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Influence of mixed feeding rate in a conventional SBR on biological P-removal and granule stability while treating different industrial effluents.

Influence of the feeding rate and COD/P ratio on bio-P activity and granule stability.

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Abstract

In this study the influence of the anaerobic mixed feeding rate on granule stability and reactor performance in a conventional SBR (C-SBR) was investigated while treating various industrial wastewaters. A lab-scale SBR fed with malting wastewater rich in phosphorus, was operated for approx. 250 days which was divided into two periods: (I) mixed pulse feed and (II) prolonged mixed feed. Initially, no bio-P activity was observed. However, by lowering the feeding rate biological P-removal was rapidly established and no effect on the AGS characteristics were observed. Additionally, to investigate the effect of the mixed feeding rate when treating an industrial effluent poor in phosphorus, i.e. brewery wastewater, a lab-scale reactor was operated for approx. 400 days applying different mixed feeding rates. Morphological and molecular analysis indicated that a low substrate concentration promoted the enrichment of anaerobic carbon storing filaments when fed with brewery wastewater. Findings suggest that a prolonged mixed feeding regime can be used as a tool to easily establish bio-P removal in a C-SBR system for the treatment of phosphorus rich wastewaters.

It should however be considered that under P-limiting conditions, enrichment of poly-P storing filaments may occur possibly due to their higher substrate affinity under anaerobic conditions.

Keywords: Biological phosphorus removal; Conventional SBR; Industrial wastewaters; Mixed feeding rate; Phosphate accumulating organisms (PAO)

Introduction

The European brewing sector is a major contributor to the European economy providing up to 2.3 million direct and indirect jobs. The agriculture malting industry provides products and serves the beer sector. Europe is responsible for one third of the worldwide malt production resulting in an increased employment within the overall beer sector (Europe Economics & The brewers of Europe 2016). Globally, efforts are made towards reducing water usage and carbon footprint. Brewery wastewater typically contains high amounts of easily biodegradable compounds (Driessen & Vereijken 2003) while wastewater produced by malting activities typically contains high amounts of particulate matter (Schwartzenbeck et al. 2004). Successful full-scale applications of the aerobic granular sludge (AGS) technology have been reported for the treatment of municipal wastewater resulting in a reduction of energy consumption between 20-40% and a footprint reduction up to 33% (Pronk et al. 2015). Due to biomass with excellent settling characteristics, sludge separation is improved drastically resulting in systems with higher biomass concentrations. In addition, simultaneous nitrification/denitrification (SND) and biological phosphorus removal contribute to the design of compact AGS bioreactors (STOWA 2013). It is generally considered as an attractive technology for the treatment of various industrial wastewaters. However, research using complex and/or particulate wastewaters such as real municipal wastewater (STOWA 2013) and industrial effluents (Schwarzenbeck et al. 2004; Caluwé et al. 2017; Stes al. 2018) show that more complex substrates may lead to filamentous outgrowth on the granule surface and the co-existence of flocculent and granular sludge. Stable aerobic granule formation when treating industrial wastewaters with a particulate content still remains challenging. The enrichment of slow growing organisms such as glycogen accumulating organisms (GAO) and phosphate accumulating organisms (PAO) appears to be critical to obtain stable aerobic granulation (STOWA 2013). PAOs and GAOs have the ability to anaerobically convert volatile fatty acids (VFA) into intracellular storage polymers which are used for microbial growth during aeration. The energy source for anaerobic VFA uptake is different for both groups of organisms; for PAOs this energy originates primarily from the hydrolysis of the intracellularly stored poly-P. For GAOs the main energy source results from the degradation of intracellular glycogen (Oehmen et al. 2007). Tu & Schuller (2013) found that the in-reactor substrate concentration influenced the PAO-GAO competition, favouring PAO over GAO when the mixed feeding rate was decreased (synthetic wastewater). It is suggested that PAOs are able to apply active transport for anaerobic carbon uptake, giving PAOs a competitive advantage when in-reactor substrate concentrations are low (Tu & Schuler 2013). Accordingly, growth of GAOs is negatively influenced by lowering the feeding rate pointing out the importance of excess P to allow PAOs to proliferate under these operational conditions. Based on these findings, it should be considered that a high COD/P ratio of the brewery wastewater will prevent proliferation of granule forming PAOs due to limited P availability. At the same time a lower feeding rate will prevent GAO growth due to an increased energy demand for anaerobic carbon uptake possibly hampering enrichment of granule forming organisms. The COD/P ratio may therefore have a substantial impact on the overall granule characteristics when the feeding rate is lowered. Short anaerobic pulse feeding strategies (several minutes) are mainly tested in lab-scale experiments to promote granulation (Val del Río et al. 2012, Caluwé et al. 2017; Stes et al. 2018). Applying this pulse feeding strategy is technically unfeasible in industrial SBR systems due to the enormous required feeding flows. Inevitably, existing or newly build WWTP will maintain longer mixed feeding times. This should be considered as an important operational parameter because of the direct impact on the in-reactor substrate concentration during the anaerobic feeding phase. It is of high relevance to investigate the influence of a prolonged anaerobic mixed feeding rate on the overall aerobic granule stability, reactor performance and the bio-P removal activity in an AGS system. It is assumed that three operational factors will be influencing the GAO/PAO competition, i.e. (1) the COD/P ratio (Oehmen et al. 2007), (2) the pH (Filipe et al. 2001b) and (3) the anaerobic mixed feeding rate (Tu &Schuler 2013). As described by Oehmen et al. (2007) it is generally assumed that a high influent COD/P ratio favours growth of GAO over PAO due to the limited availability of phoshpate. This study focusses on two main objectives. The influence of the anaerobic mixed feeding rate on the AGS stability, reactor performance and bio-P activity was investigated while treating (1) an industrial wastewater with a high phosphorus content, i.e. malting wastewater and (2) and low phosphorus content, i.e. brewery wastewater. It is hypothesised that when lowering the feeding rate, the bio-P removal activity will increase when COD/P ratios are low and PAOs are able to proliferate. However, deterioration of the granular sludge characteristics may occur when the feeding rate is lowered while treating a wastewater characterised by a high COD/P ratio.

