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Volatile fatty acids production using waste activated sludge and food waste to produce bioplastics.

Producció d'àcids grassos volàtils mitjançant residus de fangs actius i de menjar per a produir bioplàstics.

Sergi Peña Picola

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"Those who can imagine anything, can create the impossible."

Alan Turing

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REPORT

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SUMMARY

Fossil fuel exhaustion, increasing greenhouse gases emissions and population growth, among many other issues, are leading to many environmental problems which require a transformation of the production system and waste management including wastewater treatment plants (WWTPs). Hence, the end-of-pipe processes for organic wastes treatment are being converted into resource recovery facilities that produce value-added products.

Anaerobic biological processes using mixed cultures can handle the variability of organic wastes: for example, these wastes could be initially converted to volatile fatty acids (VFAs) through acidogenic fermentation and the remaining part (non-VFA organic matter) could be directed to anaerobic digestion to produce biogas. VFAs have multiple applications and one of them is its use as carbon source for polyhydroxyalkanoates (PHA) production. PHA are value-added products mainly composed by polyhydroxybutyrate (PHB) and/or polyhydroxyvalerate (PHV), that can be obtained using mixed microbial cultures in 4 phases: acidogenic fermentation, selection of PHA-storing microorganisms, accumulation of PHA using selected biomass and PHA extraction.

In this Master thesis, the first 3 stages of the PHA production process using organic wastes (namely, the acidogenic fermentation, the selection of PHA-storing microorganisms and the PHA accumulation) are studied. The effect of pH on food waste (FW) and wasted activated sludge (WAS) co-fermentation was studied using batch tests and semi-continuous experiments at mesophilic conditions (35 ºC). The pH control in semi-continuous fermenters was difficulted by continuous foaming events. Moreover, PHA production using VFA-rich wastewater (simulating the effluent resulting from an acidogenic fermentation of the organic fraction of municipal solid wastes (OFMSW)) and different selection strategies (one using aerobic feast-famine with nitrogen depletion during feast and the other applying only aerobic feast-famine regime). Furthermore, the VFA profile obtained along the suitable operation of co-fermenters is compared with the synthetic stream used to assess PHA production.

The acidogenic fermentation unit was monitored with VFA profile and distribution, pH, chemical oxygen demand and soluble chemical oxygen demand (COD and sCOD), total ammonium nitrogen (TAN) concentration, total and volatile solids (TS and VS) and alkalinity. Batch tests were performed in glass-bottles filled with 150 mL of fixed proportions of WAS and FW (75:25 on VS basis) and using a lab-scale semi-continuous fermenter's effluent to test the influence of pH in substrates and an operating fermenter, respectively. Furthermore, 2 semi-continuous fermenters with 1.75 L of working volume were operated for 46 days using

fixed proportions of WAS and FW (~65:35 on VS basis), organic loading rate (OLR) (~11 g VS kg^{-1} d^{-1}), hydraulic retention time (HRT) (~3 d) and mixing at 80 rpm. Main VFAs resulted from batch tests and fermenters were acetic acid (~30%), butyric acid (~30%) and propionic acid (~20%). Higher pH showed an increased VFA yield as the solubilisation of organic matter (hydrolysis) was enhanced. Acetic acid consumption in fermenters was experimented and reduced by changing operational parameters (reduction in FW and HRT). Due to foam formation, pH control in semi-continuous operation could not been studied although different strategies were tested to minimise foam (better homogenisation, discharging foams using effluent tubes and lowering HRT and FW proportion).

Regarding to lab-scale PHA production, the monitoring of cycles was performed by recording dissolved oxygen (DO) profile, TAN and VFA concentrations, total and volatile suspended solids (TSS and VSS) and pH. VFA-rich synthetic influent with a concentration of 3.5 g COD L^{-1} for both selection and accumulation phases was used. Biomass selection was performed with a selection sequential batch reactor (sSBR) at 35 ºC and 80 rpm agitation with aerobic conditions using diffusors connected to air pumps and net-air. Furthermore, for accumulation tests a 1 L capacity glass reactor at 35 ºC, agitation at 80 rpm and air supply system were used. Experimental results showed that if double selection strategy (aerobic feast-famine plus nitrogen decoupling in feast) higher PHA content in purge was obtained (~30% on suspended solid (SS) basis) along with higher contents of PHA during accumulation (50% on SS basis) compared to a single selection strategy (only aerobic feast-famine regime) with PHA contents of 11% and 38% (SS basis), respectively. Similar PHA compositions were obtained through selection and accumulation phases with the ~90% in PHB and ~10% in PHV.

To sum up, raising pH increases VFA yields in acidogenic fermentation as consequence of hydrolysis and organic matter solubilisation enhancement. Moreover, increasing pH earlier in fermentation batch tests derives in higher VFA production. Due to PHA production, although both strategies selected the biomass successfully, double selection results in higher PHA accumulation potential. It is expected a suitable PHA production if fermenter's effluents are used (removing previously the nitrogen content) because of the higher ratios COD_{VFA} $sCOD^{-1}$ and the similar composition of VFAs.

1. INTRODUCTION

Current economic situation is ruled by fossil fuel reserves declining, greenhouse gases emissions, costly and problematic recycling and implacable population growth which demands more energy and products worldwide. A real alternative to the traditional oil economy is biotechnology-based strategies resulting in environmentally friendly biobased products. These biobased strategies can be conceived as biorefineries which use waste materials as raw source promoting circular economy (Octave & Thomas, 2009). Consequently, the role of wastewater treatment plants (WWTPs) is being transformed from removing of organic matter and nutrients such as nitrogen (N) and phosphorus (P) into an integrated resource recovery and pollution control (Faragò et al., 2021). Thus, the implementation of the circular economy requires a transition of waste treatment systems from an end-of-pipe to an integrated resource recovery facility (Perez-Esteban et al., 2022; Puyol et al., 2017).

Microbial mixed cultures can handle the complexity and variability of organic wastes producing carboxylates which can be efficiently converted to useful bioproducts (Agler et al., 2011). There is a wide range of technologies to valorise the organic wastes through anaerobic treatments such as anaerobic digestion to obtain biogas and the consequent use of the digested sludge as soil fertiliser (Morero et al., 2017), or obtaining carbon source as volatile fatty acids (VFAs) performing acidogenic fermentation with different substrates as food waste (Dahiya et al., 2015) or waste activated sludge (Xu et al., 2020) among others. Main valorised products obtained from these anaerobic treatments are methane (CH_4), VFAs (i.e., acetic, butyric, propionic, valeric and caproic acids) and hydrogen.

Acidogenic fermentation stands as an essential biotechnology in waste processing biorefineries since it allows converting organic waste into easily assimilable organic compounds such as VFAs, lactic acid and alcohols (Dahiya et al., 2015). The acidogenic fermentation process is implemented by controlling the operational conditions (pH, organic loading rate (OLR), hydraulic retention time (HRT)) to suppress methanogenesis phase on the anaerobic digestion process (Agler et al., 2011; Peces et al., 2021).

For waste streams, acidogenic mixed-culture fermentation is more readily achievable than other processes because of the complexity and variable composition of wastes besides the presence of inherent microorganisms in these. Thus, unlike pure-cultures and co-cultures fermentations, mixed-culture fermentation has limited control on the microbial community structure and dynamics, increasing the complexity towards achieving a full-scale process (Perez-Esteban et al., 2022).

1.1. Mixed culture acidogenic fermentation

Acidogenic fermentation is an anaerobic redox process in which organic matter is partially oxidized because of microbial enzymes and the resulting products are organic matter easily assimilable compounds as VFAs, alcohols, lactic acid, etc (Agler et al., 2011). Acidogenic fermentation applied to wastes is composed by three phases: (i) hydrolysis (hydrolytic-fermentative bacteria), (ii) acidogenesis (acidogenic bacteria) and (iii) acetogenesis (acetogenic bacteria).

Hydrolysis stage is based on the transformation of insoluble organic matter as carbohydrates, proteins and lipids into soluble organic matter as amino acids, long chain fatty acids (C_{12}-C_{22}) and sugars as poli-saccharides and mono-saccharides. Furthermore, the acidogenesis stage transforms soluble organic matter into VFAs as acetic (C_2), propionic (C_3), butyric (C_4) and valeric (C_5) acids although, other subproducts are obtained in this stage such as hydrogen (H_2), carbon dioxide (CO_2) as electron acceptor and pyruvate among many others. Finally, the acetogenesis stage is based on the conversion of the VFAs into acetate. Hydrogen is produced during all phases as electron exchanging donor (figure 2.1. Anaerobic mixed culture pathway (Dahiya et al., 2015)).

VFAs are the main product obtained from wastes' mixed-culture fermentation. VFAs are defined as short-chain fatty acids consisting of six or fewer carbon atoms which can be distilled at atmospheric pressure (Lee et al., 2014). Furthermore, VFAs can be used as easily assimilable carbon source and raw material for other bioprocesses such as biological nutrient removal-recovery or bioplastic production as polyhydroxyalkanoates (PHA) explained in this final master thesis.

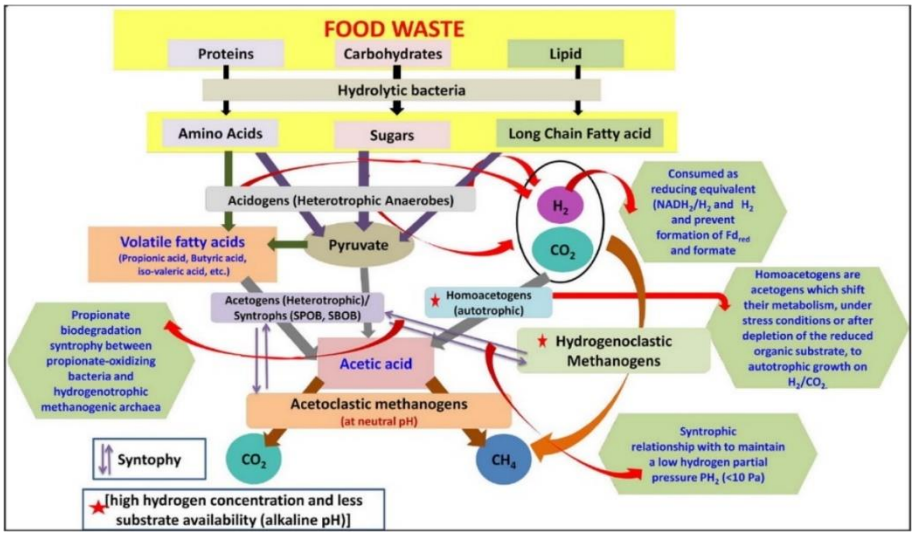


Figure 1.1 – Anaerobic mixed culture pathway (Dahiya et al., 2015).

1.2. WAS-FW co-fermentation

Organic biodegradable substrates used to produce VFAs through fermentation are sewage sludge (Fang et al., 2020; Garcia-Aguirre et al., 2017), waste activated sludge (WAS) (Y. Chen et al., 2007; Pang et al., 2020), food waste (FW) (Dahiya et al., 2015; Strazzera et al., 2021), agricultural residues (Guo et al., 2015; Potdukhe et al., 2021), animal manure (Lian et al., 2021; Saritpongteeraka et al., 2014), organic fraction of municipal solid wastes (OFMSW) (Cheah et al., 2019; Colombo et al., 2017). Although, most used substrates are sewage sludge, WAS and FW (Perez-Esteban et al., 2022).

Sewage sludge and WAS are largely produced in WWTPs and are characterised by being relatively enriched in total chemical oxygen demand (COD) (68-90 g COD L^{-1} and 52-58 g COD L^{-1} for sewage sludge and WAS) (Peces et al., 2020). Moreover, soluble chemical oxygen demand (sCOD) is much lower with values in the range of 1.7-2.3 g COD L^{-1} and ~0.3 g COD L^{-1}, respectively (Peces et al., 2020). VFA yields obtained using these substrates in Peces et al. (2020) were between 32-89 mg COD (g VS)$^{-1}$ for sewage sludge and 20-41 mg COD (g VS)$^{-1}$ for WAS in mono-fermentation batch tests. The high concentrations on particulate COD (difference between total COD and sCOD) induces hydrolysis as the limiting stage of the acidogenic fermentation process (Ji et al., 2010). Otherwise, some authors had demonstrated that the co-fermentation of FW and WAS lead to higher VFA yields reaching 480 mg COD (g VS)$^{-1}$ for 50:50% (VS basis) of WAS:FW as result of the increased sCOD and hydrolytic activity as consequence of FW presence, with higher VFA yield as FW proportion increased (Vidal-Antich et al., 2021).

Co-fermentation is defined as the simultaneous fermentation of two or more substrates. This strategy allows to overcome the limitations of a single-substrate fermentation by (i) presenting an increased OLR, (ii) providing additional buffer capacity and preventing pH drops or alkali consumption, (iii) modifying the organic matter composition, (iv) balancing macronutrients as C/N ratio, (v) diluting toxic and potential inhibitory compounds and (vi) providing an active fermentative microbial community (Perez-Esteban et al., 2022). Thus, co-fermentation is a suitable choice to boost fermentation yields and drive the fermentation product profile using the same mono-fermentation's infrastructure without the need of major capital and operating costs.

WAS is the main substrate used in co-fermentation research and the most studied mixture is WAS and FW (Perez-Esteban et al., 2022). These two substrates' mixtures are suitable for biorefineries located near high populated metropolitan areas because of the constant amounts generated of both wastes.

WAS stands as an ideal main substrate for co-fermentation when it is mixed with a highly biodegradable organic waste with limited (or null) alkalinity (FW or crop residues) or an organic waste that cannot sustain a microbial community (lignocellulosic waste, crude glycerol) (Perez-Esteban et al., 2022).

FW is a highly biodegradable waste with low alkalinity (Li et al., 2013). Certain benefits are achieved if FW is mixed with WAS for fermentation purposes as (i) WAS buffer capacity to maintain pH nearby 5.0 and to prevent fermentative bacteria inhibition due to pH drop (Zhou et al., 2018) and (ii) the high biodegradability of FW to boost fermentation yields (Dahiya et al., 2015).

1.3.Effect of pH on WAS-FW co-fermentation

Operational parameters in fermentation as temperature, pH, HRT or OLR have impact on VFA yields and product profiles in a mixed-culture fermentation (Perez-Esteban et al., 2022). This work targets the effect of pH in WAS-FW co-fermentation to maximize VFA production.

Feng et al. (2009, 2011) performed batch co-fermentation tests using WAS and FW which ranged pH 4 to 11 by using unit increments at room temperature (~20 ºC). Highest co-fermentation yields were obtained in tests at pH 8 and 9. Within pH 7-9, the higher fermentation yields observed were related to the elevated activity of some metabolic enzymes (Feng et al., 2009). However, lower co-fermentation yields were obtained at pH 6 and above pH 9, probably related to the lower enzymatic activity of fermentative bacteria. Co-fermentation yield at pH 8 was higher than the yields obtained from pH 8 control mono-fermentations of WAS and FW. These results are associated to the organic carbon and nutrients provided by FW and the alkalinity related to WAS (Feng et al., 2011). VFA profile (on COD basis) between pH 6 and 9 was mainly composed by propionic acid (~50%), acetic acid (~30%) and butyric acid (~10%) agreeing with most of the mixed-culture fermentation publications (Cheah et al., 2019; Esteban-Gutiérrez et al., 2018; Ma et al., 2017).

Moretto et al. (2019) also studied the impact of pH on WAS and FW co-fermentation using batch assays at 37 and 55 ºC and, for both temperatures, pH was adjusted at the beginning of the test (not through time as Feng et al. (2009, 2011)) at 5, 7 and 9 pH values. The results obtained were agreed with those reported by Feng et al. (2009, 2011) obtaining the highest co-fermentation yields at initially pH 7 and 9 conditions (the final pH were ~5.5 and ~6.0, respectively within 10 days) similarly for both temperatures 37 ºC and 55 ºC. Tests with initial pH 5 at 37 ºC and 55 ºC dropped quicker to pH values of ~4.0 inhibiting the acidogenic fermentation activity resulting in a non-VFA accumulating profile. Moretto et al. (2019) demonstrated that pH

had a higher influence than the temperature on fermentation yield and product profile. As happened in Feng et al. (2011), VFA profile on COD basis was composed mostly by acetic (~30%), propionic (~50%) and butyric (~10%) acids.

Moretto et al. (2019) also performed a semi-continuous stirred-tank reactor (CSTR) using WAS and FW substrates at 37 ºC in which pH was fixed between 8 and 9 values by adjusting the pH in the feedstock. High fermentation efficiency was achieved operating with OLR of 7.7 and 9.3 kg VS m^{-3} day^{-1} associated with HRTs of 6 and 5 days, respectively. Nevertheless, reactor suffered an overload at OLR of 11.3 kg VS m^{-3} day^{-1} with an HRT of 4 days although pH was kept between 8 and 9.

Furthermore, Chen et al. (2013) operated 5 semi-continuous co-fermenters using WAS and FW (12% and 88% in VSS basis), pH fixed to 5, 7, 9, 11 and a condition without pH control. The highest VFA yield was achieved in fermenters which operated at pH 7 and 9 with no substantial difference. Both fermenters had VFA concentrations 2 and 10 times higher than those produced at pH 11 and 5, respectively. The lowest VFA production was found in the fermenter without pH control.

Chen et al. (2013) distinguished the impact of pH on the solubilisation of organic matter (hydrolysis) and its conversion to VFA (acidogenic fermentation) as pH 11 fermenter showed the highest hydrolysis rate and pH 7 fermenter the lowest. Also, Feng et al. (2011) reported higher accumulation of sCOD non-associated to VFA between pH 10-11 compared to assays with pH fixed at 7, 8 and 9. Superior organic matter solubilisation occurs at alkaline conditions (pH ≥ 10). Even so, because of the acidifying profile due to fermentation and the elevated buffer capacity of WAS, high quantities of alkaline chemicals would be required to maintain a fermenter in alkaline conditions. Hence, would be reflected in increased operation costs and process complication, limiting the application of biosolids as fertilizer due to the elevated salinity content (Perez-Esteban et al., 2022).

