Formation of aerobic granular sludge and the influence of the pH on sludge characteristics in a SBR fed with brewery/bottling plant wastewater

Hannah Stes, Sven Aerts, Michel Caluwé, Thomas Dobbeleers, Sander Wuyts, Filip Kiekens, Jolien D'aes, Piet De Langhe and Jan Dries

ABSTRACT

A laboratory-scale sequencing batch reactor (SBR) was operated for 450 days to assess aerobic granule formation when treating brewery/bottling plant wastewater by consistent application of a feast/famine regime. The experiment was divided into three major periods according to the different operational conditions: (I) no pH control and strong fluctuations in organic loading rate (OLR) (1.18 ± 0.25 kgCOD·$(m^3 \cdot day)^{-1}$), (II) pH control and aeration control strategy to reduce OLR fluctuations (1.45 ± 0.65 kgCOD·$(m^3 \cdot day)^{-1}$) and (III) no pH control and stable OLR (1.42 ± 0.18 kgCOD·$(m^3 \cdot day)^{-1}$). Aerobic granule formation was successful after 80 days and maintained during the subsequent 380 days. The aerobic granular sludge was characterized by SVI_5 and SVI_{30} values below 60 mL.g^{-1} and dominated by granular, dense structures. An oxygen uptake rate based aeration control strategy insured endogenous respiration at the end of the aerobic phase, resulting in stable SBR operation when the influent composition fluctuated. The quantitative polymerase chain reaction results show no significant enrichment of *Accumulibacter* or *Competibacter* during the granulation process. The 16S rRNA sequencing results indicate enrichment of other, possibly important species during aerobic granule formation while treating brewery wastewaters.

Key words | glycogen accumulating organisms (GAO), industrial wastewater, oxygen uptake rate (OUR), pH control, phosphate accumulating organisms (PAO)

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INTRODUCTION

The brewing sector is considered a major contributor to the European economy providing over 2 million jobs and producing approximately 40 million m^3 of beer per year (The Brewers of Europe 2016). Awareness and interest in the development of sustainable water and wastewater management, improving environmental performance and reducing water and energy usage is increasing within the sector. Globally, great effort is put into decreasing water usage for beer production and reducing the carbon footprint by sourcing renewable energy (Modic *et al.* 2015). Research into sustainable wastewater treatment systems contributes to decreasing the environmental impact of the brewing industry and its waste and wastewater management.

Generally, brewery wastewater has a high organic matter content due to the presence of starch, sugar, volatile fatty acids (VFA), etc. which are typically easily

doi: 10.2166/wst.2018.132

biodegradable compounds (Driessen & Vereijken 2003). For this type of wastewater, both biological anaerobic and aerobic wastewater treatment plants (WWTP) are used globally. The main advantages of anaerobic systems are the energy production (biogas) and the low sludge production and space requirements. To insure a higher degree of effluent quality an aerobic treatment is required. Both conventional activated sludge systems (CAS) and sequencing batch reactors (SBR) can be used for aerobic wastewater treatment (Driessen & Vereijken 2003; Wang et al. 2007). To date, the aerobic granular sludge (AGS) technology in a SBR is accepted as a sustainable alternative to the CAS system for treatment of municipal wastewater and is already applied in many full-scale applications (Niermans et al. 2014; Pronk et al. 2015a). The technology provides strong improvements in settleability of the activated sludge (SVI_5–$SVI_{30} < 100$ mL·g^{-1}) due to the presence of large, dense granular structures. The excellent settling characteristics of aerobic granules implicate the possibility for higher biomass concentration resulting in the design of more compact bioreactors. In addition, full-scale references show more energy-efficient SBR operation and nutrient removal capacities (Niermans et al. 2014; Pronk et al. 2015a). More recently, laboratory-scale studies showed successful aerobic granulation while treating a variety of industrial wastewaters, some examples being: petrochemical (Caluwé et al. 2017), malting (Schwarzenbeck et al. 2004), potato industry (Dobbeleers et al. 2017) and brewery (Wang et al. 2007; Corsino et al. 2017) suggesting great potential of the AGS technology for industrial applications. Stable aerobic granule formation is based on the enrichment of phosphate accumulating (PAO) and glycogen accumulating (GAO) organisms. The selection for these bacteria is conducted by applying a feast/famine regime consisting of an anaerobic feeding, during which conversion of VFA into intracellular storage polymers, i.e. poly-hydroxyalkanoates (PHA), occurs. The subsequent aerobic phase involves consumption of these polymers for microbial growth and maintenance. GAO obtain energy for anaerobic VFA uptake through intracellular glycogen degradation (Oehmen et al. 2007). The PAO metabolism typically shows anaerobic phosphate release as a result of intracellular poly-phosphate degradation, generating additional energy for VFA uptake. Intracellular phosphate accumulation (poly-P) takes place during aeration, resulting in an net phosphate removal. Previous research showed that pH has a direct influence on the PAO/GAO ratio, favouring PAO under alkaline conditions (pH > 7.2). This is explained by the fact that additional energy is required for VFA conversion when pH increases. Stored poly-P provides a secondary energy source favouring PAO growth over GAO at higher pH (Filipe et al. 2001). The influent P/COD ratio, as well as the temperature and carbon source are considered to be a less decisive factor regarding the competition between PAO and GAO. To our knowledge, research concerning the influence of the pH on the microbial composition has so far only been conducted using synthetic wastewater (Oehmen et al. 2007; Weissbrodt et al. 2013).

The objective of this study consists of (1) stable aerobic granule formation and long term maintenance while insuring good effluent quality and (2) investigation of the influence of the pH on the selection of GAO and PAO while treating a complex industrial wastewater, i.e. brewery/bottling plant wastewater.