Material and Methods

Reactor set-up

Two fully automated lab-scale SBRs were used during this study. The SBR fed with malting wastewater, SBR_M, had a working volume of 15L (H/D=3.5) and was operated at room temperature (18-22°C) for approximately 200 days. The SBR_B, fed with brewery wastewater had a working volume of 13L (H/D=1.1) and was operated at room temperature (18-22°C) for 400 days. Each reactor was provided with a mechanical stirrer (IKA RW20 digital), a DO (Endress+Hauser, Oxymax W COS51D) and pH (Endress+Hauser, Orbisint CPS11-7AA21) sensor and an aeration system consisting of an aeration pump (Ubbink Air 1000) and an air diffuser at the bottom of the reactor (AngelAqua DY 104-A). A Siemens PLC (LOGO! Logic Module) was used for process control. Sensor data was recorded and visualized by an Ecograph T RSG35 graphic display recorder (Endress+Hauser). Monitoring of the DO setpoints was done by the same recorder. A schematic overview of the experimental set-up can be found in supplementary data I.

Industrial wastewaters and seed sludge

The SBR_M was fed with wastewater from a local malting company. The wastewater used for SBR_B originated from a local brewery/bottling plant. Sufficient nutrient availability in the brewery wastewater was ensured by manual dosage of nitrogen (urea, 30%) and phosphorus (phosphoric acid, 75%) resulting in a final COD:N:P ratio of approximately 100:2:0.5. To avoid clogging of the feeding tubes, all wastewater was sieved (pore size: 1mm) to remove grains and large particulate matter. Minimum, maximum and average influent concentrations are summarised in **Table 1**.

Table 1 Malting and brewery (incl. N and P dosage) wastewater composition

	CODt $(mgO_2.L)^{-1}$	CODs $(mgO_2.L)^{-1}$	TN $(mgN.L^{-1})$	TP $(mgP.L^{-1})$	COD/P	pH	EC (µS/cm)
Malting							
Min	1256	1079	59.6	19.8	36	5.50	1668
Max	3661	3350	118	47.2	165	7.07	3140
Av	2403	1964	85.4	33.7	76	6.38	2289
Stdev	491	443	12.6	7.54	30	0.44	357
Brewery							
Min	1768	1046	23	12.7	90	4.9	802
Max	7934	6430	166	43.7	433	7.2	3280
Av.	4473	3663	77	24.6	200	5.9	1949
Stdev	1212	1160	24	7.7	82	0.6	452

All influent and effluent concentrations were measured using Hach test kits (Mechelen, Belgium); total COD (CODt) and soluble COD (CODs): LCK014, LCK514; TN-N: LCK 338, LCK138; NH_4^+-N: LCK305, NO_2^--N: LCK342, NO_3^--N: LCK339 ,TP-P: LCK350 and LCK348, respectively. All samples were filtered using glass microfibre filters (particle retention: 0.6µm, Macherey-Nagel MN GF-3) before measuring the CODs and PO_4^{3-}-P concentrations. Seed sludge for SBR_M originated from a parent lab-scale SBR which was operated to promote aerobic granulation while treating malting wastewater. An overview of the operational parameters of the parent lab-scale SBR can be found in supplementary data II. SBR_B was seeded with flocculent sludge from the existing local WWTP treating the brewery wastewater.

Reactor operation

In this study, aerobic granulation was promoted by applying a metabolic selection pressure to enhance growth of specific groups of slow growing micro-organisms associated with successful AGS formation, i.e. PAOs and GAOs. Therefore, an anaerobic feast/aerobic famine regime was applied for both SBR systems which is known to favour growth of granule forming organisms over floc forming and/or filamentous organisms. The SBR_M was operated for approx. 200 days, divided into two periods based on changes in anaerobic mixed feeding rate. The SBR_B was operated for approx. 400 days during which the feeding rate was lowered on day 182. To quantify the difference in feeding rate from a substrate loading point of view, the ratio of the organic loading rate (OLR) to the feeding time per cycle, i.e. $OLR_{feeding}$, was calculated using subsequent formula:

$$OLR_{feeding}\ [kg\ COD.m^{-3}.h^{-1}] = \frac{C_{COD,influent}.V_{influent}}{V_{SBR}.t_{feeding}}$$

This parameter is introduced to compare different anaerobic mixed feeding rates since it will result in different in-reactor substrate concentrations. An overview of the adjustments in SBR operation during the experiment are shown in **Table 2**.

