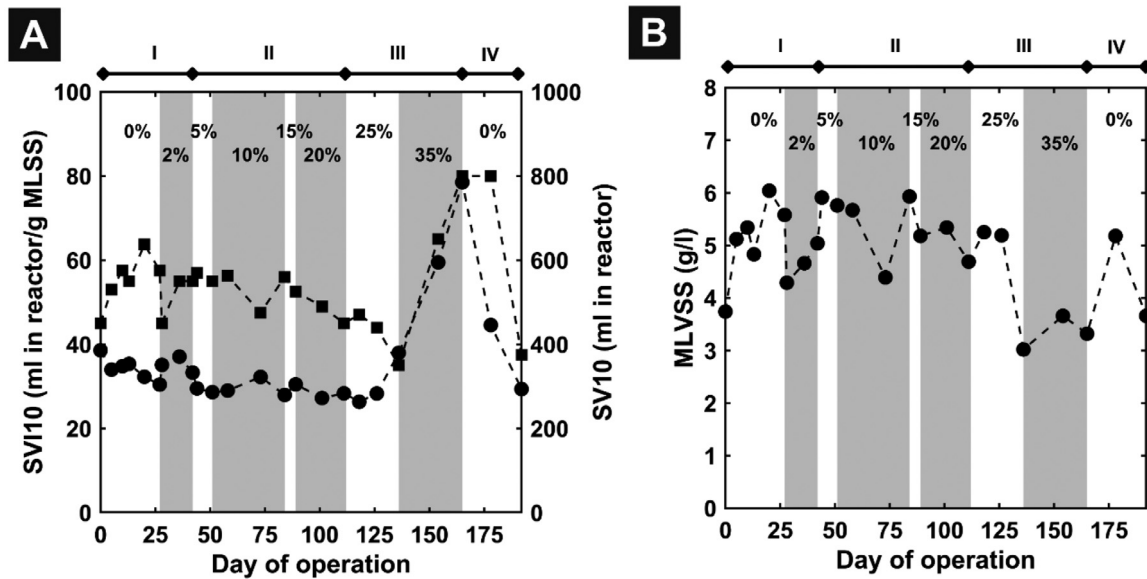
##### 3.2.1. Stage II (<20% aerobic acetate load)

The effect of an increasing fraction of the aerobic acetate load on the sludge volume (SV<sub>10</sub>) and sludge volume index after ten minutes of settling (SVI<sub>10</sub>) is shown in Fig. 2A. The applied aerobic acetate loading rate had no noticeable effect on settleability, nor on the MLVSS concentration up to dosing 20% of the acetate load in the aerobic period (Fig. 2B). The sudden decrease in MLVSS on days 28 and 73 of operation were caused by too much mixed sludge withdrawal for manual SRT control. The sludge morphology in the reactor remained smooth as well, with a small amount of filamentous bacteria extending from the surface of some granules. Suspended growth was not observed in the reactor (Fig. 3A, stage II). The settling speed (indicated by the SV<sub>10</sub>) and the packing density (indicated by the SVI<sub>10</sub>) were unaffected by the filamentous bacteria attached to the granules. The SV<sub>10</sub> and SVI<sub>10</sub> remained similar to the reference period with complete anaerobic acetate feeding (i.e., SV<sub>10</sub> = 400–500 ml, SVI<sub>10</sub> = 30–40 ml/g). As the aerobic acetate load increased to 20%, an increasing amount of suspended filamentous bacteria entangled with flocs and small granules were found in the effluent in addition to granules from the smallest size fraction (Fig. 3B, stage II). The selective wasting based on settling speed was sufficient to prevent accumulation of the filamentous bacteria in the reactor and a potential negative impact on the SVI.

##### 3.2.2. Stage III (>20% aerobic acetate load)

Sludge morphology in the reactor changed after the increase to 25% aerobic acetate load combined with an increase in aerobic acetate feeding rate (Table 1), although it did not immediately lead to an increase in SV<sub>10</sub> or SVI<sub>10</sub> in the reactor during the two weeks. The surface coverage as well as the length of the outgrowth on granules increased and affected the majority of the granules (Fig. 3A, stage III). The outgrowth's shape changed from individual filaments to intertwined, finger-like structures that formed a shell around existing granules (Fig. 3B, morphological comparison). A small fraction of suspended filamentous bacteria was also observed in the reactor, but insufficient to affect overall sludge settleability. Suspended, tape-like films were the dominant morphology besides granules in sludge sampled from the effluent discharged after the settling period. Furthermore, small granules embedded in larger films were observed (Fig. 3B, stage III). Despite the change in sludge morphology, it had not altered the settling speed of the sludge fraction growing in suspension, and thus the sludge load discharged through the effluent. Therefore, the MLVSS in the reactor did not decrease compared to the reference period.

