Influence of free ammonia on the performance of a polyhydroxyalkanoate (PHA) microbial enrichment sequential batch reactor (SBR) at pilot scale

Miki Segami

Abstract

A pilot-scale PHA microbial enrichment reactor fed on OFMSW leachate was monitored in order to assess the influence of free ammonia nitrogen (FAN) on its performance. The enrichment reactor consisted of a SBR with a 12-hours cycle which included a feast phase, a settling phase and a famine phase with external nitrogen addition. Based on the microscope pictures and FISH analysis, at least two different PHA producing bacteria were identified: small (< 2.5 µm) and big PHA producing bacteria (< 5 µm). Big PHA bacteria appeared from FAN concentrations higher than 50 mg/L, but concentrations higher than 150 mg/L were highly toxic for the whole PHA microbial enrichment. Finally, it was found that a FAN concentration around 60-70 mg/L might produce a microbial enrichment with good settling properties (SVI_{30} < 80 mL/g and BLAS < 5%) and maximum PHA yields (around 0.50 g PHA/g sCOD), possibly due to the presence of big PHA producers which are assumed to be more efficient and heavier after feast. However, this FAN concentration may lead to a lower settleable biomass production yield (around 0.30 g VSS/g sCOD). Since these results are not conclusive, it is suggested to test the observations of this pilot study in lab-scale experiments to evaluate the potential of FAN as an additional selective pressure in a PHA microbial enrichment.

Nomenclature

ATU	Allylthiourea
BLAS	Biomass loss after settling
sCOD	Soluble chemical oxygen demand (mg O_2/L)
DO	Dissolved oxygen (mg/L)
EPS	Extracellular polymeric substances
FAN	Free ammonia nitrogen (mg/L)
FISH	Fluorescence in situ hybridization
OFMSW	Organic fraction of municipal solid waste
PHA	Polyhydroxyalkanoates
q_{Scod}	Biomass specific substrate uptake rate (g sCOD/g VSS.h)
SBR	Sequential batch reactor
SRT	Sludge retention time
SVI	Sludge volume index (mL/g)
TAN	Total ammonia nitrogen (mg/L)
TSS	Total suspended solids (g/L)
VFA	Volatile fatty acids
VSS	Volatile suspended solids (g/L)
Y_{PHA}	PHA production yield (g PHA/g sCOD)
$Y_{VSS\ sett}$	Settleable biomass production yield (g VSS/g sCOD)

1. Introduction

Polyhydroxyalkanoates (PHA) are polymers of hydroxy fatty acids synthetized by bacteria as an intracellular carbon and energy storage compound. When the external carbon source has been depleted, PHA gives a competitive advantage to the bacteria that can produce it, since these microorganisms will be able to grow and produce energy by oxidizing this intracellular carbon reserve[1], [2].

In the past few years, PHA has been used to produce bioplastics as they are biodegradable, non-toxic and exhibit thermoplastic and elastomeric properties [1], [2]. As of 2009, the commercial production of PHA was based on the use of genetically engineered bacteria in a pure culture fed with specific substrates (e.g. sugars) and via aseptic processes, because these cultures can reach up to 90 wt% cellular PHA content [2] which makes the production more efficient. However, this biotechnological process has a high energy consumption and requires the expenditure on expensive substrates and equipment, thus, hindering the large-scale production of bioplastics [1].

An alternative to the pure culture approach production is the use of open cultures. This kind of culture can be exploited by following eco-biotechnology principles and its main advantage is that it does not require aseptic conditions nor pure and expensive substrates[1]–[3]. Open cultures have been generally used for the biological treatment of waste water and organic solid waste, as well as for the production of bioenergy [2]. Nowadays, open cultures are being studied for the commercial production of PHA from municipal or industry wastewater[4], [5] or from the leachate of fermented organic fraction of municipal solid waste (OFMSW)[6], [7].

The open-culture production of PHA relies on the ecological principles of selection and competition. The selective environmental pressure to obtain a desired microorganism is achieved by controlling the feeding and operating conditions of a bioreactor, i.e. engineering the ecosystem rather than the microorganism. When aiming to select PHA producing organisms in a mixed culture, the application of a cyclic feast (presence of substrate) and famine (no substrate presence) regime is one possible strategy. This dynamic feeding conditions will allow the proliferation of PHA producers, as their ability to store carbon will be their competitive advantage over other microorganisms. [2]

The production of PHA via open cultures has the potential to recover waste and reuse it for a sustainable production of bioplastics. However, PHA production from waste streams is still not commercially feasible due to, among other factors, the lower PHA cellular content of waste-fed mixed cultures. Despite the progress during the last few years, currently, the highest reported PHA cellular content in an open-mixed culture on waste streams has been 70-80 wt% [8] which is lower than the previously mentioned 90 wt% PHA cellular content in pure cultures. Therefore, the optimization of this production method is crucial to make it industrially affordable.

Orgaworld B.V., in collaboration with Paques B.V. and TU Delft, is testing the upscaling possibilities of a PHA production system based on organic waste leachate in a pilot plant in Lelystad, The Netherlands. The goal of the PHA pilot plant is to mimic the industrial conditions that would be present in a full-scale system and to evaluate the effect of these conditions on the PHA production performance. The PHA production comprises three main processes in the following order: (i)

hydrolysis of OFMSW, (ii) selective enrichment of a PHA producing microbial culture, and (iii) accumulation of PHA. Effluents from the enrichment reactor and the accumulation reactor were recirculated and reused in the hydrolysis process.

The core process of the plant is the selective enrichment which consists of a sequential batch reactor (SBR) fed with the OFMSW leachate from the hydrolysis process. The selective enrichment reactor works based on a feast-famine regime without pH control nor allylthiourea (ATU) addition (contrary to earlier studies) with the aim of minimizing costs. Besides the selective pressure from the dynamic feeding conditions, this SBR sequence adds an additional selective pressure by means of a settling phase and supernatant removal step after feast. During this phase, the heavier PHA biomass or the larger flocs settle to the bottom while the lighter bacteria (i.e. poorly efficient PHA producers or non PHA producers) or the smaller flocs are removed alongside the non-consumed carbon compounds [9], [10]. In order to ensure biomass growth during the famine phase, a nutrient solution is dosed after the supernatant is removed.

Nitrogen concentrations in a selective enrichment reactor are crucial to enable growth during the famine phase and to produce sufficient PHA producing biomass which will later be used in the PHA accumulation process. Minimum nitrogen requirements for a good enrichment have previously been studied. For instance, a laboratory study in 2010 concluded that nitrogen-limited conditions (medium C/N ratio ≥ 15-24 Cmol/Nmol) can hinder the PHA storage capacity [11]. However, the effect of excessive nitrogen conditions on the selective enrichment of PHA producers has not been fully studied. Solely, a study in 2016 reported that ammonium concentrations higher than 1 g N/L at pH 7 decreased the specific activity of *Plasticicumulans acidivorans* [7].

Among the nitrogen species, the most critical compound is free ammonia as it might be toxic for PHA producers. It is believed that free ammonia can penetrate the cell membrane and inhibit bacterial growth by causing a proton imbalance that increases maintenance energy requirements [12]. In aerobic granular sludge systems, it has been reported that free ammonia nitrogen (FAN) concentrations higher than 23.5 mg/L hindered the formation of granules due to the decrease of cell hydrophobicity and cell polysaccharides production [13]. On the other hand, it has also been reported that a reactor dominated by heterotrophic nitrifiers could withstand free ammonia concentrations up to 110 mg/L while treating leachate from municipal solid waste incineration plants [12].

In the present study, the influence of free ammonia on the performance of the selective enrichment reactor in the pilot plant was evaluated for three months. For this purpose, the SBR was monitored through the different phases of the cycle and simultaneously, five performance indicators were proposed and evaluated. At the beginning, the nutrient addition was set to a constant dose, and through the research period, the residual total ammonia and free ammonia concentrations at the end of feast were monitored.

2. Materials and methods

2.1. Pilot plant in Orgaworld

The PHA pilot plant comprises three main processes: (i) hydrolysis of OFMSW, (ii) selective enrichment of a PHA producing microbial culture, and (iii) accumulation of PHA. A more detailed description of the pilot plant composition is shown in Figure 1. The hydrolysis process unit is where the OFMSW is fermented and consequently, a volatile fatty acids (VFA) rich leachate is produced. This leachate is key for the selective enrichment and the accumulation since it constitutes the substrate stream to feed the PHA bacteria. The substrate buffer tank is prepared by diluting the hydrolysis leachate which can have different soluble chemical oxygen demand (sCOD) concentrations every day. For the purpose of this research it was planned to prepare daily a sCOD concentration of 6 g/L in the substrate buffer tank. Finally, the effluents of the selective enrichment and the accumulation process are recirculated and reused in the hydrolysis unit and for the preparation of the substrate buffer tank.