Table 2 Different operational conditions considering the SBR cycle

SBR cycle phase	SBR_M		SBR_B	
	Period I	Period II	Period I	Period II
	Day 1-85	Day 86-200	Day 1-181	Day 182-400
Mixed idle phase (min)	10	10	10	10
Total anaerobic phase (min)	90	90	120	120
Aerobic phase (min)	155	205	205	195-205
Anoxic phase (min)	50	30	0	0
Settling (min)	15	15	15	15-25
Effluent withdrawal (min)	10	10	10	10
Total cycle time (min)	360	360	360	360
Feeding time (min)	10±4	49±19	22±8	41±13
FT/TT* (%)	4±1	16±3	6±2	13±4

*FT/TT: anaerobic mixed feeding time to total cycle time ratio

For both reactors, a relatively constant F/M ratio of 0.15kgCOD.(kgMLSS.day)$^{-1}$ was applied by adjusting the feeding time with respect to the influent COD concentration and the MLSS concentration. As a result the ratio of the SBR anaerobic mixed feeding time to the total cycle time (FT/TT) varied during the experiment. For both lab-scale experiments, the DO concentration was maintained between 1.0-1.5mgO_2.L^{-1} using an on/off aeration control strategy and no pH control was applied. For SBR_M and SBR_B, the sludge retention time was kept constant at 43 and 63 days by the automatic removal of 353mL and 380mL, respectively, of the sludge mixture during effluent withdrawal.

Sludge characteristics and sludge staining

All sludge samples used for analyses were taken at the end of the anoxic (SBR_M) or aerobic (SBR_B) cycle phase. The MLSS concentration was measured by filtering 5mL of homogeneous sludge mixture over a glass microfibre filter which was subsequently washed with demineralized water and dried for 24h at 105°C. The sludge volume (SV) was determined as described by APHA/AWWA/WEF (1998). The median particle size distribution by volume, DV_{50}, was measured using a Malvern Mastersizer 3000 (Malvern, UK) as described by Stes et al. (2018). Weekly analysis of the sludge was performed to investigate the evolution of the sludge morphology using a CX21FS2 Olympus microscope. Gram (modified Hücker method) and Neisser staining's were performed according to the methods described by Jenskins et al. (2004) in the attempt to identify specific filamentous organisms present in the system.

Microbial community composition by 16S rRNA gene amplicon sequencing

Genomic DNA was purified according to the NaTCA method (McIllroy et al. 2008) from sludge samples taken in triplicate from the reactor on regular time intervals. A sequencing library pool targeting the V1-3 region of the 16S rRNA gene was generated as described by Karst et al. (2016), with minor modifications. Briefly, Phusion High-Fidelity DNA polymerase (Thermo Scientific) and barcoded primers (IDT) were used for library PCR with 10-20ng of DNA as template and the denaturation step was carried out at 98°C. The resulting library pool was submitted to a final purification step by gel extraction using NucleoSpin Gel and PCR Clean-up (Macherey Nagel), and diluted to obtain a 4 nM library pool. Amplicon sequencing was carried out on a Illumina Miseq system at the Centre for Medical Genetics (Edegem, Belgium) with the MiSeq Reagent Kit v3 (Illumina). The obtained paired-end reads were processed with the UPARSE pipeline (Edgar et al. 2013). As a reference database for taxonomy prediction, 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 phylum- and genus-level taxa present in activated sludge, anaerobic digesters and influent wastewater (McIlroy et al. 2015).

In-situ cycle measurements during SBR operation

Due to the SBR operational strategy and subsequently the presence of granule structures, the enrichment of GAO/PAO like organisms is expected. In-situ cycle measurements during SBR operation are performed to determine the anaerobic carbon uptake and the degree of anaerobic phosphorus release and the aerobic phosphorus uptake rate. This was done to investigate the impact of the feeding rate on the GAO/PAO competition, favoring PAOs when the feeding rate was lowered. To determine the carbon and phosphate profiles, grab samples were taken (1) before and (2) after feed, (3) before aeration, (4) during aeration and (5) at the end of the SBR cycle. Grab samples were filtered immediately followed by the analytical measurements. From these results, the anaerobic carbon uptake [%], anaerobic P release [mg P.g MLSS^{-1}] and aerobic P uptake rates [mg P (g MLSS.h)$^{-1}$] were calculated.

Results and Discussion

Low COD/P malting wastewater

Reactor performance

The feed to mass ratio (F/M) was kept relatively constant by adjusting the feeding volume in function of the influent COD concentration and the MLSS concentration. For SBR_M the average F/M ratio was 0.15±0.03kg COD.(kg MLSS.day)$^{-1}$. Due to changes in MLSS concentration and the aim to work at a constant F/M ratio, the organic loading rate (OLR) varied strongly with an average OLR of 0.99±0.43kg COD.(m^3.day)$^{-1}$. To investigate the influence of the anaerobic feeding rate on the sludge characteristics and metabolic processes associated with the presence of slow-growing organisms, the feeding rate was lowered resulting in an increase of the average anaerobic mixed feeding time from 10±4min up to 49±19min. This adjustment in feeding regime implicates a lower in-reactor substrate concentration during the anaerobic mixed feeding phase. The average $OLR_{feeding}$ was 0.69±0.18 and 0.34±0.09 kgCOD(m^3.h)$^{-1}$ during period I and II, respectively.