MATERIAL AND METHODS

Reactor set-up

A fully automated laboratory-scale SBR with a working volume of 11 L, a diameter of 24 cm and a H/D ratio of 1.7 was operated at room temperature (18–22 °C) for 450 days. A mechanical stirrer (100 rpm) was used to keep sludge in suspension during mixed phases of the SBR operation. Process operation of the SBR was controlled by a Siemens PLC. Process settings and visualisation of the online measurements of the dissolved oxygen (DO) concentration, pH, oxidation reduction potential (ORP) and conductivity were controlled by LabView™ (National Instruments, Austin, TX, USA).

Industrial wastewater and seed sludge

Wastewater from a local brewery/bottling WWTP was regularly collected and instantly stored at 4 °C to minimize chemical oxygen demand (COD) degradation. To insure sufficient nutrient availability for microbial growth, NH_4Cl and K_2HPO_4 were added to the raw wastewater providing a COD:N:P ratio of 100:2:0.2. During the first 250 days of the experiment, strong fluctuations in wastewater composition were observed due to the absence of an equalization tank at the existing WWTP. Periodically, high strength brewery wastewater with a COD concentration up to 13,540 mg O_2·L^{-1} is added to the medium strength wastewater from the bottling plant with a COD concentration of 1,200 mg O_2·L^{-1}. On day 252, an equalization tank was taken into service at the full scale site resulting in a slightly more constant wastewater composition. Table 1 gives an overview of the

Table 1 | Wastewater composition before and after presence of the equalization tank – incl. NH_4Cl and K_2HPO_4 dosage

	Equalization tank absent						Equalization tank present					
	n	Min.	Max.	Av.	Stdev	CV (%)	*n*	Min.	Max.	Av.	Stdev	CV (%)
CODt (mg O_2·L^{-1})	37	2,195	13,540	6,846	2,461	35.94	20	1,690	6,495	4,037	1,476	36.55
CODs (mg O_2·L^{-1})	37	2,025	9,940	6,053	2,005	33.13	20	1,540	5,010	3,606	1,294	35.88
TOC (mg C·L^{-1})	26	365	2,908	1,580	562	35.6	13	282	1,444	806	432	53.6
NH_4^+-N (mg N·L^{-1})	37	5.74	198	103	45.8	44.3	22	4.00	148	76.7	49.2	64.1
PO_4^{3-}-P (mg P·L^{-1})	38	2.0	100	19	17	89	22	1.7	19	9.2	3.7	41

wastewater composition before and after the presence of the equalization tank.

Seed sludge from the same local brewery/bottling WWTP was used to inoculate the laboratory-scale SBR. The full-scale installation consists of a side-stream membrane bioreactor (MBR) where high sludge concentrations between 12–15 g·L^{-1} are used. Initial mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) concentrations were 14.0 and 12.6 g·L^{-1}, respectively. The morphology was characterised by a filamentous/flocculent structure with $DSVI_{30}$ of 285 mL·g^{-1} indicating very poor settling capacities. The full-scale installation is operated at a sludge retention time (SRT) of approximately 60 days and a highly variable food to mass ratio (F/M) varying from 0.01 to 0.30 kg COD·(kg MLSS·day)$^{-1}$.

Reactor operation

The laboratory-scale SBR was operated for approximately 450 days which were divided into three major periods due to significant changes in operational conditions. Table 2 gives an overview of the different experimental periods. During period I and III, the total cycle length of the SBR was fixed at 12 and 8 h, respectively, consisting of the following phases: an aerated middle phase (60–30 min), followed by a short non-mixed, non-aerated feed preparation period (10 min) insuring anaerobic conditions. Subsequently, a non-mixed anaerobic pulse feed (10 min) was imposed during which 1 L of brewery/bottling wastewater was fed on top of the SBR resulting in a constant volume exchange ratio (VER) of 9%. Thereafter, an extended mixed anaerobic phase was prolonged from 90 up to 120 min on day 25 to promote anaerobic COD storage. The duration of the subsequent aerobic phase was fixed at 500–545 and 225 min during periods I and III, respectively. Aeration times were only adjusted to insure a constant total cycle length due to shortening of the settling phase from 60 down to 15 min. To finalize, a withdrawal phase (5 min) is imposed for effluent discharge. During period II, all phases remained unchanged with the exception of (1) the settling phase which was reduced to 10 min and (2) the aeration phase during which an aeration control strategy was applied as described in the following paragraph. At the end of period II, i.e. from day 319 to 346, the reactor was mixed without feeding. This was in agreement with the period during which brewing and bottling activities were ceased and therefore no wastewater was produced. Due to

Table 2 | Overview of experimental periods

	Day	Period I 1–128	Period II 129–325	Period III 325–450
Full-scale WWTP	Equalization tank	Absent	Absent/present	Present
Laboratory-scale SBR	Aeration strategy	Fixed duration DO: 1–3 mgO_2·L^{-1}	OUR regulation DO: 1–3 mgO_2·L^{-1}	Fixed duration DO: 1–3 mgO_2·L^{-1}
	Total cycle time	12 h	5.3–15.9 h	8 h
	Aeration time	500–545 min	120–720 min	225 min
	Settling time	60–15 min	10 min	10 min
	pH control	No pH control	pH control at 7.0 ± 0.2	No pH control

During period I and II, SRT was kept constant at 30 days by removing a volume of 330 mL excess sludge every day. During period III no sludge was removed.

the lack of wastewater production, it was of importance to determine the resistance of the AGS to non-active periods.