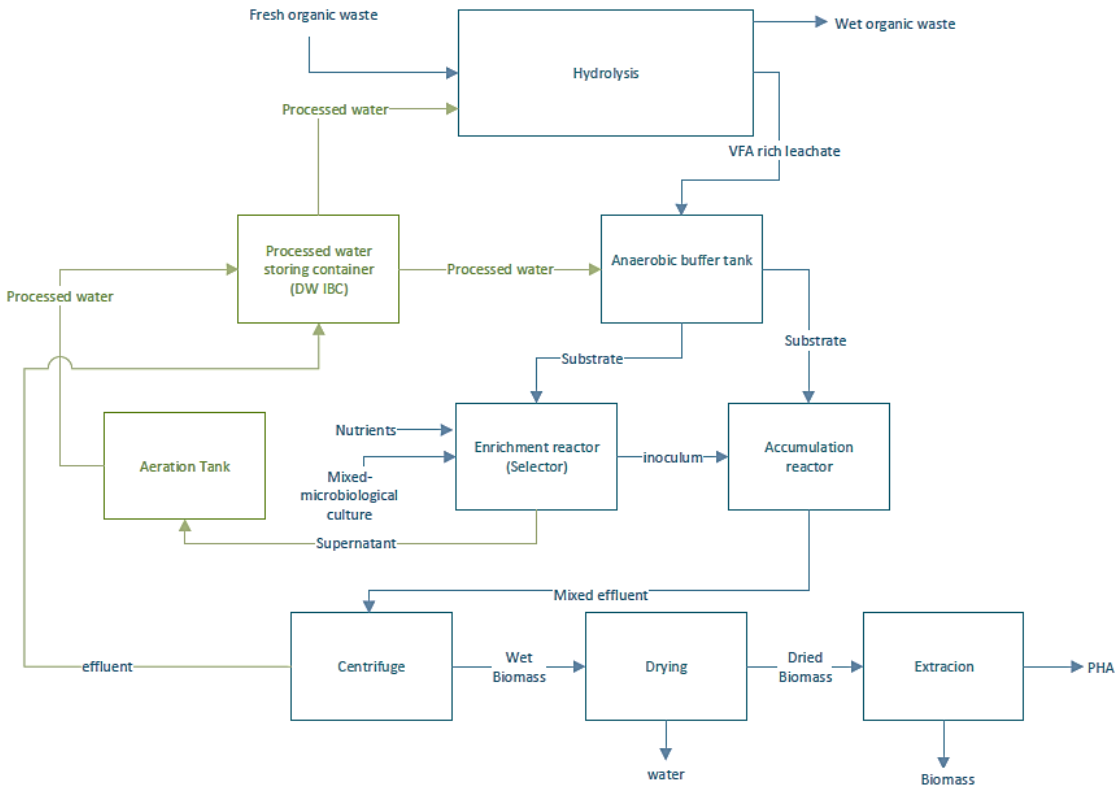


Figure 1. PHA pilot plant process flow diagram.

2.2. Operation of the selective enrichment reactor in the pilot

The selective enrichment reactor is a sequential batch reactor (SBR) with a cycle length of 12 hours and a sludge retention time (SRT) of 24 hours approximately. The cycle consists of the following sequential steps: (i) substrate addition (15 min), (ii) feast phase (70 min), (iii) settling phase (25 min), (iv) supernatant removal (10 min), (v) nutrient dosing + famine phase (580 min), and (vi) biomass removal (5 min) (see Figure 2). The nutrient solution was composed of 3 M of nitrogen in the form of urea, 0.3 M phosphate, 0.3 M $MgSO_4$, 0.2 M K_2SO_4, and trace elements (64 mM $FeCl_3$, 3 mM $ZnSO_4$, 2.7 mM H_3BO_3, 2.1 mM $NiCl_2$, 1.5 mM $CoSO_4$, 0.6 mM $CuSO_4$ and 0.8 mM Na_2MoO_4). The nutrient dosing step starts simultaneously with the famine phase. At the beginning of this research, the nutrient dosing was fixed to last 9500 seconds (equivalent to 13.6 g of nitrogen) since at this rate, residual ammonia nitrogen was detected at the end of cycle, meaning the enrichment was not nitrogen limitated.

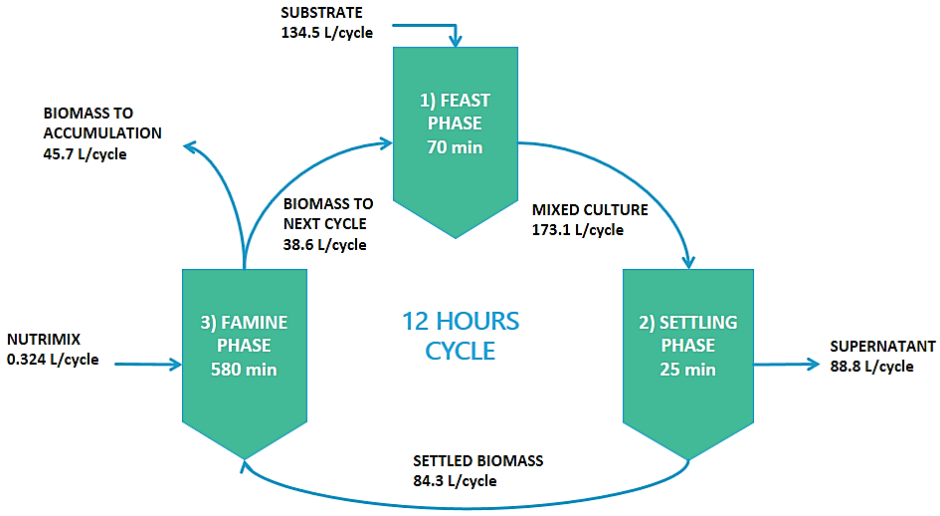


Figure 2. PHA selective enrichment sequential batch reactor.

The SBR was aerated and mixed by a fine bubble diffuser on the bottom which supplied an air flow of 200 L/min in order to ensure no oxygen limitation (dissolved oxygen > 2 mg/L) and completely mixed condition. The air compressor was only turned off during the settling phase and supernatant removal. Additionally, the inner temperature was constantly controlled to be between 28-32 °C by means of a thermostat compartment of warm water that surrounded the reactor.

2.3. Sampling and analytical methods

The SBR was continuously monitored (24-hours) using an online graphic user interface that shows the pH, temperature and dissolved oxygen (DO) concentration inside the reactor (see Figure 3). The DO was measured by an LDO sensor, while the pH, by a Ag/AgCl electrode. The dissolved oxygen

profile was used to determine the actual end of feast: when the DO would suddenly rise at a very fast rate, it was assumed that the VFA were completely taken up (see example in Figure 3).

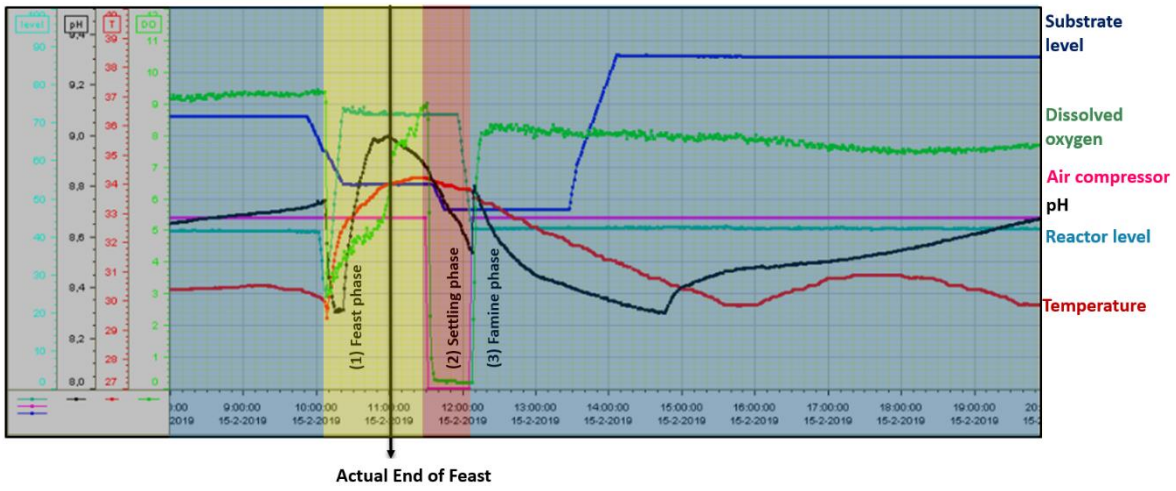


Figure 3. Online monitoring of pH, temperature and dissolved oxygen.

Every morning cycle, a sampling routine was performed in order to measure a list of offline parameters in the substrate, at the end of cycle, at the end of feast and after the settling phase (see Figure 4). For the total suspended solids (TSS), volatile suspended solids (VSS) and PHA analysis, duplicate samples of 50 mL were prepared in falcon tubes to which 1 mL of formaldehyde (37%) was added for preservation. For the sCOD and total ammonia nitrogen (TAN) analysis, two samples of 2 mL were centrifuged for 3 minutes with a relative centrifugal force (RCF) of 20238 x g, later, filtered with a 0.45 µm pore size PVDF membrane (Millipore, Ireland) and finally, stored in 2 mL Eppendorf tubes. Additionally, 2 mL samples of biomass at the end of feast were taken to make phase-contrast microscope pictures at 10X100X.

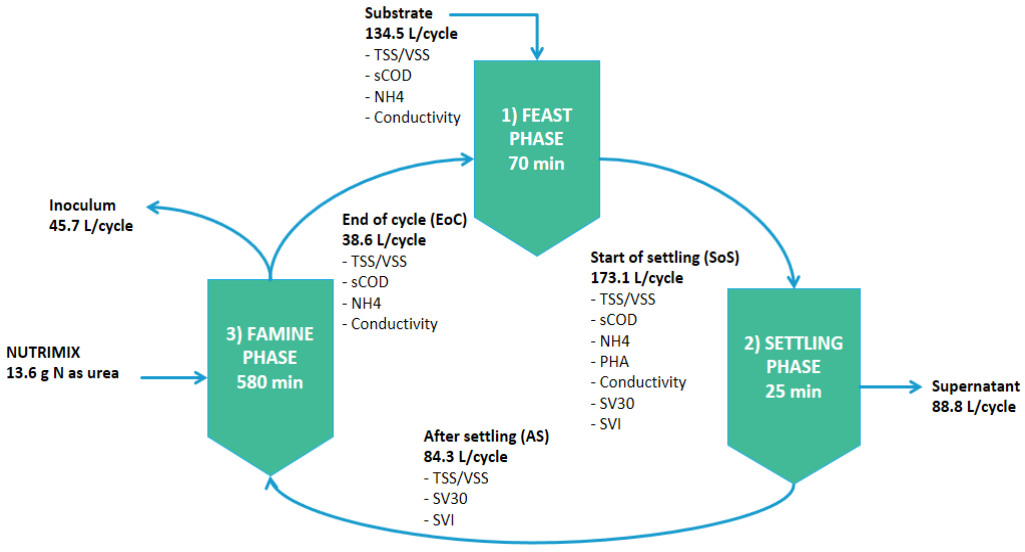


Figure 4. Offline parameters and its sampling points.