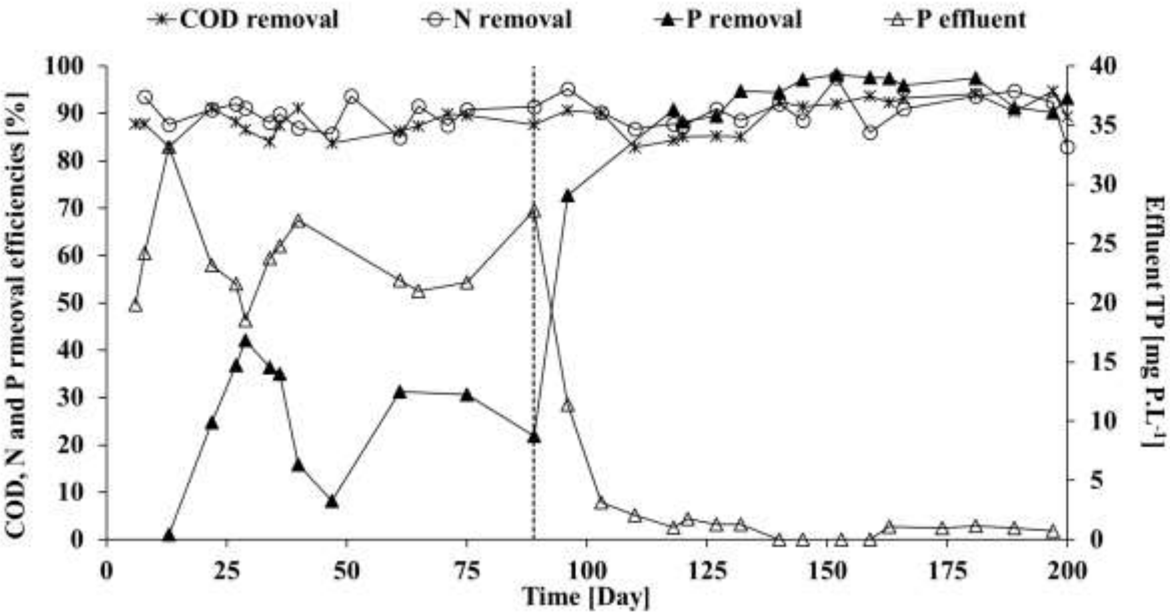

Figure 1 Evolution of the COD, nitrogen and phosphorus removal efficiencies and effluent phosphorus concentrations for SBR_M (dotted line: introduction of prolonged feeding phase)

The COD, N and P removal efficiencies were calculated for both operational periods showing stable carbon and nitrogen removal efficiencies throughout the experiment. Nitrogen removal was obtained through the two-step nitrification/denitrification process showing high removal efficiencies during the complete experiment. Effluent total nitrogen (TN) concentrations were consistently below 15mg $N.L^{-1}$ (Belgian discharge limit) with NO_3^- as the main effluent nitrogen compound with a maximum concentration of 5.1mg NO_3^--$N.L^{-1}$. Introduction of the prolonged feeding strategy did not have any impact on the N removal process. However, the adjustment of the feeding pattern had a major impact on the P removal efficiency due to the rapid increase of bio-P activity during period II. As can be seen in **Figure 1**, high effluent phosphate concentrations (>20mg PO_4^{3-}-$P.L^{-1}$) were measured during period I while during period II the effluent phosphate concentrations were consistently below 2mg PO_4^{3-}-$P.L^{-1}$. The average P removal efficiency was 26±13% during period I which is remarkably low compared to 89±8% during period II. For SBR_M the pH varied between 6.8-7.2 within the anaerobic phase so it was assumed that phosphorus removal may not occur during operation of SBR_M, especially during period I (short feeding time) due to the competitive advantage for anaerobic carbon uptake by GAOs. The results considering the absence of bio-P activity during period I confirm the findings by Filipe et al. (2001a) that GAOs show a competitive advantage for anaerobic carbon uptake when pH values are below 7.25. Additionally, the strong increase of bio-P removal when the anaerobic mixed feeding was prolonged is in line with results described by Tu et al (2013). They found that lowering the feeding rate in a lab-scale SBR fed with synthetic wastewater led to a shift in the GAO/PAO competition, favouring PAOs over GAOs even when pH is below 7.2. This was explained by the fact that PAOs contain an additional energy source for anaerobic carbon uptake, i.e. internally stored poly-P. Our results seem to confirm the statement made by Liu et al. (1997) that the PAO/GAO competition is an energy based competition for anaerobic carbon uptake which tends to favour poly-P containing PAOs over GAOs when in-reactor substrate concentration are low. To gain more insight in the shift in metabolic processes occurring within the system, carbon and phosphate concentrations were profiled by the use of in-situ cycle measurements.

In-situ cycle measurements

In-situ cycle measurements were performed showing minor anaerobic phosphate release during period I while anaerobic COD uptake from 74 up to 94% was observed. Together with the sludge settling characteristics and morphology (see further), the results indicate the presence of GAO like organisms in the system during period I. This is in line with previous findings described by Filipe et al. (2001a) showing that when pH is below 7.25, GAOs tend to be more dominant when a mixed anaerobic pulse feed is applied. During period II, anaerobic carbon uptake varied between 74% and 89% while a drastic increase of the anaerobic P-release and aerobic P-uptake rates was observed, as can be seen in **Table 3**.