Aeration control strategy and pH control

Online oxygen uptake rate (OUR) calculations were conducted during the aerobic phase. Aeration was provided using an automatic on-off regulation between two DO setpoints. When the maximum DO setpoint was reached, aeration stopped and oxygen levels dropped due to oxygen usage by the microorganisms, resulting in a decreasing linear DO versus time curve. From these data, the OUR is automatically calculated as the absolute value of the slope from the linear curve between the two DO set-points. Due to the combination of strong variations in organic load of the wastewater and a constant volume exchange ratio during period I, feeding of low strength wastewater resulted in prolonged famine conditions characterised by constant and low specific OUR (sOUR) measurements ($<$2mg O_2.(g MLVSS.h)$^{-1}$). By introducing an aeration control strategy during period II, sOUR values variated between 2–5 mgO_2·(gMLVSS·h)$^{-1}$ at the end of the aerobic phase so prolonged aeration was avoided. Aeration continued until the occurrence of an inflection point in the OUR curve indicating the degradation of all internal and external carbon. Therefore, a minimum OUR value was set at 20 mg O_2·(L·h)$^{-1}$, corresponding with sOUR values between 2–5 mg O_2·(g MLVSS·h)$^{-1}$. When reaching the aimed setpoint, the next cycle phase was initiated.

The pH in the full-scale installation treating the brewery/bottling wastewater typically varies between 8.0–8.5 which is considered to influence GAO growth negatively and therefore is expected to favour PAO growth when applying anaerobic feeding followed by an aerobic famine period (Filipe *et al.* 2001; Oehmen *et al.* 2007; Weissbrodt *et al.* 2013). The composition of the used brewery wastewater requires addition of phosphate to insure sufficient nutrient availability for microbial growth. Since PAO are known to accumulate excess phosphate intracellularly but only limited amounts are present in the wastewater, enrichment of PAO could be influenced negatively and therefore complicate enrichment of slow growing microorganisms. The influence of the pH on sludge characteristics is investigated by controlling pH at 7.0 ± 0.2 during period II. The correction was applied by the automatic addition of a 1 M HCl solution.

Analytical methods

Total and soluble chemical oxygen demand concentrations, i.e. CODt and CODs, were measured with test tubes (HI 93754A-25 and HI 93754B-25) from Hanna Instruments (Temse, Belgium). Also, NH_4^+-N (HI 93715-01), NO_2^--N (HI 93707-01), NO_3^--N (HI 93766-50) and PO_4^{3-}-P (HI 93717-01) concentrations were measured using test kits from Hanna Instruments. Sulphate concentration of the influent was measured using a LCK153 test kit from Hach (Mechelen, Belgium). Dissolved organic carbon (DOC) concentrations were measured using a Sievers InnovOx Laboratory Total Organic Carbon Analyzer (GE Analytical instruments). Before measurement of the DOC, CODs, NH_4^+-N, NO_2^--N, NO_3^--N and PO_4^{3-}-P concentration, samples were filtered using 1.2 μm glass microfibre filters (VWR International, Belgium). Effluent suspended solids (SS) concentrations were calculated by the ratio of the difference between effluent CODt and CODs concentrations to the conversion factor of 1.42 as presented by Eckenfelder *et al.* (2008).

All sludge samples were taken at the end of the aerobic phase to insure endogenous conditions for all analyses. The sludge volume (SV) and mixed liquor (volatile) suspended solids concentration (ML(V)SS) were determined according to the standard methods described by (APHA/AWWA/WEF 1998). Particle size distribution by volume, DV, measurements of the sludge mixture were performed using a Malvern Mastersizer 3000 (Malvern, UK). Settings: refractive index: 1.52, absorption index: 1.0, particle size: non-spherical, dispersant: water, both blue and red measurement duration: 30 s, number of measurements: 10, obscuration: 8–12%, stirrer speed: 600 rpm. To investigate evolution of the sludge morphology, microscopic analysis was performed using a MOTIC BA310 microscope (Opti-service, Belgium).

In-situ cycle measurement during SBR operation

In order to evaluate anaerobic DOC uptake and phosphate release, associated with the presence of GAO and PAO bacteria, in-situ cycle measurements were performed during multiple SBR cycles. Grab samples were taken every 30 min during the anaerobic phase and at the end of the SBR cycle in order to obtain DOC and phosphate profiles.

$$\textit{Anaerobic DOC removal}\ (\%) = 100 \times \frac{DOC_{t0} - DOC_{t1}}{DOC_{t0} - DOC_{te}}$$

With t_0 just after anaerobic feed, t_1 at the end of the anaerobic phase and t_e at the end of the SBR cycle. Grab samples were filtered using glass microfibre filters before analytical measurements were performed.

DNA extraction

To investigate the evolution in abundance of PAO and GAO and perform 16S rRNA gene sequencing analysis, sludge samples were taken in triplicate on different days during the experiment and preserved at −80 °C. Subsequently, DNA extractions were performed according to the method described by McIlroy *et al.* (2008). A Qubit 3.0 Fluorometer (ThermoFisher Scientific, Massachusetts, USA) was used for quantification of the resulting DNA concentrations.

Molecular quantification by quantitative polymerase chain reaction (qPCR)

Target genes were quantified as described in (Caluwé *et al.* 2017) by the use of PAO541f/PAO846r primers for PAO (16S rRNA *Candidatus* Accumulibacter phosphatis), GAOQ989f/GAM1278r primers for GAO (16S rRNA *Candidatus* Competibacter phosphatis) and Universal1055f/Universal1392r primer for the total amount of bacteria (16S rRNA Universal bacteria). From the measured DNA concentrations, the amount of target cells per g MLVSS was calculated.

Microbial community composition by 16S rRNA gene amplicon sequencing

The 16S rRNA gene amplicon sequencing analysis was performed on two sludge samples, i.e. seed sludge and a grab sample taken at the end of operational period II (day 302). Amplicons targeting the V4 region of the 16S rRNA gene were generated with barcoded primers (IDT), and Phusion High-Fidelity DNA polymerase (Thermo Scientific) as described by Kozich *et al.* (2013). PCR products were purified using the SequalPrep Normalization plate kit (Invitrogen), and pooled. The resulting library was further purified by gel extraction using NucleoSpin Gel and PCR Clean-up (Macherey Nagel), and diluted to obtain a 2 nM library. Amplicon sequencing was carried out on a Illumina Miseq system at the Centre for Medical Genetics (Edegem, Belgium) with the MiSeq Reagent Kit v2 (Illumina).