The TSS, VSS and sludge volume (SV_{30}) measurements were performed according to the standard methods [14]. The PHA content was measured following a method described by Johnson et al. [2]. The sCOD and TAN concentrations were measured using Hach (Germany) test kits and spectrophotometer. Free ammonia nitrogen (FAN) concentrations were calculated based on TAN and pH measurements at the end of cycle and using Equation 1, where the constant K_a was assumed to be 9.3.

$$FAN\ (\frac{mg}{L}) = \frac{[TAN] * 10^{-Ka}}{(10^{-pH} + 10^{-Ka})} \quad \text{... (Equation 1)}$$

FAN was decided to be measured at the end of cycle since at that moment the pH rises to its maximum value, which means that the FAN concentration is also at its maximum value.

2.4. Performance indicators

The following five performance indicators were proposed and calculated to assess the influence of FAN on the enrichment reactor.

2.4.1. Biomass specific substrate uptake rate (q_{sCOD})

The biomass specific substrate uptake rate was defined as the amount of soluble COD (sCOD) converted during feast per amount of biomass and per hour (feast length expressed as hours). For the calculation, the converted amount of sCOD was estimated via a mass balance approach, while the amount of active biomass was estimated as the grams of VSS minus PHA at the end of cycle (assuming PHA content is negligible) minus the grams of inert VSS at the end of cycle which came from the substrate of the previous cycle (see Equation 2). It is worth mentioning that the VSS originated from the substrate were assumed to be permanently in suspension, so a fraction of the total will either go into the supernatant, go into the effluent to the accumulation reactor or remain inside the enrichment reactor for the next cycle.

$$q_{sCOD}\left(\frac{g\ sCOD}{g\ VSS.h}\right) = \frac{g\ sCOD_{EoC} + g\ sCOD_{substrate} - g\ sCOD_{SoS}}{\left(g\ VSS_{EoC} - g\ VSS_{EoC_{inert}}\right) * feast\ length\ in\ hours} \quad \text{... (Equation 2)}$$

Where

$$g\ VSS_{EoC_{inert}} = g\ VSS_{substrate} * (\frac{vr1}{1-vr1}) \text{ and } vr1 = volume\ ratio\ 1 = \frac{Liters_{EoC}}{Liters_{SoS}} = \frac{38.6\ L}{173.1\ L}$$

2.4.2. PHA production yield (Y_{PHA})

The PHA production yield was defined as the amount of PHA that is produced during feast per amount of sCOD converted during feast. For the calculation, the amount of PHA was estimated from the measured PHA content at start of settling and the amount of sCOD was estimated via a mass balance approach (as it was conducted for q_{sCOD}) (see Equation 3). It is important to note that the

PHA yield can decrease if less efficient PHA producers or non-PHA producers proliferate in the enrichment reactor.

$$Y_{PHA}\left(\frac{g\ PHA}{g\ sCOD}\right) = \frac{\frac{PHA}{TSS}\%_{SoS} * g\ TSS_{SoS}}{g\ sCOD_{EoC} + g\ sCOD_{substrate} - g\ sCOD_{SoS}} \quad \text{... (Equation 3)}$$

2.4.3. Settleable biomass production yield ($Y_{X\,sett}$)

The settleable biomass production yield was defined as the amount of produced biomass that goes to the accumulation per amount of sCOD converted during the whole cycle and it can be interpreted as an "economical" yield of produced PHA biomass per unit of carbon fed to the reactor. It is important to note that the amount of biomass that could be lost into the supernatant is not considered in the calculation of this indicator, thus, it represents an underestimation of the real biological yield. For its calculation, the amount of biomass was estimated as grams of VSS in the inoculum, i.e. biomass to the accumulation reactor, (assuming a steady state enrichment reactor and negligible PHA content) minus grams of VSS originated from the substrate that goes into the accumulation reactor as part of the inoculum stream. The amount of sCOD converted during the cycle was estimated via a mass balance approach (see Equation 4).

$$Y_{X_{sett}}\left(\frac{g\ VSS}{g\ sCOD}\right) = \frac{g\ VSS_{inoculum} - (gVSS_{substrate} * vr2)}{g\ sCOD_{substrate} - g\ sCOD_{supernatant} - g\ sCOD_{inoculum}} \quad \text{... (Equation 4)}$$

Where

$$vr2 = volume\ ratio\ 2 = \frac{Liters_{\ Inoculum}}{Liters_{\ Substrate}} = \frac{45.7\ L}{134.5\ L}$$

2.4.4. Biomass loss after settling percentage (BLAS%)

The biomass loss after settling percentage is the fraction of biomass that is lost into the supernatant after the settling phase is over. This was estimated using the amount of VSS after settling and the amount of VSS at the start of settling. The first value was corrected by subtracting the amount of VSS originated from the substrate that remains in the reactor after settling and the amount of inert VSS at the end of cycle, while the second value was corrected by subtracting the total amount of VSS originated from the substrate and the amount of inert VSS at the end of cycle (see Equation 5).

$$BLAS\ (\%) = \left(1 - \frac{g\ VSS_{AS} - (\left(g\ VSS_{substrate} + g\ VSS_{EoC_{inert}}\right) * vr3)}{g\ VSS_{SoS} - g\ VSS_{substrate} - g\ VSS_{EoC_{inert}}}\right) * 100 \quad \text{... (Equation 5)}$$

Where

$$g\ VSS_{EoC_{inert}} = g\ VSS_{substrate} * \left(\frac{vr1}{1 - vr1}\right) \text{ and } vr\ 3 = volume\ ratio\ 3 = \frac{Liters_{\ AS}}{Liters_{\ SoS}} = \frac{84.3\ L}{173.1\ L}$$

2.4.5. Sludge volume index (SVI_{30})

Conceptually, the sludge volume index corresponds to the inverse of the sludge density. In this study, the proposed SVI_{30} characterizes only the biomass that settles to the bottom as sludge right after the feast ("start of settling" in Figure 4) and it can be interpreted as biomass fluffiness: a higher value indicates a lighter sludge while a low value indicates a heavier sludge. For its calculation, the dry weight of the settled sludge after 30 minutes was measured: first, the sludge was separated via a valve at the bottom of a sedimentation Imhoff cone, then, it was dried for 24 hours at 110 °C and lastly, it was weighed. Finally, the SVI_{30} was calculated by dividing the volume of settled sludge after 30 minutes (SV_{30}) by the dry weight of the settled sludge (see Equation 6).

$$SVI_{30}\left(\frac{mL}{g}\right) = \frac{\frac{mL}{L}\, SV_{30\ SoS}}{\frac{g}{L}\, dry\ solids_{30\ SoS}} \quad \text{... (Equation 6)}$$

3. Results and discussion

3.1. Feeding conditions of the enrichment reactor

3.1.1. Soluble COD concentrations

From the beginning of the research period, it was aimed to have a sCOD concentration of 5.5 – 6.5 g/L in the substrate. However, due to unforeseen operational problems in the hydrolysis pilot, this concentration was not always in the desired range. As shown in Figure 5,from the 11th till the 25th of March, the soluble COD in the substrate was significantly below the expected concentration. This event was caused by a batch of poor-quality waste that was used in the hydrolysis pilot, thus producing leachate with less VFA and overall sCOD concentration. The poor performance was corrected by adding a new batch of fresh waste around the 20th of March; that way, the leachate quality improved after a week.

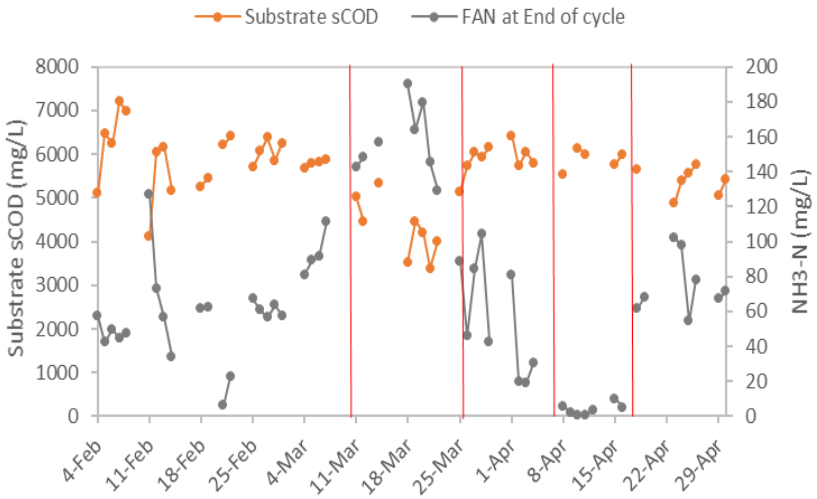


Figure 5. Historical profile of soluble COD in the substrate and free ammonia at the end of cycle.

3.1.2. Total ammonia and free ammonia concentrations

At the beginning of this research period, the nitrogen addition during the famine phase was fixed to 13.6 grams of nitrogen as urea. On the first day, the TAN and FAN concentrations at the end of cycle were around 160 mg/L and 60 mg/L respectively (see Figure 6). From these initial values, TAN and FAN increased due to the recirculation of the enrichment reactor effluents which contain residual ammonia nitrogen (see Figure 1).

From the 4th of March, this increasing TAN and FAN trend became significantly steeper, which could be related to the decrease of sCOD in the substrate: since the microbial enrichment had less carbon available, it grew less and used less nitrogen, thus, causing the accumulation of nitrogen in the SBR. The highest concentration was observed on the 18th of March, and after the nutrient pump was turned off the 20th of March, TAN and FAN started to decrease. It is important to note that due to

the high pH during this period (see Figure 7), the FAN concentration did not decrease at the same ratio as TAN.

The nutrient pump was turned on again the 26th of March in order to avoid nitrogen-limited conditions but since the biomass content in the reactor did not increase at FAN concentrations around 100 mg/L, the nutrient pump was turned off again on the 1st of April. Finally, the biomass content increased and recovered its characteristic white color on the 8th of April when TAN and FAN concentrations were close to 0 mg/L. This concentration was kept for a week in order to confirm the recovery of the biomass content. From the 18th of April, the nutrient pump was turned on in order to avoid nitrogen-limited conditions and the FAN concentration was kept around 70 mg/L. Under these conditions, the biomass content remained stable.