After two weeks (stage III), short circuit flows during anaerobic feeding were becoming more frequent and the anaerobic storage capacity of acetate was diminished (Section 3.3.2, Fig. 5C). The increased aerobic conversion of acetate resulted in more poor-settling suspended growth and a subsequent decrease in MLVSS through an increased sludge load discharged through selective wasting. No manual SRT control was applied to prevent the average SRT from decreasing below 10 days. Although selective wasting stabilized the SV<sub>10</sub> and SVI<sub>10</sub> initially, the sludge volume started to increase noticeably after one month. The SVI kept deteriorating after the subsequent step to 35% aerobic acetate load, ultimately leading to a twofold increase to 80 ml/g (stage III, Fig. 2A). The thin



**Fig. 2.**  $SV_{10}$  (squares, dashed line) and  $SVI_{10}$  (circles, dashed line) as measured in the reactor at the end of a cycle (A). MLVSS concentration in reactor (circles, dashed line) (B). Alternating shading indicates the different aerobic acetate loads. Percentage annotations in graphs indicate the aerobically dosed acetate as fraction of the total acetate load. Roman numerals indicate stage in morphological transition mentioned in the text.

**Table 2**

Stages in morphological development of aerobic granular sludge as a result of an increase in aerobic acetate load dosed at a rate forcing transport-limited substrate uptake, linked to the observed overall settleability and effect on biological phosphorus removal performance.

Phase	Aerobic acetate load	Settleability	Sludge morphology	Granular	Biological phosphorus removal
I	0%, 2%	$SVI_{10} = 30\text{--}40\text{ ml/g}$	Not present	Smooth surface	No effect on aerobic phosphate uptake rate. Full P-removal.
II	5%, 10%, 15%, 20%	$SVI_{10} = 30\text{--}40\text{ ml/g}$	Flocs with filamentous bacteria in effluent, not in reactor	Mainly smooth, with some filamentous bacteria attached to surface	Sharp decrease, then slight recovery of biomass specific phosphate uptake rate. Full P-removal.
III	25%, 35%	Increase of $SVI_{10}$ to 80 ml/g in one month	Finger-like flocs made of tape-like films	Surface covered with finger-like outgrowth	Complete loss anaerobic phosphate release and aerobic uptake rate.
IV	0%	Recovery of $SVI_{10}$ to 40 ml/g in one SRT	Finger-like flocs made of tape-like films	Binary: new smooth granules and older granules with finger-like outgrowth.	Partial recovery of specific phosphate uptake rate. Sufficient for full P-removal.

films now were the main suspended sludge morphology in the reactor and covered remaining granules in finger-like structures to a varying degree (Fig. 3A, stage III). Although not smooth surfaced, granular morphology remained the dominant morphology in terms of mass.

### 3.2.3. Stage IV (recovery with complete anaerobic acetate dosage)

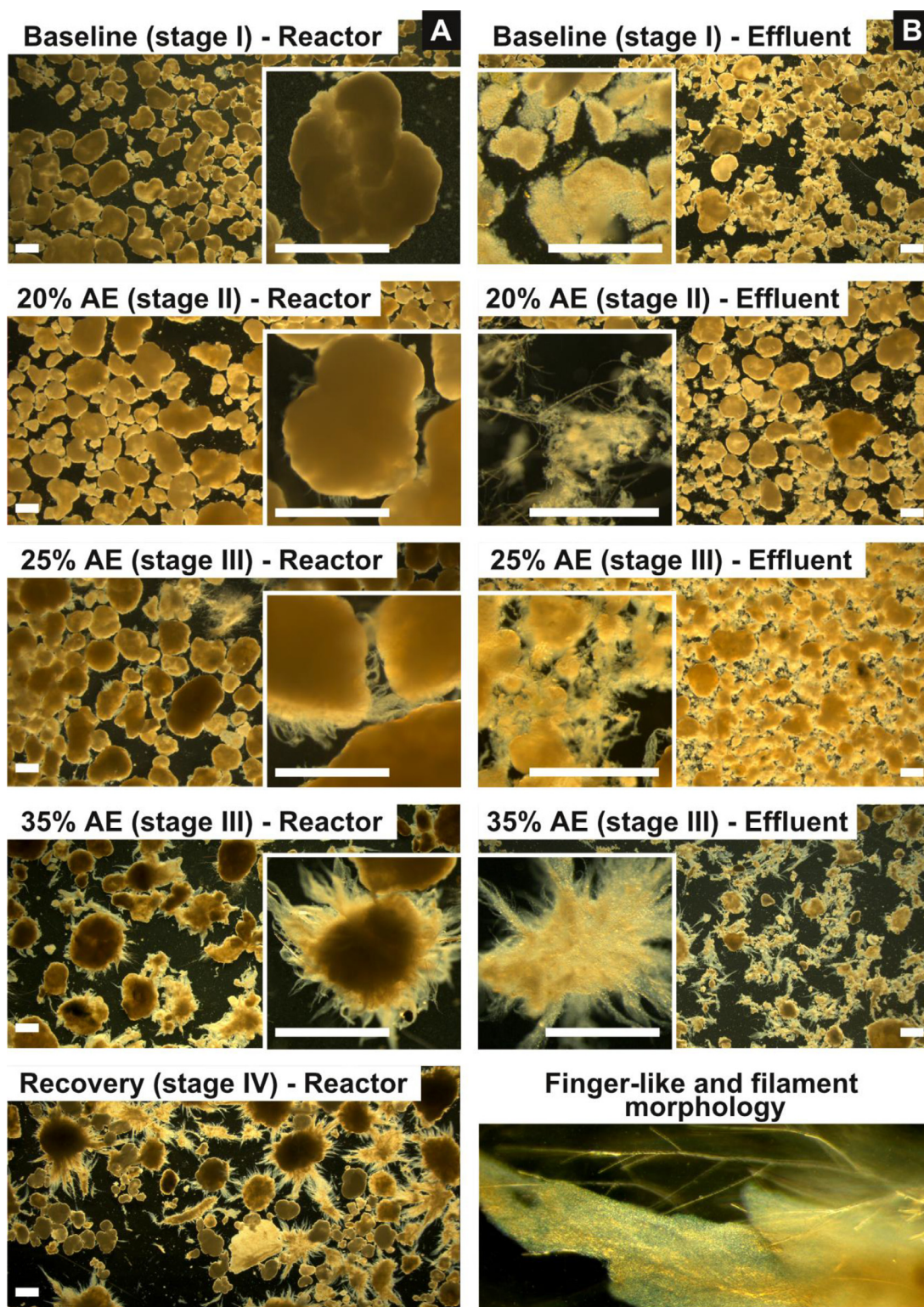
Within one SRT after switching back to the reference conditions the MLVSS concentration went back to the reference stage level by the formation of new smooth granules (Fig. 3A, stage IV). This improved the  $SVI_{10}$  to 40 ml/g as well, but the sludge volume took between 2–3 SRTs to completely recover to 400 ml. Short circuit flows during the anaerobic contact phase kept occurring on an irregular basis up to one SRT after the switch. This likely resulted in transport-limited acetate uptake during the aeration phase and delayed the recovery. Gradually granules covered in finger-like outgrowth decreased in number over time, either through wasting or disintegration. Newly formed smooth granules increased in number (Fig. 3A, stage IV), reminiscent of the original morphology at the start of the experiment. An overview of identified stages in morphological development is presented in Table 2.

