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Formation of aerobic granular sludge during the treatment of petrochemical wastewater

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Abstract

In this study, petrochemical wastewater from the port of Antwerp was used for the development of aerobic granular sludge. Two different reactor setups were used, (1) a completely aerated sequencing batch reactor (SBR_{ae}) with a feast/famine regime and (2) a sequencing batch reactor operated with an anaerobic feast/aerobic famine strategy (SBR_{an}). The seed sludge showed poor settling characteristics with a sludge volume index (SVI) of 285 mL.gMLSS⁻¹ and a median particle size by volume of 86.0 µm ± 1.9 µm. In both reactors, granulation was reached after 30 days with a SVI of 56 mL.gMLSS⁻¹ and median granule size of 264.7µm in SBR_{an} and a SVI of 71 mL.gMLSS⁻¹ and median granule size of 307.4µm in SBR_{ae}. The chemical oxygen demand (COD) and dissolved organic carbon (DOC) removal was similar in both reactors and above 95%. The anaerobic DOC uptake increased from 0.13% to 43.2% in 60 days in SBR_{an}.

Keywords: monoethyleneglycol; GAO; methanol; PAO; PCR; sequencing batch reactor

1 INTRODUCTION

Aerobic granular sludge (AGS) technology has been proposed as an innovative technique for the treatment of municipal and industrial wastewater (Henriet et al., 2016). It is a particular type of biofilm, defined as aggregates of microbial cells which do not coagulate (de Kreuk et al., 2007). Microbial cell-to-cell self-immobilization involving biological, physical and chemical phenomena leads to densification of the aggregates (Lee et al., 2010). Various advantages have been attributed to AGS compared to floc based activated sludge such as (i) good biomass retention which leads to higher biomass concentrations inside the reactor and the ability of higher substrate loading rates (Adav et al., 2008), (ii) lower energy requirements (absence of a sludge recycling pump) and a higher aeration efficiency if the operation is at increased height, (iii) lower investment and operational costs (de Bruin et al., 2004), (iv) the presence of aerobic and anoxic zones inside granules allowing simultaneous nitrification/denitrification (Dobbeleers et al., 2017), (v) impressive settling velocities (Adav et al., 2008), (vi) good endurance to shock loadings and high-strength wastewater (Liu and Tay, 2004). (vii) ability for biological phosphorus removal (Cassidy and Belia, 2005) and (ix) potential for resource recovery, for example as alginate-like exopolysaccharides (Lin et al., 2010).

The first descriptions regarding successful formation of aerobic granular sludge employed a completely aerobic feast/famine period (Morgenroth et al., 1997). Later, different researchers illustrated the benefits of an anaerobic feast/aerobic famine period to stimulate the growth of slow growing organisms, which improves granule formation and stability (Gao et al., 2011).

The development of slow growing organisms is influenced by the feeding strategy. As discussed in Pronk et al. (2015a), depending on the substrate, a slow or a fast fill, which can both be aerobic or anaerobic, can be applied. According to Pronk et al. (2015a), an anaerobic fill phase promotes substrate uptake and diffusion throughout the whole granule. If possible, storage polymers are formed and slow growing organism dominate the granule. This results in stable, dense and compact granules. When substrate is added with a pulsed (fast) aerobic feeding two different types of granules can be formed. If the substrate promotes a slow growth such as methanol, stable granules are formed. If the substrate promotes high growth rates, aerobic heterotrophic growth on the outside of the granules occurs which results in unstable granules (Pronk et al., 2015a).

The formation of AGS has been demonstrated successfully for a limited number of industrial wastewaters, such as: winery (López-Palau et al., 2009), pig manure (Figueroa et al., 2011 and Liu et al., 2017), brewery (Wang et al., 2007 and Corsino et al. 2017), abattoir (Cassidy and Belia, 2005), rubber (Rosman et al., 2013), soybean (Su and Yu, 2005), malting (Schwarzenbeck et al., 2004, 2001), dairy (Arrojo et al., 2004), pharmaceuticals (Amorim et al., 2014), textile (Muda et al., 2010), dyeing (Manavi et al., 2017), agro-based (Abdullah et al., 2013), palm oil mill (Gobi et al., 2011) and potato industry (Dobbeleers et al., 2017).

However, only two studies describe the treatment of petrochemical wastewater with aerobic granular sludge. Milia et al. (2016) discussed granulation based on petrochemical wastewater originating from an integrated gasification cycle which was characterised by high ammonia concentrations (up to 630 mg.NH₄-N L⁻¹). Granulation was successfully obtained but acetate was added to the waste stream as additional carbon source to obtain a volumetric organic loading rate of 3 gCOD.(L.d)⁻¹. Lowering the acetate concentration resulted in destabilization of the granules. Also Zhang et al. (2011) observed granulation during the treatment of petrochemical wastewater. The wastewater used originated from

an oil refining company. Granulation was first obtained using synthetic wastewater (glucose). Thereafter, petrochemical wastewater (average COD: $282 \pm 20 \text{ mgO}_2\cdot\text{L}^{-1}$) was added to the influent. Increasing this proportion to 100% led to poor granules. Stable granules were only obtained again when sodium propionate was added as a co-substrate in a COD ratio of 1:1.