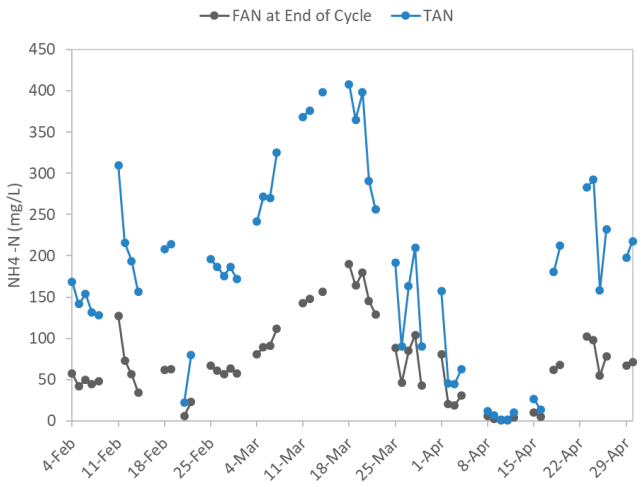


Figure 6. Historical profiles of total ammonia and free ammonia at the end of cycle.

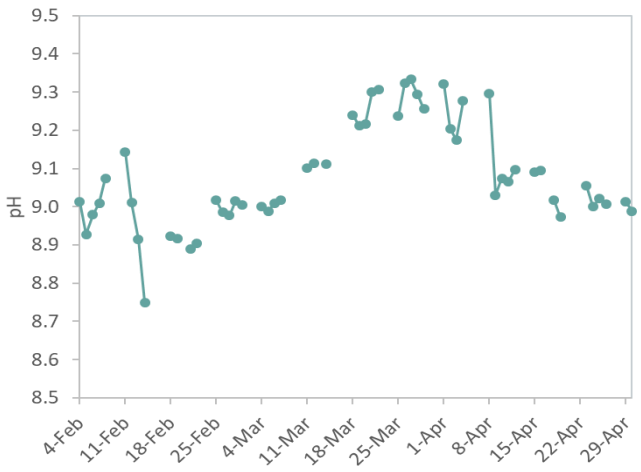


Figure 7. Historical profile of the pH at the end of cycle.

3.1.3. Pilot-study subperiods

As the feeding conditions were shifting during this pilot study, five subperiods were identified according to the different sCOD and FAN concentrations and biomass conditions (see Table 1 and Figure 5). These subperiods were used for the correlation analysis between FAN and the different performance indicators (see section 3.2).

Table 1. Periods for correlation analysis.

#	TIME PERIOD	SUBSTRATE	FAN concentration	Biomass condition
1	4 Feb - 7 Mar	5 - 7 g sCOD/L	Increasing	Stable
2	11 Mar – 25 Mar	< 5.5 g sCOD/L	Peak of 190 mg/L and decline	High loss of biomass
3	26 Mar – 5 Apr	5 - 7 g sCOD/L	Declining	Recovery
4	8 Apr – 17 Apr	5 - 7 g sCOD/L	Stable (avg 4 mg/L)	Recovery
5	18 Apr – 30 Apr	5 - 7 g sCOD/L	Stable (avg 75 mg/L)	Stable

3.2. Performance of the enrichment reactor and the influence of free ammonia

The performance of the enrichment reactor was evaluated based on the microscope pictures and the five performance indicators. The influence of free ammonia on each of the indicators is discussed based on correlation analysis.

3.2.1. Microbial community

The microbial community of PHA producers shifted during the three months of experimentation. During the first week of February, the community was dominated by small PHA producing bacteria that could reach a maximum size of 2.5 µm after feast (see Figure 8A). Later, in the middle of February, bigger PHA producing bacteria appeared in the sludge. These bigger microorganisms could reach a size of 4-5 um after feast (see Figure 8B) and via a fluorescence in situ hybridization (FISH) analysis (see Figure 9), they were identified as a different species (named *Candidatus gubernatori,* which has not been described in literature prior to this study).

It is hypothesized that the shift in the microbial community composition was due to the slight increase of FAN at the end of cycle (to around 70 mg/L) (see Figure 6). As a comparison, Neumann et al. [15] found that *Pseudomonas putida* would increase their cell size and therefore, decrease its relative surface (relative to its volume) in order to reduce the toxic effect of aromatic compounds. Similarly, a bigger PHA bacteria could resist the toxic effect of higher FAN concentrations.

By late March, the microbial community was dominated by small and big PHA producing bacteria, and it was noticed that the big bacteria were losing its ability to aggregate in flocs (see Figure 8D), which could be due to less extracellular polymeric substances (EPS) production. This event coincided with the highest peak of FAN registered during this research (190 mg/L) and the massive loss of biomass content in the reactor (see Table 1), which could be related to the disaggregation of the big

bacteria. Past studies about aerobic granular sludge concluded that high free ammonia concentrations could repress EPS production and therefore hinder the formation of granules [13], [16].

During the last week of March and first week of April, the FAN concentration at the end of feast was decreased in order to recover the biomass content and overall performance of the reactor. From Figure 8E, it was observed that the aggregated flocs were smaller and the PHA bacteria looked different from the previous weeks. While sampling the sludge, it was also observed that the sludge after feast changed and half of it looked like tiny precipitates (possibly struvite).

From the 8th of April, the sludge after feast increased its volume and recovered its characteristic white color. From Figure 8F, it was observed that the flocs size was recovered, and that the community was dominated by the small PHA producing bacteria. This event coincided with the low FAN concentrations, which was kept low in order to fully recover the biomass content in the reactor. From the 18th of April, the FAN concentration was increased to concentrations around 80 mg/L and from Figure 8G it was observed that the big PHA bacteria appeared once again.

This last observation suggests that the big PHA bacteria require a higher minimum FAN concentration compared to the small PHA bacteria. Small bacteria have a larger relative surface (relative to its volume) which could facilitate the absorption and consumption of ammonia for growth [15]. Meanwhile, bigger bacteria have a smaller relative surface, making them less competitive for the absorption and consumption of ammonia. Therefore, it can be hypothesized that the big PHA producing bacteria might be nitrogen limited at low FAN concentrations. Free ammonia could diffuse through the cell membrane and then facilitate its consumption by the big bacteria.

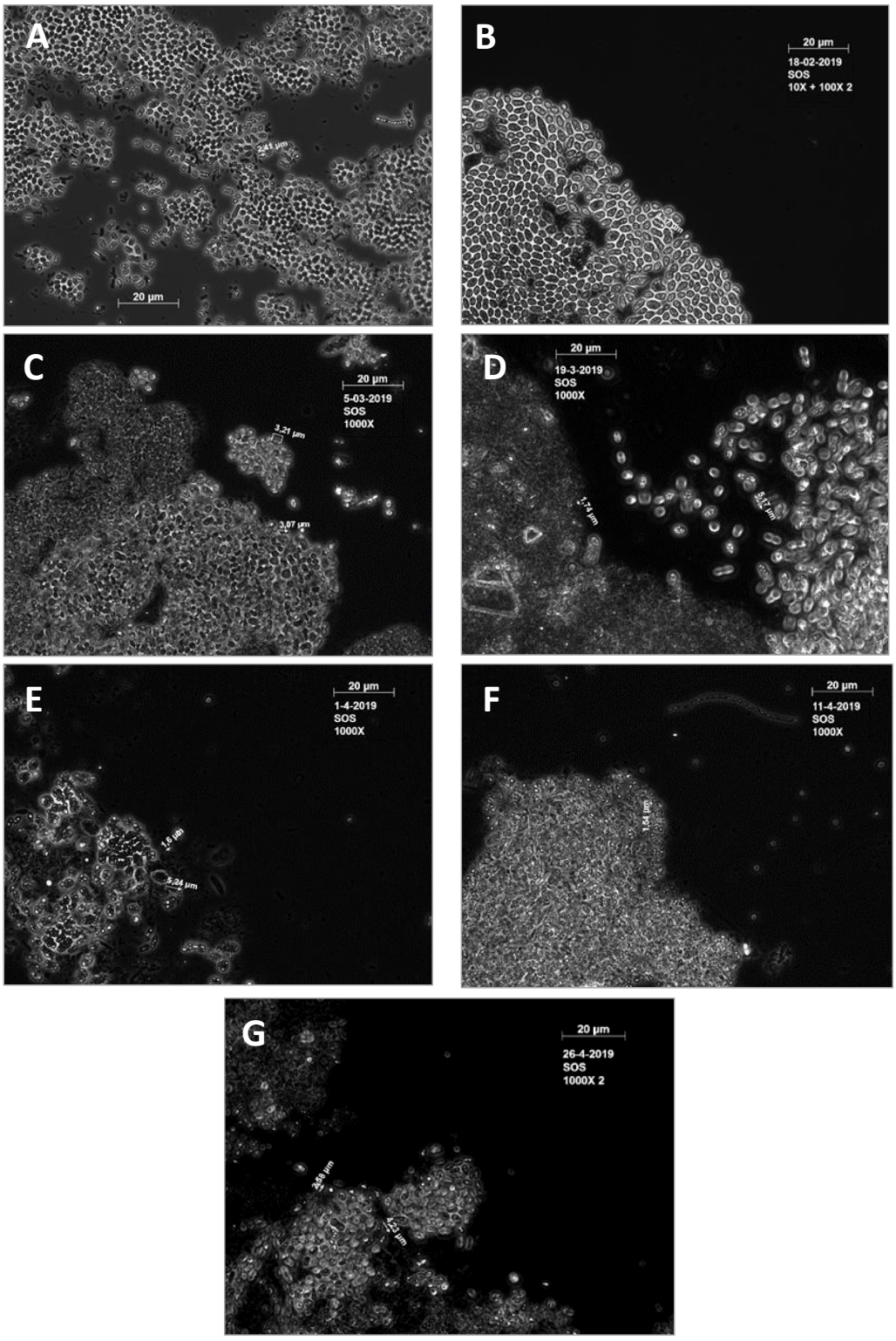


Figure 8. Biomass flocs at the end of feast: A) 5th of February, B) 18th of February, C) 5th of March, D) 19th of March, E) 1st of April, F) 11th of April and G) 26th of April.