WAS-FW co-fermentation research has shown higher fermentation yields within pH 7-9 with mostly acetic, propionic and butyric acids in VFA profile as consequence of the hydrolysis increment as pH raises although HRT must be large enough to ensure hydrolysis stage. Increasing OLR carries the risk of overloading the fermenter (decreasing fermentative activity). Moreover, HRT and pH are the key parameters to inhibit the growth of methanogenic archaea (Perez-Esteban et al., 2022).

1.4.PHA production using VFAs

VFAs produced through acidogenic fermentation can be used as raw material to synthetise polyhydroxyalkanoates (PHA) because of the easily biodegradable property associated to VFAs and by performing an aerobic feast (presence of carbon source as VFAs) and famine (absence of VFAs due to total consumption) in mixed cultures (Colombo et al., 2017). The aerobic feast-famine regime promotes the growth of biomass capable to synthetise energy reservoirs as PHA during the abundance phase (feast) to use it in the absence period (famine). Furthermore, if an essential nutrient as nitrogen (N) is decoupled during feast phase, higher PHA-producer biomass selection is achieved due to growth inhibition during feast (Kourmentza et al., 2017).

Polyhydroxyalkanoates (PHA) are biodegradable polymers, mainly composed by polyhydroxybutyrate (PHB) and/or polyhydroxyvalerate (PHV), that could be synthesized intracellularly by some microorganisms as a carbon and energy reservoirs. This valuable bioplastic could be produced using mixed microbial cultures and treating organic wastes in a four-stage process (Albuquerque et al., 2011; Conca et al., 2020): (i) the first stage is an acidogenic fermentation to the production of volatile fatty acids (VFAs) that would be subsequently fed to the following stages as C source, (ii) the second stage of the process is the selection of PHA storing organisms to produce a biomass enriched in microorganisms able to accumulate PHA using the combination of periods of high availability of VFAs (feast phase) and periods of absence of easily biodegradable carbon source in the mixed liquor (famine phase), while favouring the proliferation of those microorganisms able to store PHA and to grow using their PHA reservoirs under famine conditions, (iii) the third stage of the process is the accumulation of PHA which consists of the increase of PHA content in the purged biomass from the selection reactor by maintaining feast conditions, thanks to the feeding of the liquid VFA-rich stream produced in the first fermentation stage. Then, (iv) the extraction of the PHA accumulated inside the cell is performed by using different techniques as solvent extraction (Kunasundari & Sudesh, 2011). Figure 1.2 shows a scheme of the PHA production process

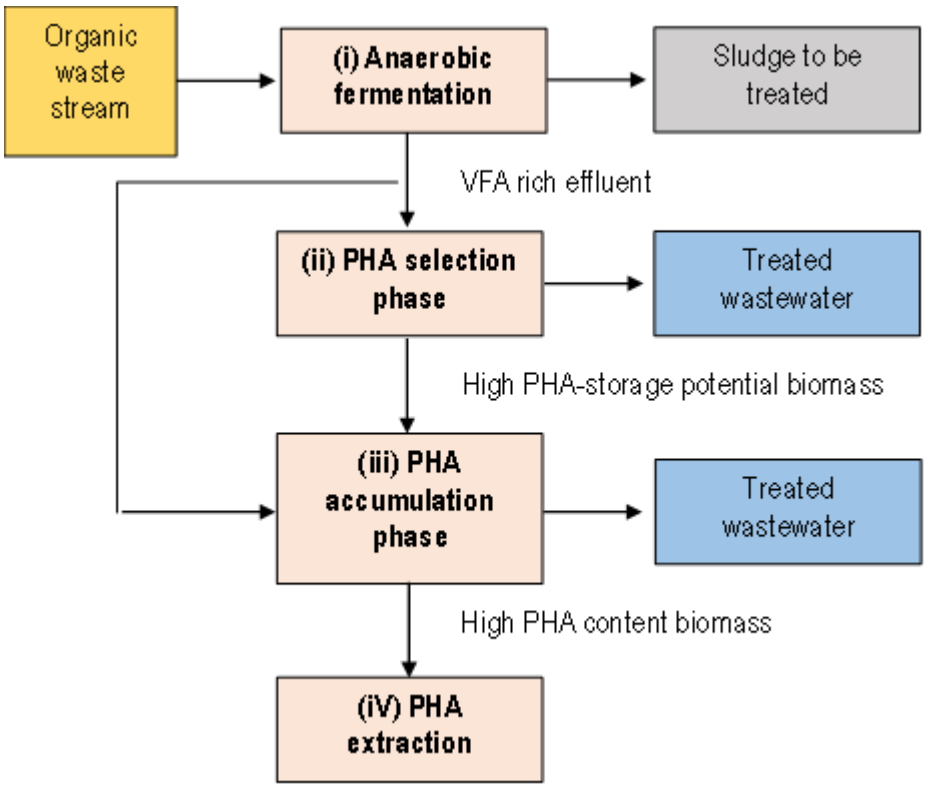


Figure 1.2 – PHA production scheme adapted from Albuquerque et al., (2011).

2. JUSTIFICATION AND OBJETIVES

This project aims to study the influence of pH in acidogenic co-fermentation by performing 3 set of batch tests, using WAS and FW as co-substrates besides the VFA production in WAS-FW semi-continuous co-fermentation operating 2 lab-scale fermenters. Furthermore, the effectiveness of mixed cultures storing bioplastic (PHA) using synthetic feed in a sequential process composed by a selection reactor and accumulation equipment is evaluated.

The objectives to be covered are specified in below:

- To study the effect of pH treating FW and WAS in discontinuous batch tests to maximize VFA production.
- To perform 2 semi-continuous fermenters and determine the VFA production and distribution along the operation.
- To assess the selection and accumulation of PHA using VFA-rich synthetic wastewater while testing different selection strategies as double selection implementation (nitrogen decoupling during feast and aerobic feast-famine phases) and single selection (only aerobic feast-famine regime).

- To compare the VFA profile obtained in semi-continuous fermenters and the synthetic feed used to produce PHA.

3. MATERIALS AND METHODS

In this chapter, the experimental set up used to carry out this study is detailed. Basically, four lab-scale reactors were operated. For VFA production, two fermenters were run to produce VFAs and compare different operating strategies. On the other hand, a selection reactor and an accumulation reactor were used to obtain an enriched PHA-storing biomass and subsequently increase its PHA content, respectively. Moreover, the substrates and inoculum used besides the standardized methods used are presented.

3.1. Experimental set-up

As described previously, four reactors were operated in this study, two of them related to VFA production and the other two for the downstream processing of the VFA-rich fermentation liquids. In the next section, the experimental devices used for acidogenic fermentation and for PHA production are described.

3.1.1. Acidogenic fermentation experimental devices

To test the VFA production, batch fermentation tests were carried out to assess the short-term effect of operating conditions on VFA production, while two lab-scale reactors were operated to assess the long-term effect of these conditions.

Fermentation batch tests. Batch fermentation tests were performed in 250 mL glass bottles with 150 mL of working volume to quantify the effect of pH changes in VFA profile and distribution (see figure 3.1 a). These bottles were equipped with a cap and a septum on the top, where samples were taken. Each bottle was incubated at 35 ºC (Memert UF 750 incubator). Influents used contained a fixed proportion of WAS:FW on VS basis in each bottle. Mass measurements were done using Sartorius TE214S scientific balance. After filling the bottles with substrates, a gentle flow of nitrogen was sparged for 1 minute in the gas phase of the bottle to avoid the presence of oxygen and assure anaerobic conditions.

Those experiments were monitored by extracting 4 mL of sample with a 5 mL BD Plastipak™ syringe to measure pH (METRIA glass body pH electrode with semi micro applications with CRISON MultiMeter MM 41). Then the samples were centrifuged (SIGMA MODEL 1-14) for 5 minutes at 14800 rpm and filtered with 45 μm syringe-filters (Simsi Syringe Filter with pore size of 0.45 μm and diameter of 0.25 mm) to analyse TAN, sCOD and VFAs concentration and distribution. Depending on the batch assay, pH could be modified by the manual addition of

NaOH (5 M). For each experiment, a previous characterization of substrates was performed by measuring pH, TAN, COD, sCOD and VFA.

Lab-scale semi-continuous fermenters. Two sealed-glass jacketed reactors were used as fermenters (figure 3.1 b) with 2 L of capacity and 1.75 L of working volume. Those reactors had an agitation system of 80 rpm (multiple level pallets shaker using IKA®-WEKE RW 16 basic motor) and temperature was controlled at 35 ºC using a thermostatic bath. Also, these fermenters were equipped with a glass-lid with 4 entrances. Two slots were dedicated to the emptying and filling of volumes using neoprene tubes connections with two peristaltic pumps (Masterflex® L/S® Precision Variable-Speed Console Drives with Masterflex® L/S® Easy-Load® 3 Pump Heads for Precision Tubing) which were used to fill the feed stream (mixture of FW and WAS) and to extract the desired mixed liquor. Therefore, one slot had a pH probe (Mettler Toledo HA405-DPA-SC-S8/225) and there was an entrance dedicated to gas extraction by nylon tubes connected to bag recovery gas (SKC TEDLAR® Sample Bag 1-Liter Tedlar Sample Bag with Polypropylene Fitting).

Monitorization of the semi-continuous operation was based on TS, VS, pH, COD, sCOD, TAN and VFAs concentration. These analysis were applied to the inlets and outlets of the reactors three times per week. The WAS was characterized using the same assays once per week and FW characterization was performed twice per week.

(a) (b)

Figure 3.1 – Glass bottles used in batch fermentation tests (a) and Semi-continuous lab-scale fermenters set-up (b).

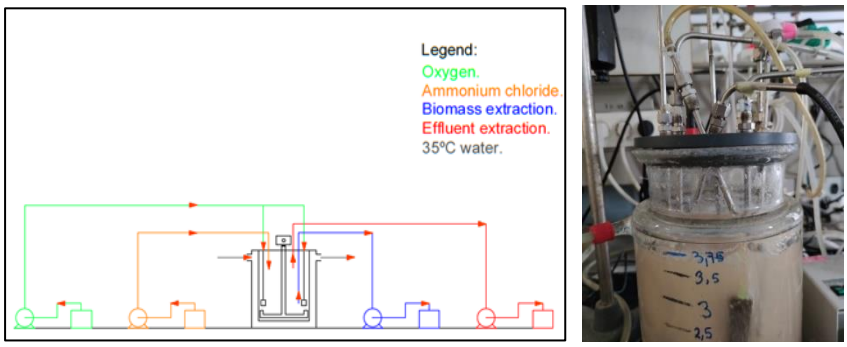
3.1.2. PHA storing biomass selection in a sequential batch reactor (sSBR)

The selection SBR (sSBR) was a glass reactor of 5 L (3.75 L of effective volume) equipped with a heating jacked set-up at 35 ºC. This equipment had an agitation system at 80 rpm conformed by a pallet shaker and IKA®-WEKE RW 16 basic motor. It had a lid with six entrances for accessories in which a pH probe (Mettler Toledo HA405-DPA-SC-S8/225), an oxygen probe (CellOx 325, WTW) with a dissolved oxygen portable meter (Oxi 3310, WTW) and tubes related

with the pumps were settled. Figure 3.2 a and b shows a scheme and a photography of this reactor.

Air was supplied to the reactor using 12 air blowers (Moure air pump 5, Epsilon) and net-air system that derives in three tubes connections with three porous stone diffusers into the reactor. Filling and drawing operations were performed by 4 peristaltic pumps (PERCOM-I) and 5 timers (Smartwares 10.047.65 with programable mechanical temporizer) connected to each pump and the oxygen-agitation system to perform the sSBR cycle.

Two feed streams were used in the sSBR cycles of this reactor, one enriched in VFAs and the other in ammonium nitrogen. The VFAs-rich feed was stored in two closed tanks of 10 L to prevent the degradation of the organic matter because of light exposure, microorganism's growth, or algae proliferation. These tanks were connected to a peristaltic pump (PERCOM-I) to fill it in the reactor. Otherwise, the ammonium chloride feeding solution was stored in two glass bottles of 1 L and connected to the same type of peristaltic pump as feed, purge extraction and effluent withdraw. Purge and effluent were collected into 12 L recipients.



(a) (b)

Figure 3.2 – Scheme of the selection SBR set up done by AutoCAD software (a) and Real photograph of the selection reactor (b).

The sSBR followed cycles of 6 h (4 cycles per day) controlled by the timers (Smartwares 10.047.65 with programable mechanical temporizer). Table 3.1 shows the operating conditions and time distribution of the sSBR cycles in the working periods of this project.

Table 3.1 – Operating parameters of the selection reactor.

Parameter	Value	Units
Temperature	35	ºC
Hydraulic retention time (HRT)	1.12	d
Solid Retention Time (SRT)	4.21	d
Organic loading rate in first period	2.51	g COD L^{-1} d^{-1}
Organic loading rate in second period	2.51	g COD L^{-1} d^{-1}

Parameter	Value	Units
Nitrogen loading rate in first period	0.094	g NH_4^+-N L^{-1} d^{-1}
Nitrogen loading rate in second period	1.09	g NH_4^+-N L^{-1} d^{-1}
Cycle length	6	h
Time distribution in each operating cycle (steps 1 – 8)		
1 -VFAs rich wastewater feeding	15	min
2 - Agitation + air supply	135	min
3 – Agitation + air supply + sludge purge	7	min
4 - Agitation + air supply	8	min
5 - Agitation + air supply NH_4^+-N rich wastewater feeding	2	min
6 - Agitation + air supply	148	min
7 – Settling (no agitation nor air supply)	30	min
8 – Effluent withdrawal	15	min

As it could be seen in table 3.1, in each sSBR cycle the following operations were performed:

1) **VFA-rich wastewater feeding:** for 15 minutes, the synthetic feed composed by only carbon sources (VFAs) and nutrients (except ammonium nitrogen) was fed to the selection reactor.
2) **Agitation and air supply:** agitation and air supply were performed during 5 h. Dissolved oxygen was supplied using air compressors and/or compressed air service connected to diffusors, to ensure the dissolved oxygen not being a limiting factor in the growth of microorganisms.
3) **Sludge purge:** as the reactor was under agitation, biomass purge was performed to maintain the desired solid retention time (SRT) in the reactor.
4) **Ammonium-rich stream feeding:** for 2 minutes the necessary concentration of ammonium nitrogen was given to the reactor as ammonium chloride (NH_4Cl).
5) **Biomass settling**: after the reaction phase, the agitation and oxygen pumps were turned off to settle the biomass in the reactor for 30 minutes.
6) **Effluent withdrawal:** for 15 minutes, the desired volume of clarified effluent was removed of the system to establish an operating HRT.

Characterization of a sSBR cycle was based on the extraction of 15 samples of 20 mL. Each sample was extracted with 20 minutes of difference, centrifuged for 5 min (SIGMA MODEL 1-14) and filtered using 45 μm syringe-filters (Simsi Syringe Filter with pore size of 0.45 μm and diameter of 0.25 mm). The filtered sample was used for TAN and VFAs measurements as defined before. Moreover, three PHA samples were taken, the first at 20 minutes after feed

addition, the second 20 minutes before the NH_4Cl addition and the last at 240 min of the cycle operation. Moreover, the PHA content of the purged biomass and VFAs of the effluent were also analysed. Finally, DO profile was recorded every minute using MultiLab Importer software and pH was monitored every 20 minutes.

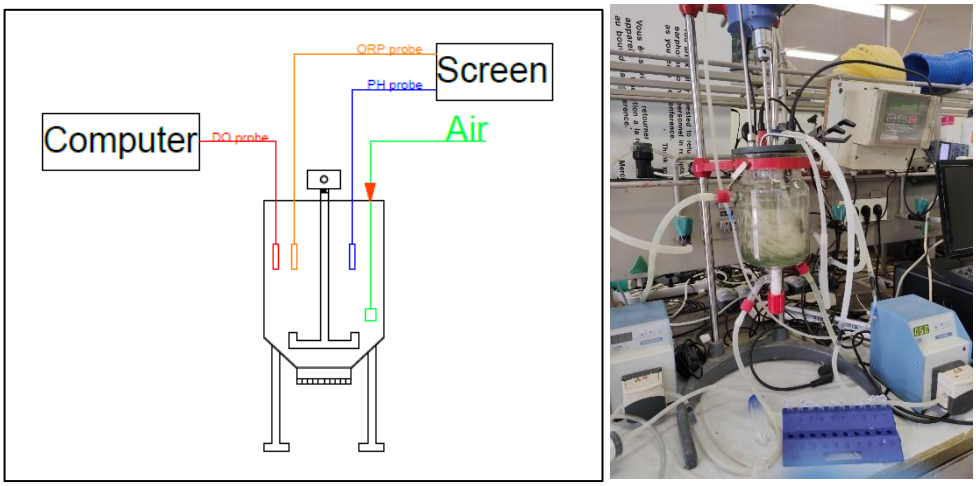
3.1.3. PHA accumulation batch tests equipment:

The PHA accumulation batch tests were performed using a jacketed glass lab-scale reactor with a working volume of 1 L. Operating temperature was controlled by a heating system that maintains the temperature at 35 ºC. Stirring was performed by an agitation system at 80 rpm and an IKA®-WEKE RW 16 basic motor. The reactor was also equipped with a pH probe (Mettler Toledo HA405-DPA-SC-S8/225) and an oxygen probe (CellOx 325, WTW) connected to a dissolved oxygen portable meter (Oxi 3310, WTW). The reactor had a bottom discharge system to empty all the working volume. Aeration was carried out using a net-air system connected with a porous diffusor by plastic tube. Operational parameters are summarized in table 3.2 and the scheme and photography of this experimental set-up is presented in figure 3.3 a and b. An identical test-checking as selection reactor was done before starting the operation.

The feed stream used was the same VFA-rich wastewater used in the selection reactor (sSBR) and was stored in a closed tank of 10 L to prevent the degradation of organic matter, the entrance of air and the exposition to external light (avoiding the proliferation of algae and microorganisms). Hence, the feed was added into the reactor using a previously calibrated peristaltic pump (PERCOM-I) to control the volume filled. Ammonium chloride was not supplied to this reactor to avoid microorganism's growth by consuming PHA reservoirs.