This paper investigates the development of aerobic granules starting from floccular seed sludge (with poor settling characteristics) during the treatment of industrial petrochemical wastewater without addition of an external carbon source. The aim of the present study was to compare two different feast/famine strategies to obtain granulation: (i) a complete aerobic feast/famine regime and (ii) an anaerobic feast/aerobic famine strategy. The industrial wastewater was produced by a chemical company which is located in the harbour region of Antwerp (Belgium).

2 MATERIALS AND METHODS

2.1 Reactor Set-up

Two identical lab-scale activated sludge sequencing batch reactors (SBRs) were used. The reactors had an internal diameter of 240 mm and a height of 400 mm and were operated with an effective working volume of 14L (H/D = 1.29). Each reactor was equipped with a Heidolph® RZR 2020 mechanic mixer (Heidolph Instruments GmbH & Co. KG, Germany) to keep sludge in suspension. A Super Fish Koi Flow 60 (Aquadistria, China) air pump was connected to a ceramic disc air diffuser (Angel Aqua, Korea) with a diameter of 132 mm to supply the reactor with oxygen. An electric ER-PLUS® valve (Watts Water Technologies, The Netherlands) was used to withdraw the effluent. Every cycle, 10L of sludge was kept in the reactor. The sludge retention time (SRT) was kept at 20 days. Each reactor was equipped with an oxygen (LDO sensor - Hach, United States) and pH sensor (Jumo TecLine, Germany). Data logging of the sensors occurred with a Lange sc1000 (Hach, United States). Influent was added to the reactors via Watson-Marlow 300 OEM peristaltic pumps (Falmouth, Great-Britain). Furthermore, the reactors were automatically controlled with a programmable logic controller (PLC) type CPU 319-3PN/DP (Siemens, Germany) which was used in order to perform hardware actions such as pump and valve activities. LabView™ software from National Instruments (Austin – Texas, United States of America) was used as interface for process visualisation, control and data transfer to MS Excel (Washington, United States).

Aeration was regulated by an on/off air control. The dissolved oxygen (DO) concentration in the reactors varied between 1 and 4 mgO₂.L⁻¹ which made it possible to calculate the oxygen uptake rate (OUR).

2.2 Reactor Operation

The reactors were operated at room temperatures (19-22°C) for a total operation time of 60 days and were inoculated with seed sludge originating from the full scale treatment plant of the petrochemical company. Seed sludge was sampled in two 10 L plastic barrels and immediately inoculated in the reactor after transportation. The full-scale industrial installation is operated at a SRT of 10 days at 30 – 35°C. The sludge is aerated with pure oxygen and polyelectrolyte is used to improve the settling characteristics.

The lab scale reactors were operated with 2 different feeding strategies. The first reactor, SBR_{ae}, was operated under a fully aerobic regime. The second reactor, SBR_{an}, was operated with an anaerobic feast and aerobic famine regime. Reactors were pulse fed at the top, with a feeding rate of 1L.min⁻¹, and mixing was carried out during feeding. The SBR cycle consisted of a pre-aeration phase, a feeding step, a reaction step (aerobic or anaerobic/aerobic depending on the reactor), settling and effluent withdrawal (operation times are summarized in the supplementary material (1)). During reactor operation, the feeding volume, aerobic reaction time and settling time were adjusted in order to promote granulation. Initially, the reactors were fed with a feeding volume of 1L. In 3 days, the feeding volume was increased to 4L. As a result, the volume exchange ratio (VER) increased from 16.6% to 28.6%. The aerobic reaction time was gradually shortened from 22 to 10.7h in SBR_{ae}. The anaerobic time of SBR_{an} was increased from 60 to 90 minutes on day 45 to promote substrate uptake during this period. The aerobic reaction time in SBR_{an} was gradually shortened from 21 to 9.2h. The settling time in both reactors was gradually shortened from 60 to 10 minutes from day 0 until 22 to decrease the cycle time. The time was only shortened if the sludge settling rate was high enough, so no washout of the sludge occurred. Hence, the reactors were not subjected to hydraulic pressure. The total cycle time of both reactors was always equal.

2.3 Industrial Wastewater

Two different influents were collected from the buffer tank of the petrochemical company and used during this study. In between the sampling, there was a period of one month. The collected influents were named batch 1 and batch 2 respectively. The composition and pollution load of both influent batches was similar and in range with the long-term daily average composition of the influent from the petrochemical company (not shown). To avoid degradation of the organic compounds during the storage period, the wastewater was stored in a refrigerated tank (4-5°C).

The 3 most common compounds in the influent were monoethyleneglycol (MEG), methanol (MeOH) and ethanol (EtOH). The average MEG, MeOH and EtOH concentration accounted respectively for 30.1 %, 12.4 % and 1.8 % of the influent TOC (Figure 1 (a)).