### 3.3. Biological phosphorus removal during stepwise increase of aerobic acetate load

#### 3.3.1. Stage II ( $\leq 20\%$ aerobic acetate load)

The stepwise increase of the aerobically dosed acetate to 20% of the total acetate did not affect sludge settleability, and the overall removal performances of nitrogen (data not shown) and phosphorus were also not affected. Acetate concentrations were below the detection limit ( $<1\text{ mg COD/l}$ ) during the aerobic acetate feeding phase under all experimental conditions  $\leq 20\%$  aerobic acetate load, sampled in the first cycle after the aerobic acetate load had been increased. The addition of acetate under aerobic conditions did have a clear negative impact on the phosphate uptake rate. The development of the biomass specific phosphate uptake rate over time is shown in Fig. 4A. Rates were calculated based on measured concentrations from samples taken during a cycle within an experimental phase (sample interval similar to depicted in Fig. 1). The biomass specific phosphate uptake rate decreased sharply after increasing the aerobic acetate load to 5%. The uptake rate increased again during the stepwise transition from 5% to 20% aerobic acetate load, as well as the anaerobic P/COD-ratio (Fig. 4B).



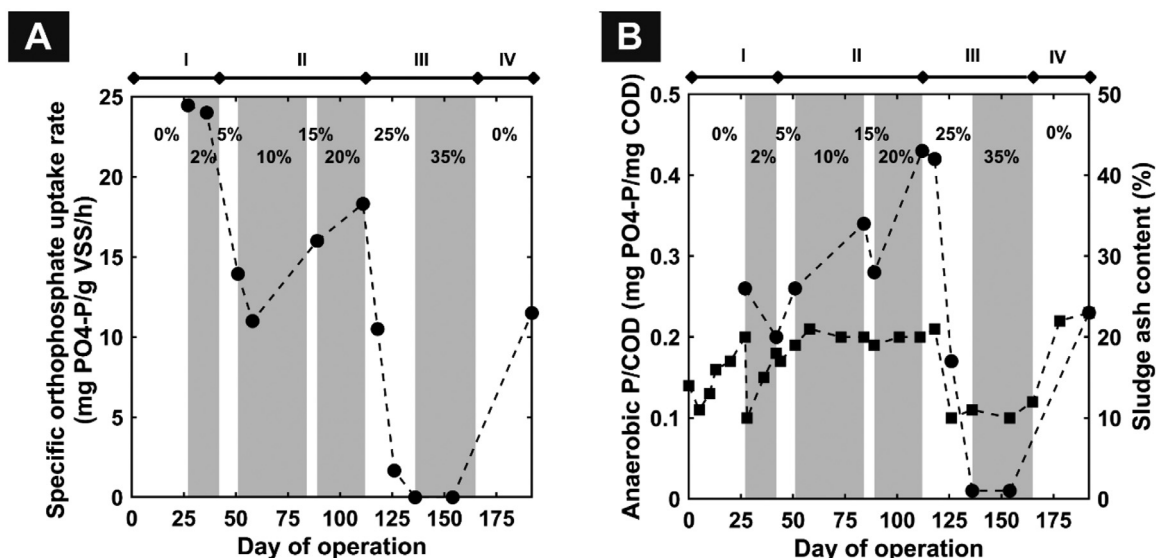


**Fig. 3.** Granular morphology at various stages in the experiment as recorded using stereo-zoom microscopy. Label denotes percentage aerobic acetate load of total acetate load. (A) Overview of morphology in reactor and inset of best settling fraction. (B) Overview of morphology in effluent and inset with worst-settling sludge fraction. Effluent images taken from effluent collected from several cycles and concentrated via decanting. Bottom-right images depict difference between filamentous bacteria and finger-like growth films. Roman numerals indicate stage in morphological transition mentioned in the text. Length represented by the white scale bars is 1 mm.