Table 3.2 – Operating parameters of the PHA accumulation tests

Parameter	Value	Units
Temperature	35	ºC
Feeding COD concentration	3.5	g COD L^{-1}
Nitrogen loading rate	0	g NH_4^+-N L^{-1} d^{-1}
Biomass volume	450	mL
Feed-spikes	5-6	spikes
Volume feed-spike	80	mL
Cycle length	7-8	h
Time distribution in each operating cycle		
- Agitation + air supply	continuously	-



(a) (b)

Figure 3.3 – Scheme of the PHA Accumulation reactor using AutoCAD software (a) and photography of the real accumulation reactor (b).

Accumulation reactor operation cycles were based in the addition of 450 mL selected biomass (resulting the volume of 2 selection reactor purges involving 7:30 h of continuous operation). Then, a pulse-feeding strategy was implemented with 150 mL of feed in each addition. When a total consumption of the carbon source was observed due to a sudden rise in the monitored DO, the following addition was performed. This process was repeated until the fifth addition. The approximate duration of the cycle took 7-8 h.

To characterize the accumulation cycle, a continuous monitorisation of DO (oxygen portable meter connected to a computer via USB) and lectures of pH each 20 minutes besides before and after the additions were taken as well as the off-line analysis of VFAs, TSS, VSS and PHA content. A previous characterization of the initial biomass used in the assay applying the same analysis was performed.

3.2. Inoculum and substrate

This section gathers the inoculums and substrates used in both types of operation (fermentation and PHA production).

3.2.1. Fermentation: inoculum and substrates

The inoculum used in fermentation was WAS from the WWTP of Barcelona metropolitan area. A volume of 1.75 L of WAS was initially filled on the reactors. Then, the substrate used to feed the reactor was a mixture of WAS eluted to a VS concentration of 2.5% (%w) and FW with proportions of 65:35, 75:25 and 80:20 (WAS:FW) on VS percentage. Moreover, the substrates used to perform the first two batch tests were 75:25 (WAS:FW) on VS basis and for the third batch test, a semi-continuous fermenter's effluent treating 65:35 (WAS:FW in VS basis) was

used. The FW used was synthetic based on previous experimentations (Vidal-Antich et al., 2021) (see composition of FW in table 3.3).

Table 3.3 – Synthetic FW composition.

Component	Percentage (% w)
Pasta	10.0
Rice	10.0
Apple	20.0
Banana	10.0
Potato	20.0
Onion	10.0
Turkey	10.0
Surimi	10.0

*16 % (% w) of Water is added on the mix.

3.2.2. PHA production unit: inoculum and substrate

For PHA production, the inoculum used to start-up the selection SBR was from a previously operated lab-scale reactor (Pérez, 2019), which consists in the set-up of bioreactors to produce bioplastic using organic waste and where the selection of PHA-producer biomass had been done. Approximately 750 mL of that sludge was filled on the reactor at the set-up operation which had a VSS concentration of 2.06 g L^{-1}. The inoculum for the accumulation reactor was two sludge purges of the selection reactor resulting in a selection operation of 7:30 h.

The substrate used for the selection and accumulation reactors was a synthetic VFA-rich wastewater containing 3.5 g COD L^{-1}, as stated in table 3.4. Macronutrients and micronutrients concentrations ensuring a suitable growth of the biomass were obtained from an adaptation of Dapena-Mora et al. (2004) and VFA distribution was based on acetic, propionic and butyric acids with percentages of 62.4%, 18.8% and 18.8% on COD basis, respectively. Those percentages represent the typical composition of fermented OFMSW (Dosta et al., 2018). Table 3.4 summarizes the composition of the synthetic substrate (it is not represented the ammonium nitrogen as a macronutrient because it was uncoupled from the feed in the selection operation). Calculations carried out to define the wastewater characteristics are shown on annex 1: sSBR synthetic VFA composition in feed and concentration of ammonium chloride.

Ammonium nitrogen concentration was theoretically calculated using the heterotrophic ratio (Yobs) of the organic loading (g COD L^{-1} d^{-1}) which is destinated to microorganism's growth as well as considering the pump flowrate as it has been represented on annex 1: sSBR synthetic VFA composition in feed and concentration of ammonium chloride.

The nitrogen loading rate used in the first period was 0.094 g NH_4Cl L^{-1} d^{-1} which represents an increase of a 26% from the theoretically calculated to ensure nitrogen not to be a limitation in the growth of biomass. In a second stage of the work, it was increased to 0.16 g NH_4Cl L^{-1} d^{-1} (an increase of 18% from the first period). As previously described, nitrogen addition was uncoupled from the feed and added independently.

The substrate used for the accumulation reactor was the same used in selection reactor (table 3.4) to operate without increasing the OLR designed to selection. In the accumulation reactor there was no addition of external nitrogen preventing the reproduction of the microorganisms and the PHA reservoirs reduction.

Table 3.4 – Synthetic wastewater composition.

Compound	Concentration	Units	Compound	Concentration	Units
Propionic acid	0.51	g L^{-1}	$FeCl_3 \cdot 6H_2O$	1.50	mg L^{-1}
Butyric acid	0.51	g L^{-1}	H_3BO_3	0.15	mg L^{-1}
Acetic acid	1.69	g L^{-1}	$CuSO_4 \cdot 5H_2O$	0.03	mg L^{-1}
K_2HPO_4	0.58	g L^{-1}	KI	0.03	mg L^{-1}
KH_2PO_4	0.23	g L^{-1}	$MnCl_2 \cdot 4H_2O$	0.12	mg L^{-1}
$MgSO_4 \cdot 7H_2O$	0.09	g L^{-1}	$Na_2MoO \cdot 2H_2O$	0.06	mg L^{-1}
$CaCl_2 \cdot 2H_2O$	0.07	g L^{-1}	$ZnSO_4 \cdot 7H_20$	0.12	mg L^{-1}
EDTA	0.02	g L^{-1}	$CaCl_2 \cdot 2H_2O$	0.12	mg L^{-1}

*$NaHCO_3$ dosage of 25 g L^{-1} was required to ensure a buffer capacity into the reactor.

3.3.Analytical methods

The analytical methods used in this work were performed according to the Standard Methods for the Examination of Water and Wastewater (APHA, 2012). Otherwise, PHA extraction (analytical method 3.3.7) has no standardized procedure but was done as described in Lanham et al. (2013). In this section the analytical methods applied are shortly explained.

3.3.1. Total solids (TS) and volatile solids (VS)

Total solids are the material residue left in the vessel after the evaporation in a sample by drying in an oven at 100 ºC. TS includes total suspended solids which are the portion retained by a filter of 2.0 μm or smaller nominal pore size and the total dissolved solids, the portion that passes through the filter. Volatile solids are the weight part of the TS that is lost on ignition due to decomposition or volatilization as occurs in an oven at 550 ºC. (Standard Methods for the Examination of Water and Wastewater, 2012). The process followed is explained below:

Total and volatile solids are obtained by a previous melting pot weight (M_p) and then weighting a determined volume of a sample in this melting pot (M_0). Secondly, the sample takes

24 h in an oven at 100 ºC and the weigh is measured (M_s). Finally, sample is putted 2:30 h in an oven at 550 ºC to obtain the last measurement (M_f).

The formula used to calculate TS and VS are described below (3.1, 3.2 and 3.3 formulas):

$$M_m\ (\mathrm{g}) = M_0 - M_p \quad (3.1)$$

$$\mathrm{TS}\ (g\ L^{-1}) = \frac{M_m - M_s}{V} \quad (3.2)$$

$$\mathrm{VS}\ (g\ L^{-1}) = \frac{M_s - M_f}{V} \quad (3.3)$$

*Weight percentages can be obtained by dividing TS and VS with the sample mass added (M_m).

M_p (g) is the melting pot mass; M_0 (g) is the mass associated to the sample and the melting point; M_m (g) is the sample weight added; M_s (g) is the sample mass after 24 h at 100 ºC; M_f (g) is the weight after 550 ºC during 2:15 h and V (L) is the volume of the sample used.

3.3.2. Total suspended solids (TSS) and volatile suspended solids (VSS)

Solid particles larger than 2 microns remaining in suspension into a solvent are considered the total suspended solids (TSS). Depending on the wastewater origin, the presence of suspended solids (SS) could be related to bacteria, algae and inorganic materials (Fondriest Envoirmental Learning Center, 2021). Total volatile suspended solids are the organic part of the TSS, so it is a used to characterize the concentration of biomass into the reactor or a stream. Lab procedures followed to assess 25440D and 25440E are detailed below:

1) Total and volatile suspended solids (25440D):

Firstly, a quantitative paper filter (MilliPore of 0.45 μm) is put on a metal plate and then goes in a stove at 100 ºC for 24 h to eliminate impurities and humidity and ensure a constant weight. After this time, the plate with filter is weighted (M_0). Then, some volume (V) of the biomass purge is vacuum filtered by using the filter putted into the metal plate, a Kitasato and a vacuum bomb. The filter goes into a 100 ºC stove for 24 hours to eliminate the water presence in the sample. And the weight is measured passed the 24 hours (M_f). The difference between the weight of the plate with biomass and the weight without it and divided by the volume added at point 3 the TSS concentration is obtained (see equation 3.4).

2) Volatile suspended solids (VSS) (2540E):

The metal plate used to TSS analysis is putted into an oven at 550 ºC for 2:15 h. Then the weight of the plate (M_L) is measured. The difference between the 24 h at 100 ºC mass

obtained and M_L divided by the volume added is the concentration of the biomass into the reactor (see equation 3.5).

$$\text{TSS } (g\ L^{-1}) = \frac{M_o - M_f}{V} \quad (3.4)$$

$$\text{VSS } (g\ L^{-1}) = \frac{M_f - M_L}{V} \quad (3.5)$$

M_o [g] is the initial sample mass; M_f [g] is the final mass after 24 h in an oven at 100 ºC; V [L] is the volume of sample used; M_L [g] is the mass weighted after 2:15 h in an oven at 550 ºC.

3.3.3. Total ammonium nitrogen (TAN or [NH_4^+-N])

The analysis of total ammoniacal nitrogen (4500-NH_3D) have been done by using a selective ammonia probe (Thermo Scientific ISE membrane for gas detection Orion™ and OrionTM Dual StarTM mV measurer) and implementing a logarithmic calibration based on potential values (mV) given using different patron concentrations of ammoniacal nitrogen: 10, 25, 50 and 100 mg N L^{-1}. Few drops of NaOH (10 M) must be added before the measurements to favour the acid-base equilibrium to NH_3 formation and to measure the conductivity associated to the proton liberation. A previously 0.45 μm filtration must be done by using a syringe and a filter (Simsi Syringe Filter with pore size of 0.45 μm and diameter of 0.25 mm).

3.3.4. Total chemical oxygen demand (COD) and soluble chemical oxygen demand (sCOD)

Chemical oxygen demand (COD) is defined as the amount of a specified oxygen that reacts with a sample under controlled conditions. The consumed quantity of oxidant is expressed in terms of oxygen equivalence. Because of its unique chemical properties, dichromate ion ($Cr_2O_7^{-2}$) is the specified oxidant in method 5220B where it is reduced to chromic ion (Cr^{3+}). Organic and inorganic components are subject to oxidation. Moreover, the organic component predominates. Hence, COD is used to quantify the biodegradable matter in the media. The detailed procedure to follow 5520B is described beneath:

Total chemical oxygen demand (COD) and soluble chemical oxygen demand (sCOD):

2.5 mL of a determined elution of the sample were added in COD test tubes (Spectroquant® Empty cells 16 mm). Then, 1.5 mL of potassium dichromate 0.04 mol L^{-1} 0.24 N solution in 80 g L^{-1} mercury (II) sulphate for COD determination (PanReac AppliChem) and 3.5 mL of silver sulphate solution 10 g L^{-1} in sulfuric acid for volumetric analysis (PanReac AppliChem) are added on each tube. After that, a digestion at 150 ºC for 120

minutes are applied in Nesslab Eco25 Thermoreactor equipment. Finally, the absorbance is measured using a Jenway 7200 Visible Spectrophotometer with 600 nm of wavelength. Thus, calibrating the spectrophotometer with patrons of 0, 50, 250, 500 and 100 mg O_2 L^{-1} with their respective absorbances, COD in samples can be obtained.

3.3.5. Alkalinity

The alkalinity of a water stream is its acid-neutralizing capacity as the sum of all the titratable bases. Principal alkalinity properties in a water are function of carbonate, bicarbonate and hydroxide content and it is taken as an indication of the concentration of these constituents. Otherwise, the measured values may include contributions of borates, phosphates, silicates or other bases. Alkalinity is given in concentration of the $CaCO_3$ in mg L^{-1}. Alkalinity analysis has been done by weighting a determinate volume of sample (around 20 g) an using a Mettler Toledo pH electrode InLab Routine with a Crison Mutlimeter MM 41 to obtain the concentration of $CaCO_3$ titrated. Then partial alkalinity and total alkalinity is distinguished at pH 4.3 and 5.75, respectively. Applying equation 3.6 the total and partial alkalinities are calculated:

$$\text{Alkanlinity}\left[\frac{mg\ CaCO_3}{L}\right] = \frac{2\ B\ N\ 50000}{mL\ sample} \qquad (3.6)$$

*Where B is the volume to reach the determined pH and N is the normality of the acid.

3.3.6. Volatile fatty acids (VFAs)

The standard method 5560D is followed by taking a volume of 1 mL of each sample previously filtered using a syringe-filter (Simsi Syringe Filter with a pore size of 0.45 μm and diameter of 0.25 mm) are pipetted in different chromatography vials and 100 μl of an internal standard patron (solution composed by 15% of phosphoric acid 85% and 1 g of a SIGMA-ALDRICH volatile free acid mix standard) is added on each vial to acidify the media under 2 pH values besides to maintain a constant concentration of internal patron according to the internal chromatograph calibration. Then VFA concentration and distribution are analysed by gas chromatography technique (Shimadzu GC 2010 plus) equipped with a capillary column (Nukol™) and a flame ionization detector (FID). The chromatograph uses helium as carrier gas, hydrogen as fuel gas and synthetic air as the oxidizing gas. Gas chromatography configuration and procedure is performed as Astals et al. (2012). The chromatograph is calibrated to detect acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic, caproic and heptanoic acids. Moreover, VFA isomers are considered as single species. Concentrations obtained using this analytical method are given in mg VFA L^{-1}.

3.3.7. Polyhydroxyalkanoates (PHA) content

The content of PHA is determined by a solvent extraction technique as a simple and rapid methodology used in laboratory (Kunasundari & Sudesh, 2011). Involving two steps, firstly the modification of the cell membrane permeability is induced allowing PHA solubilization and, secondly a non-solvent precipitation. This method avoids the degradation of polymers. The PHA extraction can be done by using different solvents as chlorinated hydrocarbons or cyclic carbonates (Jacquel et al., 2008). In the analysis performed, chloroform is used as a chlorinated solvent. Moreover, the precipitation of PHA is induced by a non-solvent such as methanol and ethanol (Ramsay et al., 1994). In this case methanol is used as the non-solvent-cause precipitation. The analysis of standards and samples of the bioreactor are detailed below and applies the procedure described in Lanham et al. (2013).

1) PHA content. Standards preparation:

6.0 g of the PHA patron containing 88% PHB and 12% PHV in mass percentage is dissolved using 1 L of chloroform with a constant concentration of benzoic acid as an internal patron and indicator of chromatogram peaks. The peak caused by this acid will be constant and then the PHB and PHV peaks will experiment changes in peaks area (y label in chromatogram with intensity as unit) in function of the concentration but distances (x label in chromatogram in minutes) between benzoic acid, PHB and PHV are maintained. Moreover, if some changes in benzoic acid area peaks are experimented it will indicate dilutions or some sample changes in composition invalidating the results of the sample.

To prepare the patrons, a Hamilton 1000 μL syringe is used as well as ventilated vitrine conditions. Then, different volumes (100 μl, 200 μl, 300 μl, 400 μl, 500 μl and 600 μl) of the dissolved PHA patron are added into different test tubes and after, some chloroform is added reaching 1 mL volume.

Next step consists in adding 1 mL of methanol (20% sulphuric) as non-solvent in each tube resulting in a total volume of 2 mL. Then test tubes must be closed to avoid evaporation.

These test tubes are putted into a COD digestor for 5 hours at 100 ºC. After that, samples suffer a temperature decrease using water and ice mixture for 30 minutes. Then, 0.5 mL of water are added into test tubes producing two phases one aqueous phase which water is the dissolvent and the other is the organic phase where methanol is the solvent. Bottom phase is the aqueous phase which contains chloroform and the PHA dissolved. Then the tubes suffer a vortex centrifugation for a minute.

About 2-3 molecular sieves are used as water adsorbent and put into chromatography vial. Using a Pasteur pipe, 0.5 mL of the aqueous phase are taken and putted into the chromatography vials and closed hermetically. It is important to preserve the vials into a fridge until their analysis.

Analysis of the samples are proceeded by a gas chromatography technique (Shimadzu GC 2010 plus) equipped with a capillary column (Nukol™, 15 m x 0.53 mm x 0.5 μm) and a flame ionization detector (FID). The chromatograph uses helium as carrier gas, hydrogen as fuel gas and synthetic air as the oxidizing gas. The temperature of the capillary column starts at 80 ºC and is heated by 10 ºC·min^{-1} to 110 ºC. From then on, the temperature increases 15 ºC·min^{-1} until 145 ºC and, finally, it is increased 20 ºC·min^{-1} to 190 ºC.

By the integration of the peaks resulted from chromatography and relating each one to the different PHB and PHV concentrations, a lineal calibration is obtained for each compound.

2) PHA content. Samples analysis:

Biomass is frozen during 24 h at -80 ºC producing a deactivation state. Then a lyophilization of the samples are applied taking 24 h at -52 ºC releasing water by sublimation.

After the lyophilization process, 2 mg of biomass samples are taken using a scientific balance with +/- 0.0001 precision and putted into a test tube.

Next step consists in adding 1 mL of methanol (20% sulphuric v/v) as non-solvent and as bacterial wall rupture. Then 1 mL of chloroform with the constant benzoic acid concentration is put on the tube obtaining 2 mL total volume and PHA solubilization. It is important to close the tubes to avoid evaporation.