The average chemical oxygen demand (COD) and dissolved organic carbon (DOC) of the influent were 2233 mgO₂.L⁻¹ and 2028 mg C.L⁻¹ respectively (Figure 1 (a)). Nitrogen and phosphorus were added to the influent to satisfy the nutrient requirement. Nitrogen was added as NH₄Cl in a COD/N ratio of 40. Phosphorus was added as K₂HPO₄ in a COD/P ratio of 300. Effluent measurements of NH₄-N and PO₄-P were conducted to verify the nutrient uptake.

2.4 Analytical Methods

Biomass concentrations measured as mixed liquor (volatile) suspended solids (ML(V)SS) and sludge volume index (SVI) were determined according to the Standard Methods (APHA, 1998). The sludge morphology was observed using a MOTIC BA310 microscope (Opti-service, Belgium) with an EF-N Plan 4x0.25 ocular. Floc/granule size of the activated sludge was measured with a Malvern Mastersizer 3000 (Malvern, United Kingdom) with the following settings: non-spherical, refractive index 1.52, absorption index 1.0, dispersant water, for both red and blue light sample measurement 30 seconds, 10 measurements, obscuration 8-12% and a stirrer speed of 600 RPM. All measurements were executed at the end of the aeration step and before the settling phase which means sludge was always in an endogenous state.

Commercial test tubes of Hanna Instruments (Temse, Belgium) were used to measure total COD and soluble sCOD, both medium range (HI 93754A-25) and low range (HI 93754B-25). DOC concentration was measured with a Sievers InnovOx Laboratory Total Organic Carbon Analyzer (General Electric

Company, Colorado, United States). NH₄-N was measured with the HI 93715-01 test kit and PO₄-P with the HI 93717-01 both from Hanna Instruments. Samples were filtered before being analysed (except for total COD) using 1.2µm glass microfiber filters from VWR international (Belgium, Leuven).

2.5 qPCR Procedure for Molecular Quantification

DNA extraction from activated sludge samples was carried out according to the NaTCA method (McIlroy et al., 2009). Extracted DNA concentrations were measured with a fluorometric quantification using a Qubit™ 3.0 Fluorometer (ThermoFisher Scientific, Waltham, Massachusetts). On those samples, Phosphate accumulating organisms (PAO), glycogen accumulating organisms (GAO) and total bacteria (table 1) were quantified through SYBR Green quantitative polymerase chain reaction (qPCR) assays. qPCR was performed on a CFX96 Touch™ (BioRad, Hercules, California, USA). During the analysis, standard curves were constructed with specific target gene plasmid DNA, which was diluted between 1.0 x 10³ and 1.0 x 10⁸ ng.µL⁻¹. Only standard curves with an R square higher than 0.99 were withheld. For each DNA sample, three technical replicates were measured and during sampling of the reactors also three biological replicates were taken so in total 9 measurements were done for every sampling point. Furthermore, all qPCR assays contained three no-template control reactions. For every sample, the average target copy numbers per genome (table 1), MLVSS and specific DNA concentration, were used to calculate the amount of target cells per gram biomass.

2.6 In-situ measurements

2.6.1. Anaerobic DOC uptake in SBR_{an}

In-situ measurements were conducted to measure the DOC uptake during the anaerobic period of SBR_{an}. Samples for DOC analysis were taken at the beginning of the anaerobic step, right after the feeding, and at the end of the anaerobic step before the start of the aeration. The DOC uptake was defined as follows:

$$\% \text{ anaerobic DOC uptake} = \frac{S_F - S_{e,a}}{S_F - S_e} \cdot 100 \quad (1)$$

Where S_F is the DOC concentration at the start of the anaerobic phase just after the feeding, $S_{e,a}$ is the DOC concentration at the end of the anaerobic phase and S_e is the effluent DOC concentration. All

concentrations were expressed as mg.L⁻¹. (An example of the anaerobic DOC uptake is shown in the supplementary material (2)).

2.6.2. In-situ PAO activity in SBR_{an} and SBR_{ae}

In both reactors, PAO activity was investigated by following up the phosphate concentration in the reactor during a whole cycle.

2.6.3. Feast/famine ratio in SBR_{ae}

As shown by López-Palau et al. (2012), both the DO concentration (OUR) and the DOC or COD removal profile can be used to determine the feast/famine ratio. In this study, the evolution of the feast/famine ratio was examined using OUR profiles of SBR_{ae}. The feast phase is characterized by high OUR values reflecting high microbial activities. Low OUR values correspond to the famine phase where sludge activities are low. (An example of the relation between the OUR and DOC concentration to obtain the feast/fame ratio in this study is shown in the supplementary material (3)).