### 3.3.2. Stage III ( $\geq 20\%$ aerobic acetate load)

The transition from 20% to 25% aerobic acetate load resulted in a further decrease of the phosphate uptake rate. The phosphate uptake was completely lost over the course the next 25 days (first part of stage III).

Upon closer inspection, a series of events occurred after the aerobic acetate load was increased from 20% to 25% that resulted in the twofold increase in  $SVI_{10}$  during stage III. The transition from 20% to 25% aerobic acetate load had to be accompanied by a twofold increase in aerobic acetate dosage rate (i.e., to 116 mg



**Fig. 4.** Biomass specific aerobic orthophosphate uptake rate during operation of the reactor (A). Ratio of PO<sub>4</sub>-P released per amount of acetate fed in the anaerobic phase (circles, dashed line) and ash content as indicator for loss of polyphosphate storage polymer content (squares, dashed line) (B). Alternating shading represent a change in aerobic acetate load. Percentage values in graphs indicate the aerobically dosed acetate as fraction of the total acetate load. Roman numerals indicate stage in morphological transition mentioned in the text.

COD/h), since the aerobic acetate load of 25% could not be achieved at the initial aerobic dosage rate of (i.e., 58 mg COD/h) while maintaining the same total organic loading within the fixed total cycle time. Although this dosage rate still forced a transport-limited acetate uptake rate, the increase in the aerobic acetate dosage rate had a negative effect on the biomass specific phosphate uptake rate. This was initially observed during stage II as well.

Additionally, the concentration profiles of phosphate during the final cycle before the transition (Fig. 5A) and a cycle on the fourth day after the transition (Fig. 5B) showed that the initial biomass specific phosphate uptake rate rapidly decreased as the aeration phase progressed. It increased again after the dosage of acetate had finished, but the remaining aeration time was insufficient for complete removal of phosphate. Biological phosphorus removal became kinetically limited due to increased aerobic acetate dosage rate.

The phosphorus removal during the remaining cycles was less than the combined phosphate load from the influent and the anaerobic phosphate release, resulting in a net decrease of the intracellular polyphosphate storage pool. In between day four (Fig. 5B) and day twelve (Fig. 5C) after the increase to 25% aerobic acetate load and increased feeding rate (see Table 1), two changes were observed in the reactor operation.

- First, the anaerobic ratio of phosphate release over the anaerobic acetate load decreased. The decreasing intracellular polyphosphate pool likely resulted in insufficient anaerobic uptake capacity for acetate, as it coincided with a 10 percent point decrease in the inorganic sludge fraction depicted in Fig. 4B. The increase in oxygen uptake rate (OUR, first seen on day 6) at the start of aeration indicated the presence of a residual acetate concentration, as was deduced from the sudden change in dissolved-oxygen concentration measured in the bulk liquid. This increase came on top of the acetate load dosed as part of the experimental set-up (Fig. 5C).
- Second, the anaerobic uptake of acetate was further decreased by a shorter anaerobic contact time due to short circuit flows. This was most likely caused by increased granular surface roughness due to outgrowth. The short circuit flows increased dispersion in the flow through and above the sludge bed, which was indicated by a non-zero dissolved oxygen concentration at the end of anaerobic feeding (Fig. 5C). The short circuit flows

first occurred on an irregular basis (first seen on day 10 after increasing the aerobic acetate load to 25%), but increased in frequency as the experiment progressed. The aerobic acetate load was thereby increased higher than intended.

Both of these mechanisms shifted more of the anaerobic acetate load to the aeration phase besides the already imposed aerobic acetate load. The absence of anaerobic release of phosphate and negligible effective anaerobic contact time resulted in mainly aerobic conversion of the complete acetate load. Biological phosphorus removal remained negligible throughout this period.

### 3.3.3. Stage IV (recovery with complete anaerobic acetate dosage)

The recovery of granular morphology was studied after letting it deteriorate at an aerobic acetate load of 35% prior to switching to complete anaerobic feeding (stage IV). Both the ash fraction and the ratio of anaerobic phosphate release over acetate uptake returned to baseline levels after one SRT (Fig. 4B) and complete biological phosphorus removal was restored. Short circuit flow channels still occurred on an irregular basis during anaerobic feeding after the switch, until sludge morphology had significantly improved. The remaining effective anaerobic contact time was sufficient to restore the intracellular poly-phosphate pool (restored ash-fraction, Fig. 4B), thus recovering biological phosphorus removal. The combined observations of settleability, morphology and nutrient removal for all stages are summarized in Table 2.