These tubes are putted into a COD digestor for 5 hours at 100 ºC. After that time, samples are cooled with water and ice for 30 minutes. After those 30 minutes, 0.5 mL of water are added into test tubes producing two phases one aqueous phase which water is the dissolvent and the other is the organic phase where methanol is the dissolvent compound. Bottom phase is the aqueous phase which contains chloroform and PHA dissolved. Then the tubes suffer a vortex centrifugation for a minute.

Analysis of the samples are done by the same gas chromatography technique as standards. Then, by using the lineal calibration in standards preparation PHB and PHV mass compositions can be obtained from the area given by the chromatography.

4. EXPERIMENTAL RESULTS AND DISCUSSION

In this section, the experimental results of both acidogenic fermentation and PHA production are described. In section 4.1 the results related to acidogenic fermentation (both at discontinuous and semi-continuous mode) are discussed, focusing on the VFA yield and the distribution of VFAs. Section 4.2 is devoted to the operation of the selection SBR to discuss the enrichment of the sludge purge in PHA-storing microorganisms and the accumulation batch tests, where the increase in the PHA content of the previously selected biomass is quantified and discussed.

4.1. Acidogenic fermentation unit

First set of fermentation batch tests. Initially, a fermentation batch test was performed during 10 days with a proportion 75:25 of WAS:FW (%VS basis) without an external inoculum. The characteristics of each substrate are summarised in table 4.1. To evaluate the pH influence on VFAs concentration and distribution, 4 different conditions were established:

i) reference without pH control.

ii) initial pH of 10.

iii) no pH control and pH regulation to 10 at day 6.

iv) no pH control and pH regulation to 7 at day 6.

Each condition was performed by quadruplicate (16 bottles were used in total). The weights filled in each bottle were 150 g of WAS and 8.8 g of FW (the specific weights added are summarized in the supplementary material: annex 2: supplementary material of the experimental results).

Table 4.1 – Characteristics of the WAS and FW in the first set of batch fermentation tests.

Parameter (units)	WAS	FW	WAS:FW Mixture
pH (-)	7.70 ± 0.05	-	7.07 ± 0.33
COD (mg L^{-1})	32441 ± 8721	19553 ± 5568	19553 ± 5568
sCOD (mg L^{-1})	5474 ± 39	20888 ± 292	20888 ± 292
Total VFA (mg COD L^{-1})*	1221	2246	
Acetic acid (mg COD L^{-1})	400	879	-
Propionic acid (mg COD L^{-1})	148	111	-
Butyric acid (mg COD L^{-1})	164	512	-
Valeric acid (mg COD L^{-1})	190	211	-
Caproic acid (mg COD L^{-1})	250	298	-
Heptanoic acid (mg COD L^{-1})	69	235	-
TAN (mg NH_4^+-N L^{-1})	166 ± 7	34 ± 1	-
Total alkalinity (mg $CaCO_3$ L^{-1})	1696 ± 375	-	1400 ± 475
Partial alkalinity (mg $CaCO_3$ L^{-1})	4143 ± 153	-	3960 ± 50
TS (g L^{-1})	43.7 ± 0.2	186.3 ± 1.5	49.2 ± 0.9

Parameter (units)	WAS	FW	WAS:FW Mixture
VS (g L^{-1})	31.2 ± 0.1	177.3 ± 1.4	37.1 ± 0.9

*The VFAs analysis was performed once in an external laboratory (no standard deviation available)

The evolution of pH, sCOD, TAN, VFA yield and VFA $sCOD^{-1}$ ratio is presented in figure 4.1, while figure 4.2 depicts the evolution of VFA concentration and its profile. As it can be observed in the monitored data, in the reference test (control) pH progressively decreased down to 5.5 in 8 days and then, a slight increase of pH was observed. This fact has been related to VFA consumption due to methanogens (Bolzonella et al., 2005). The same trend was recorded in bottles with initial pH 10, where pH decreased in 8 days to 5.6 and then remained constant or even slightly increased. In these assays where pH was regulated to 10 and 7 at the sixth day of experiment (conditions iii and iv, respectively), decreased from an initial pH of 7.7 down to pH 5.5 at day 6, before the pH was modified. After the new change of pH, iii condition lead to a pH decline from 9.9 to 8.5 in the following 4 days and in iv condition, the pH dropped from 6.7 to 6.6 within 2 days and then raised to 6.7 at day 10.

Regarding VFA production, maximum VFA concentrations were reached on day 10 and the highest VFA content (13.6 g COD_{VFA} L^{-1}) was observed when working with an initial pH value of 10. The control test produced 13.2 g COD_{VFA} L^{-1}, without external reagents consumption. Slightly lower values were obtained in iii and iv conditions with 12.7 and 12.1 g COD_{VFA} L^{-1}, respectively, although the pH regulation at day 6 lead to a clear enhancement of VFA production. In all the studied cases, acetic and butyric acids were the predominant VFAs produced, with percentages in the range of 30-50% and 20-40% (COD basis), respectively. These results are in accordance with those obtained by Vidal-Antich et al. (2021), who reported that higher butyric acid percentages were obtained when the proportion of FW in the WAS:FW mixture was increased to the detriment of propionic acid percentage. In fact, in this study propionic and valeric acids showed similar percentages (nearby 10% on COD basis) and heptanoic had the lowest proportion (<10% on COD basis).

The VFA yield, expressed as the quantity of VFAs (COD basis) produced per unit of VS fed, reached maximum values within the range of 329-369 mg COD_{VFA} (g VS fed)$^{-1}$ at day 10. Furthermore, in control bottles the proportion of the soluble COD associated with VFAs (COD basis) raised from 0.39 up to 0.79 mg COD_{VFA} (mg sCOD)$^{-1}$. However, in bottles initially controlled at pH 10, this ratio increased from 0.29 to 0.89 mg COD_{VFA} (mg sCOD)$^{-1}$, indicating higher solubilisation due to the alkaline pH which led to a higher VFA production. Lower values in

the range of 0.46 and 0.68 mg COD_{VFA} (mg sCOD)$^{-1}$ were reached when pH was controlled at day 6 at conditions iii and iv, respectively.

Moreover, sCOD and NH_4^+-N increased along the experiment, reaching maximum values at the end of each test. Higher sCOD values were obtained in those conditions with major pH, since alkaline pH values are related to higher hydrolysis of suspended organic matter (Cheah et al., 2019). Besides, TAN concentration at the end of each test was higher when maximum value of sCOD was monitored (due to organic nitrogen ammonification) and it was in the range of 1.1-1.5 g NH_4^+-N L^{-1}, depending on the working conditions.

Second set of fermentation tests. Analysing the first batch performed, it is seen that modifying pH at sixth day of operation slightly increase VFA production due to the fermentative activity reduction through time. To study the effect of pH in different time, 4 different conditions were tested in a second fermentation batch test using same proportion of WAS:FW of 75:25 (%VS basis) without external inoculum:

i) reference without pH control.

ii) initial pH of 10.

iii) no pH control and pH regulation to 10 at days 2 and 5.

iv) no pH control and pH regulation to 7 at days 2 and 5.

Each condition was done by quadruplicate (16 bottles were used in total). The weights filled in each bottle were 150 g of WAS and 7.6 g of FW (specific weights are summarized in the supplementary material: annex 2: supplementary material of the experimental results). Characteristics of substrates are collected in table 4.2.

Table 4.2 – Characteristics of the WAS and FW in the second set of batch fermentation tests.

Parameter (units)	WAS	FW	WAS:FW Mixture
pH (-)	7.15 ± 0.03	5.06 ± 0.03	6.90 ± 0.05
COD (mg L^{-1})	40272 ± 45	187564 ± 12691	-
sCOD (mg L^{-1})	561 ± 203	118219 ± 2402	-
Total VFA (mg COD L^{-1})	83 ± 45	6350 ± 81	-
Acetic acid (mg COD L^{-1})	83 ± 45	5479 ± 45	-
Propionic acid (mg COD L^{-1})	n.d.	96 ± 17	-
Butyric acid (mg COD L^{-1})	n.d.	672 ± 35	-
Valeric acid (mg COD L^{-1})	n.d.	n.d.	-
Caproic acid (mg COD L^{-1})	n.d.	103 ± 1	-
Heptanoic acid (mg COD L^{-1})	n.d.	n.d.	-
TAN (mg NH_4^+-N L^{-1})	142 ± 8	164 ± 21	-
Total alkalinity (mg $CaCO_3$ L^{-1})	2722 ± 9	-	3064 ± 26

Parameter (units)	WAS	FW	WAS:FW Mixture
Partial alkalinity (mg $CaCO_3$ L^{-1})	1164 ± 46	-	1299 ± 53
TS (g L^{-1})	37.7 ± 0.2	189 ± 1	-
VS (g L^{-1})	27.6 ± 0.2	182.0 ± 0.8	-

*n.d. refers to non-detected concentration.

Evolution of pH, sCOD, TAN, VFA yield and VFA $sCOD^{-1}$ ratio is presented in figure 4.3, while figure 4.4 collects the evolution of VFA concentration and its profile. As expected by previous results, pH decreased within 0-7 days and then increased between 7-9 days as acetic consumption phase increased in these conditions without pH control and initial pH 10 (conditions i and ii, respectively) as happened for 2 days and after the pH regulation in day 2 in conditions with pH regulation during the experiment (conditions iii (pH 10 regulation) and iv (pH 7 regulation)). Moreover, after pH regulation in day 5, pH slightly decreased in condition iii and increased in condition iv as consequence of methanogenic enhancement in pH nearby 7 (Clark & Speece, 1971).

As was foreseeable, highest VFA production was found in day 9 in all conditions tested. Maximum VFA concentration was achieved in test with pH 10 regulated twice (18.3 g COD_{VFA} L^{-1}). Lower values were obtained in condition ii and iv with 14.8 and 14.4 g COD_{VFA} L^{-1}, respectively as control test produced 12.2 g COD_{VFA} L^{-1}. Modifying pH in day 2 clearly improves VFA production by enhancing hydrolysis and organic matter solubilisation while higher fermentative activity occurred (Maspolim et al., 2015). Furthermore, VFA composition and VFA yield reached similar values as previous fermentation batch although the proportion of VFA contributed to soluble COD was higher (0.91-0.99 mg COD_{VFA} (mg sCOD)$^{-1}$ in day 9). Soluble COD and TAN concentrations evolution through time were similar as the first set of fermentation batch.

Third set of fermentation tests. Due to study the effect of initial pH in a lab-scale semi-continuous fermenter, 3 different conditions were tested during 8 days in a third fermentation batch test using as substrate the effluent from a semi-continuous fermenter working with a proportion WAS:FW of 65:35 (%VS basis) without external inoculum:

i) reference without pH control.

ii) initial pH of 10.

iii) initial pH of 7.

Each bottle was filled with 150 g of effluent (see specifics weights in annex 2: supplementary material of the experimental results) and conditions were studied for quadruplicated (12 bottles in total). Effluent's characteristics are collected in table 4.3.

Table 4.3 – Characteristics of the semi-continuous fermenter's effluent in the third set of batch fermentation tests.

Parameter (units)	**Fermenter's effluent**
pH (-)	5.09 ± 0.02
COD (mg L^{-1})	20177 ± 1456
sCOD (mg L^{-1})	11198 ± 193
Total VFA (mg COD L^{-1})	9997 ± 55
Acetic acid (mg COD L^{-1})	3211 ± 45
Propionic acid (mg COD L^{-1})	1653 ± 2
Butyric acid (mg COD L^{-1})	3280 ± 3
Valeric acid (mg COD L^{-1})	1616 ± 3
Caproic acid (mg COD L^{-1})	211 ± 1
Heptanoic acid (mg COD L^{-1})	26 ± 1
TAN (mg NH_4^+-N L^{-1})	285 ± 8
Total alkalinity (mg $CaCO_3$ L^{-1})	2722 ± 9
Partial alkalinity (mg $CaCO_3$ L^{-1})	1164 ± 46
TS (g L^{-1})	43 ± 1
VS (g L^{-1})	34 ± 1

Evolution of pH, sCOD, TAN, VFA yield and VFA $sCOD^{-1}$ ratio is shown in figure 4.5 as figure 4.6 depicts the evolution of VFA concentration and its profile. As foreseeable, pH 10 enhanced VFA production (16.9 g COD_{VFA} L^{-1}) and had the highest VFA yield (496 mg COD_{VFA} L^{-1} (g VS fed)$^{-1}$). Furthermore, pH 7 had the lowest VFA concentration (12.6 g COD_{VFA} L^{-1}) demonstrating to be the condition with most acetic consumption due to acetotrophic methanogenic enhancement (Dahiya et al., 2015). The proportion of VFA contributed to soluble COD reached >0.90 mg COD_{VFA} (mg sCOD)$^{-1}$ as occurred in the second set of batch fermentation tests.

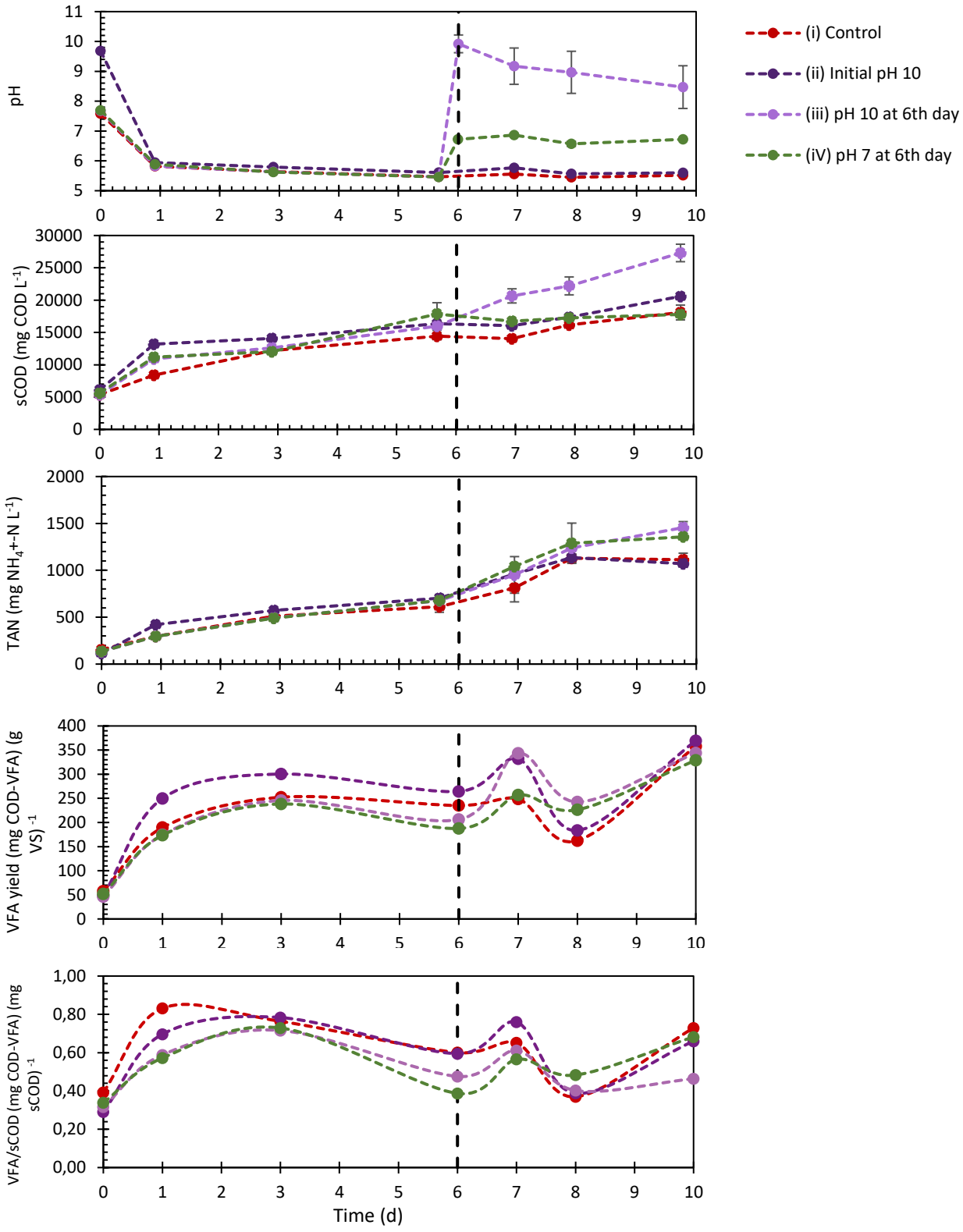


Figure 4.1 – Evolution of pH, sCOD, TAN concentration, VFA yield and VFA $sCOD^{-1}$ ratio in the first batch of fermentation tests treating a mixture of WAS:FW of 75:25 % (on VS basis).

(---- is referred to day 6 where a modification of pH was performed in some assays – see pH evolution).