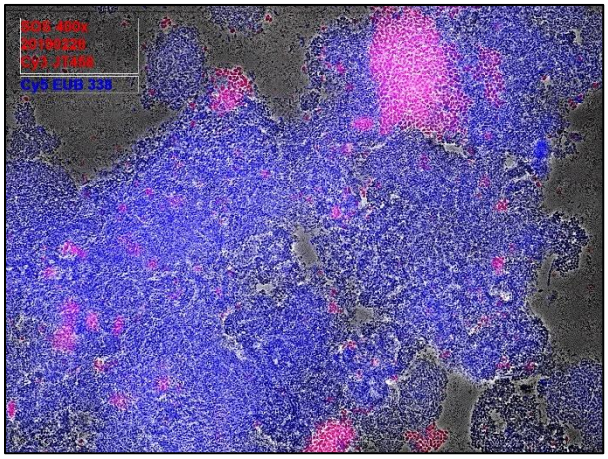


Figure 9. Biomass flocs at the end of feast composed of unidentified bacteria (blue) and Candidatus gubernatori (purple).

3.2.2. Biomass specific substrate uptake rate (q_{sCOD})

During the first research subperiod, the biomass specific substrate uptake rate started around 3 g sCOD/g VSS/h and then gradually increased up to 5.3 g sCOD/g VSS/h (see Figure 10A). This slight increase was poorly correlated to the increasing FAN concentration, as shown in Figure 10B. During the second subperiod, the specific uptake rate increased up to 6.3 g sCOD/g VSS/h, however, this increase was also poorly correlated to FAN. During the third subperiod, the specific uptake rate reached its maximum value (8.9 g sCOD/g VSS/h) and then started to decrease. The high specific uptake rate during this subperiod could be related to the poor aggregation among cells, providing a larger specific surface for carbon uptake. As previously observed, there was no apparent correlation between the rate and FAN as the green correlation dots in Figure 10B are scattered. During the last two subperiods, the specific uptake rate stabilized around 4 g sCOD/g VSS/h and it showed no apparent trend when plotted against FAN. The obtained rates during this study are comparable to the maximum VFA uptake rates obtained in other pilot studies: 7.8 g COD/g biomass/h using paper-mill wastewater [8] and 2.8 g COD/g biomass/h using effluent of a Mars candy bar factory [5].

The poor correlation between the specific substrate uptake rate and FAN suggests that regardless of any possible effect of FAN on the biomass during the famine phase, the specific uptake rate during feast will not be affected by the increasing or decreasing concentration of TAN/FAN at the end of cycle. Other factors could be responsible for the shifting specific uptake rate, such as the pH which was found to have a significant correlation with it (see Appendix 1). Thus, these results suggest the need of future research about the influence of other factors on the specific uptake rate.

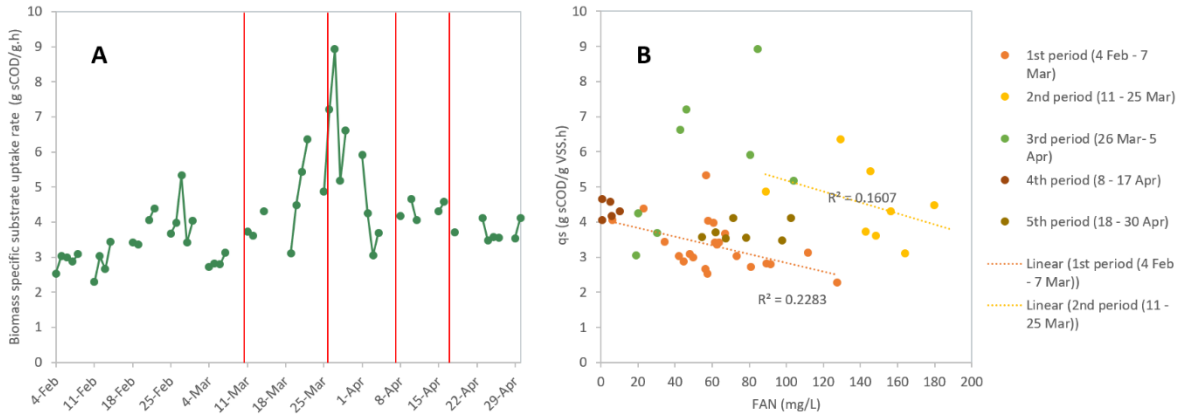


Figure 10. Biomass specific substrate uptake rate results: A) Historical profile and B) Correlation with FAN.

3.2.3. PHA production yield (Y_{PHA})

During the first subperiod, the PHA production yield remained around 0.55 g PHA/ g sCOD despite the gradual and slight increase of FAN (see Figure 11A). Later, from the start of the second subperiod, the yield started to decrease up to 0.17 g PHA/ g sCOD which coincided with the pronounced increase of FAN at the end of feast. This event could be due to the loss of PHA producers which is possibly related to high FAN concentrations. Once the FAN concentrations were decreased during the third subperiod, a noticeable recovery of the PHA yield is observed. However, the yield remains unstable and it stabilizes during the fourth subperiod (around 0.45 g PHA/ g sCOD) and increases slightly during the last subperiod (around 0.50 g PHA/ g sCOD).

Based on the correlation analysis between the PHA yield and FAN concentrations during the whole research period, there is a noticeable trend with a coefficient of determination of 0.44 (see Figure 11B). It is worth mentioning that this correlation with FAN was found to be independent from the pH since no significant correlation was found between pH and the PHA yield (see Appendix 2). The regression line in Figure 11B shows that the PHA yield reaches its maximum around a FAN concentration of 60 mg/L. With lower FAN concentrations, as during the fourth subperiod, the yield decreases slightly, which might be related to a dominance of small PHA producers, as it was described in section 3.1.2. Therefore, it is hypothesized that small PHA bacteria have a lower PHA production yield.

On the other hand, with higher FAN concentrations, as during the second subperiod, the PHA yield decreases dramatically possibly due to the loss of PHA producers in the reactor. Since the SBR empties itself around half of its volume at the end of cycle, the PHA bacteria need to duplicate each cycle in order to maintain a stable PHA producer population in the reactor [3]. The stored PHA during the feast phase is used for growth and maintenance during the famine phase [17], [18]. However, if the FAN concentration rises during the famine phase, the PHA bacteria would be forced to use the stored polymer for solely maintenance rather than cell duplication [12], [13]. Thus, a high FAN concentration during the famine phase could hinder PHA bacterial growth, which would decrease

the PHA producer population for the next cycle, and as a result, the PHA production yield during next cycle's feast would also decrease.

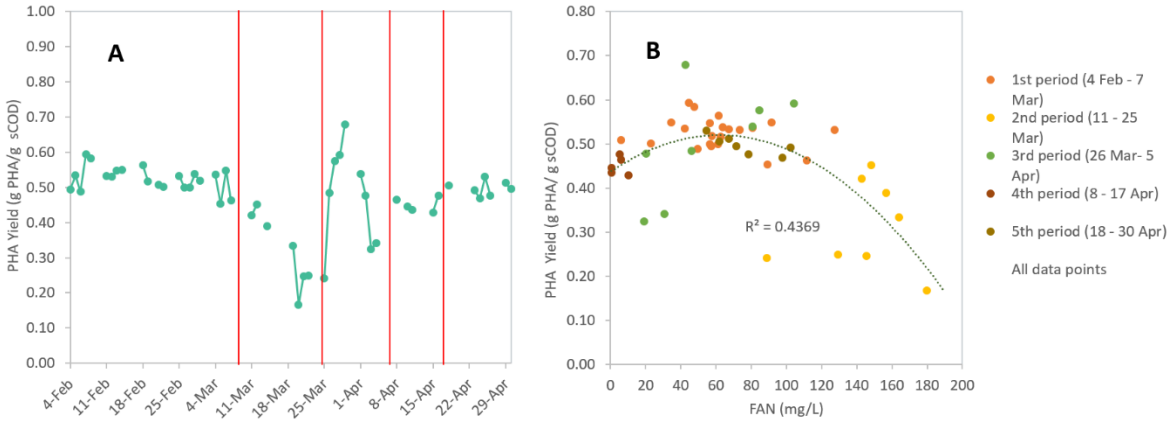


Figure 11. PHA production yield results: A) Historical profile and B) Correlation with FAN.

3.2.4. Settleable biomass production yield ($Y_{x\,sett}$)

During the first research subperiod, the settleable biomass yield shifted every day around 0.33 g VSS/ g sCOD (see Figure 12A). During the second subperiod, the yield has a decreasing trend which coincided with the increasing FAN concentrations at the end of cycle. Later, during the third and fourth subperiod, the yield has an increasing trend which happened right after the FAN concentration at the end of cycle was gradually decreased. Lastly, during the fifth subperiod, the FAN concentration was risen to around 70 mg/L, and at the same time, the settleable biomass yield stopped increasing and remained around the value of 0.25 g VSS/ g sCOD.

Based on the correlation analysis between the settleable biomass yield and FAN concentrations, a significant correlation (R^2 = 0.62) was found solely during the first subperiod: at higher FAN concentrations during the famine phase, the settleable biomass yield decreases (see Figure 12B). High FAN concentrations could hinder biomass growth by diverting energy storage (PHA) to mainly maintenance, i.e. detoxification of free ammonia, instead of bacterial growth [12], [13]. It is important to note that this correlation with FAN was found to be independent from the pH since no significant correlation was found between pH and the settleable biomass yield (see Appendix 3).

It is important to note that by following the dots according to the colors in Figure 12B, it is possible to observe how the biomass yield shifted in time. From high yields at the beginning of the first subperiod (first orange dots), to lower values when FAN increased (orange and yellow dots), followed by a recovery subperiod (green dots) and then a rapid yield increase when FAN was nearly 0 mg/L (dark red dots). Lastly, the biomass yield recovered to similar values as during the first subperiod (brown dots).