The obtained paired-end reads were processed with the UPARSE pipeline (Edgar 2013). As a reference database for taxonomy predictions of the OTY sequences, MiDAS (version 2.1) was used, which is a manually curated SILVA 16S rRNA taxonomy (release 1.23 Ref NR99) that proposes a name for all the abundant genus-level taxa present in activated sludge, anaerobic digesters and influent wastewater (McIlroy *et al.* 2015).

RESULTS AND DISCUSSION

Reactor performance

A constant applied volume exchange ratio (VER) of 9% and strong variations of the influent COD concentration resulted in distinct variations in organic loading rate (OLR) during period I. Low strength wastewater resulted in prolonged famine conditions causing possible unnecessary aeration. Since aeration is one of the main operational costs in full-scale installations, excess aeration should be avoided when assessing industrial applicability of the technology (Niermans *et al.* 2014). During period I, the average OLR was 1.18 ± 0.25 kg COD·$(m^3 \cdot day)^{-1}$ resulting in strong fluctuation of the F/M ratio between 0.11–0.36 kg COD·$(kg\ MLVSS \cdot day)^{-1}$. During period II, an aeration control strategy based on the OUR was introduced to reduce fluctuation of the OLR resulting in an average OLR of 1.45 ± 0.65 kg COD·$(m^3 \cdot day)^{-1}$. After the equalization tank was taken into service at the full-scale WWTP (day 252), a slightly more constant wastewater composition was observed so, again, fixed aeration times were applied during period III (Tables 1 and 2). As a result, OLR and F/M ratio varied between 1.1–1.9 kg COD·$(m^3 \cdot day)^{-1}$ and 0.13–0.25 kg COD·$(kg\ MLVSS \cdot day)^{-1}$, respectively. Similar OLR were applied by Corsino *et al.* (2017), resulting in stable granule formation. Figure 1 shows the evolution of the F/M applied during operation and the results of the total and soluble effluent COD concentration throughout the experiment.

Adjustment of the seed sludge to the new operation strategy and the distinct variations in OLR caused poor effluent quality with CODt concentrations up to 141 mg $O_2 \cdot L^{-1}$ during start-up. Thereafter, CODt and CODs concentrations differed strongly indicating wash-out of suspended solids up to 56 mg $SS \cdot L^{-1}$ on day 84. However, at the end of period I, good effluent quality was already achieved. From day 129, i.e. start of period II, an aeration control strategy was introduced to handle strong fluctuations of the influent COD concentration and, additionally, pH control was imposed. During the first 40 days of period II, the presence of suspended solids in the effluent decreased with an average of 8.1 ± 5.7 mg $SS \cdot L^{-1}$, far below the discharge limit of 60 mg $SS \cdot L^{-1}$, so thereafter only effluent CODs concentration was measured. After a possible adjustment period of 40 days, stable effluent quality well below the Flemish discharge limit (125 mg $O_2 \cdot L^{-1}$) was established. Low effluent COD concentrations were maintained throughout the remainder of the experiment even though strong variations

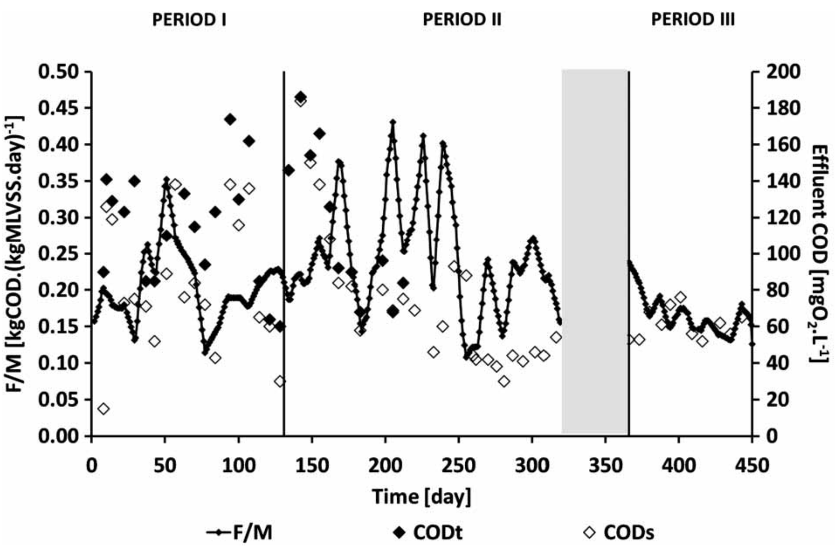

Figure 1 | Evolution of the F/M and effluent CODs concentration during the experiment (grey zone: 30 days mixed without feeding).

in applied F/M still occurred and pH control was switched off again during period III. Due to the lack of wastewater production, the resistance of the AGS to non-active periods was investigated. Therefore, a 30 day period of mixing without feeding was imposed as a strategy to preserve AGS. Results in Figure 1 show high effluent quality and therefore high COD removal efficiencies up to 99% immediately after reinitiating normal SBR operation at the start of period III. Therefore, mixing without feeding can be assumed as an appropriate strategy to preserve AGS during periods where no wastewater is produced. Throughout the experiment, the CODs removal efficiency varied between 95–99%. Results show improvement in effluent quality as granule formation evolved and large structures started to dominate the sludge mixture (see below). An OUR based aeration control strategy is proposed to result in proper famine conditions, i.e. sOUR values <5 mg $O_2 \cdot (\text{g MLVSS} \cdot \text{h})^{-1}$, at the end of the aeration period. Consequently, stable SBR operation and effluent quality were obtained.

Sludge characteristics and morphology

By applying a feast/famine regime consisting of an anaerobic pulse feeding followed by prolonged anaerobic and aerobic phase, sludge characteristics changed significantly during the experiment (Figure 2). Figure 3 shows the evolution of the MLSS, the median particle size by volume (DV_{50}) and the SVI after 5 and 30 min of settling.