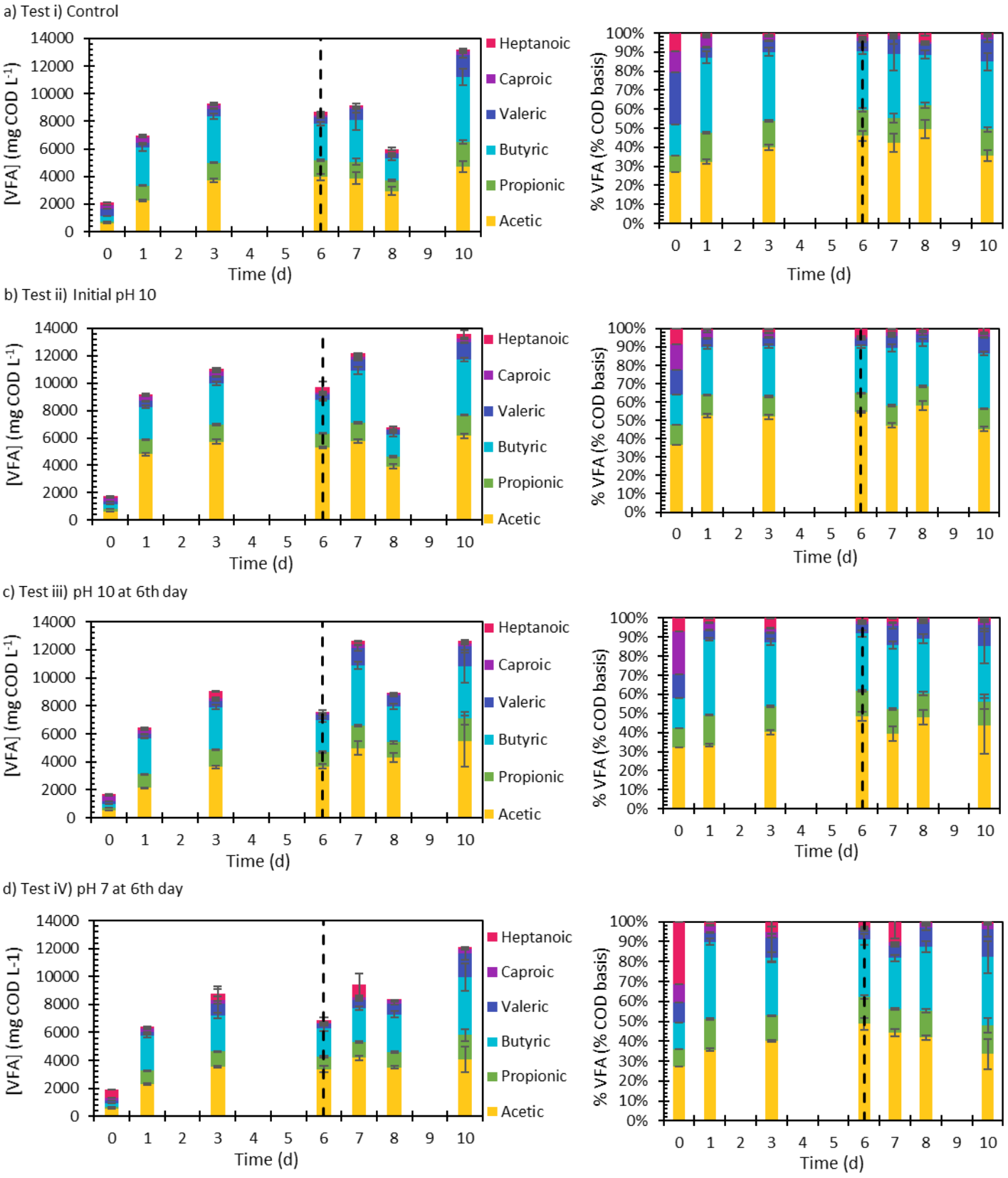


Figure 4.2 – Evolution of the VFA concentration and distribution (% on COD basis) for the conditions i (a), ii (b), iii (c) and iv (d) of the first set of batch fermentation tests (---- is referred to day 6 where a modification of pH was performed in some assays – see pH evolution).

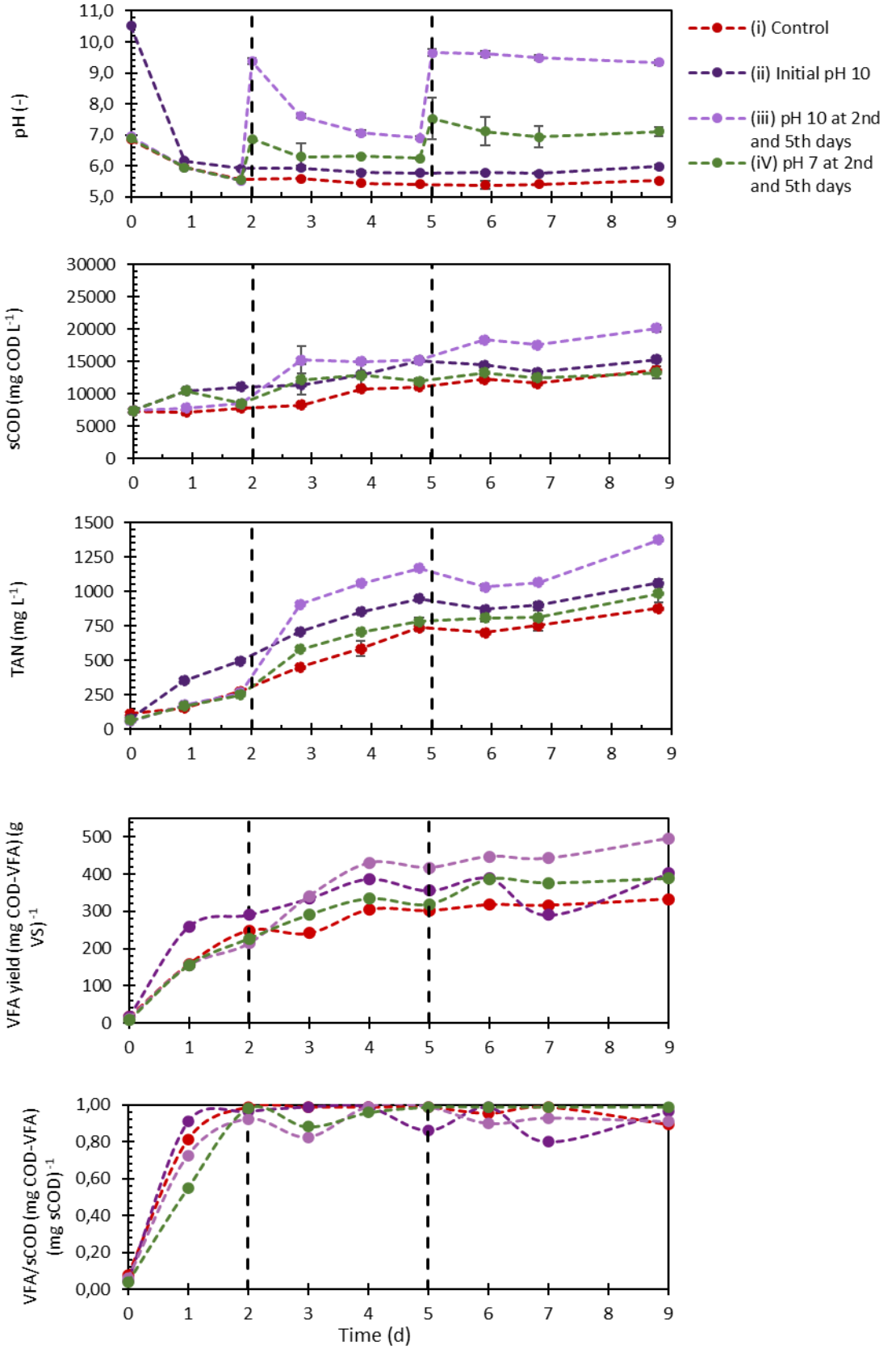


Figure 4.3 – Evolution of pH, sCOD, TAN concentration, VFA yield and VFA sCOD^{-1} ratio in the second batch of fermentation tests treating a mixture of WAS:FW of 75:25 % (on VS basis) (---- is referred to days 2 and 5 where a modification of pH was performed in some assays – see pH evolution).

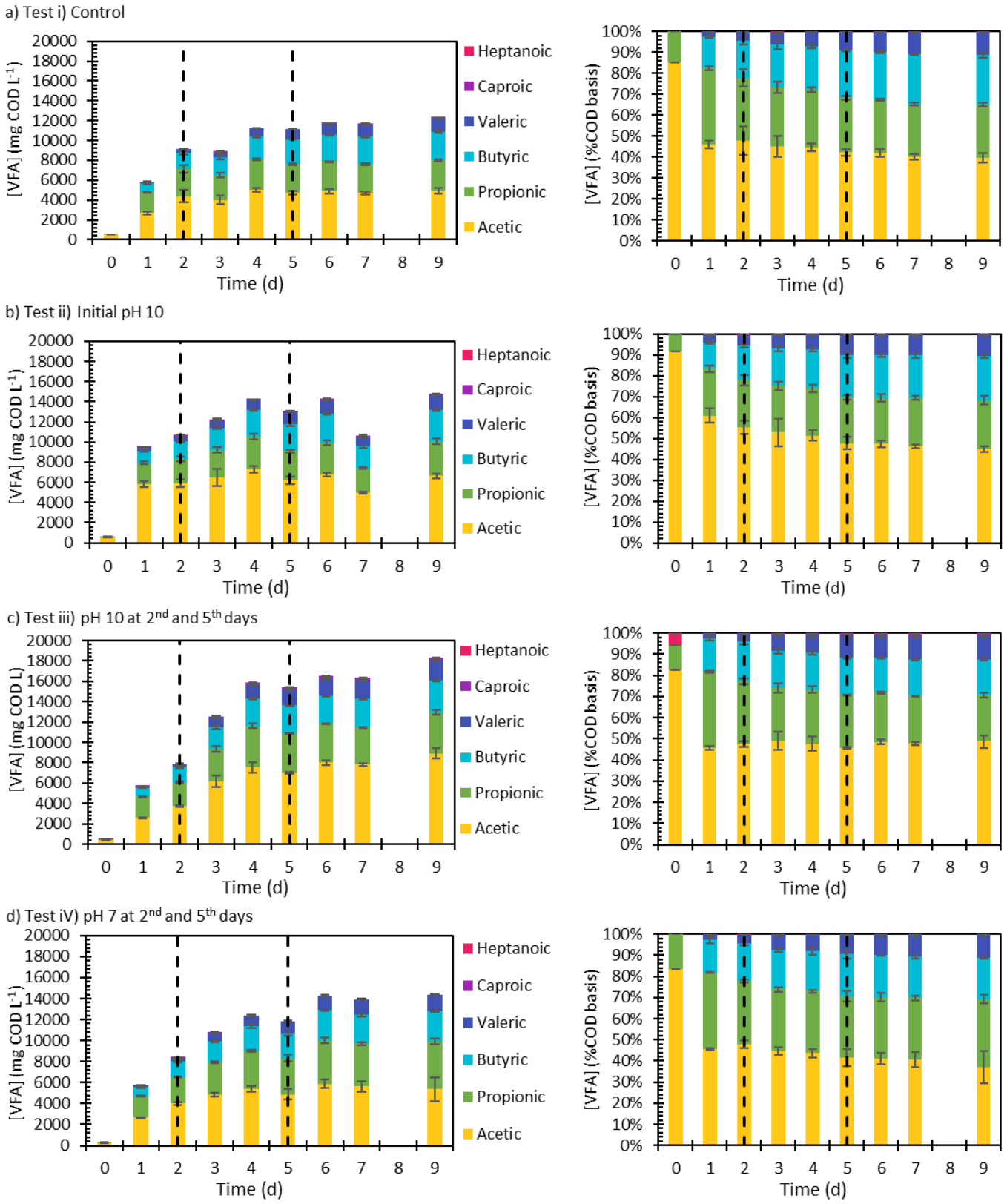


Figure 4.4 – Evolution of the VFA concentration and distribution (% on COD basis) for the conditions i (a), ii (b), iii (c) and iv (d) in the second set of fermentation batch tests (---- is referred to days 2 and 5 where a modification of pH was performed in some assays – see pH evolution).

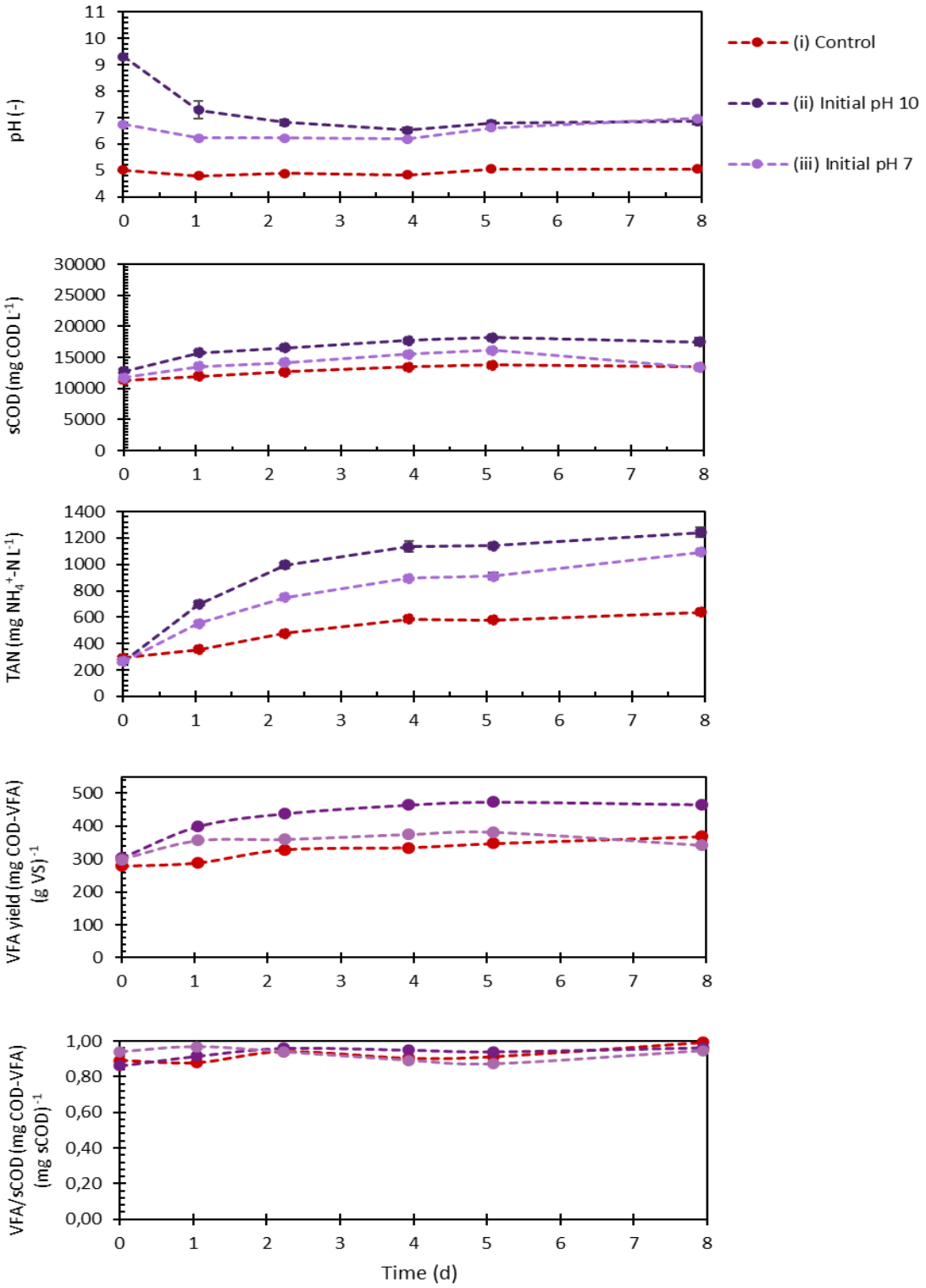


Figure 4.5 – Evolution of pH, sCOD, TAN concentration, VFA yield and VFA sCOD^{-1} ratio in the third batch of fermentation tests treating the effluent of a semi-continuous fermenter.

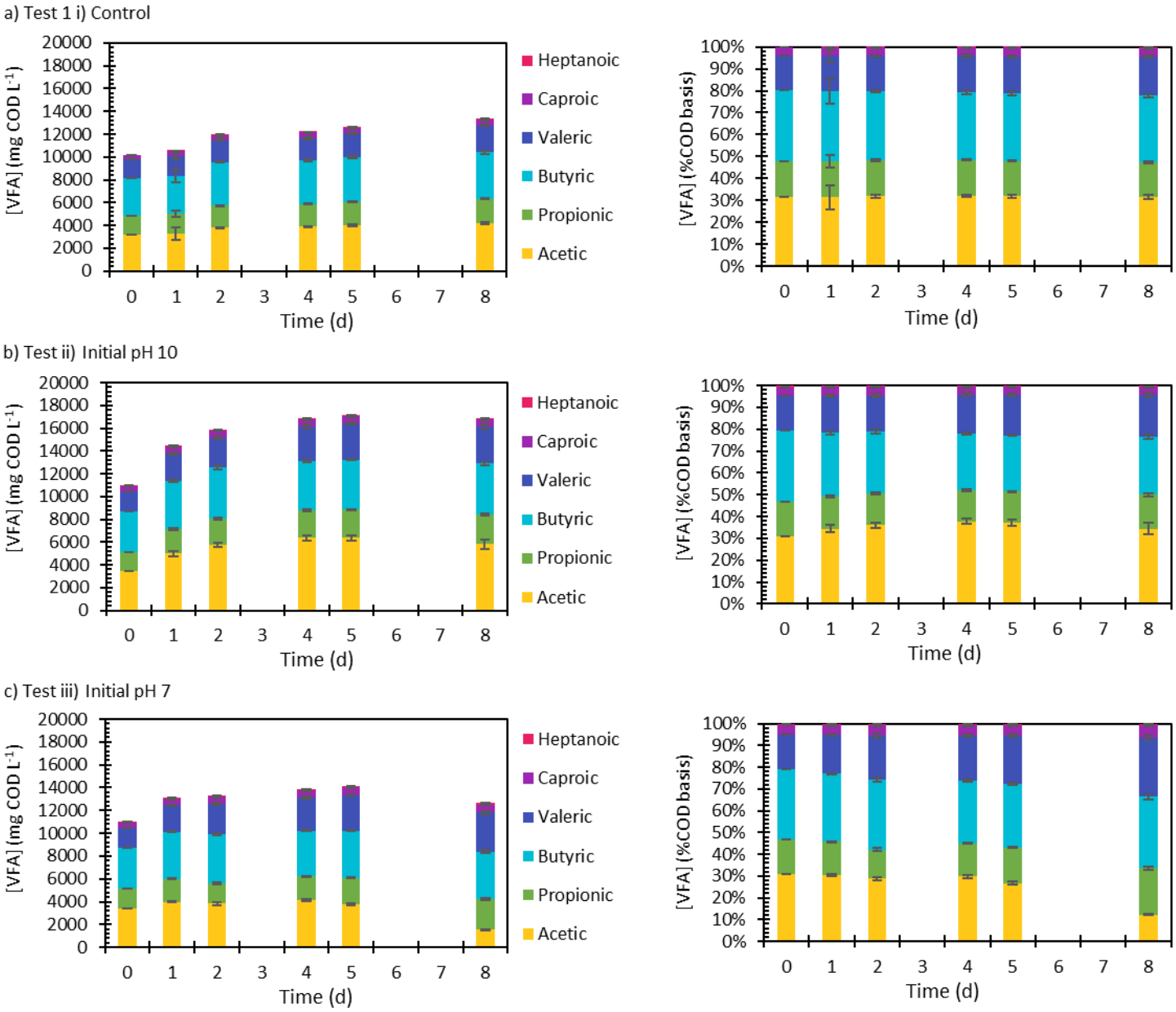


Figure 4.6 – Evolution of the VFA concentration and distribution (% on COD basis) for the conditions i (a), ii (b), iii (c) in the third set of batch fermentation tests.