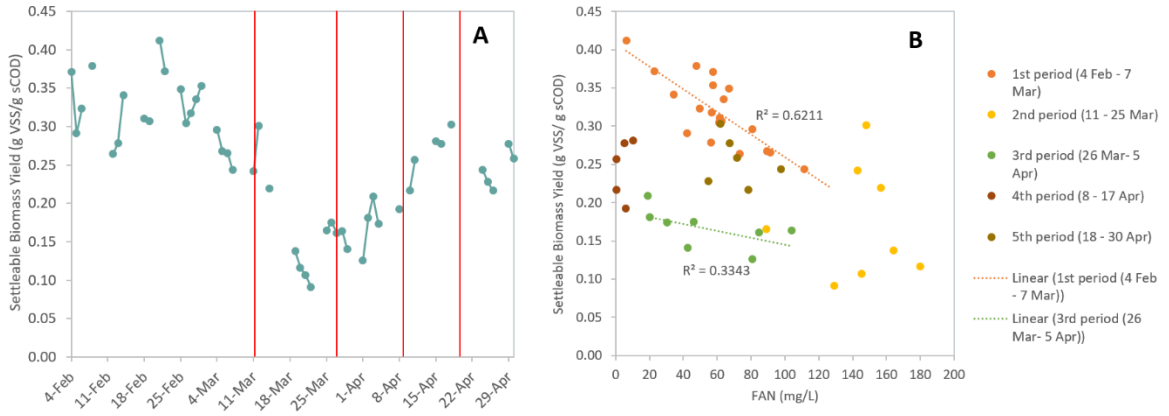


Figure 12. Settleable biomass production yield results: A) Historical profile and B) Correlation with FAN.

3.2.5. Biomass loss after settling percentage (BLAS%)

The biomass loss after settling (BLAS) percentage represents the suspended biomass that is not able to settle and that is withdrawn from the reactor as part of the supernatant. According to Figure 13A, during the first subperiod, BLAS ranged from -2.8% to +13.1% which is an acceptable loss considering that the settling phase purpose is to select the heavier PHA bacteria after feast, which are assumed to have stored more PHA [9]. Additionally, N_2 gas could have contributed to the poor settleability since during this research, nitrifying activity was detected some days (NO_2-N < 6 mg/L and NO_3-N < 11 mg/L) which means that denitrification could have occurred simultaneously.

During the second subperiod, the BLAS percentage had an overall increasing trend which coincided with the increasing FAN concentrations. During the third subperiod, the BLAS% reached its peak (50%) a few days after FAN also reached its peak. Similarly, the BLAS% decreased a few days after the FAN concentrations were decreased. During the fourth subperiod, BLAS% kept a decreasing trend around the value of 20%. It is important to note that during this subperiod FAN was close to 0 mg/L which could enhance the dominance of small PHA bacteria (see section 3.2.1). Smaller cells are assumed to have a lower density and therefore, settle poorly, inducing a higher BLAS percentage [9]. Finally, during the fifth subperiod, when the FAN concentration was controlled around 70 mg/L, BLAS shifted mostly around the value of 12%.

According to Figure 13B, there is an observable trend (R^2= 0.43) between FAN and BLAS% when considering all the subperiods but the third one. The third subperiod's green dots are mostly scattered above the trend line and represent the time when the PHA yield was unstable and the biomass yield was recovering. Nonetheless, the curvilinear trend suggests that at very low FAN concentrations, BLAS is around 20%, possibly due to the dominance of smaller PHA bacteria which settle poorly, while at extremely high FAN concentrations (yellow dots), BLAS increases up to 33.4% possibly due to the toxic effect of FAN on the bacteria which might die off during the famine phase and later be washed-out on the next cycle's settling phase and supernatant removal. Around an intermediate FAN concentration of 80 mg/L, BLAS seems to be minimized to a value around 5%,

possibly thanks to a co-dominance of small and big PHA bacteria in the microbial community. These observations about the influence of free ammonia on the BLAS% are not conclusive and other factors could have had a more important role on this mechanics. For instance, the increasing pH could have also caused the loss of biomass (see Appendix 4).

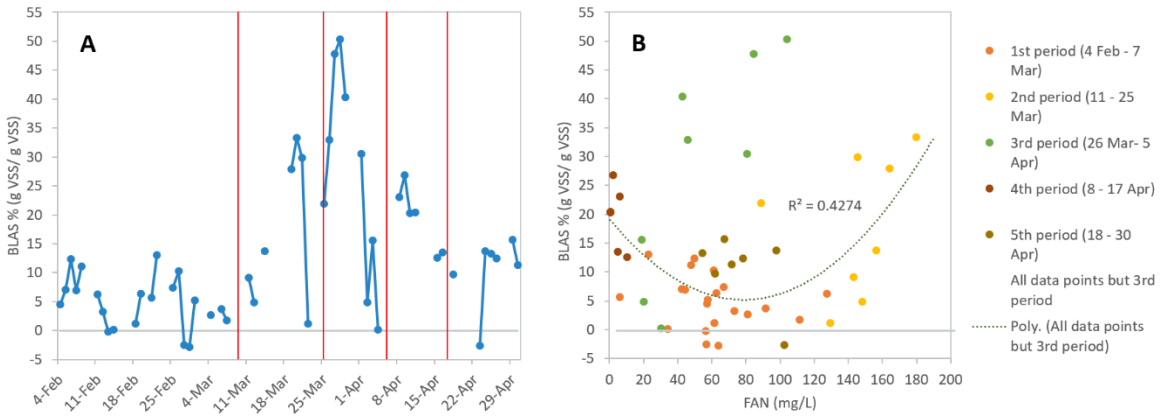


Figure 13. Biomass loss after settling results: A) Historical profile and B) Correlation with FAN.

3.2.6. Sludge volume index (SVI_{30})

The sludge volume index can be interpreted as fluffiness of the sludge that achieves to settle to the bottom within 30 minutes. It is important to note that during that settling period, other compounds different from biomass can also settle such as precipitates of struvite (NH_4MgPO_4), thus, the sludge is a mixture of both. As a result, it is important to interpret this indicator while considering other indicators such as BLAS%. For instance, if the biomass loss after settling is high (around 30%), the measured SVI_{30} will be an indicator of fluffiness for the combination of 70% of the VSS at the end of feast (which is the portion that achieved to settle) and precipitates. In the following analysis, the precipitates content in the sludge is assumed to be relatively constant (compared to BLAS% variations) based on the historical profile of ash content (see Appendix 6).

During the first research subperiod, the BLAS percentage was less or equal to 13.1% (see Figure 13A) so the SVI_{30} was fairly representative for the biomass at the end of feast. At the beginning of this subperiod, the SVI_{30} remained around 75 mL/g, later, it decreased to around 50 mL/g, and then, increased to around 80 mL/g (see Figure 14A). As a comparison, Nereda aerobic granular sludge can reach an SVI of 50 mL/g which translates to compact and very fast settling biomass [19]. Therefore, the SVI_{30} of the sludge during the first subperiod indicates an overall low fluffiness i.e. a good settleability.

During the second subperiod, the BLAS increased up to 33.4% while SVI_{30} decreased from 60 to around 20 mL/g (see Figure 14A). By interpreting these two indicators together, it can be deduced that the low SVI_{30} values during this subperiod might correspond to a higher precipitates fraction rather than a more compact biomass. Sludge volume index around 20-30 mL/g for biomass has not

been reported earlier, as the best values correspond to Nereda reactors (50 mL/g). During the third subperiod, BLAS increased even further up to 50.3%, which indicates a second increase in the precipitates fraction in the sludge, but the SVI_{30} remained at low values around 30 mL/g. The slight increase in SVI_{30} despite the high BLAS% could be related to the moderate improvement in the settleable biomass yield during that same subperiod (see Figure 12A).

During the fourth period, the BLAS decreased and had an average value of 20% while the SVI_{30} increased noticeably up to 105 mL/g and FAN was close to 0 mg/L. The BLAS value indicates that the precipitates fraction in the settled sludge decreased considerably and the high SVI represents a very fluffy biomass which could be related to the fact that the microbial community was most likely dominated by the small PHA producers, as it was shown in Figure 8F. Lastly, during the fifth subperiod BLAS decreased to the value around 12% and simultaneously, the SVI_{30} decreased. Both indicators changed when FAN concentration was risen around 70 mg/L. Just as previously discussed in section 3.2.5, both indicators changed possibly due to the recovery of the big PHA producers which presumably are heavier and thus, constitute a less fluffy sludge.

According to Figure 14B, there is an inverse proportional relationship between FAN and SVI (R^2= 0.43) if all the data but the third subperiod (green dots) is considered (just as the correlation between BLAS and FAN was conducted). It is important to note that this correlation was found to be independent from the pH since no significant correlation was found between pH and SVI_{30} (see Appendix 5). This result suggests that low FAN concentrations could enhance a fluffier biomass, possibly due to a dominance of smaller PHA bacteria, while an intermediate FAN concentration could produce a less fluffy biomass because of the appearance of big PHA bacteria (assumed to be heavier and more compact) in the microbial community. Extremely high FAN could produce an apparent very compact sludge; however, it would have a higher precipitates fraction, most likely because the toxic effect of FAN can reduce the settleable biomass content in the reactor (previously discussed in section 3.2.4 and 3.2.5).

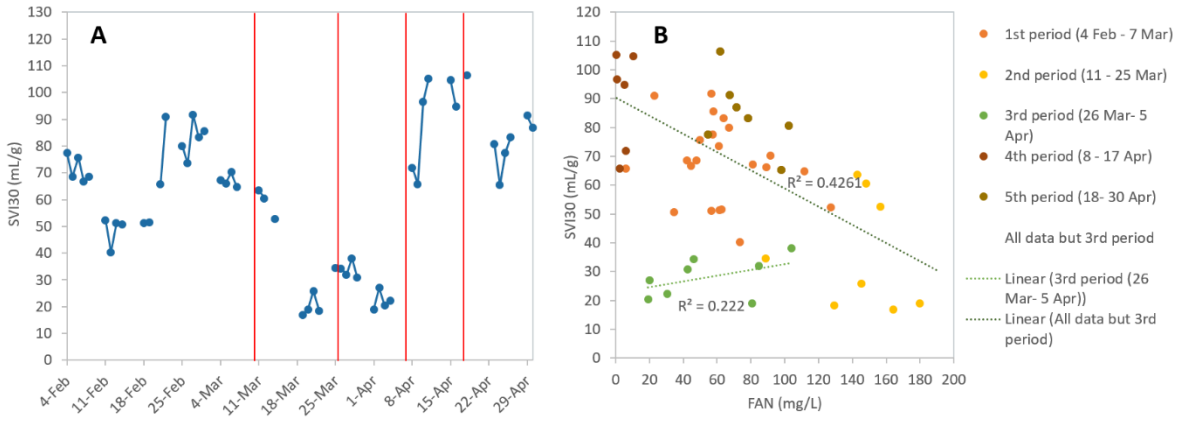


Figure 14. Sludge volume index results: A) Historical profile and B) Correlation with FAN.