## 4. Discussion

In this study, we investigated the effect of an increasing fraction of the total acetate load on the morphology of AGS enriched for anaerobic PHA formation through EBPR (stage I). Acetate was dosed aerobically at a rate that forced transport-limited uptake. This simulated rbCOD leaking through an anaerobic phase as well as the release of rbCOD from slowly biodegradable COD during aeration. These conditions are known to result in the proliferation of filamentous bacteria and poor-settling activated sludge (Chudoba, 1985). Smooth granular sludge morphology and good settleability ( $SVI_{10}=30-40$  ml/g) up to and including 20% aerobic acetate load (stage II) were maintained. PAOs were able to



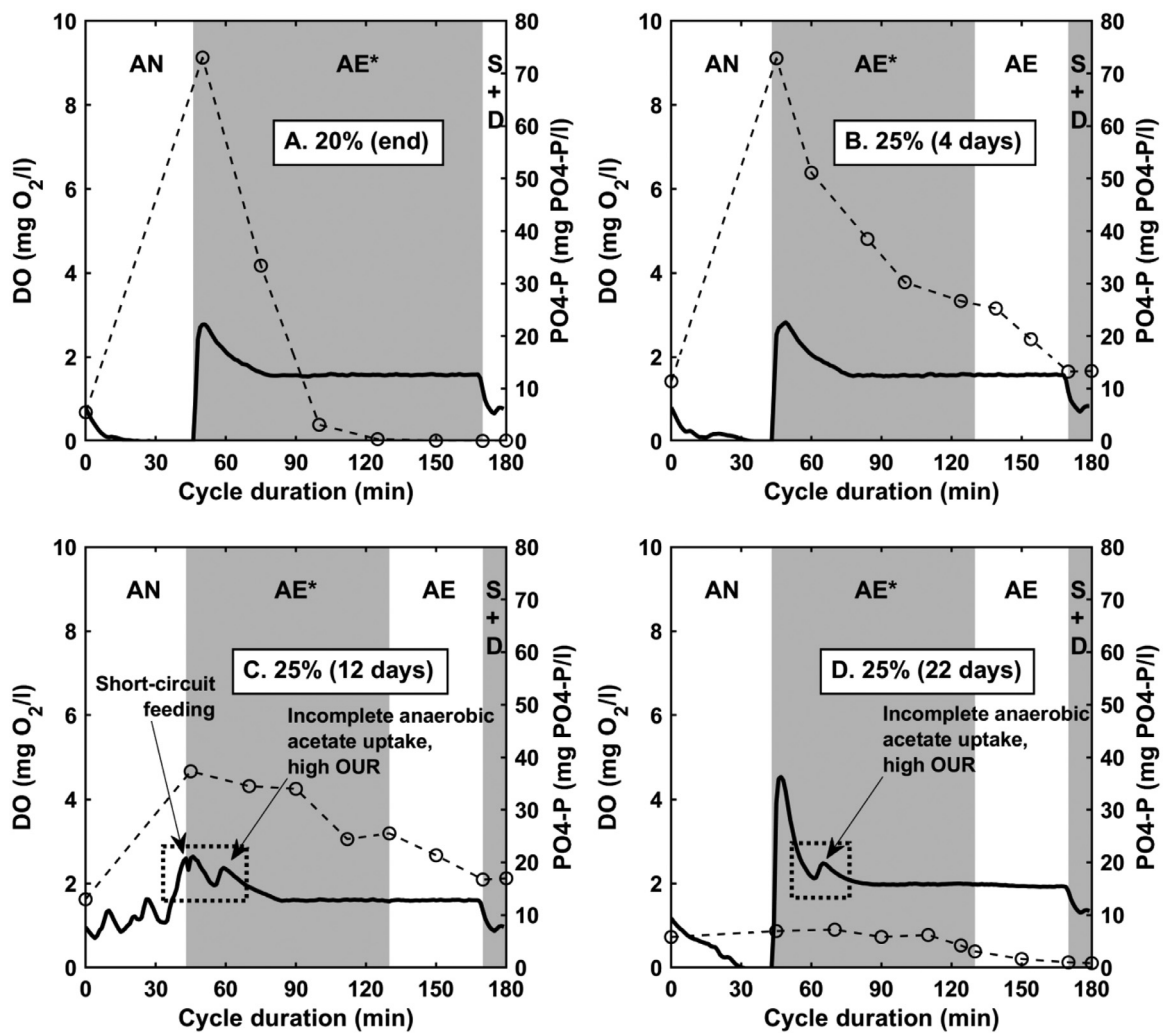


Fig. 5. Concentration profiles during a cycle for PO<sub>4</sub>-P (circles, dashed line) and dissolved oxygen (solid line) for respective cycles on the last day operating at 20% aerobic acetate load (A), after 4 days of 25% aerobic acetate load, (B) after 12 days (C) and after 22 days (D). Alternating shading denote phase changes within the cycle (AN = anaerobic phase, AE\* = aerobic phase with acetate dosage, AE = aerobic phase without acetate dosage, S + D = settling and discharge).

aerobically compete with aerobic heterotrophic organisms for acetate uptake and oxygen. This ability of PAOs has been previously described for flocculent sludge (Kuba et al., 1994; Pijuan et al., 2005) and shown to aid in controlling the SVI in flocculent sludge (Martins et al., 2004a). Although a flocculent sludge fraction with filamentous bacteria formed when acetate was partially fed aerobically, the fraction in the reactor remained negligible due to selective wasting based on settling speed. Additionally, bottom-feeding favoured PAOs growing in well-settling granules and contributed maximally to the ability to aerobically compete for acetate. This work underlines that the selection principles that result in AGS at the same time contribute to mitigation of the adverse effects of an aerobic rbCOD load on sludge morphology.