2.6.4. In-situ DOC removal kinetics in SBR_{ae}

During reactor operation, filtered DOC samples were analysed to follow up the DOC removal in SBR_{ae}. Samples were taken right after the feeding phase, followed by sampling on regular time base. The multicomponent DOC removal kinetics were described by an exponential decreasing curve (Eckenfelder et al., 2008):

$$\frac{S}{S_0} = e^{\frac{-K.X.t}{S_0}} \quad (2)$$

where X is the biomass concentration (mgMLVSS.L⁻¹), t is time (d), K is a first order rate coefficient (d⁻¹). S₀ = start DOC concentration at t₀ (mg.L⁻¹) and S = DOC concentration at time t (mg.L⁻¹). This equation describes the removal of substrate (expressed as DOC) in a multicomponent mixture and was experimentally verified by Caluwé et al. (2017). K values were estimated via non-linear regression (equation 2) using GraphPad software. (An example of the correlation between the measured DOC removal and the model is shown in the supplementary material (4)).

2.7 Ex-situ measurements

As described earlier, the influent wastewater contained a significant amount of MEG, MeOH and EtOH (figure 1 (a)). The specific anaerobic substrate uptake rate for those three components was measured in ex-situ batch experiments with the sludge of SBR_{an} . Each component was individually added to endogenous sludge. DOC measurements were conducted to measure the anaerobic DOC removal of each individual component to determine the maximum substrate uptake. In addition, also phosphorus release was investigated to measure the activity of PAO with MeOH, MEG and EtOH as substrate. No additional phosphate was added during these experiments.

Also for SBR_{ae} an ex-situ experiment was carried out. Wastewater was pulse fed anaerobically to the sludge, taken from SBR_{ae} in the endogenous phase, in order to investigate the anaerobic phosphorus release and DOC removal.

3 RESULTS and DISCUSSION

3.1 Reactor operation

The MLSS concentration of the seed sludge was 3.2 g.L^{-1} and the SVI after 30 minutes settling (SVI_{30}) was $285 \text{ mL.gMLSS}^{-1}$. Those measurements clearly illustrate poor settling characteristics of the sludge. During the whole reactor operation, MLSS (results not shown) and MLVSS (figure 2 (a)) concentrations were measured frequently. The MLVSS concentration in both reactors was minimum 86% of the MLSS concentration during the whole experimental period and varied between 1.8 and 3.4 mg.L^{-1} . After the start-up of the reactors, a drop in the MLVSS concentration was observed from approximately 3.2 to $1.8 \text{ gMLVSS.L}^{-1}$ on day 30 in both reactors. This significant decrease can be explained by the food to microorganism ratio (F/M). In contrast to the industrial installation which is operated at $30 - 35^\circ\text{C}$ and F/M ratio of $0.45 \text{ kgCOD. (kgMLVSS.day)}^{-1}$, the lab-scale experiments were performed at an initial F/M ratio of $0.11 \text{ kgCOD. (kgMLVSS.day)}^{-1}$ (figure 2 (b)). The cycle time was shortened step-by-step (from 24h to 15h), which resulted in an increased F/M to $0.45 \text{ kgCOD. (kgMLVSS.day)}^{-1}$ at day 30. The organic loading rate (OLR) (figure 2 (c)) increased during the same period from 0.37 to $1.0 \text{ kgCOD. (m}^3\text{.d)}^{-1}$.

On day 31, a new influent batch of wastewater was fed to the reactors. Sludge growth was observed during this period from 1.9 to 3.4 gMLSS.L^{-1} in both reactors. The F/M ranged between 0.34 and 0.5

kgCOD.(kgMLVSS.day)⁻¹ during the last 30 days (Figure 2 (b)). The OLR ranged between 1.0 and 1.12 kgCOD.(m³.d)⁻¹ from cycle 30 to 55. During the last 5 cycles, OLR was equal to 1.35 kgCOD.(m³.d)⁻¹ (Figure 2 (c)).

3.1 Reactor performance

During the experimental period, differences in effluent quality are observed between influent batch 1 and 2. The results are shown in table 2. Lower effluent COD, sCOD and DOC values were observed for batch 1, although similar removal efficiencies for the two batches were measured. The removal efficiency of all the observed parameters was higher than 95%. A Similar effluent quality was obtained in both reactors. The maximum concentration measured for COD and sCOD was obtained in SBR_{ae} with influent batch 2 and were respectively 94.0 ± 44.2 and 66.4 ± 29.8 mgO₂.L⁻¹. Effluent COD and sCOD were well below the Flemish discharge limits of 125 mgO₂.L⁻¹ for petrochemical companies.

3.2 The aerobic feast/famine strategy in SBR_{ae}

3.2.1 Performance of the granulation

During the first 30 days, a significant improvement of the sludge settling was observed. Within 30 days the sludge reached a SVI₃₀ of 56 mL.gMLSS⁻¹ (Figure 3 (a)). The reactor reached a SVI₃₀/SVI₁₀ ratio equal to 1 at this stage. During the first 30 days, the median granule particle size by volume (DV₅₀) evolved from $86 \mu\text{m} \pm 1.86 \mu\text{m}$ to $307.4 \mu\text{m} \pm 29.8 \mu\text{m}$ (figure 3 (b)). Also the morphology of the sludge significantly improved, from an irregular sludge to a sludge with a clearly defined granular structure (as shown in the supplementary material (5)).