Semi-continuous fermenters. To test the long-term effect of FW and WAS co-fermentation, 2 semi-continuous fermenters (A and B) were operated for 46 days. The operation of both reactors started using the same proportion of WAS:FW used in third batch test (see section 4.1), namely 65:35 (%VS basis). The organic loading rate (OLR) was established at 11 g VS L^{-1} d^{-1} and an HRT of 3.2 d (**period 1 / 0-34 days**).This working conditions were implemented since an HRT of 3.2 days is considered low to promote the proliferation of methanogens and an OLR of 11 g VS L^{-1} d^{-1} has been reported as an optimum value for VFA production (Strazzera et al., 2021). It

is important to highlight that at day 15, the agitation blades of the reactors were changed to improve the contact between the feed stream and the microorganisms present in the reactor. Due to foaming issues, during **period 2 (34-43 days)** the WAS:FW composition of the feed stream of reactor A was changed to 75:25 of WAS:FW (%VS basis), while maintaining the OLR (11.0 g VS L^{-1} d^{-1}) and slightly decreasing the HRT (at 2.9 d), as an attempt to avoid these foaming issues enhancing the withdrawal of acetic consumers and the FW associated protein-lipidic components reduction. Since the operation of the reactor was improved but foaming was not completely avoided, during **period 3 (43-46 days)**, the influent of reactor A was changed again to work with a WAS:FW proportion of 80:20 (%VS basis), an OLR of 12.5 g VS L^{-1} d^{-1} and HRT of 2.4 d. Reactor B was operated during the whole experiment using the operating conditions applied during period 1. Figure 4.7 shows the evolution of pH, sCOD, TAN concentration, VFA yield and VFA $sCOD^{-1}$ ratio in continuous fermenters operation of both reactors and figure 4.8 collects the total VFA concentration and the VFA production and distribution for both fermenters.

Regarding to TS and VS, increasing evolution from both parameters in effluents indicated that there were solids accumulation as foam phase and so, undesirable operation of the reactors until modifications in agitation were performed at day 15. After the upgrading, TS and VS stabilized in the effluents (~30 and 20 g L^{-1}, respectively) as result of the better homogenization in mixed liquor and higher contact between fermenters' microorganisms and feed although it can be distinguished a slightly accumulation of solids as foam (inlets' solids 10% higher than output solids). To reduce this behaviour, during period 1, the effluents were withdrawn by adjusting tube heigh to extract by level the same volume as fed and maximize foam removal besides operation parameters changed in period 2 and period 2b. These strategies did not solve the foam formation and pH control could not being applied, although period 2b reduced foam at suitable levels to control pH, this period produced a foam overflow in day 46 that forced to stop the operation of reactor A and the experiment.

As observed in collected data, pH in fermenters decreased (5.1-6.9) compared to inlets (6.7-7.6) indicating VFA production (11-3.5 g COD_{VFA} L^{-1}) during the operation and the consequently acidification of the mixed liquor. Moreover, pH in the mixed liquor increased along time as VFA consumption occurred reducing acetic acid from 40% to 2% (COD basis) and butyric acid from 30% to 10% (COD basis) as propionic acid increased from 15% to 50% (COD basis) as consequence of the total VFA reduction. Furthermore, period 2 and period 2b increased the proportion of acetic acid to 6% and 16% (COD basis), respectively as in fermenter B remained in 3% (COD basis). A reduction of the acetic acid degraders is observed as HRT was reduced

(period 2 and 2b) as result of the smaller times that these microorganisms remained in the fermenter (Vidal-Antich et al., 2021).

The VFA yield and the proportion of VFA in soluble COD were into the range of 200-385 mg COD_{VFA} (g VS Fed)$^{-1}$ and 0.40-0.99 mg COD_{VFA} (mg sCOD)$^{-1}$, respectively for both reactors.

As seen, sCOD values in fermenters were kept between 5.3-10.9 g COD L^{-1} through the operation decreasing as acetic and butyric acids were consumed. Soluble COD values in effluents were higher than inlets until day 15, indicating the hydrolysis phase in fermenters by increasing the soluble organic matter. Moreover, since day 16, sCOD values in effluents were slightly lower than inlets as consequence of the acetic consumption due to biogas production (Westerholm et al., 2016). On the other hand, total COD was similar in inlets and effluents in a range of 28.1-69.2 g COD L^{-1} along the operation.

Furthermore, TAN concentration in effluents increased along the operation (compared to inlets) in both reactors as consequence of the ammonification of the organic matter.

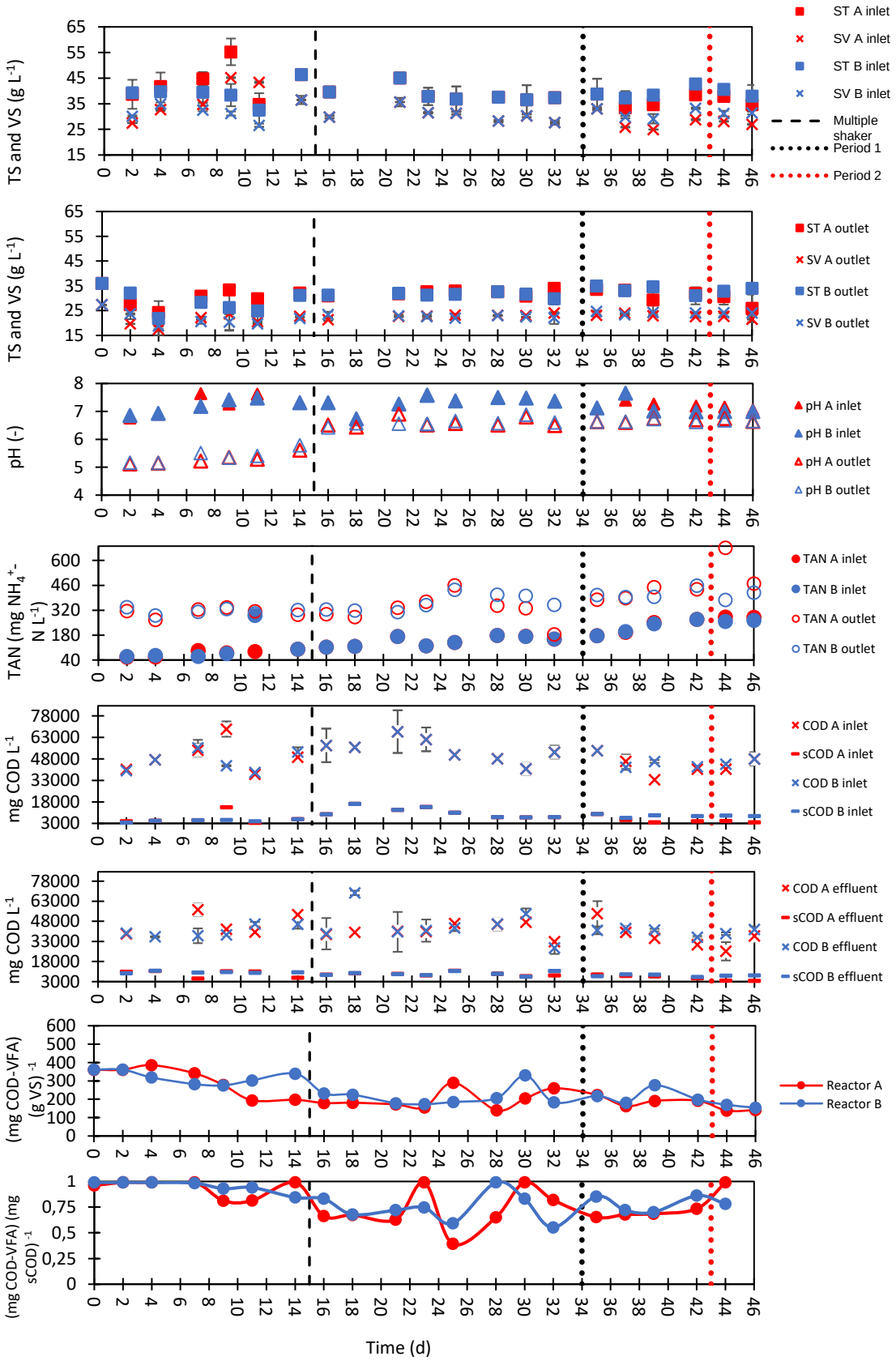


Figure 4.7 – Evolution of pH, TAN concentration, sCOD, COD and sCOD concentrations, VFA yield and VFA sCOD^{-1} ratio in continuous fermenters operation.

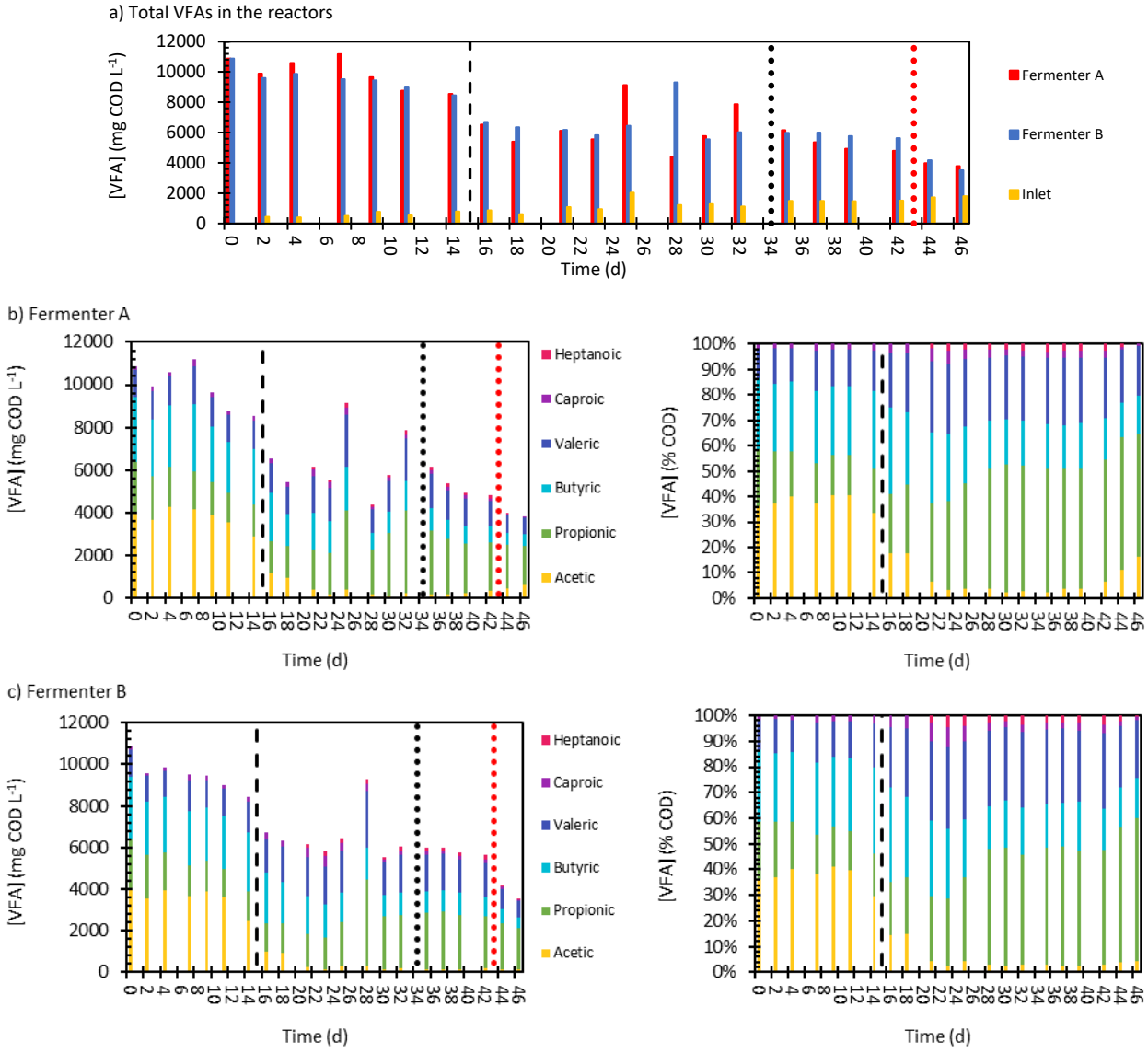


Figure 4.8 – Evolution of the total VFA concentration in inlets and fermenters (a). Evolution of the VFA concentration and distribution (% on COD basis) for the fermenter A (b) and fermenter B (c).

4.2.PHA production unit

Considering the results of Serra-Toro et al. (2022), who completely recovered the TAN content of a fermentation effluent using gas-permeable membranes, in this project the operation of a selection reactor treating a VFA-rich synthetic wastewater without nitrogen content was studied. The selection reactor was operated for 30 days, during which 2 different stages could be distinguished according to the OLR and NLR applied. Moreover, the biomass purge of this reactor was introduced in an accumulation batch equipment where pulses of the VFA-rich synthetic wastewater were performed to increase the PHA content of this selected biomass. Figure 4.9

shows a scheme of the proposed process. In the next section, the results obtained in the selection SBR operation and the accumulation batch tests are discussed.

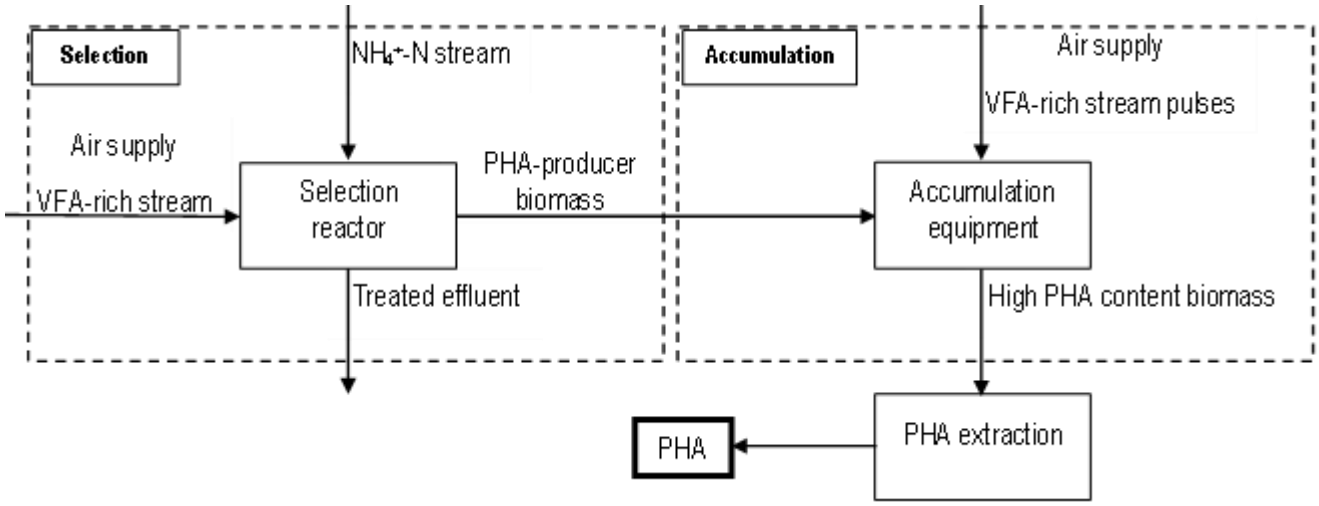


Figure 4.9 – Scheme of the PHA production process using VFA-rich stream without N content.

The performance of a sSBR was monitored for 30 days and different periods could be distinguished by analysing VFA and TAN concentrations in the effluent: **(i) start-up (days 1-8)**, **(ii) operating period with a COD N^{-1} ratio of 3.08 g COD_{VFA} (g NH4+-N)$^{-1}$ (days 9-17**) and **(iii) operating period with a COD N^{-1} ratio of 1.09 g COD_{VFA} (g NH4+-N)$^{-1}$ (days 18-30)**. Figure 4.10 shows the monitoring of VFA and TAN concentration in the treated effluent, the duration of the feast conditions with respect to the total cycle length, the TSS, VSS and ratio VSS TSS^{-1} in the biomass purge of the sSBR, besides the PHA content and the percentage of PHB of this biomass. Moreover, average data of TSS, VSS, PHA content, PHB PHA^{-1} proportion on the purge and VFA, TAN concentrations, pH on the effluent as well as the feast to cycle length time ratio are summarized in table 4.4.

As collected in data, set-up period was characterized to present a high variation on VFA concentration, PHA content and feast-cycle relation. Furthermore, first operational period had the lowest VFA and TAN concentration (1.48 mg COD_{VFA} L^{-1} and 0.48 mg NH_4^+-N L^{-1}, respectively) and higher PHA content (29.84% on SS basis) indicating that nitrogen decoupling in an aerobic feast-famine regime increases the PHA content in biomass as demonstrated in Oliveira et al. (2017). The second period had the lowest PHA content (11.23% on SS basis) and feast $cycle^{-1}$ proportion while TAN reached the highest concentration (13.46 mg $N\text{-}NH_4^+ \cdot L^{-1}$). Moreover, no substantiable differences were observed in TSS, VSS, PHB proportion and pH in the three operational phases.