#### 4.1. Theoretical aspects of sludge morphology

Selection for anaerobic rbCOD conversion to storage polymers results in well-settling flocculent sludge (Martins et al., 2004a). These slow-growing organisms generally form dense biofilms (De Kreuk and Van Loosdrecht, 2004; van Loosdrecht et al., 1995) and transport-limitation of the electron acceptor in a subsequent stage does not affect the growth morphology. It was hypothesised that the ability of these slow-growing organisms to aerobically convert rbCOD also reduces the fraction consumed for growth of

filamentous bacteria when part of the rbCOD load was available under aerobic conditions (Martins et al., 2004a). Similarly, the majority of the growth of filamentous bacteria was found in suspension in this study (stage II, Fig. 3B), while filamentous outgrowth on granules remained limited (stage II, Fig. 3A). At least up to 20% aerobic acetate load could be sustained without adverse effect on settleability of flocculent sludge enriched for EBPR (Martins et al., 2004a), but the upper limit was not reported. At least the same extent of stability was observed for AGS in this study. Mainly anaerobic acetate uptake capacity thus ensures the aerobic competition for substrate uptake with ordinary heterotrophs.

The morphological fate of aerobically consumed acetate by ordinary heterotrophs, observed in this study, was in line with Martins et al. (2003b, 2003a), who formulated the hypothesis that transport-limited aerobic uptake of substrates drives the formation of poor-settling sludge. Transport-limited uptake rates of either rbCOD or oxygen due to low bulk concentrations favours one-dimensional growth in the direction of the concentration gradient. The specific morphology that causes the poor settleability depends on the type aerobic presence of rbCOD, which can be divided into two categories:

- negligible bulk rbCOD concentrations during a large part of the aerated phase (e.g., from hydrolysis of slowly biodegrad-

able COD or continuous supply or rbCOD to the aeration zone in continuous flow reactors from incomplete anaerobic uptake), or

- high bulk rbCOD concentrations during a short pulse in the first part of the aerated phase (e.g. from incomplete anaerobic uptake in SBRs)

For the first category, mainly suspended filamentous bacteria were found in this study, aerobically growing on the low concentration of acetate from active feeding during the majority of the aerated time (stage II, Fig. 3B). This is similar as previously observed for acetate fed CSTRs, where the low concentration of acetate also resulted in growth of mainly filamentous bacteria (van Niekirk et al., 1987).

The second category was observed when the significant decrease in the anaerobic acetate uptake after loss of EBPR (Section 4.3) caused a pulse of acetate at the start of aeration. During this initial pulse of acetate, the transport-limitation switched to oxygen. This led to overgrowth of the filamentous bacteria in suspension and attached to granular surfaces. The finger-like flocs that formed in this study resembled observations from work on flocculation by Crabtree et al. (1966) and selects for aerobic storage of acetate rather than direct growth. The similarly shaped flocs and finger-like films that formed on granular surfaces closely resembled those from work performed on granular sludge with completely aerobic pulse feeding. There, the relatively low DO (all between 2 and 4 mg O<sub>2</sub>/l) led to transport-limitation and high shear stress was required to erode this outgrowth and achieve well-settling granular sludge (Beun et al., 1999; Morgenroth et al., 1997; Mosquera-Corral et al., 2005).

These observations emphasize that aerobic transport-limitation in substrate uptake drives irregular growth in activated sludge as well as granular systems, as was shown in a recent modelling study by Wu et al. (2020) as well. Combined with the level of shear and mixing, the bulk concentration and duration of aerobic presence of rbCOD thus determine the extent of the transport-limitation and overall effect of sludge morphology.

#### 4.2. AGS reactor operation and morphological effect of rbCOD

Minor filamentous outgrowth on granular surfaces was observed at 20% aerobic acetate load, supporting a good resilience towards acetate presence (stage II, Fig. 3A). Consequently, the majority of the aerobic growth on acetate formed suspended filamentous bacteria (stage II, Fig. 3B). The large difference in settling speed of both fractions allowed for efficient wasting of most filamentous bacteria and prevented an increase in SVI. Furthermore, anaerobic bottom-feeding favours PHA storage in the granular sludge fraction, in this study leading to a significant PAO-fraction in the granules (Layer et al., 2019). The PAO-fraction therefore competed substantially for aerobically available acetate with filamentous bacteria. The selection for anaerobic PHA formation and selective wasting contribute to maintaining a smooth granular morphology and good settleability. The effect of aerobic conversion of rbCOD on granular sludge morphology previously enriched for anaerobic storage has been generalized in Fig. 6A.

In stage III, the loss of significant anaerobic storage leads to mainly aerobic conversion of rbCOD. As was observed in this study, the aerobic competition for rbCOD will be more in favour of flocculent growth or filamentous bacteria (Fig. 6B). Since this becomes the main growth morphology, selective wasting is insufficient to maintain stable granulation and eventually results in loss of granulation.

When granular sludge morphology has deteriorated or is not well established, it can strongly hinder the start-up or recovery of granular sludge morphology. In line with this reasoning, the SVI<sub>10</sub>

did not improve in this study until after the active aerobic feeding was stopped and complete anaerobic acetate feeding was applied. In stage IV, the SVI<sub>10</sub> recovered substantially within one SRT with the formation of new granules and increase in MLVSS. The recovery of the sludge volume took approximately 2–3 SRTs. In lab-scale reactors the waste sludge withdrawal is less optimised for selection compared to full-scale plants, which could result in different time scales for recovery. On the other hand, the less favourable substrate composition of sewage (i.e., not only rbCOD) might result in slower recovery of the sludge volume.