A small improvement of the settling characteristics was observed after switching to a new influent batch on day 31, with an average SVI₃₀ of 48.7 ± 8.1 mL.gMLSS.L⁻¹. Microscopic observation of the sludge showed an increase of the granule size. The median granule size increased during this period to a DV₅₀ of $471.0 \pm 15.4 \mu\text{m}$. 10 % of the granules (DV₉₀) were larger than $984 \pm 45.3 \mu\text{m}$.

3.2.2 DNA analysis of the PAO and GAO concentration and PAO activity in SBR_{ae}

On several occasions, DNA samples from activated sludge were extracted and analysed using PAO, GAO and universal probes. The results are shown in figure 4 (a). Analysis of those samples showed

the lowest amount of PAO and GAO in the seed sludge. PAO were more abundant than GAO and the concentration increased slowly during the experimental period. The highest concentration of PAO and GAO were measured at day 58, although these concentrations were still very low and less than 0.10 % compared with the concentrations obtained with the universal probes.

The activity of PAO in the reactor was investigated at the end of the experimental period (day 60). Results are shown in table 3. No phosphate release was seen during in-situ measurement. In an additional ex-situ experiment, wastewater of batch 2 was anaerobically added to the sludge of SBR_{ae}. A total P release of 4.4 mgP.L⁻¹ was measured. This suggests the presence of a small amount of PAO which was also confirmed with the qPCR analysis.

3.2.3 Evolution of the sludge kinetics in SBR_{ae}

The evolution of the DOC removal kinetics, described by equation 2, was examined at regular intervals during the entire study. As shown in the present research and also mentioned before by Grau et al. (1975) and Caluwé et al. (2016) this equation can be used to describe the kinetics of a multicomponent substrate removal by a mixed culture. The first order rate coefficient (K) is shown in figure 4 (b). Kinetics measured for the seed sludge showed a K value of $0.75 \pm 0.14 \text{ d}^{-1}$. This K values gradually increased to $2.2 \pm 0.52 \text{ d}^{-1}$ at the end of the experimental period. This corresponds to an increase of 2.9 times, which indicates a higher activity of granular sludge compared with the floccular seed sludge.

3.2.4 Evolution of the feast/famine ratio in SBR_{ae}

The feast/famine ratio was calculated based on the changes of the OUR values during the SBR cycle. For each cycle, the period with high respiration rates was compared with the period with the low respiration rates to calculate the feast/famine ratio. The results are shown in figure 4 (b). A ratio varying between 0.11 and 0.38 was observed. The feast/famine ratio plays a significant role in the stability of granules. Corsino et al. (2016) and López-Palau et al. (2012) stated that stable granules were formed with a feast/famine ratio of 0.5 or less. However, this ratio was lower in the present research which means that the sludge was subjected to a starvation phase that was sufficiently long for stable granulation.

3.3 The anaerobic feast/aerobic famine strategy in SBR_{an}

3.3.1 Performance of the granulation

After start-up of the reactor, a fast and significant improvement of the sludge settling characteristics was observed. After 30 days, granulation was obtained in the reactor. At day 30, SVI was equal to 71 mL.gMLSS^{-1} with a SVI_{30}/SVI_{10} ratio of 1 (figure 5 (a)). During the same period, the DV_{50} evolved to $264.7 \mu\text{m} \pm 19.9 \mu\text{m}$, which is an increase of $187.7 \mu\text{m}$ compared with the seed sludge (figure 5 (b)). Also the morphology of the sludge improved significantly. The irregular structure of the seed sludge was replaced by a clearly defined granular structure in 30 days (as shown in the supplementary material (6)). After switching to a new influent batch on day 31, SVI improved further to a value of $52.8 \pm 10.1 \text{ mL.gMLSS.L}^{-1}$ and median granule size increased to a DV_{50} equal to $350 \pm 7.2 \mu\text{m}$ (figure 5 (b)).

3.3.2 Determination of the GAO and PAO concentration

The results of the DNA analysis are shown in figure 6 (a). An increasing concentration of PAO and GAO was observed during the experimental period. The concentration amounted to 0.21% and 0.002% at day 58 for PAO and GAO respectively.

3.3.3 Anaerobic substrate uptake in SBR_{an}

On day 1, an anaerobic DOC uptake of 0.13% was observed. After 9 and 31 days, the substrate uptake increased to 12.2 % and 17 % was observed. No difference in the substrate uptake occurred when the influent batch was changed (on day 32). In the subsequent 30 days, the anaerobic DOC uptake increased further to 43.2 %. Different ex-situ tests (table 4) were conducted to measure the anaerobic uptake of the most common carbon sources present in the influent, MeOH, MEG and EtOH. (figure 1 (a)). MeOH showed the lowest ability for anaerobic uptake. The uptake of EtOH was 3.3 to 4.7 times higher and the uptake of MEG 5.6 times. An increasing evolution was observed in the anaerobic substrate uptake for all components.