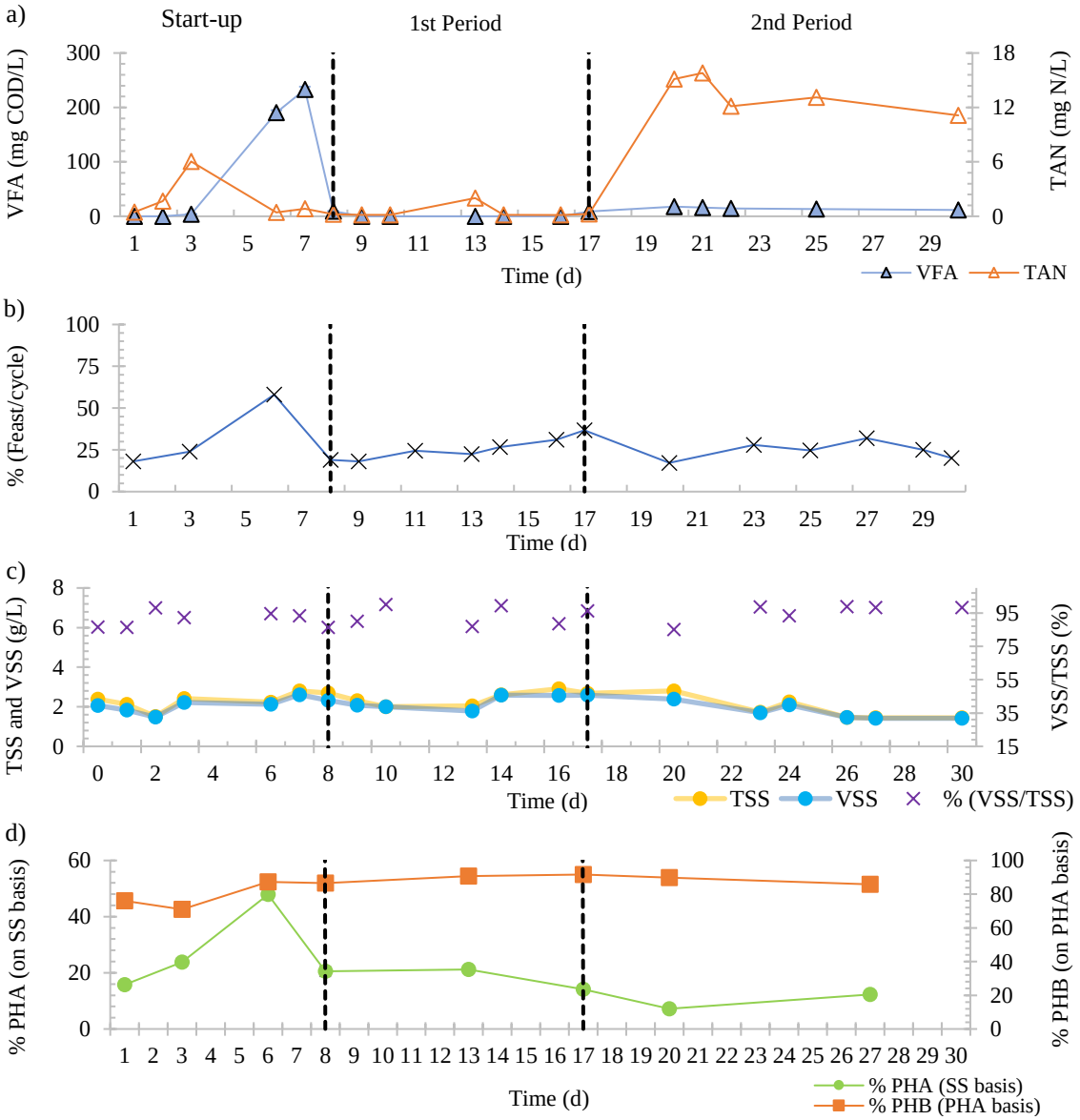


Figure 4.10 – (a) sSBR VFA and TAN concentrations in effluent, (b) sSBR ratio feast cycle^{-1}, (c) sSBR TSS and VSS in purge and (d) sSBR PHA content in the purge.

Table 4.4– sSBR operational parameters.

Parameter (units)	Start-up period (days 1-8)	1st operational period (days 9-17)	2nd operational period (days 18-30)
Temperature (ºC)	35	35	35
HRT (d)	1.12	1.12	1.12
SRT (d)	4.21	4.21	4.21
TSS ($g \cdot L^{-1}$)	2.31 ± 0.42	2.42 ± 0.36	1.94 ± 0.58
VSS ($g \cdot L^{-1}$)	2.09 ± 0.36	2.26 ± 0.36	1.81 ± 0.41
% PHA (% SS basis)	29.15 ± 16.69	29.84 ± 0.51	11.23 ± 3.60
% PHB (% PHA basis)	78.11 ± 8.38	88.66 ± 2.90	89.11 ± 3.00
VFA concentration in effluent (mg COD L^{-1})	72.78 ± 108.50	1.48 ± 4.46	14.72 ± 2.45
TAN concentration in effluent (mg N-NH_4^+-N·L^{-1})	1.61 ± 2.23	0.48 ± 0.74	13.46 ± 1.97
Ratio COD N^{-1} (mg COD (mg N-NH_4^+-N)$^{-1}$)	-	3.08 ± 4.55	1.09 ± 0.31
OLR (g COD L^{-1} d^{-1})	-	2.51 ± 0.01	2.51 ± 0.01
NLR (g NH4+-N L^{-1} d^{-1})	-	0.09 ± 0.01	0.16 ± 0.01

Parameter (units)	Start-up period (days 1-8)	1st operational period (days 9-17)	2nd operational period (days 18-30)
Ratio OLR/NLR (g COD (g NH4+-N)$^{-1}$)	-	26.70 ± 0.01	15.69 ± 0.01
pH effluent (-)	8.65 ± 0.35	8.22 ± 0.04	8.37 ± 0.29
feast to cycle length time ratio (-)	0.30 ± 0.19	0.29 ± 0.11	0.24 ± 0.06

Representative sSBR cycles of both first and second operational periods are described with the monitored data (DO, TAN and VFA concentrations, pH, PHA content and PHB proportion) as shown in figure 4.11 a and b.

The monitoring of the sSBR cycle under pseudo steady-state operation in the first period (ratio OLR NLR^{-1} 26.70 g COD_{VFA} (g $NH4^{+}$-N)$^{-1}$) as represented in figure 4.11 a, revealed that VFAs were completely depleted within the first 140 min of feast phase (aerobic react) of the operating cycle, decreasing initial concentration from 0.5 to 0.0 g COD_{VFA} L^{-1}. TAN concentration remained nearby 0 mg NH_4^{+}-N L^{-1}, indicating a suitable performance of a double selection strategy. 25 min after the consumption of VFAs, TAN was supplied in the mixed liquor according to the applied NLR and was consumed at the end of the cycle (<2 mg NH_4^{+} L^{-1}). Regarding to PHA content of the biomass, it increased during feast conditions up to 16-32% (SS basis) and then, decreased to 27% (SS basis) because of famine period. Sludge purge was programmed at the 150 min of operation, 10 min after the maximum PHA content was reached. Besides, the PHB proportion in PHA followed the same trend as PHA, increasing from 77 up to 87% (SS basis) during feast conditions. As it can be observed, pH had an increasing trend in the range of 7.66-8.82 because of VFAs consumption and CO_2 stripping, while a sudden rise of the DO concentration profile clearly showed the time when VFAs were completely depleted.

Figure 4.11 b collects the monitoring of a representative sSBR cycle (pseudo-stationary state operation) in the second period, which shows the VFAs completely depletion produced within the first 120 min of aerobic reaction (feast phase) of the operating cycle, decreasing VFAs concentration from 800 to 11 mg COD_{VFA} L^{-1}. TAN was added after the sludge purge, when VFAs were completely consumed. However, this nutrient was not completely consumed during famine conditions and the decoupling of VFAs and NH_4^{+}-N availability was not met during this period, since the OLR NLR^{-1} ratio was lower (15.69 g COD_{VFA} (g $NH4^{+}$-N)$^{-1}$) than in the previous operating period. Therefore, the selection of PHA-storing organisms in this period only relied on the alternation of feast and famine conditions. Nevertheless, PHA content increased during feast phase from 12 up to 18% (SS basis) and then decreased to 14% (SS basis) as famine phase took place. PHB proportion in PHA remained stable nearby 92% (SS basis) and pH increased from 7.3 to 9.1, which is attributed to VFAs consumption and CO_2 stripping. The DO

concentration profile followed the same pattern recorded in the previous period, with a sudden rise of its value when VFAs were consumed.

By analysing these results, it was clearly observed that when a double selection strategy was established successfully, ensuring that low values of residual TAN and VFA concentrations were recorded at the end of the feast and famine stages, respectively, biomass more enriched in PHA was obtained as consequence of the higher growth inhibition of those microorganisms incapable to accumulate carbon reservoirs during feast as lack of an essential nutrient as N and enabling the sSBR to treat higher OLR without decreasing selection yields (Oliveira et al., 2017).

Nevertheless, the PHA potential of accumulation associated to biomass is an important fact to determinate a suitable selection and to study that, different accumulation tests were performed with purges of each operational periods. The results of the accumulation tests performed are shown in figure 4.12 a and b (first and second operational periods, respectively). Moreover, detailed information for each accumulation test besides the repetitiveness of these tests can be found in tables ii.4-ii.7 in annex 2: supplementary material of the experimental results.

Representative accumulation test for each sSBR operational period were analysed by monitoring DO, TAN and VFA concentrations, pH, PHA content and PHB proportion. All data are collected in figure 4.12 a and b.

As it can be observed in the representative accumulation test of the first operation period (figure 4.12 a), in each VFA addition (discontinuous grey lines in Y axis) pH remained within the range of 7.1-9.1 and DO profile clearly indicated the time when each VFA pulse was consumed by showing a sudden rise in its profile. To confirm this consumption, VFAs were analysed before and after each pulse. As expected, the initial VFA concentration after each pulse feeding was in the range of 280-174 mg COD_{VFA} L^{-1} and the VFA concentrations recorded after the rise in the DO profile were in the range of 0-16 mg COD_{VFA} L^{-1}. Furthermore, VFA degradation was lower in each pulse-feeding performed demanding more time to the complete consumption. This VFA degradation led to an increase in PHA content from 14 up to 51% (SS basis), while the PHB proportion remained nearby 84-87% (SS basis). Not substantiable difference in PHA content was observed between the fifth and sixth additions increasing less than 1% (SS basis). Also, TAN concentrations were analysed to confirm the absence of nitrogen during the test.

Furthermore, in a representative accumulation test of the second operational period (figure 4.12 b), PHA content increased along the experiment increasing from 11% to 40% (SS basis) with a stable PHB composition closer to 82-87% (SS basis). As expected, total VFAs consumption was reached slowly after each spike as pH stabilized in the range of 7.1-8.7

depending on the VFA concentration. TAN concentration was also analysed to ensure the absence of nitrogen.

As data collected suggests, double selection strategy obtained a PHA content nearby 30% (SS basis) after feast phase. Thus, is reflected in accumulation tests which PHA in biomass reached the 50 % (SS basis) within 5 spikes. Besides, if the selection was based only in feast-famine regime, the PHA content after the aerobic react was substantively lower decreasing to an 11% (SS basis) which, by performing accumulation tests, raised to a PHA content of 40% (SS basis).

It is demonstrated that if more quantity of organic matter is wanted to be eliminated as PHA production is applied and thus, reducing PHA selection yields (Oliveira et al., 2017), a substrate and nitrogen decoupling during carbon abundance in the mixed liquor should be applied to increase selection efficiency in sSBR in consequence reaching higher PHA content in accumulation tests.

Moreover, it is corroborated that DO profile is an acceptable indicator of VFAs depletion for both selection and accumulation operations and a pseudo-stationary state operation can be identified by analysing the stability of TAN and VFA concentrations in effluent and SS and PHA content in the purge as well as the relation between the time taken by the feast and the cycle length.

Referring to feast $cycle^{-1}$ relation, double selection strategy increases this variable as consequence of the microorganism cultures incapability to accumulate C reservoirs during famine. Observed with the average difference between period 1 (double selection) and period 2 (non-double selection) obtaining in period 2 a 17.26% lower feast $cycle^{-1}$ proportion.

Aerobic dynamic feeding strategy with effluent withdrawal and nitrogen decoupling seems to be a suitable PHA production strategy as mentioned in Kourmentza et al. (2017) allowing to operate with major OLR and thus, to perform the process treating major organic matter.

If fermenter's effluent is used instead of the synthetic feed, it is expected a suitable PHA production implementation due to the elevated ratio COD_{VFA} $sCOD^{-1}$ (>80%) and similar VFA components while correct acidogenic fermentation operation was achieved (0-14 days). Furthermore, Albuquerque et al. (2010) showed that PHV increased to 20-30% depending on the VFA composition of the fermented molasses clarified and thus, sightly modifying PHA composition.

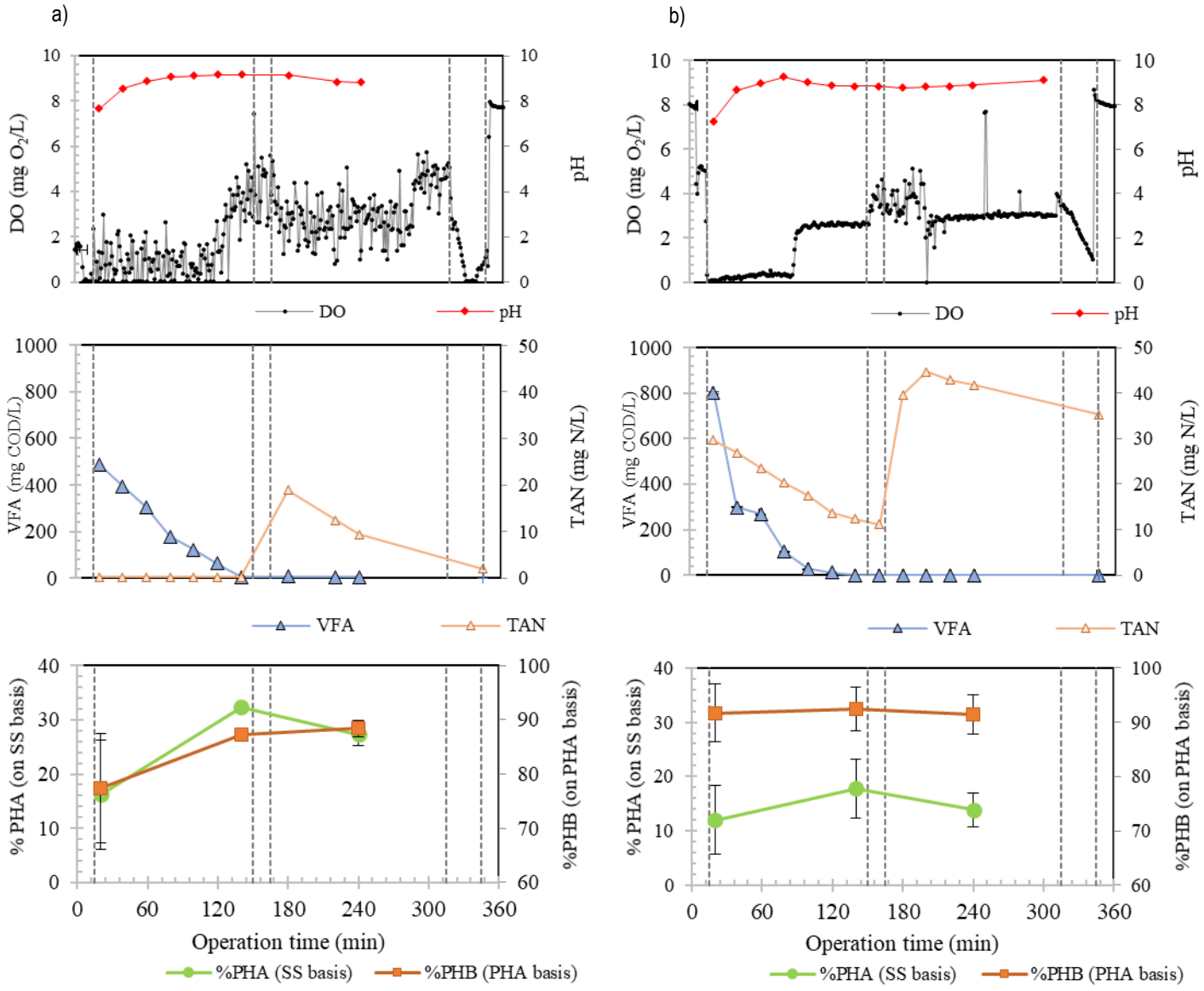


Figure 4.11 – Monitored DO, pH, VFA, TAN, %PHA and %PHB in (a) representative selection cycle of the first operational period and (b) representative selection cycle of the second operational period.