3.3. Implications for the pilot plant and prospective research

During this pilot-scale research different factors could not be completely controlled, such as the shifting sCOD concentration of the substrate or the possible presence of unidentified toxic compounds in the substrate. Hence, it is difficult to dismiss other factors that could have been influencing the performance indicators at the same time as FAN. Despite not having conclusive results, this study suggests that controlling free ammonia concentrations around 70 mg/L at the end of cycle could enable a high PHA yield (0.50 g PHA/g sCOD) and good settling properties (SVI_{30} < 80 mL/g and BLAS < 5%) but a relatively lower settleable biomass production yield (around 0.30 g VSS/g sCOD) (see Figure 15).

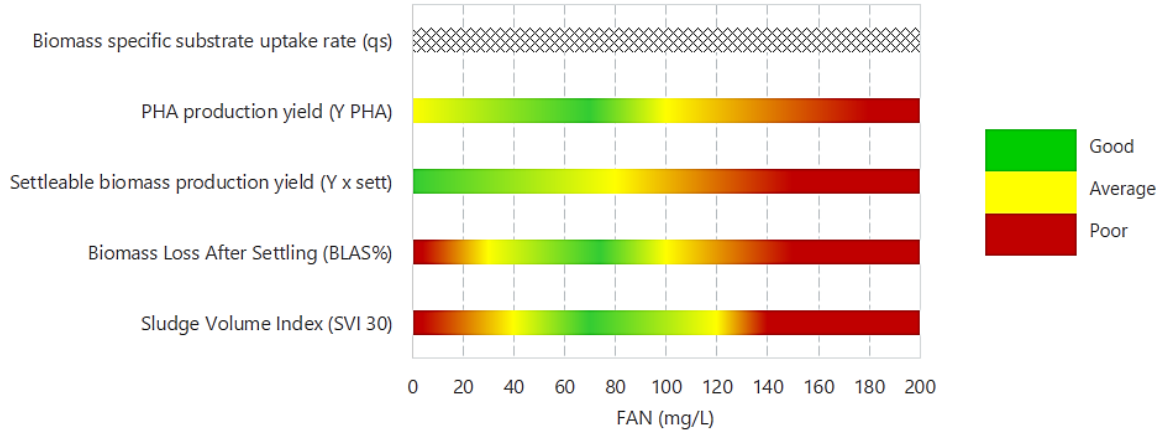


Figure 15. Free ammonia influence on the five microbial enrichment reactor performance indicators.

One of the uncontrolled factors that could have affected the performance was the conductivity of the substrate and effluent. This parameter could not be made independent from TAN concentrations: as a fact, it was found that TAN and FAN concentrations at the end of cycle had a good correlation with conductivity values. However, it is likely that the conductivity, i.e. osmotic pressure, did not have a significant effect on the performance since values in the SBR did not exceed 10 mS/cm. This conductivity is less than the value reported by Korkakaki et al. [7] who stated that a conductivity of 40 mS/cm (286mM NaCl) decreased *P.acidivorans* biological activity by 40%.

The pH was another parameter that was not controlled in the reactor and, in this study, it was found that it could have influenced the specific substrate uptake rate (q_s), the settleable biomass yield ($Y_{x\ sett}$) and the biomass loss after settling (BLAS%) (see Appendix 1, 3 & 4). Oehmen et al. [20] studied the effect of pH control on a PHA microbial enrichment and concluded that a controlled pH of 8 led to a particular microbial community and enabled an increase in the biomass growth rate and the PHA storage capacity. Therefore, it would be worth evaluating the influence of pH control on this pilot enrichment reactor while using the same five performance indicators used in this study.

Overall, the five performance indicators proved to be useful since each described a different feature of the enrichment reactor. The biomass specific substrate uptake rate indicates whether the

bacteria are inhibited by a toxic compound during the feast phase. Meanwhile, the PHA production yield indicates how efficient the microbial community is to accumulate PHA, which could depend on the presence of a toxic compound, the efficiency of the individual PHA microbial species or the ratio of PHA producers and non-PHA producers in the microbial community. The settleable biomass production yield is very important as it indicates how much enriched biomass is produced for the accumulation reactor (the main purpose of the enrichment reactor). Lastly, the BLAS% and the SVI_{30} indicate the settleability of the sludge and it is important to determine the operational parameters (settling phase settings) and future up-scaling of the process.

Finally, this study allows the proposal of future research projects to test these pilot-scale observations in a lab-scale environment. It would be worth testing again the influence of FAN but in a completely controlled reactor fed with a fixed substrate composition and while controlling the conductivity and pH. This way, it would be possible to obtain more conclusive results regarding the influence of FAN and its possible use as an additional selective pressure for a PHA microbial enrichment reactor.

4. Conclusions

The influence of free ammonia on the performance of the PHA microbial enrichment reactor in the pilot plant was found to be both positive and negative. Increasing TAN and FAN concentrations led to a shift in the microbial community. Bigger PHA bacteria (4-5 µm after feast) appeared from FAN concentrations higher than 50 mg/L, however, concentrations higher than 150 mg/L were highly toxic for the whole PHA microbial enrichment.

From the correlation analysis, it was suggested that FAN concentrations around 60 mg/L lead to maximum PHA yields (around 0.50 g PHA/g sCOD), possibly due to the presence of big PHA producers which are assumed to be more efficient. Meanwhile, increasing FAN concentrations may lead to a decreasing settleable biomass yield, most likely caused by the diversion of energy for maintenance rather than growth during the famine phase. On the other hand, the specific substrate uptake rate was found to be independent from FAN concentrations at the end of cycle.

The correlation analysis with BLAS and SVI_{30} suggests that low FAN values (< 60 mg/L) might lead to a fluffier biomass (SVI_{30} > 80 mL/g) that is not able to settle efficiently and therefore, increase the biomass loss through the supernatant. On the contrary, higher FAN concentration (> 80 mg/L) may lead to high BLAS% as a result of its toxic influence on the biomass during the famine phase. In conclusion, it was suggested that a FAN concentration around 70 mg/L might produce a microbial enrichment with good settling properties (SVI_{30} < 80 mL/g and BLAS < 5%) and a high PHA yield (0.50 g PHA/g sCOD), but a lower settleable biomass production yield (around 0.30 g VSS/g sCOD).

This study found all the proposed performance indicators to be useful for monitoring the pilot-scale enrichment reactor. These indicators shall be used for the future evaluation of other parameters influence on the reactor performance, for example, while controlling the pH in the reactor. Finally, it is suggested to test the observations of this pilot study in lab-scale experiments in order to get more conclusive results about the potential of FAN as an additional selective pressure in a PHA microbial enrichment.

Acknowledgements

This study was possible thanks to the collaboration of all the Lelystad Innovation Center (LIC) team members, both supervisors and interns. Also, the author would like to thankfully acknowledge Jeffrey Croonenberg for all the TSS, VSS and PHA analytical measurements and the microscope pictures, as well as, Diego Doornbos for the FISH analysis.

References

[1] M. G. E. Albuquerque, M. Eiroa, C. Torres, B. R. Nunes, and M. A. M. Reis, "Strategies for the development of a side stream process for polyhydroxyalkanoate (PHA) production from sugar cane molasses," *J. Biotechnol.*, vol. 130, no. 4, pp. 411–421, Jul. 2007.

[2] K. Johnson, Y. Jiang, R. Kleerebezem, G. Muyzer, and M. C. M. Van Loosdrecht, "Enrichment of a mixed bacterial culture with a high polyhydroxyalkanoate storage capacity," in *Biomacromolecules*, 2009, vol. 10, no. 4, pp. 670–676.

[3] Y. Jiang, L. Marang, R. Kleerebezem, G. Muyzer, and M. C. M. van Loosdrecht, "Polyhydroxybutyrate production from lactate using a mixed microbial culture," *Biotechnol. Bioeng.*, vol. 108, no. 9, pp. 2022–2035, Sep. 2011.

[4] S. Bengtsson *et al.*, "A process for polyhydroxyalkanoate (PHA) production from municipal wastewater treatment with biological carbon and nitrogen removal demonstrated at pilot-scale," *N. Biotechnol.*, vol. 35, pp. 42–53, Mar. 2017.

[5] J. Tamis, K. Lužkov, Y. Jiang, M. C. M. va. Loosdrecht, and R. Kleerebezem, "Enrichment of Plasticicumulans acidivorans at pilot-scale for PHA production on industrial wastewater," *J. Biotechnol.*, vol. 192, no. Part A, pp. 161–169, Dec. 2014.

[6] B. Colombo *et al.*, "Enhanced polyhydroxyalkanoate (PHA) production from the organic fraction of municipal solid waste by using mixed microbial culture," *Biotechnol. Biofuels*, vol. 10, no. 1, Aug. 2017.

[7] E. Korkakaki, M. Mulders, A. Veeken, R. Rozendal, M. C. M. van Loosdrecht, and R. Kleerebezem, "PHA production from the organic fraction of municipal solid waste (OFMSW): Overcoming the inhibitory matrix," *Water Res.*, vol. 96, pp. 74–83, Jun. 2016.