The objective of this study was to stimulate aerobic granule formation while respecting the required effluent quality. Therefore, a long initial settling time (60 min) was imposed. Due to the extremely poor settling characteristics of the membrane bioreactor (MBR) seed sludge, i.e. $DSVI_{30} = 285$ $mL \cdot g^{-1}$, which was dominated by flocculent and filamentous sludge structures (Figure 3), biomass washout occurred during start-up resulting in a decrease of the MLSS concentration. The second drop of the MLSS concentration (day 38) was caused by removing half of the sludge volume for start-up of a second experiment. The MLVSS/MLSS ratio remained stable during the entire experiment at $92 \pm 3\%$. A rapid increase of DV_{50} is observed from 87.5 ± 9.4 μm up to 349 ± 7.6 μm during period I. Concurrently, strong improvement of the sludge settling characteristics were observed: SVI_5 and SVI_{30} decreased from 285 $mL \cdot gMLSS^{-1}$ down to 66 and 40 $mL \cdot gMLSS^{-1}$, respectively. These results correspond to the significant reduced presence of filamentous and flocculent sludge structures and the development to a more dense granule morphology (Figure 3). From day 102, decreased particle size and a temporary reduction of sludge settling capacities was observed. Nevertheless, results show successful aerobic granule formation after approximately 80 days of SBR operation. Similar results were observed by Wang *et al.* (2007) where mature granule formation also fed with brewery wastewater was observed after nine weeks of operation.

After introducing the pH control and the OUR based aeration control strategy (period II), the effluent quality and settling characteristics temporary worsened. The sudden lowering of the pH_{max} from 8.1 to 7.2 may have created an excessive change in environmental conditions. After

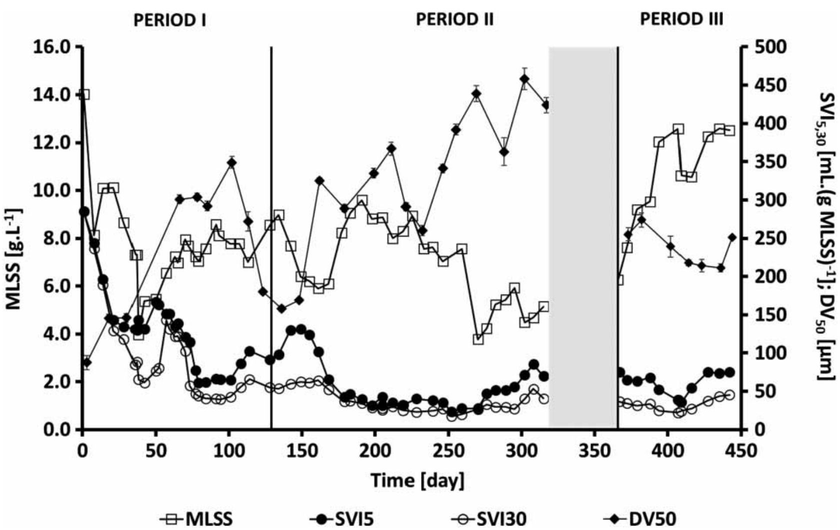

Figure 2 | Evolution of the MLSS, SVI_5 and SVI_{30} and DV50 during the experiment (grey zone: 30 days mixed period without feeding).

a 30 day adjustment period, stable SBR operation was recovered. Thereafter, significant increase of DV_{50} was observed from $180 \pm 3.8\,\mu m$ up to $424 \pm 10\,\mu m$ on day 317. In addition, large, dense granules dominated the biomass mixture (Figure 3), while settling capacities improved further to stable SVI_5 and SVI_{30} values of 35 and 30 $mL{\cdot}gMLSS^{-1}$, respectively. Subsequently, 30 days of mixing without feeding was applied. When SBR operation

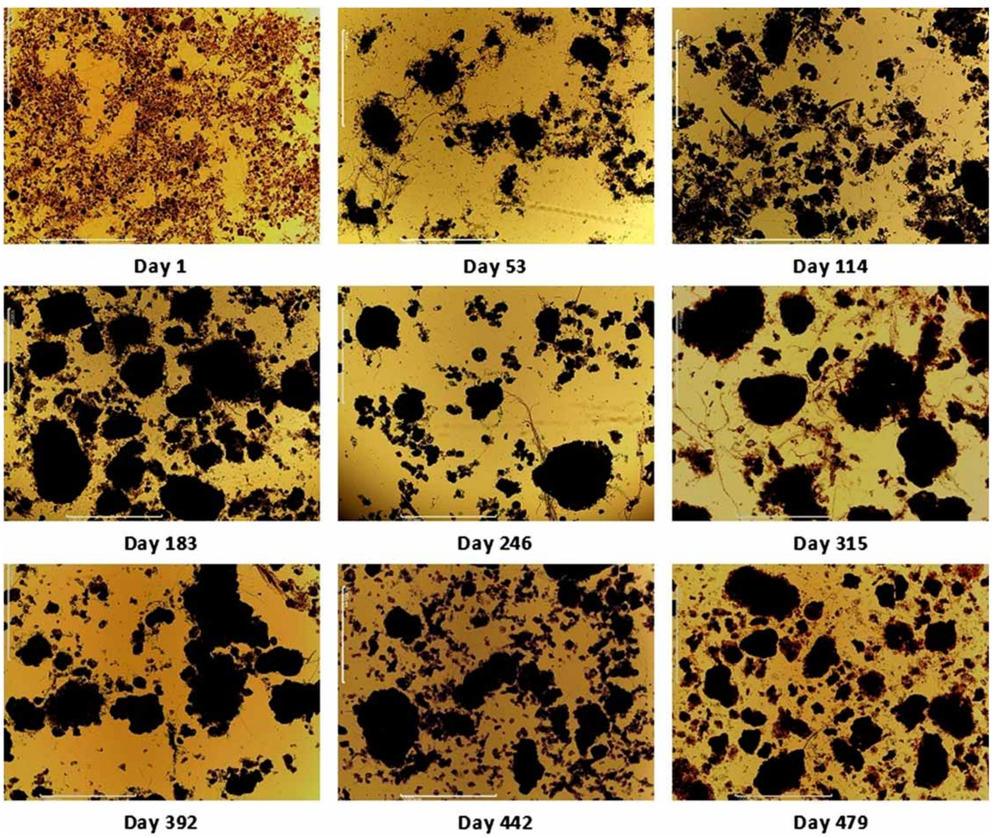

Figure 3 | Microscopic analysis of sludge morphology (calibration curve: 1,000 µm).