Table 3 Evolution of the anaerobic P-release and aerobic P-uptake rate for SBR_M

	Day	Anaerobic P-release [mg P.(g MLSS)$^{-1}$]	Aerobic P-uptake rate [mg P.(g MLSS.h)$^{-1}$]
Period I	6	1.19	0.36
	20	0.61	0.41
	34	1.27	0.60
Period II	96	6.12	3.16
	159	5.99	9.42
	196	5.71	5.70

Together with the increased phosphorus removal efficiencies up to 98%, it can be concluded that lowering the feeding rate has induced the enrichment of PAOs over GAOs in the system. Our results confirm the findings of Tu & Schuller (2003) that applying a low feeding rate promotes bio-P activity and seems to be more critical than the pH. This is, to our knowledge, the first time that the influence of the feeding time on the bio-P activity is investigated while treating an industrial wastewater in a C-SBR.

Granule characteristics

Evolution of the granule settling characteristics, DV_{50} and sludge morphology were investigated to determine the influence of the feeding regime on the overall granulation state and stability. During the first period of the experiment, the settling characteristics showed SVI values below 30mL.g^{-1}. In addition, the average DV_{50} was 209±3μm suggesting good selection for granule forming organisms. Meanwhile, filamentous outgrowth at the granule surface gradually decreased.

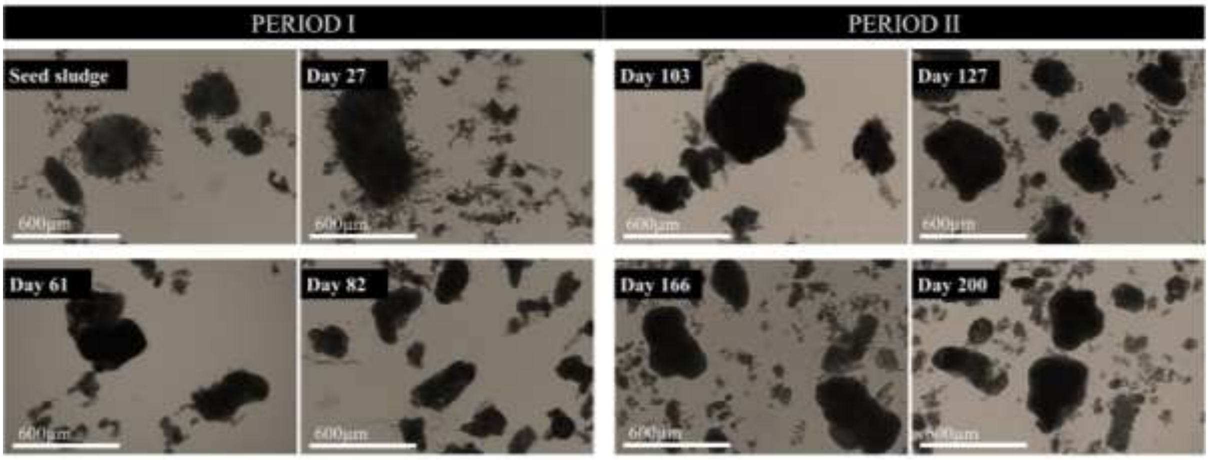

Figure 2 Overview of the sludge morphology for SBR_M during Period I and period II

Granule settleability only slightly decreased during period II, resulting in final SVI_{10} and SVI_{30} values of 78mL.g^{-1} and 47mL.g^{-1}, respectively on day 200 of the experiment. These are still considered as values representing good settling granular sludge. Lowering the anaerobic feeding rate did not influence the overall granule morphology as shown in **Figure 2**. However, it can be observed that smaller granular structures developed during period II. The final DV_{50} was only 153±1μm on day 189. This phenomenon can be explained by the fact that lower in-reactor substrate concentrations may lead to substrate diffusion limitations and subsequently the development of smaller but still compact granule structures. It can be concluded that lowering the feeding rate from 10±4min to 49±19min had no major impact on the granule settling or morphological characteristics. However, the feeding rate had a major impact on the reactor performance considering the increased bio-P activity during period II of the experiment. These results point out that applying a low anaerobic mixed feeding rate may be a easily applicable operational strategy to promote biological phosphorus removal in an AGS system for the treatment of malting wastewater in a compact C-SBR. Considering all results, a low in-reactor substrate concentration is thought to select for (smaller) PAO like granules over GAO which is assumed to be due to the presence of an additional energy source, i.e. poly-P, for anaerobic carbon uptake. This, however, raises some questions concerning the applicability of a low feeding rate for the treatment of wastewaters without excess phosphorus, like brewery wastewater.

High COD/P brewery wastewater

Reactor performances

For this experiment, a lab-scale SBR_B treating brewery wastewater was operated for approx. 400 days, divided into two periods based on the anaerobic mixed feeding rate. The average F/M ratio and OLR were 0.20±0.09kg COD.(kg MLSS.day)$^{-1}$ and 0.93±0.42kg COD.(m3.day)$^{-1}$, respectively. On day 182 the mixed feeding rate was decreased resulting in an increase of the average feeding time from 22±8min during period I up to 41±13min during period II. Consequently, the average $OLR_{feeding}$ decreased from 0.78±0.18 to 0.25±0.07kg COD.(m^3.h)$^{-1}$. Stable COD, N and P removal efficiencies of 98±1%, 99±1 and 96±4%, respectively, were obtained throughout the experiment. When comparing period I and II, the overall removal efficiencies were stable and the differences between both periods were found to be negligible.