In the SBR, 70 mg C.L^{-1} influent carbon was taken up anaerobically. Based on the influent composition shown in figure 1 (a)), approximately 50 mg.L^{-1} of the added DOC originated from MEG. These results suggest that all MEG can be taken up during the anaerobic feast phase. Future work should be done to confirm this uptake of MEG.

342

343 **3.3.4 PAO activity**

344 The activity of PAO was investigated in-situ at the end of the experimental period (day 60). Results are
345 shown in table 3. A total P release of 11.4 mgP.L^{-1} was measured after adding the influent (shown in
346 the supplementary material (7)). A mol P release/mol C uptake ratio of 0.05 was observed. A total P-
347 release of $5.1 \text{ mg PO}_4\text{-P.gVSS}^{-1}$ was measured. This is significantly lower than the amounts presented
348 in enhanced biological phosphorus removal systems. For example Dobbeleers et al. (2017) reported
349 release values of $20.6 \pm 6.4 \text{ mg PO}_4\text{-P.gVSS}^{-1}$ during the treatment of potato wastewater.

350

351 MeOH, MEG and EtOH were all tested as a possible carbon source to induce PAO activity (table 3).
352 No phosphate release occurred when MeOH was added anaerobically. A low activity was seen with
353 EtOH and the highest activity (5.7 times higher than for EtOH) was observed with MEG. A total
354 concentration of 9.5 mg P.L^{-1} was released with MEG. This corresponds to the anaerobic substrate
355 uptake of MEG which was also the most significant. The results suggest that MEG was the main
356 component that triggered the activity and presence of PAO. Grady et al. (2011) stated that 1.01 mol P
357 can be released for the uptake of 1 mol C. Comparing this P/C ratio with the ratio observed in the
358 reactor (0.05 mol/mol), suggests the presence of other carbon uptake mechanisms besides PAO
359 activity. According to the correlation between % PAO and the P/C ratio proposed by Oehmen et al.,
360 (2007), PAO concentrations below 5% are expected which corresponds to the qPCR results in figure
361 6 (a).

362

363 **3.4 Comparison of both strategies**

364 **3.4.1 Performance of the granulation**

365 After 30 days, criteria for granulation were fulfilled in both reactors, i.e. the size of the granules was
366 larger than 0.2 mm, the settling rate was higher than 10 m.h^{-1} and the $\text{SVI}_{30}/\text{SVI}_{10}$ ratio was equal to 1
367 without addition of coagulant or carrier material (de Kreuk et al., 2007). Earlier research by Liu and
368 Tay (2015) and Morgenroth et al. (1997) stressed the use of a short settling time to obtain granulation.
369 This is in contrast with this research in which hydraulic selection pressure was not an important
370 parameter to obtain granulation since almost no wash out of the sludge occurred. Effluent suspended
13

solids were measured as difference between sCOD and COD. The highest difference in SBR_{ae} was 66 $mgO_2.L^{-1}$, the smallest 4 $mgO_2.L^{-1}$. If those values are divided by 1.42 (1 gMLVSS corresponds with 1.42 $gO_2.L^{-1}$ (Eckenfelder et al., 2009)) 46.5 and 2.8 $mg VSS.L^{-1}$ were washed out respectively. In SBR_{an} a washout of minimum 4.2 $mgVSS.L^{-1}$ and maximum 52.1 $mgVSS.L^{-1}$ was observed.

The time to reach granulation in this study was about 30 days. In literature, a wide variation in the time needed to obtain granulation with industrial wastewater is reported. Cassidy and Belia (2005) obtained granulation in 4 days with abattoir wastewater, Arrojo et al. (2004) formed granules of dairy wastewater in 21 days, Dobbeleers et al. (2017) needed 64 days (potato wastewater), Abdullah et al. (2013) 17 days (agro-based wastewater), Rosman et al. (2013) 7 days (rubber wastewater), Manavi et al. (2017) 94 days (dyeing wastewater), Liu et al. (2017) 18 days (piggery wastewater), Wang et al. (2007) 63 days and Corsino et al., (2016) 7 days with brewery water, Liu et al. (2010) needed 400 days with a mixture of municipal and industrial wastewater (In a 40/60 ratio). To the best of our knowledge, the formation of granular sludge with 100% petrochemical wastewater is not reported in literature before.

3.4.2 Development of GAO and PAO during granulation

In both reactors, an increase in the concentration of PAO and GAO was observed, but it was more significant in SBR_{an} . In both reactors, PAO were more present than GAO. An explanation for the dominance of PAO over GAO can be found in the reactor pH which was equal to 7.8 as an average during the whole reactor operation. Fukushima et al. (2010) and Oehmen et al. (2005) showed that the optimum pH for the growth of PAO is between 7.5 and 8. At lower pH (6.5 – 7.0), GAO would be more dominant since they assimilate carbon sources faster under anaerobic conditions, compared to PAO (Oehmen et al., 2007).

The measured quantities of both organisms were very low compared with the numbers presented in other studies. Fukushima et al. (2010) quantified GAO and PAO using FISH during the treatment of synthetic wastewater with acetate as main carbon source. PAO and GAO concentrations ranged between 3 and 52% of the total population. Winkler et al. (2011) detected 0.9% - 24.5% PAO and 0.2% - 5.3% GAO during the treatment of domestic wastewater by aerobic granular sludge.