was reinitiated (period III), the median granule size was smaller which may indicate that this period had a slightly negative influence on granule stability. However, the median granule size remained stable between 211 and 255 μm during the subsequent 84 days indicating good resistance of the AGS to periods without feeding. Sludge settling characteristics were good during this period with a final SVI_{30} value of 46 mL·g $MLSS^{-1}$ while MLSS concentrations were as high as 12.5 g·L^{-1}. At this point, biomass consisted of a mixture of large and small granules.

Considering the overall duration of the experiment, our results indicate the good resistance to variable operational conditions to which the AGS was exposed, such as significant fluctuations in F/M ratio, the variations in pH (with and without control) and the 30 day period of mixing without feeding. These variable conditions had no negative influence on carbon removal efficiencies, settling characteristics and the overall sludge morphology. Only the sudden lowering of the pH at the start of period II seemed to negatively influence both the sludge settling capacities and effluent quality. Generally, all results indicate that aerobic granule formation was successful and long term stability was maintained under changing operational conditions during the treatment of brewery wastewater.

It should be mentioned that minor filamentous outgrowth was constantly present at the granule surface (Figure 3). This corresponds to previous findings on the use of more complex wastewaters containing large organic compounds (i.e. starch, polysaccharides, etc.) and particulate matter, which are also expected to be present in brewery wastewater. Slowly biodegradable compounds are found to adsorb on the granule surface where hydrolytic conversion take place. Hydrolytic products diffuse into the granule structure and subsequently, anaerobic conversion into storage polymers can take place. Depending on the anaerobic hydrolysis rate and duration of the anaerobic phase, aerobic hydrolysis may occur at the granule surface which is suggested to favour filamentous growth (de Kreuk *et al.* 2010; Pronk *et al.* 2015b). In contrast to our findings, no filaments and larger granules were observed by Wang *et al.* (2007) and Corsino *et al.* (2017) using brewery wastewater as substrate for granule formation. In both studies, influent was fed at the bottom of the reactor into the sludge bed to insure high substrate concentrations to stimulate anaerobic carbon uptake. In our study, it was decided to feed influent on top of the SBR since this feeding strategy is believed to be more easily applicable. Other possible reasons for these varying results may be found in the high initial hydraulic selection pressure applied by Wang *et al.* (2007) (18 min of settling) and Corsino *et al.* (2017) (4.5 min of settling). In our study, the initial hydraulic selection was low due to the 1 h settling time, so complete initial wash-out of the biomass was avoided. The difference in feeding strategy and/or hydraulic selection pressure may have had an impact on the overall sludge composition and morphology. It is, however, unclear which factor is more decisive. Since the wastewater composition was highly variable, the 2 h anaerobic phase may have periodically been insufficient to hydrolyse all carbon compounds, resulting in possible aerobic hydrolysis which may explain the minor but constant presence of filaments.

To summarize, without feeding at the bottom of the reactor and without significant hydraulic selection pressure, aerobic granule formation and long-term maintenance was successful. Consistent submission of a fixed anaerobic feast phase, followed by a dynamically regulated (OUR based) aerated famine phase, seems to be a promising strategy to maintain a stable AGS system using wastewater with a highly fluctuating composition.

Evolution of the anaerobic DOC removal

Multiple in-situ cycle measurements were performed during the SBR cycle in order to evaluate the anaerobic DOC removal.

The gradual increase of anaerobic DOC uptake during period I is shown in Figure 4. Initially, only 41 ± 3% of the DOC was removed from the bulk liquid during the 90 min anaerobic phase. At that point, sludge mixture was

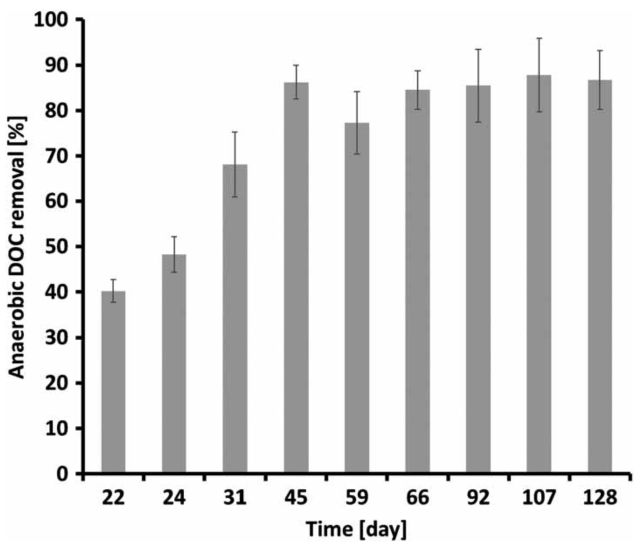

Figure 4 | Evolution of the anaerobic DOC uptake during period I of SBR operation.

dominated by flocculent and filamentous structures and settling characteristics were poor ($SVI_{5,30} > 100\ mL.g^{-1}$). To promote anaerobic DOC uptake, the duration of the anaerobic phase was increased up to 120 min. Consequently, anaerobic DOC removal rapidly increased up to 87 ± 7%, suggesting strong improvement in anaerobic conversion of biodegradable substrate into storage polymers. Additional analysis of the evolution of the internal PHA content is necessary to confirm these findings. During period II, anaerobic DOC removal increased further up to 94 ± 7% while sludge structures developed to mature granules. During period III, the percentage of anaerobic substrate removal from the bulk liquid remained stable with an average of 97 ± 2%. The high percentage of DOC removal during the anaerobic phase indicates that substrate was converted into storage polymers, resulting in a DOC removal from the bulk liquid, creating stable operational conditions favouring growth of slow growing microorganisms associated with aerobic granulation and additionally avoiding filamentous outgrowth.