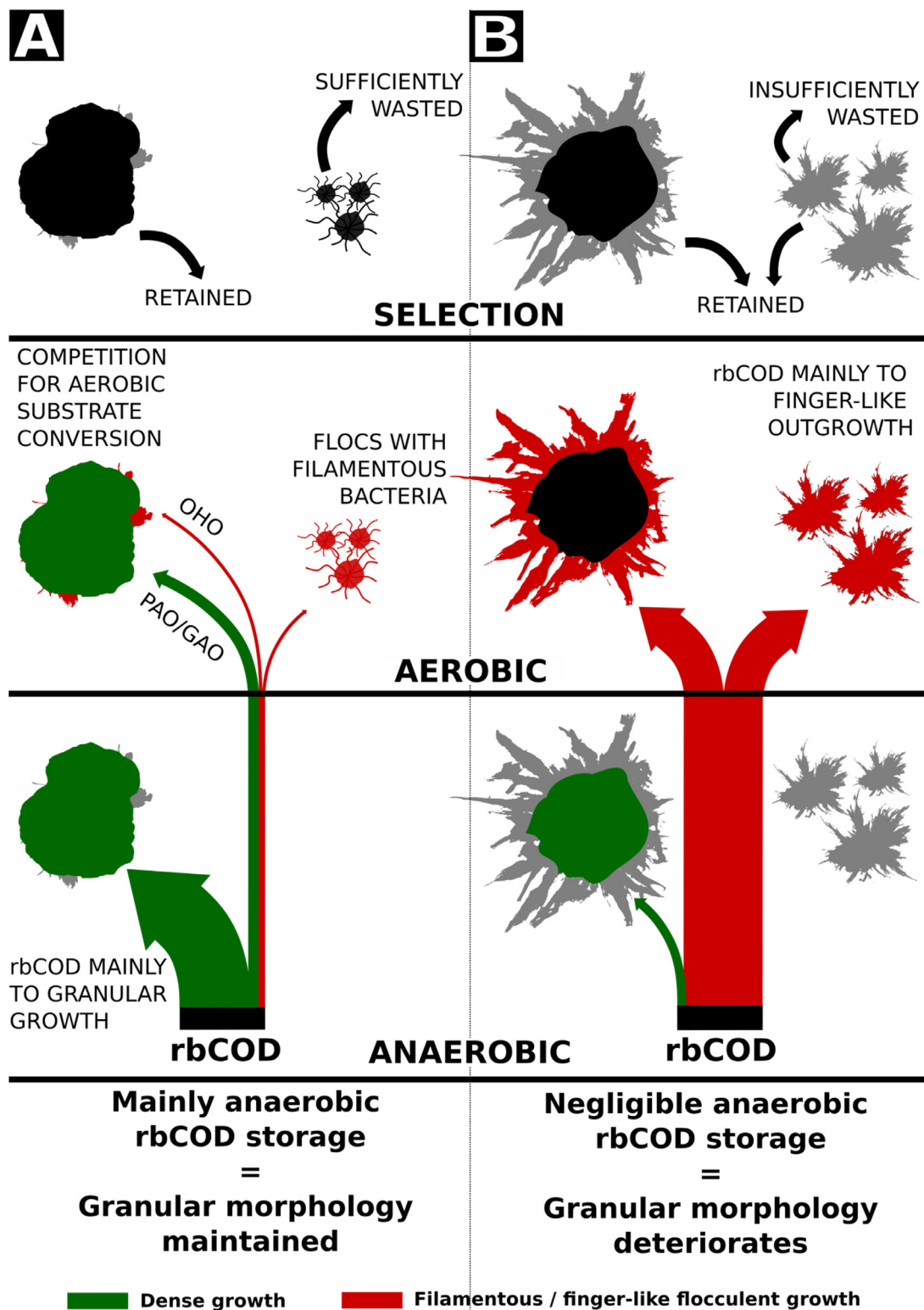
#### 4.3. Limits of morphological stabilization of AGS by anaerobic PHA storage

The experimental results showed that phosphate uptake was negatively influenced each time by an increase in the aerobic feeding rate of acetate. Likely, the decreased phosphate uptake rate was the result of the PAOs modulating their metabolism towards storage in the presence of an electron acceptor (Kuba et al., 1994). The simultaneous aerobic acetate uptake seems to limit the phosphate uptake rate (Guisasola et al., 2004; Pijuan et al., 2005). The incomplete aerobic phosphate uptake initiated the cascaded collapse of biological phosphorus removal (Fig. 5) through the gradual decrease in polyphosphate storage pool (Fig. 4B). As reported also by Pijuan et al. (2006), the latter resulted in complete loss of P-removal. Good granular morphology due to anaerobic storage of rbCOD can thus be maintained if the combination of the aerobic rbCOD uptake rate and the aerobic load allows for sufficient phosphate uptake and glycogen synthesis. In principle, granular stability is not dependant on PAOs or EBPR (De Kreuk and Van Loosdrecht, 2004), and stable granular systems with glycogen accumulating organisms have been regularly reported (Meyer et al., 2003; Pronk et al., 2015b). The short circuit flows observed during anaerobic feeding shortly after loss of P-removal, likely minimized the effective anaerobic contact time and prevented continued selection for anaerobic conversion of acetate to PHA by glycogen accumulating organisms. Future studies should focus on the impact of rbCOD availability on the phosphate removal process and the risk of GAOs outcompeting PAOs to ensure good phosphate removal.