In-situ cycle measurements

Throughout the experiment, in-situ cycle measurements were performed to evaluate the influence of the feeding rate on the bio-P activity, i.e. the anaerobic P release and the aerobic P uptake rate. When treating brewery wastewater the in-reactor pH is expected to be above 7.25 promoting minor bio-P activity as described by Stes et al. (2018). Since the pH is known to play a key role in the competitions between GAO/PAO (Filipe et al. 2001b), this parameter was taken into account during the study. The average pH was 8.1±0.1 which is generally known to favour PAOs over GAOs. As can be seen in **Table 4** an increase of the anaerobic P-release was observed during period I. These results suggest that even though the COD/P ratio of the influent was high, minor enrichment of some granule forming PAO like organisms may have occurred during period I. These findings are in line with the results reported by Stes et al. (2018) where stable aerobic granules showed minor bio-P activity when the pH>7.2 while treating brewery wastewater. Due to the alkaline conditions it is believed an additional energy source is required to overcome the pH gradient across the cell membrane in order to take up carbon anaerobically. Since PAOs can provide this additional energy requirement through poly-P degradation, they tend to have a competitive advantage over GAOs (Filipe et al. 2001a). It is clear that the in-reactor pH can not be neglected in an anaerobic-aerobic operated SBR system.

Table 4 Evolution of the anaerobic P-release and the aerobic P-uptake rate for SBR_B

	Day	Influent COD/P [%]	Anaerobic P release [mg P.g $MLSS^{-1}$]	Aerobic P-uptake rate [mg P.g $MLSS^{-1}$]
Period I	8	117	0.29	0.20
	15	189	0.47	0.36
	29	109	0.36	0.17
	34	138	0.15	0.08
	43	138	0.20	0.13
	57	135	0.38	0.15
	119	204	1.48	-
	142	92	1.68	0.37
	162	195	2.79	1.26
	182	126	3.15	2.54
Period II	211	270	1.34	1.98
	247	226	3.39	-
	283	370	1.49	1.98
	289	199	2.14	1.80
	379	222	1.50	1.67
	393	177	0.88	1.87
	400	190	1.88	2.48

In addition, results show that anaerobic P release as well as the aerobic P-uptake rates are somewhat higher during period II compared to period I. These results illustrate that when treating brewery wastewater with a high COD/P ratio, the anaerobic mixed feeding rate has no significant influence on the bio-P activity. These findings are less clear compared to those in SBR_M which showed a strong increase in the bio-P activity when the feeding rate was decreased. It is suggested that during this experiment, next to the pH and the feeding rate, the high COD/P ratio had a major contribution on the competition between GAO and PAO like organisms, possibly preventing PAOs to proliferate.

Sludge characteristics

The evolution in granule settleability, granular size and morphology were investigated to determine the influence of the feeding regime on granule characteristics and stability when treating brewery wastewater. When comparing the sludge settling characteristics, results show a significant difference between the two operational periods. **Figure 3** shows the evolution of the DV_{50} and (D)SVI values during the 400 days of operation.

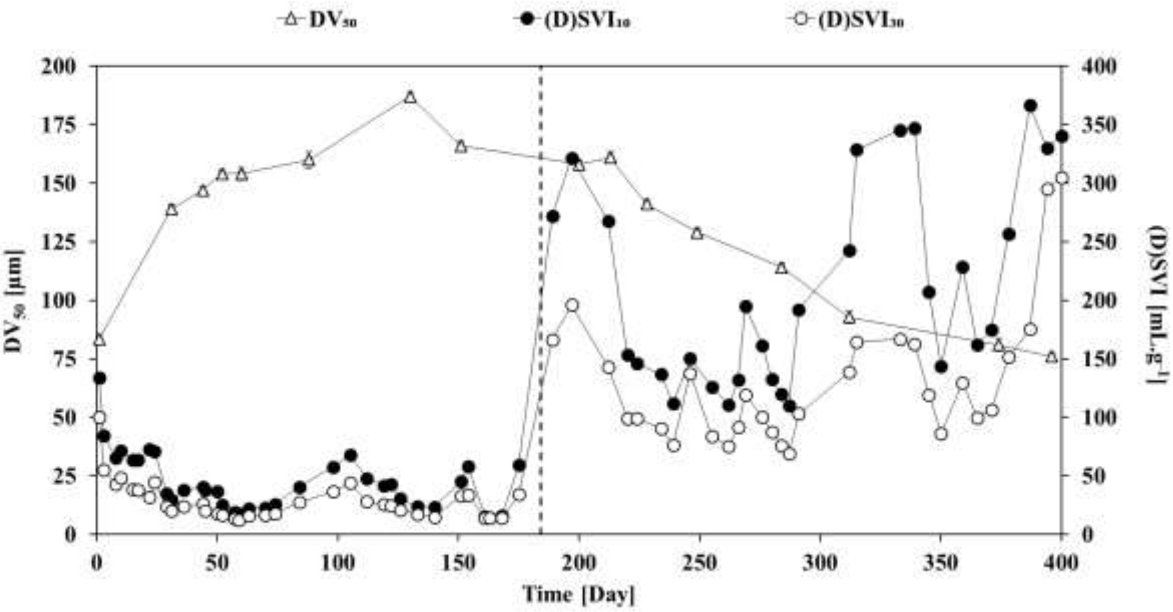

Figure 3 Evolution of DV_{50} and (D)$SVI_{10,30}$ for SBR_B. (dotted line: introduction of prolonged feeding time)