Influence of pH on selection of GAO and PAO

The pH is suggested to be one of the main factors influencing PAO/GAO competition. Due to the presence of an additional energy source (poly-P) to convert VFA into intracellular storage polymers, PAO outcompete GAO when pH > 7.2 (Filipe *et al.* 2001). Similar results, showing direct selection of PAO under slightly alkaline conditions, were obtained by Weissbrodt *et al.* (2013). Caluwé *et al.* (2017) showed an increased PAO activity and abundance treating petrochemical wastewater with a COD/P ratio of 300 for the formation of AGS. In this study, the average pH in the reactor was 7.8 which is known to stimulate PAO growth. Remarkably, even though no excess phosphate was present in the wastewater, minor enrichment of PAO was observed. These results implicate that other factors than COD/P ratio, like pH, may play a more decisive role in the selection for PAO. When treating brewery wastewater, maximum pH values (pH_{max}) are typically obtained at the end of the aeration phase due to CO_2 stripping. No pH control was applied during period I, resulting in an average pH_{max} of 8.1 ± 0.4. With this knowledge, it was suggested that growth of GAO could be affected due to unfavourable conditions which consequently may have an negative impact on the granule formation. On day 30, in-situ measurements of DOC and phosphate concentrations were conducted during the anaerobic phase. The anaerobic DOC removal was 68 ± 7% while no phosphate release was observed. Subsequently, on day 128, anaerobic DOC removal had increased to 87 ± 7% while anaerobic phosphate release was also observed from 4.8 $mgP{\cdot}L^{-1}$ after feed up to 6.5 $mgP{\cdot}L^{-1}$ at the end of the anaerobic phase. A yield of phosphate release to carbon uptake ($Y_{p/C,An}$) was 0.008 $Pmol_{PO4}{\cdot}Cmol_{DOC}^{-1}$ indicating minor enrichment of PAO since start-up of the experiment. Since anaerobic DOC removal increased significantly, enrichment of carbon-storing microorganisms is assumed to be established.

Molecular analysis by qPCR was performed to quantify enrichment of PAO (*Candidatus* Accumulibacter phosphatis) and GAO (*Candidatus* Competibacter phosphatis), commonly associated with aerobic granule formation. A slight increase of PAO abundance was observed during period I to 0.87 ± 0.12%, confirming the findings above regarding a minor increase of the $Y_{p/C,An}$ ratio due to some PAO enrichment. Abundance of GAO showed no increase, even a slight decrease from 2.91 ± 0.21% down to an average abundance of 2.00 ± 0.25% during the first experimental period. At this stage, no significant enrichment of the common PAO or GAO group was observed while the granulation process evolved. During period II, pH control was introduced using a 1 M HCl solution to lower the pH, resulting in an average pH_{max} of 7.3 ± 0.1 to influence microbial competition to stimulate GAO growth since anaerobic carbon removal is less energy consuming under these conditions. In addition, it was assumed PAO activity would not occur. In contrast to our expectations, the abundance of GAO did not increase, i.e. 2.46 ± 0.03% during period I and 2.44 ± 0.20% during period II. Introducing the pH control did not seem to enhance enrichment of this specific group of GAO. However, granulation remained stable, suggesting good selection for granule forming organisms other than the well-known PAO or GAO. In-situ measurements on day 317 showed that phosphate release occurred to a lesser extent from 1.6 $mgP{\cdot}L^{-1}$ to 2.4 $mgP{\cdot}L^{-1}$ at the end of the anaerobic phase while 94 ± 7% of DOC was anaerobically removed, resulting in a lower $Y_{p/C,An}$ of 0.006 $Pmol_{PO4}{\cdot}Cmol_{DOC}^{-1}$. The qPCR results confirm the nearly complete absence of the PAO fraction during this period with an average PAO abundance of 0.07 ± 0.03%. After switching off pH control in period III, the GAO abundance decreased down to 1.42 ± 0.22%. A slight increase in anaerobic phosphate release from 2.2 $mgP{\cdot}L^{-1}$ to 4.4 $mgP{\cdot}L^{-1}$ at the end of the anaerobic phase was once more observed while 99 ± 7% of DOC was taken up, resulting in a $Y_{p/C,An}$ of 0.009 $Pmol_{PO4}{\cdot}Cmol_{DOC}^{-1}$. The qPCR results show low percentages of PAO during period III with an average of 0.12 ± 0.02% confirming these findings.

Even when no pH control was applied (pH > 7.2), low $Y_{p/C,An}$ values were obtained compared to results reported by Weissbrodt *et al.* (2013) indicating that other carbon-storage microorganisms were more dominant compared to PAO. This can be accredited to the very high COD/P ratio of the wastewater, i.e. 419 ± 100 mgCOD/mgP. The measured GAO concentrations are all relatively low (<6%) so no decisive conclusion can be made regarding the effective enrichment of these specific groups of slow growing organisms. It is, however, unclear which group of organisms played a key role in the successful aerobic granule formation.