Dobbeleers et al., (2017) measured increasing PAO (*Accumulibacter*) and GAO concentrations during the treatment of potato wastewater with aerobic granular sludge. The concentration of PAO increased during 455 operation days from approximately 10^9 to 10^{12} cells/gMLVSS. The increase of GAO was less significant from approximately 10^8 to 10^9 cells/gMLVSS. However, extensive experimental data obtained with industrial wastewater are currently missing.

3.4.3 Aerobic feast/famine granulation in SBR_{ae}

SBR_{ae} showed good granulation although no anaerobic phase was present to stimulate the growth of slow growing organisms such as PAO and GAO. Pronk et al. (2015a) showed that stable and dense granules only can be formed during an aerobic mixed feeding if the substrate provides low growth rates (μ_{max}). Previous research by Val del Río et al. (2012) already showed the ability for the formation of granular sludge out of industrial wastewater (dairy, plant processing marine products, canning industry and pig manure) with an aerobic feast/famine regime. Mosquera-Corral et al. (2003) and Villaseñor et al. (2000) showed that methanol was degraded aerobically with low growth rates. Due to the slow growth rate of the MeOH degraders, stable granulation can be obtained even without an anaerobic feeding step. Guo et al. (2014) investigated the effect of aerobic and anaerobic pulse feeding on the settling characteristics of activated sludge during the treatment of methanol. It was shown that both strategies led to the formation of good settling sludge with a SVI around 80 mL.gMLSS⁻¹.

3.4.4 Anaerobic feast/famine granulation in SBR_{an}

The ability of bacteria to store organic substrates anaerobically plays an important role in the formation and stability of AGS (De Kreuk et al., 2005). However not every substrate can be utilized anaerobically. The most important organisms for anaerobic substrate uptake are PAO and GAO (Pronk et al., 2015a). As reported by Veltman et al. (1998), anaerobic decomposition of MEG (main component of the influent) leads to the formation of acetate and ethanol. PAO and GAO are only able to take up volatile fatty acids (VFA) so uptake of acetate is possible. The production of short-chain carbon compounds out of MEG can explain the increasing presence of PAO and GAO and the removal of substrate and the PAO activity in the reactor during the anaerobic feast phase. The concentrations of PAO and GAO however were too low to explain the high levels of substrate uptake

observed. Additional research has to be done to investigate which organisms and mechanisms were responsible for the substrate uptake.

With respect to methanol, special attention is needed when it is used as substrate since conversion to methane and carbon dioxide can occur if an anaerobic feeding period is induced to the reactor. Pronk et al. (2015b) discussed the importance of the sludge retention time in the methanogenic pathway (formation of methane). Lowering the anaerobic SRT from 17 to 8 days enabled selective removal of the methanogens and prevented undesired methane formation. In absence of methanogens, methanol was converted aerobically, while granule formation remained stable. In this study, the anaerobic SRT was between 1 and 2.5 days. This should be short enough to avoid formation of methane based on the sludge age, although this was not measured. Future work can be done to confirm this.

4 CONCLUSION

The formation of aerobic granular sludge was possible with industrial petrochemical wastewater. Two feeding strategies were tested, an aerobic feast/famine and an anaerobic famine/aerobic feast for the formation of granules from raw wastewater. Granulation was already obtained after 30 days in both reactors without hydraulic selection pressure but granules were smaller in SBR_{an}. The settling characteristics were comparable in both reactors. Both reactors reached the Flemish effluent discharge limits for COD (125 mgO₂·L⁻¹) during the whole experimental time with a COD removal efficiency of at least 95,8 ± 0.9 % and a maximum effluent COD of 94.0 ± 44.2 mgO₂·L⁻¹.

5 SUPPLEMENTARY MATERIAL

Supplementary material can be found in the electronic annex on the website of the journal.

6 ACKNOWLEDGMENTS

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- 581
- 582

583 **List of tables**

Target	Cell copy number	Primers	Conditions	Reference
16S rRNA <i>Candidatus</i> <i>Accumolibacter phosphatis</i>	2	PAO 651f PAO 846r	98°C - 3' [98°C - 15" / 57°C - 15" / 72°C - 15"] x 40	(Fukushima et al., 2007)
16S rRNA <i>Candidatus</i> <i>Competibacter phosphatis</i>	5.66	GAO Q989f GAM 1278r	98°C - 3' [98°C - 15" / 59°C - 15" / 72°C - 30"] x 40	(Crocetti R. et al., 2002)
16S rRNA Universal bacteria	4.12	Universal 1055f Universal 1392r	98°C - 3' [98°C - 15" / 53°C - 30" / 72°C - 30"] x 40	(Ferris et al., 1996)

584
585 Table 1: Target, cell copy number, primers, qPCR conditions and references used during DNA
586 analysis