When comparing the (D)SVI values it is clear that selection of well settling sludge was established during period I resulting in a decrease in (D)SVI values below 50mL.g^{-1}. The prolonged mixed feeding strategy was introduced on day 184. Hereafter, the (D)SVI_{10} values increased strongly up to 322mL.g^{-1} on day 197. Subsequently, the settling characteristics drastically deteriorated often resulting in very high SVI_{30} values above 200mL.g^{-1} during period II. It is clear that increasing the FT/TT ratio had a major negative impact on sludge settleability when treating brewery wastewater. Additionally, it became clear that during period II the settling characteristics deteriorated rapidly when the difference between the influent COD_t and COD_s concentration, i.e. ΔCOD, increased (data not shown). This is in line with findings reported by de Kreuk et al. (2010) that the presence of particulate matter in the influent has a negative influence on the overall AGS characteristics. However, the increase of influent ΔCOD had a greater impact on the settling characteristics during period II compared to period I. This suggests that lowering the feeding rate and thus the in-reactor substrate concentration had a negative impact on the overall stability of the sludge settleability. In Stes et al. (2018) successful aerobic granulation in an SBR treating brewery wastewater was reported showing very low SVI_{30} values (<50 mL.g^{-1}). They applied a non-mixed (static) pulse feeding strategy to obtain a maximum substrate concentration during the subsequent anaerobic mixed feast phase. The brewery wastewater used by Stes et al. (2018) was also characterised by strong fluctuations of the particulate matter content but showed no significant effect on the sludge settling characteristics. It is clear that an high substrate gradient during the anaerobic feast phase had a positive impact on the sludge settling characteristics and the stability of the system towards variations in influent composition.

Evolution of the sludge morphology was investigated by microscopic analysis and shown in **Figure 4**.

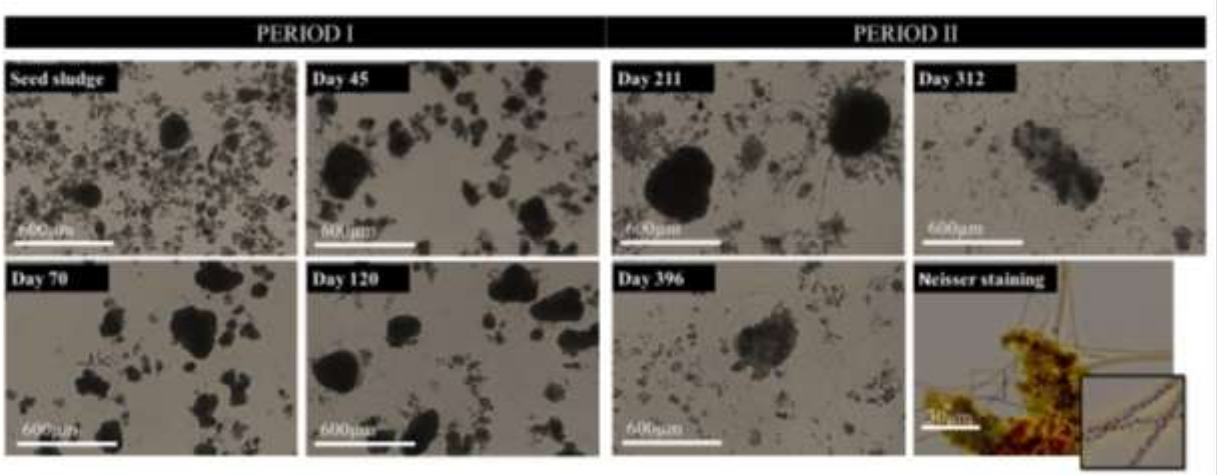

Figure 4 Evolution of the sludge morphology during period I and II from SBR_B (incl. example of a Neisser stained sludge sample).