Selection of slow growing microorganisms for aerobic granule formation

Aerobic granule formation was successfully maintained for approximately 350 days while treating brewery wastewater. The strategy used in this study was based on the selection and enrichment of slow growing microorganisms by applying a feast/famine regime. qPCR results indicate that no significant enrichment of *Accumulibacter* nor *Competibacter* took place during granule formation. Alongside, 16S rRNA gene amplicon sequencing analysis was performed which showed that read abundances for *Accumulibacter* and *Competibacter* were negligible (<0.1%) for both seed sludge and a sludge sample taken at the end of period II. An overview of all genera with read abundances >0.1% for both sludge samples can be found in the supplementary data (available with the online version of this paper). Additionally, read abundance of a second GAO genus also associated with aerobic granule formation, i.e. *Defluviicoccus* (Pronk *et al.* 2017) decreased towards the end of period II. However, results concerning anaerobic DOC uptake, settling characteristics and morphology suggest strong enrichment of slow growing microorganisms who are able to store carbon intracellularly during the anaerobic phase and show strong granule formation. According to the 16S rRNA gene amplicon data, it was only *Candidatus* Obscuribacter (PAO) which seemed to have increased in read abundance from 0.2 to 0.3 ± 0.1%. Molecular analysis does, therefore, not confirm the enrichment of GAO in the system. Interestingly, the read abundance of *Rhodobacter* and *Thiothrix* were amplified by a factor 3.5 and 10 at the end of period II compared to the seed sludge. Resulting read abundances were 1.4 ± 0.1 and 10.2 ± 0.6%, respectively. The presence of *Rhodobacter* in AGS has been reported previously, suggesting a possible role in granule formation due to the fact they have the capacity to produce and secret EPS (Lv *et al.* 2014). In addition, *Rhodobacter* is also known for its sulphate reducing capacities and since sulphur-containing compounds were present in the brewery wastewater (i.e. 18.4 mg SO_4^{2-}-S, 1.21 mg SO_3^{2-}-S·L^{-1}, 0.154 mg S^{-2}·L^{-1}) this should be taken into account. As a result, sulphate present in the brewery wastewater may be anaerobically converted into sulphide, resulting in possibly significant amounts of sulphide in the system. Recent studies showed a reduced anaerobic activity of *Accumulibacter* when total sulphide concentrations in wastewater increased from 0 to 189 mg TS-S·L^{-1}. In addition, the aerobic metabolism of PAO, i.e. phosphate uptake, was also affected negatively by the presence of sulphide at total sulphide concentrations above 42 mg TS-S·L^{-1} (Rubio-Rincón *et al.* 2017a). One specific filamentous species of the *Thiothrix* genus, i.e. *Thiothrix caldifontis*, was found to be able to convert VFA into storage polymers, i.e. PHA, indicating a competition between *T. caldifontis* and *Accumulibacter* for VFA when sulphide is present in the wastewater (Rubio-Rincón *et al.* 2017b). Since enrichment of *Rhodobacter* and *Thiothrix* was observed, these microbial groups may have had an influence on the selection of *Accumulibacter*. These results suggest that aside from the well-known GAO and PAO species, enrichment of other groups of bacteria could influence the evolution of the biomass composition and therefore the aerobic granule formation and stability. When using complex, industrial effluents, more fundamental research is needed concerning the influence of other specific compounds. For example, the influence of S-compounds on the biochemical conversions and competition between microbial groups should be looked at. During this study, this was not investigated more intensively since the main focus consisted of stable aerobic granulation and the influence of the pH on the selection for PAO and GAO. However, it is highly possible that microorganisms with similar metabolic pathways are selected by the aerobic/anaerobic SBR processes that are not yet associated with granule formation nor the operational strategy. In future research, 16S rRNA gene amplicon sequencing should be used in research concerning the treatment of industrial wastewaters to give more insight in enrichment and role of other species during the granulation process.

CONCLUSION

Aerobic granule formation in an SBR fed with brewery wastewater was successful after 80 days of operation by applying feast/famine regime, while, in our opinion, a more applicable feeding strategy was applied by feeding on top of the SBR and complete initial biomass wash-out was avoided by applying long initial settling periods. Stable sludge settling capacities characterised by SVI_5 and SVI_{30} values consistently below $60\,mL{\cdot}g^{-1}$ and COD removal efficiencies between 95–99% were maintained for approximately 280 days. With these findings the stability of AGS for the treatment of an industrial effluent under changing operational conditions was determined showing good resistance to fluctuations in F/M ratio and changing pH conditions. Results show that for industrial applications, where wastewater composition is strongly variable, the presence of a balancing tank or a dynamic system is highly recommended to insure feast/famine conditions, maintain stable reactor performances and sludge characteristics. In this study, the integration of an OUR based aeration insured endogenous respiration at the end of the aerobic phase. It is thereby found to be suitable as a strategy when strong fluctuations in wastewater composition occur. In addition, mixing without feeding is found to be a suitable strategy to preserve AGS in periods when no wastewater is produced due to periods of shutdown of the industrial activities. The selection of slow growing organisms is found to be one of the main drivers for successful granulation. The molecular analysis however do not confirm a significant enrichment of common PAO or GAO groups associated with successful aerobic granulation. The lack of enrichment of common PAO and GAO groups, associated with stable granule formation, is highly remarkable in this study. Combined with the fact that anaerobic DOC removal increased up to 100%, it should be taken into account that, when using complex, industrial effluents, it is likely that other, possibly slow growing microorganisms with similar metabolic pathways are selected by the aerobic/anaerobic SBR process who are not yet associated with granule formation nor the operational strategy. However, this was not investigated more intensively since it was not the main focus of this study. More fundamental research is needed concerning the influence of other specific compounds, e.g. S-compounds, on the biochemical conversions and competition between microbial groups.

ACKNOWLEDGEMENTS

This work was supported by the University of Antwerp, Pantarein Water BVBA and the Flanders Innovation and Entrepreneurship Agency (grant number 150723).

The authors have declared no conflict of interest.

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First received 30 October 2017; accepted in revised form 9 March 2018. Available online 23 March 2018