#### 4.4. Considerations for full-scale AGS processes

Aerobic presence of rbCOD has potential consequences for process stability in full-scale AGS reactors. The rate at which rbCOD becomes available in the bulk and the duration of availability compared to the total aerated time are the critical parameters to maintain stable granulation (Section 4.3). In practice, these are determined by the reactor design (i.e., a SBR or a continuous flow reactor) and the source of rbCOD (i.e., incomplete anaerobic uptake of rbCOD or aerobic hydrolysis of slowly biodegradable COD, see Section 4.1).

In case of incomplete anaerobic rbCOD uptake, it will result in a very dilute availability of rbCOD during the complete residence time of the mixed aeration zone in continuous flow reactors. As this study showed, dilute availability of acetate during most of the aerated time was detrimental to the anaerobic acetate uptake capacity and sludge morphology due to insufficient aerobic phosphate uptake (stage III). On the other hand, SBRs will have a higher residual rbCOD concentration at the start of the aeration phase for a relatively short time. Once active aerobic dosage of acetate was stopped in stage IV, acetate was only present during aeration for a short time (10–15 min of 110 min) since short circuit flows through the sludge bed still occurred during anaerobic feeding. Despite this aerobic acetate load, phosphate uptake and sludge morphology recovered (Fig. 3A, stage IV). Our results therefore indicate that SBRs



**Fig. 6.** Schematic representation of the morphological stabilization by anaerobic PHA storage in case of an additional aerobic rbCOD load (A) and after loss of anaerobic storage capacity (B). Colours of arrows corresponds morphological destination of rbCOD. The size of arrows indicate the relative amount of rbCOD of the load per cycle.

will be less prone to deterioration of granular morphology compared to continuous flow reactors. Thus, it is of vital importance for granular stability in continuous flow reactors to limit the rbCOD presence in the aeration zones.

Besides incomplete anaerobic uptake, aerobic availability of rbCOD will also arise from aerobic hydrolysis of slowly biodegrad-

able COD that is not converted anaerobically (De Kreuk et al., 2010; Layer et al., 2019; Pronk et al., 2015a). Particulate COD will either be consumed by protozoa or incorporated in the flocculent sludge fraction before being hydrolysed (Martins et al., 2011). The hydrolysis products will then be converted into more flocculent sludge. A good selective removal of the flocculent sludge fraction is there-



fore required for keeping a stable granular sludge bed. Note that a certain level of flocculent sludge is always present in AGS plants treating sewage and has been shown to aid in achieving good suspended solids effluent quality (van Dijk et al., 2018). The release rate of both sources of rbCOD are relatively slow and will always result in negligible residual concentrations and transport-limited uptake rates, but continuous flow reactors will still be more affected due to the intrinsic dilution in the mixed aeration zone compared to SBRs.

When granular sludge is well-established, the presence of a large PHA storing fraction (PAOs and GAOs) in the microbial community will stabilise the granular morphology by competing with more flocculent growth for rbCOD during aeration. A recent study by Ali et al. (2019) showed a three-fold higher level of enrichment of PAOs in the largest granular size fraction (>1.0 mm, 60% of biomass) than in the smaller granules and flocculent sludge (<1.0 mm, 40% of biomass) fraction in a full-scale Nereda®. The extra selection for PAO (and thus PHA storage) increases the capacity for aerobic rbCOD uptake in the largest granular sludge fraction. This limits the negative impact of aerobic presence of rbCOD on the granular morphology.

## 5. Conclusion

- Selection for anaerobic storage of rbCOD in AGS through EBPR limited the adverse effect of a partial aerobic rbCOD load on sludge morphology through aerobic competition for substrate uptake with ordinary heterotrophs ( $\leq 20\%$  aerobic acetate load at dosage rate of 4 mg COD/g VSS/h). Sufficient aerobic competition could be maintained while the combined negative effect of the aerobic rbCOD uptake rate and the aerobic rbCOD load on phosphate uptake (PAOs) still allowed for full P-removal.
- The reactor configuration with anaerobic bottom-feeding and selective wasting of formed slow-settling flocculent sludge further limited the negative effect of aerobic presence of rbCOD on the sludge morphology of AGS.
- Loss of anaerobic rbCOD uptake and aerobic competition deteriorated the granular sludge morphology due to increased aerobic growth of finger-like structures on the granules and as flocs in suspension ( $> 20\%$  aerobic acetate load at dosage rate of 8 mg COD/g VSS/h).
- Finger-like structures on the granular surfaces provoked non-uniform flow through the sludge bed during anaerobic feeding, resulting in extra acetate availability in the aerated phase and further deterioration of the granular sludge morphology.
- Recovery of the sludge morphology through the formation of new, smooth granules occurred only after active aerobic feeding of acetate was stopped. It is therefore important to ensure maximum anaerobic rbCOD removal and prevent transport-limited substrate uptake rates in the aeration phase during recovery or start-up.

In general, the sludge morphology of AGS systems with good anaerobic storage of rbCOD can be considered resilient to low concentrations of rbCOD under aerobic conditions. The selection principles that result in AGS formation also contribute to mitigation of the adverse effects of low aerobic rbCOD concentrations on settleability.

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## Declaration of Competing Interest

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

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## Supplementary materials

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