Reactor	Influent batch	Effluent			Removal efficiency		
		COD	sCOD	DOC	COD (%)	sCOD (%)	DOC (%)
		(mgO ₂ .L ⁻¹)	(mgO ₂ .L ⁻¹)	(mg.L ⁻¹)			
SBR _{ae}	1	73.4 ± 23.5	52.2 ± 20.0	18.7 ± 4.9	96.5 ± 1.1	97.1 ± 1.1	96.2 ± 1
	2	108.5 ± 34.7	75.0 ± 26.4	22.8 ± 4.8	95.5 ± 1.5	96.6 ± 1.2	95.6 ± 0.8
SBR _{an}	1	67.6 ± 18.7	53.0 ± 22.7	17.7 ± 4.0	94.8 ± 1.6	95.8 ± 1.4	95.3 ± 1.0
	2	89.5 ± 22.0	61.0 ± 23.2	24.2 ± 6.1	95.5 ± 1.5	96.6 ± 1.1	95.9 ± 0.9

587
588
589 Table 2: Summary of the average effluent quality and average removal efficiencies of both reactors
590 during the whole operational time for each influent batch.

591

592

P release (mg PO ₄ -P.gVSS ⁻¹)				
Sludge	during anaerobic feeding of:			
	MeOH	MEG	EtOH	Wastewater
SBR _{an}	0	2.9	1.5	5.1
SBR _{ae} (in-situ)				0
SBR _{ae} (ex-situ)				2.13

593

594 Table 3: PAO activity results tested for both reactors at cycle 60.

595

Day	MeOH	MEG	EtOH	Wastewater
	uptake	uptake	uptake	mg C.gVSS ⁻¹
	mg C _{MeOH} .gVSS ⁻¹	mg C _{MEG} .gVSS ⁻¹	mg C _{EtOH} .gVSS ⁻¹	
10	1.18	6.59	3.89	6.76
60	2.38	13.35	11.13	20.23

596

597 Table 4: Maximum anaerobic uptake of MeOH, MEG and EtOH for sludge of SBR_{an}. Duration of the
598 experiment at day 10 was 60min, at day 60 total anaerobic time was 90min.

599

600

List of figures

Figure 1: (a) Concentration of the 3 most common chemicals in the 2 influent batches expressed as the % carbon compared to the total organic carbon concentration of the influent. (b) COD, sCOD and DOC of the 2 influent batches.

Figure 2: (a) Evolution of the MLVSS in both reactors (b) food to mass ratio and (c) organic loading rate, in both reactors. Vertical dotted line represents the switch to a new influent batch.

Figure 3: Evolution of (a) the SVI_{10} and SVI_{30} and (b) the granule size of SBR_{ae} (Vertical dotted line represents the switch to a new influent batch).

Figure 4: (a) PAO and GAO concentration in SBR_{ae} and (b) feast/famine ratio and first order rate coefficient in SBR_{ae} . Vertical dotted line represents the switch to a new influent batch.

Figure 5: Evolution of (a) the SVI_{10} and SVI_{30} and (b) the granule size of SBR_{an} (Vertical dotted line represents the switch to a new influent batch).

Figure 6: (a) PAO and GAO concentration in SBR_{an} and (b) DOC uptake during the anaerobic feast phase of SBR_{an}. Vertical dotted line represents the switch to a new influent batch.

626 **List of supplementary data**

SBR cycle – step (h)	SBR _{ae}	SBR _{an}	(h)
Pre-aeration		1	
Feeding	0.017 – 0.067 (depending on the volume)		
Anaerobic reaction	/	Day 0 – 45: 1	
		Day 46 – 60: 1.5	
		Day 0 – 7: 22	
Aerobic reaction	Day 8 - 21: 17.7		Day 0 – 7: 21
	Day 22 – 56: 13.7		Day 8 – 21: 16.7
			Day 22 – 44: 12.7
	Day 57 – 60: 10.7		Day 45 – 56: 12.2
Settling time	Day 0 – 13: 1		Day 57 – 60: 9.2
	Day 14 – 21: 0.5		
	Day 22 - 60: 0.17		
Effluent discharge	0.033		

627 Supplementary material 1: Operating conditions of the two SBRs

628

629

630 Supplementary material 2: Typical DOC concentration progress in SBR_{an} (day 38). Time of the
631 anaerobic feast phase was equal to 60 minutes.

632

633 Supplementary material 3: Relation between the OUR and DOC concentration to obtain the

634 feast/famine ratio in SBR_{ae} (day 40).

635

636 Supplementary material 4: Example of the correlation between the experimental measured DOC

637 removal expressed as S/S_0 and the fitted model

638

639

Supplementary material 5: Evolution of the microscopic structure of SBR_{ae} (black scale bar represents 1000 μm and a number in each picture indicates the day of sampling)

Supplementary material 6: Evolution of the microscopic structure of SBR_{an} (black scale bar represents 1000 μm and a number in each picture indicates the day of sampling)

Supplementary material 7: Online phosphate release and uptake with the DOC removal in SBR_{an} during cycle 60. The end of the anaerobic phase is indicated with a vertical dotted line at 90 min.