[8] J. Tamis, M. Mulders, H. Dijkman, R. Rozendal, M. C. M. van Loosdrecht, and R. Kleerebezem, "Pilot-Scale Polyhydroxyalkanoate Production from Paper Mill Wastewater: Process Characteristics and Identification of Bottlenecks for Full-Scale Implementation," *J. Environ. Eng.*, vol. 144, no. 10, p. 04018107, Aug. 2018.

[9] Z. Chen, Z. Guo, Q. Wen, L. Huang, R. Bakke, and M. Du, "A new method for polyhydroxyalkanoate (PHA) accumulating bacteria selection under physical selective pressure," *Int. J. Biol. Macromol.*, vol. 72, pp. 1329–1334, Jan. 2015.

[10] E. Korkakaki, M. C. M. van Loosdrecht, and R. Kleerebezem, "Survival of the fastest: Selective removal of the side population for enhanced PHA production in a mixed substrate enrichment," *Bioresour. Technol.*, vol. 216, pp. 1022–1029, 2016.

[11] K. Johnson, R. Kleerebezem, and M. C. M. van Loosdrecht, "Influence of the C/N ratio on the performance of polyhydroxybutyrate (PHB) producing sequencing batch reactors at short SRTs," *Water Res.*, vol. 44, no. 7, pp. 2141–2152, Apr. 2010.

[12] X. Liu, Z. Shu, D. Sun, Y. Dang, and D. E. Holmes, "Heterotrophic Nitrifiers Dominate Reactors Treating Incineration Leachate with High Free Ammonia Concentrations," *ACS Sustain. Chem. Eng.*, vol. 6, no. 11, pp. 15040–15049, 2018.

[13] S. F. Yang, J. H. Tay, and Y. Liu, "Inhibition of free ammonia to the formation of aerobic granules," *Biochem. Eng. J.*, vol. 17, no. 1, pp. 41–48, 2004.

[14] A. D. Eaton *et al.*, *Standard methods for the examination of water and wastewater LK - https://tudelft.on.worldcat.org/oclc/40733179*, 20th ed. 1. Washington, DC SE - 1 volume (various pagings) : illustrations (some color) ; 29 cm: American Public Health Association, 1998.

[15] G. Neumann *et al.*, "Cells of Pseudomonas putida and Enterobacter sp. adapt to toxic organic compounds by increasing their size," *Extremophiles*, vol. 9, no. 2, pp. 163–168, 2005.

[16] X. Zheng *et al.*, "Inhibition of free ammonia to the granule-based enhanced biological phosphorus removal system and the recoverability," *Bioresour. Technol.*, vol. 148, pp. 343–351, 2013.

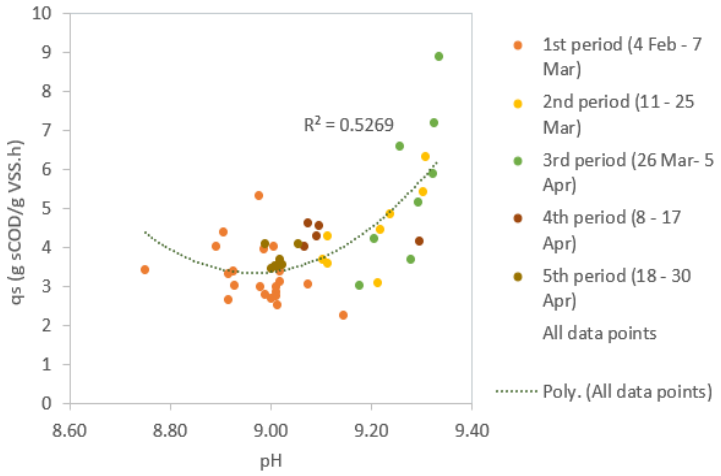
[17] Y. Jiang, M. Hebly, R. Kleerebezem, G. Muyzer, and M. C. M. van Loosdrecht, "Metabolic modeling of mixed substrate uptake for polyhydroxyalkanoate (PHA) production," *Water Res.*, vol. 45, no. 3, pp. 1309–1321, Jan. 2011.

[18] L. Marang, Y. Jiang, M. C. M. van Loosdrecht, and R. Kleerebezem, "Butyrate as preferred substrate for polyhydroxybutyrate production," *Bioresour. Technol.*, vol. 142, pp. 232–239, 2013.

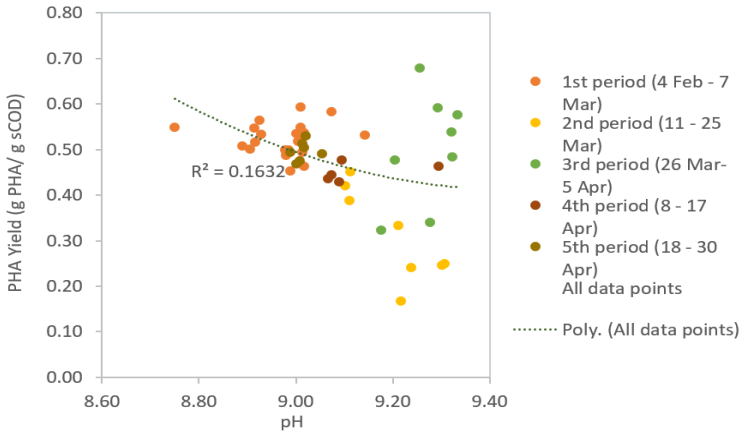
[19] M. Pronk, M. K. de Kreuk, B. de Bruin, P. Kamminga, R. Kleerebezem, and M. C. M. van Loosdrecht, "Full scale performance of the aerobic granular sludge process for sewage treatment," *Water Res.*, vol. 84, pp. 207–217, 2015.

[20] A. Oehmen, F. V. Pinto, V. Silva, M. G. E. Albuquerque, and M. A. M. Reis, "The impact of pH control on the volumetric productivity of mixed culture PHA production from fermented molasses," *Eng. Life Sci.*, vol. 14, no. 2, pp. 143–152, 2014.

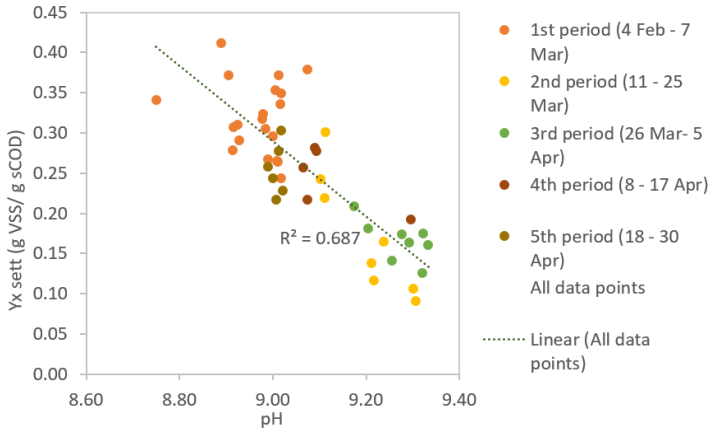
Appendices



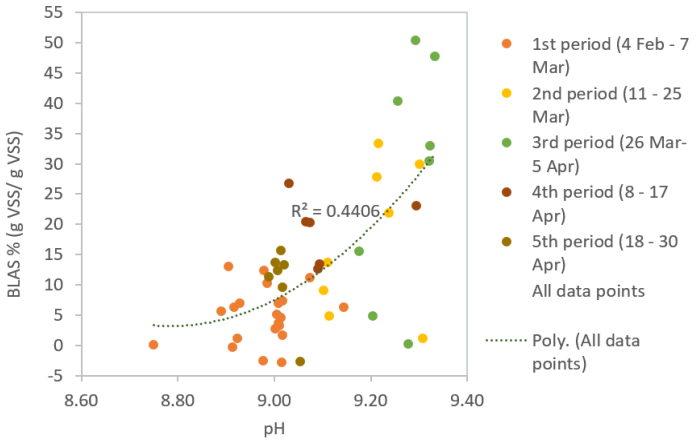
Appendix 1. pH and biomass specific substrate uptake rate correlation.



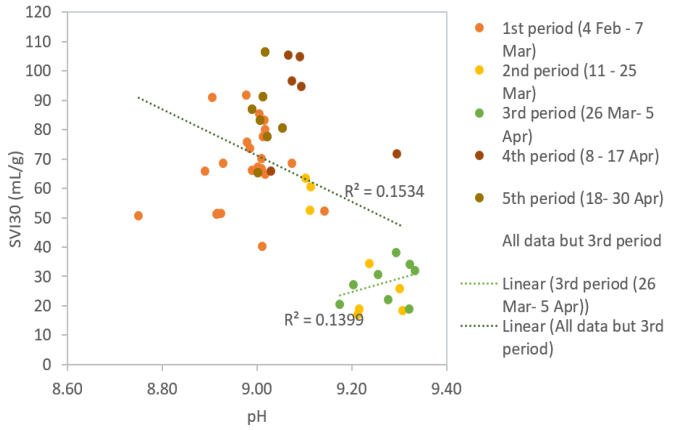
Appendix 2. pH and settleable VSS production yield correlation.



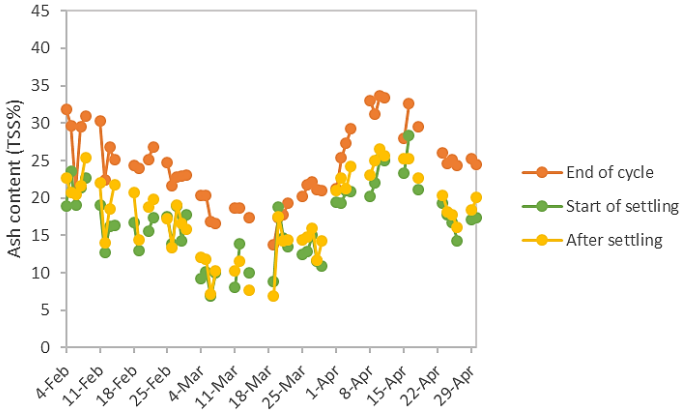
Appendix 3. pH and PHA production yield correlation.



Appendix 4. pH and BLAS percentage correlation.



Appendix 5. pH and Sludge volume index 30 correlation.



Appendix 6. Historical profile of ash content at the end of cycle and after settling.