It can be concluded that during period I dense granular structures developed and only a limited amount of filamentous organisms were present. Preservation of the large granules within the system was successful throughout period I. The morphology during period I is in strong contrast with period II where less dense, medium large granule structures, small flocs and filamentous organisms dominated the overall sludge morphology. These observations indicate that the increase of the (D)SVI values during period II are caused by the increased presence of smaller flocs and filamentous structures. In the attempt to identify the filamentous organisms and to confirm the presence of poly-P activity, i.e. intracellular poly-P granules, Neisser staining's were performed. As previously discussed, the in-situ cycle measurements showed bio-P activity within the system which was relatively high during period II considering the high COD/P ratio. Sludge samples were taken at the end of the aerobic phase to insure a maximum poly-P content within the cells. Remarkably, no accumulation of poly-P was observed within the granular sludge structures while it was clearly present within the filamentous organisms (see **Figure 4**). Only a limited number of filamentous organisms are known to have the capacity to store poly-P granules. In addition, Gram-staining responses were positive for the filamentous structures (data not shown). Considering the SBR_B operational strategy (anaerobic feast to promote carbon conversion into intracellular polymers) and based on the staining results and the filament morphological characteristics only two specific filamentous organisms are suggested to be present in SBR_B, i.e. *Thiothrix* spp. or *M. parvicella* (Jenkins et al. 2004). Both species are known to be able to accumulate carbon intracellularly as poly-hydroxyalkanoates (PHA) and have the capacity store phosphorus intracellularly (Rosetti et al. 2005; Rubio-Rincón et al. 2017). Molecular analysis by 16S-rRNA gene amplicon sequencing was performed in order to gain insight in the overall biomass composition of the seed sludge (Sample 1) and additionally identify the filamentous organisms at the end of period II (Sample 2). In supplementary data III_{a-b} a detailed overview of the microbial composition at different taxonomical levels can be found. For sample 1 and 2, up to 97% and 93%, respectively, of the resulting sequences could be classified at phylum level and up to 62% and 68%, respectively, at genus level. For sample 1, Planctomycetes and Bacteroidetes were the most abundant phyla representing 30% and 25% of all bacteria, respectively. For sample 2, Proteobacteria and Bacteroidetes were the most abundant phyla raching up to 45% and 39% of the bacteria, indicating a shift in microbial composition during this study. The read abundance for *C. Accumulibacter* (PAO), known as an anaerobic carbon storing organism associated with granule formation, slightly increased from 0.00±0.04% in sample 1 up to 0.14±0.05% in sample 2. For *Defluviicoccus* (GAO) the read abundance in the seed sludge was 0.71±0.07% and 0.76±0.06% at the end of period II. No enrichment of other known PAOs or GAOs was observed during this study (see supplementary data III_c for the resulting read abundances for all known GAOs and PAOs). The suggestion, based on microscopic observations that *M. parvicella* may be present in the system was countered by the 16S rRNA amplicon sequencing analysis indicating the complete absence in both sludge samples. However, the presence and strong enrichment of the *Thiothrix* genus (Proteobacteria) was confirmed by 16S rRNA amplicon sequencing analysis with an average read abundance of 0.0±0.0% in the seed sludge and 7.25±2.27% at the end of period II. The enrichment of *Thiothrix* in an anaerobic-aerobic granular sludge system was also observed by Stes et al. (2018) treating brewery wastewater in an anaerobically fed SBR. Since the presence of sulphur compounds was not taken into account during this study, measurements of the influent sulphate concentrations are absent. However, the anaerobic feeding strategy and the enrichment of *Rhodobacter* (Proteobactria), known as a sulphate reducing organism, indicate the presence of sulphur compounds in the brewery wastewater. The read abundance for *Rhodobacter* increased from 0.28±0.03% in sample 1 up to 1.95±0.28% in sample 2. By applying a prolonged anaerobic phase, sulphate reduction was found to be promoted and subsequently to induce growth of filamentous sulphur bacteria in an anaerobic/aerobic SBR system (Baetens et al. 2001). It is likely that enrichment of *Thiothrix* spp. was promoted by the prolonged anaerobic SBR phase which was applied to promote anaerobic carbon uptake. Rubio-Rincón et al. (2017) showed that, in the presence of sulphide, *Thiothrix caldifontis* has the capacity to store carbon anaerobically as intracellular polymers and contribute to bio-P removal using stored poly-S as an additional energy source. These findings may explain our observations showing enrichment of poly-P storing filamentous organisms, i.e. *Thiothrix*, when applying an anaerobic/aerobic SBR operational strategy for the treatment of brewery wastewater. Although, minor filamentous outgrowth was observed during period I of the experiment suggesting growth of granule forming organisms is promoted over filamentous organisms during period I. It is suggested that, like *M. parvicella,* other filamentous carbon storing organisms show a higher substrate affinity compared to granule forming organisms when in-reactor concentrations are low during anaerobic mixed feeding. It is expected that sulphur compounds were present in the brewery wastewater. This complements the explanation to why more filamentous outgrowth, i.e. *Thiothrix,* was observed during period II compared to period I due to an increased energy demand for anaerobic carbon uptake when in-reactor substrate concentrations decline.

Summary and conclusion

The aim of this study was to investigate the influence of the anaerobic mixed feeding rate on the aerobic granule stability, reactor performance and bio-P activity while treating an industrial wastewater with a relatively low (malting) and high (brewery) COD/P ratio. In both cases, selection of carbon storing organisms was promoted by applying a feast/famine regime through anaerobic/aerobic/anoxic SBR cycle operation. It can be concluded that for SBR_M a decrease of the $OLR_{feeding}$ resulted in a strong increase in bio-P activity and is therefore assumed to promote growth of PAO over GAO. The effect on the granule settleability was only minor and dense granular structures were preserved. This is to our knowledge the first time that the positive effect of a prolonged feeding time on the bio-P removal activity in an

AGS system is investigated while treating an industrial wastewater in a C-SBR. When treating brewery wastewater characterised by a high COD/P ratio, successful granule formation was achieved by applying an anaerobic mixed pulse feeding strategy. Sludge morphology was dominated by dense granule structures showing good and stable settling characteristics suggesting successful selection for granule forming organisms. A decrease of the anaerobic mixed feeding rate resulted in deterioration of the granular sludge combined with filamentous outgrowth. In-situ cycle measurements showed no increase in bio-P activity while Neisser staining showed intracellular poly-P granules within filamentous organisms. This shift in sludge morphology towards enrichment of filamentous organisms had a negative impact on the settleability of the biomass. It is suggested that the high influent COD/P ratio prevented proliferation of PAO like organisms within the system promoting enrichment of high affinity filaments over granule forming groups. Through this study, the importance of the anaerobic mixed feeding rate in a C-SBR system is confirmed. When considering application of the AGS technology in conventional mixed SBR systems, the anaerobic mixed feeding rate should be taken into account. In this study, a new operational parameter, $OLR_{feeding}$, was defined to allow quantification of the feeding rate from a substrate loading point of view.

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