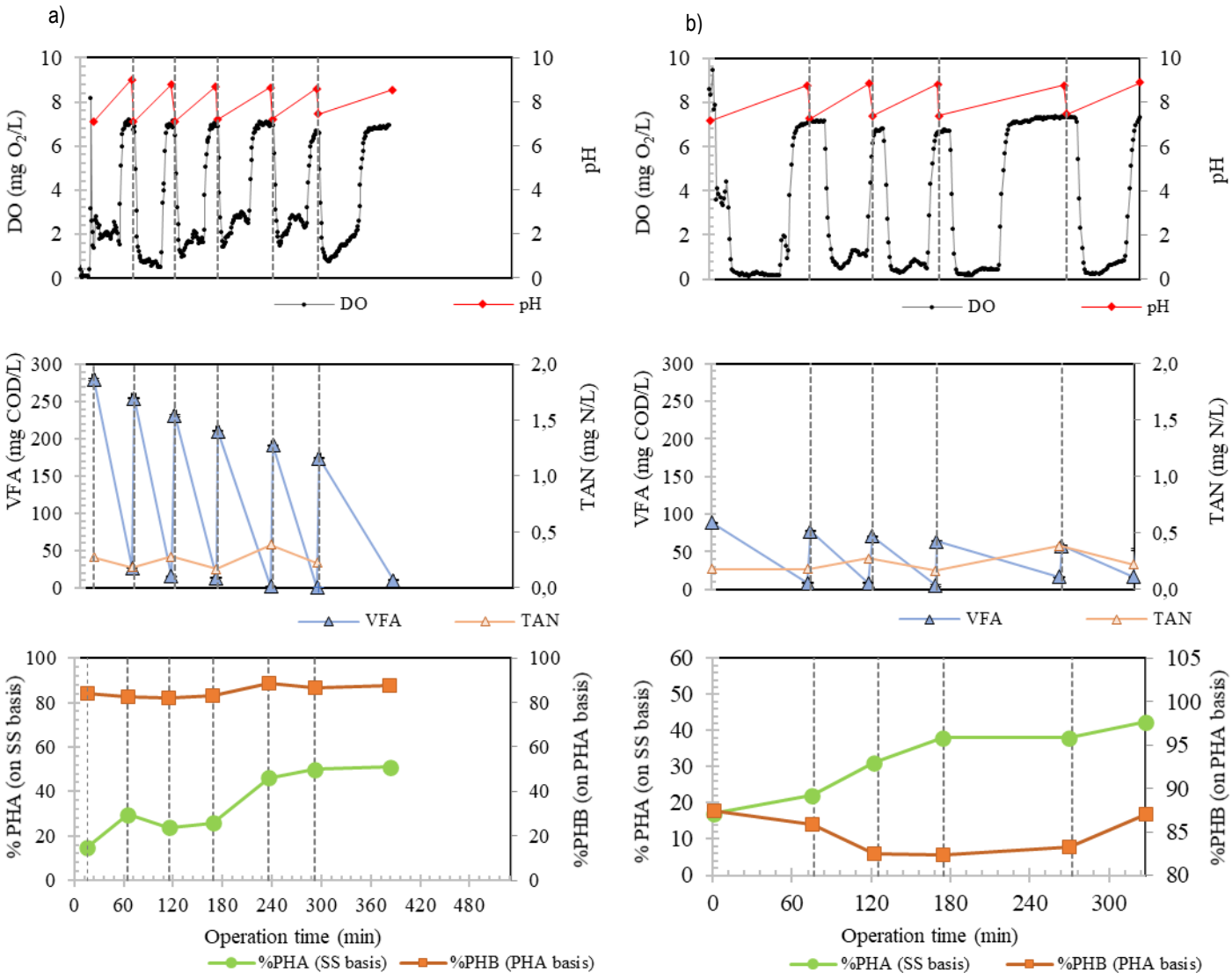


Figure 4.12 – Monitored DO, pH, VFA, TAN, %PHA and %PHB in (a) representative accumulation test of the first operational period and (b) representative accumulation test of the second operational period.

5. CONCLUSIONS AND RECOMMENDATIONS

5.1. Acidogenic fermentation unit

As observed in monitored data, main acids produced in acidogenic fermentation are acetic (~40%), propionic (30%) and butyric (20%) acids.

Increasing pH enhances hydrolysis and the consequently solubilization of organic matter reflected in higher VFA production, soluble COD and TAN concentrations. Moreover, pH in the range of 6.3-7.0 increase the acetic consumers microorganisms reducing VFA yields. Lower HRT reduces acetic consumers' activity in fermenters.

In batch tests, increasing pH after 5 days do not increase substantially VFA production as consequence of the lower fermentation activity. Moreover, if pH is modified earlier, it is seen a VFA increment.

In semi-continuous fermenters, if pH is raised and maintained through the operation increases the soluble COD which derives to reach higher VFA yields.

Foam formation in semi-continuous fermentation is an important issue to the suitable operation and measurement/control of pH. Lower foam levels can be enhanced with better homogenization, foam extraction using effluent tubes, lower HRT and FW proportion.

5.2.PHA production unit

As seen in data collected, if double selection strategy (aerobic feast-famine plus nitrogen decoupling) was applied during the biomass selection in sSBR, higher PHA content is obtained in purge (~30% in SS basis) reflected with a major PHA in accumulation tests (50% in SS basis) compared with single selection strategy (only aerobic feast-famine regime) where PHA content in purge and after accumulation tests were ~11% and ~38% on SS basis, respectively. PHA composition through operation and accumulation tests was ~90% in PHB and ~10% in PHV.

5.3. Comparison between VFA produced in fermenters and synthetic feed used to PHA production

During suitable operation of semi-continuous fermenters between 0-14 days, VFA profile was mainly composed by acetic acid (~40%), propionic acid (~20%) and butyric acid (~30%). Although VFA composition in synthetic feed was composed by acetic acid (~62%), propionic acid (~19%) and butyric acid (~19%), there is not a huge difference in product profile composition and it is not expected a malfunction in PHA selection and accumulation phases if fermenter's effluents with nitrogen removal are used. Moreover, PHB and PHV percentages probably would not change significatively.

6. ACRONYMS

COD, chemical oxygen demand; **CSTR**, continuous stirred-tank reactor; **DO**, dissolved oxygen; **FW**, food waste; **HRT**, hydraulic retention time; **NLR**, nitrogen loading rate; **OFMSW**, organic fraction of municipal solid waste; **OLR**, organic loading rate; **PHA**, polyhydroxyalkanoates; **PHB**, polyhydroxybutryate; **PHV**, polyhydroxyvalerate; **sSBR**, selection sequential batch reactor; **sCOD**, soluble oxygen demand; **SRT**, solid retention time; **SS**, suspended solids; **TAN**, total ammonium nitrogen; **TS**, total solids; **TSS**, total suspended solids; **VFA**, volatile fatty acids; **VS**, volatile solids; **VSS**, volatile suspended solids; **WAS**, wasted activated sludge; **WWTP,** wastewater treatment plant.

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ANNEXES

ANNEX 1: sSBR SYNTHETIC VFA COMPOSITION IN FEED AND CONCENTRATION OF AMMONIUM CHLORIDE

Volume required of each VFA in the synthetic feed of 3.5 g COD L^{-1}:

1) Volume required by each acid depending on mass proportions (x) (Dosta et al. 2018):

   a. Acetic acid (HAc):

$$\frac{0.625\ x\ g\ HAc}{L}\ \frac{1\ mol\ HAc}{60.05\ g\ HAc}\ \frac{2\ mol\ O_2}{1\ mol\ HAc}\ \frac{32\ g\ O_2}{1\ mol\ O_2} = 0.670\ x \frac{g\ O_2}{L}$$

   b. Propionic acid (HPr):

$$\frac{0.188\ x\ g\ HPr}{L}\ \frac{1\ mol\ HPr}{74.04\ g\ HPr}\ \frac{3.5\ mol\ O_2}{1\ mol\ HPr}\ \frac{32\ g\ O_2}{1\ mol\ O_2} = 0.284\ x \frac{g\ O_2}{L}$$

   c. Butyric acid (HBt):

$$\frac{0.188\ x\ g\ HBt}{L}\ \frac{1\ mol\ HBt}{88.11\ g\ HBt}\ \frac{5\ mol\ O_2}{1\ mol\ HPr}\ \frac{32\ g\ O_2}{1\ mol\ O_2} = 0.342\ x \frac{g\ O_2}{L}$$

2) COD balance to obtain x (mass proportion):

$$\frac{3.5g\ COD}{L} = (0.670 + 0.284 + 0.342)\ x \frac{g\ O_2}{L}$$

$$\frac{3.5g\ COD}{L} = 1.296\ x\ \frac{g\ O_2}{L}$$

$$x = 2.70$$

3) Acid volume required with x=2.70 for 10L of volume:

   a. Acetic acid (HAc):

$$\frac{0.625\ x\ g\ HAc}{L} = \frac{0.670\ x\ g\ O_2}{L} = \frac{0.670\ 2.70\ g\ O_2}{L} = 1.69\ \frac{g\ HAc}{L}\ \frac{1\ L}{1050\ g\ HAc} 10\ L = 0.016\ L\ HAc$$

$$= 16.10\ ml\ HAc$$

   b. Propionic acid (HPr):

$$\frac{0.188\ x\ g\ HPr}{L} = \frac{0.188\ x\ g\ O_2}{L} = \frac{0.188\ 2.70\ g\ O_2}{L} = 0.51\ \frac{g\ HPr}{L}\ \frac{1\ L}{990\ g\ HPr} 10\ L = 0.00515\ L\ HPr$$

$$= 5.15\ ml\ HPr$$

   c. Butyric acid (HBt):

$$\frac{0.188\ x\ g\ HBt}{L} = \frac{0.188\ x\ g\ O_2}{L} = \frac{0.188\ 2.70\ g\ O_2}{L} = 0.51\ \frac{g\ HBt}{L}\ \frac{1\ L}{960\ g\ HBt} 10\ L = 0.00531\ L\ HBt$$

$$= 5.31\ ml\ HBt$$

Concentration of ammonium chloride required per day:

1) COD destinated to the bacteria growth:

$$Y_{obs} = \frac{Y}{1 + k_d\ SRT} = \frac{0.035}{1 + 0.1\ 4.21} = 0.25\ \frac{g\ DQO\ to\ celular\ growth}{g\ DQO}$$

Where:

Y_{obs} is the heterotrophic performance on anoxic conditions.

k_d is the death constant.

2) COD concentration required per day:

a. Acetic acid (HAc):

$$\frac{0.670\ x\ g\ O_2}{L}\ \frac{1}{HRT} = \frac{0.670\ 2.70\ g\ O_2}{L}\ \frac{1}{1.12\ d} = 1.61\frac{g\ DQO}{L\ d}$$

b. Propionic acid (HPr):

$$\frac{0.188\ x\ g\ O_2}{L}\ \frac{1}{HRT} = \frac{0.188\ 2.70\ g\ O_2}{L}\ \frac{1}{1.12\ d} = 0.45\frac{g\ DQO}{L\ d}$$

c. Butyric acid (HBt):

$$\frac{0.188\ x\ g\ O_2}{L}\ \frac{1}{HRT} = \frac{0.188\ 2.70\ g\ O_2}{L}\ \frac{1}{1.12\ d} = 0.45\frac{g\ DQO}{L\ d}$$

d. Total (OLR):

$$(1.61 + 0.45 + 0.45)\frac{g\ DQO}{L\ d} = 2.51\frac{g\ DQO}{L\ d}$$

3) NH_4Cl mass required:

$$2.51\frac{g\ DQO}{L\ d}\ 3.75\ L\ \frac{0.25\ g\ DQO\ cell\ growth}{1\ g\ DQO}\ \frac{1\ g\ C_5H_7O_2N}{1.42\ DQO}\ \frac{1\ mol\ C_5H_7O_2N}{113\ g\ C_5H_7O_2N}\ \frac{1\ mol\ N}{1\ mol\ C_5H_7O_2N}$$

$$\frac{1\ mol\ NH_4Cl}{1\ mol\ N}\ \frac{53.5\ g\ NH_4Cl}{1\ mol\ NH_4Cl} = 0.785\ \frac{gNH_4Cl}{d}$$

$$\frac{1}{52\frac{ml}{cycle}\ 4\frac{cycle}{1\ d}\ \frac{1L}{1000ml}\ \frac{1\ d}{0.785\ gNH_4Cl}} = 3.77\frac{gNH_4Cl}{L}$$

ANNEX 2: SUPPLEMENTARY MATERIAL OF THE EXPERIMENTAL RESULTS

1) Specific substrate weights of the first batch fermentation test:

Bottle ID	WAS weight (g)	FW weight (g)
1.1	149.25	8.57
1.2	156.62	8.74
1.3	148.46	8.58
1.4	147.09	8.66
2.1	149.45	8.96
2.2	150.52	8.54
2.3	149.60	9.03
2.4	150.75	8.74
3.1	150.70	8.61
3.2	149.45	8.90
3.3	150.52	8.64
3.4	150.49	8.61
4.1	150.68	8.87
4.2	150.83	8.63
4.3	150.70	8.94
4.4	149.57	8.66

Table ii.1 – Weighs added in each bottle. Bottles identified as 1.x corresponds to first condition (no pH modification); 2.x bottles refers to second condition (initial pH of 10); bottles mentioned as 3.x are those which third condition was applied (pH of 10 at 5th day); 4.x bottles indicates the 4th condition (pH of 7th at 5th day).

2) Specific substrate weights of the second batch fermentation test:

Bottle ID	WAS weight (g)	FW weight (g)
1.1	149.89	7.53
1.2	149.88	7.53
1.3	149.88	7.67
1.4	149.91	7.52
2.1	149.89	7.61
2.2	149.48	7.50
2.3	150.39	7.57
2.4	150.04	7.60
3.1	150.04	7.60
3.2	153.72	7.68
3.3	149.03	7.47
3.4	149.82	7.51
4.1	149.45	7.52
4.2	155.74	7.48
4.3	149.8	7.56
4.4	150.93	7.56

Table ii.2 – Weighs added in each bottle. Bottles identified as 1.x corresponds to first condition (no pH modification); 2.x bottles refers to second condition (initial pH of 10); bottles mentioned as 3.x are those which third condition was applied (pH of 10 at 2nd and 5th days); 4.x bottles indicates the fourth condition (pH of 7 at 2nd and 5th days).

3) Specific substrate weights of the third batch fermentation test:

Bottle ID	Fermentation effluent weight (g)
1.1	159.85
1.2	159.10
1.3	159.45
1.4	163.07
2.1	159.65
2.2	160.21
2.3	159.83
2.4	160.89
3.1	162.54
3.2	170.15
3.3	159.28
3.4	159.39

Table ii.3 – Weighs added in each bottle. Bottles identified as 1.x corresponds to first condition (no pH modification); 2.x bottles refers to second condition (initial pH of 10); bottles mentioned as 3.x are those performed with third condition (initial pH of 7).

4) Accumulation tests performed:

Accumulation test 26-02-2021 (1st operational period)	
Parameter	**Value**
Feed stream composition:	
Acetic acid (mg COD L^{-1})	1.81
Propionic acid (mg COD L^{-1})	0.77
Butyric acid (mg COD L^{-1})	0.93
Total VFAs after first spike (mg COD L^{-1}):	533.24 ± 3.12
Acetic acid (mg COD L^{-1})	274.75 ± 0.76
Propionic acid (mg COD L^{-1})	119.62 ± 1.07
Butyric acid (mg COD L^{-1})	138.87 ± 1.29
Initial TSS (g L^{-1})	2.25 ± 0.53
Final TSS (g L^{-1})	2.33 ± 0.53
Initial VSS (g L^{-1})	1.95 ± 0.22
Final VSS (g L^{-1})	2.06 ± 0.22
Initial purge volume (L)	0.45 ± 0.01
Pulse-feeding volume (L $spike^{-1}$)	0.08 ± 0.01
Number of spikes	6
PHA content (% on SS basis):	
Initial	-
After 1st spike	27.87 ± 1.46
After 2nd spile	34.83 ± 0.79
After 3rd spike	33.97 ± 2.18
After 4th spike	36.75 ± 1.28
After 5th spike	39.53 ± 3.46)
After 6th spike	47.98 ± 0.83
PHB proportion in PHA (%):	
Initial	-
After 1st spike	83.02 ± 1.32
After 2nd spile	81.63 ± 0.21
After 3rd spike	78.82 ± 1.33
After 4th spike	78.84 ± 0.68
After 5th spike	78.86 ± 1.43
After 6th spike	80.83 ± 0.83

Accumulation test 02-03-2021 (1st operational period)	
Parameter	**Value**
Feed stream composition (mg COD L^{-1}):	
Acetic acid (mg COD L^{-1})	1.81
Propionic acid (mg COD L^{-1})	0.77
Butyric acid (mg COD L^{-1})	0.93
Total VFAs after first spike (mg COD L^{-1}):	119.70 ± 0.75
Acetic acid (mg COD L^{-1})	113.83 ± 0.75
Propionic acid (mg COD L^{-1})	2.14 ± 0.25
Butyric acid (mg COD L^{-1})	3.73 ± 0.25
Initial TSS (g L^{-1})	2.53 ± 0.02
Final TSS (g L^{-1})	2.61 ± 0.07
Initial VSS (g L^{-1})	2.67 ± 0.16
Final VSS (g L^{-1})	1.58 ± 0.23
Initial purge volume (L)	0.68 ± 0.01
Pulse-feeding volume (L $spike^{-1}$)	0.08 ± 0.01
Number of spikes	6
PHA content (% on SS basis):	
Initial	-
After 1st spike	28.97 ± 0.54
After 2nd spile	27.00 ± 0.01
After 3rd spike	26.62 ± 3.2
After 4th spike	28.94 ± 0.29
After 5th spike	41.39 ± 0.06
After 6th spike	36.79 ± 0.81
PHB proportion in PHA (%):	
Initial	-
After 1st spike	84.55 ± 0.56
After 2nd spile	83.36 ± 0.02
After 3rd spike	81.95 ± 3.0
After 4th spike	80.25 ± 0.35
After 5th spike	79.36 ± 0.06
After 6th spike	78.59 ± 0.74

Table ii.4 and table ii.5 – Results of the accumulation tests: 26-02-2021 (table ii.4) and 02-03-2021 (table ii.5).

Accumulation test 05-03-2021 (1st operational period)	
Parameter	**Value**
Feed stream composition (mg COD L^{-1}):	
Acetic acid (mg COD L^{-1})	1.81
Propionic acid (mg COD L^{-1})	0.77
Butyric acid (mg COD L^{-1})	0.93
Total VFAs after first spike (mg COD L^{-1}):	279.87 ± 2.34
Acetic acid (mg COD L^{-1})	175.91 ± 1.07
Propionic acid (mg COD L^{-1})	54.47 ± 0.53
Butyric acid (mg COD L^{-1})	49.48 ± 0.74
Initial TSS (g L^{-1})	2.68 ± 0.54
Final TSS (g L^{-1})	3.31 ± 1.94
Initial VSS (g L^{-1})	1.58 ± 0.24
Final VSS (g L^{-1})	2.58 ± 0.40
Initial purge volume (L)	0.50 ± 0.01
Pulse-feeding volume (L $spike^{-1}$)	0.08 ± 0.01
Number of spikes	6
PHA content (% on SS basis):	
Initial	14.97 ± 0.63
After 1st spike	29.62 ± 1.43
After 2nd spile	23.826 ± 0.98
After 3rd spike	25.81 ± 0.07
After 4th spike	46.26 ± 1.56
After 5th spike	50.04 ± 0.23
After 6th spike	50.88 ± 0.63
PHB proportion in PHA (%):	
Initial	84.19 ± 0.54
After 1st spike	82.68 ± 1.23
After 2nd spile	82.05 ± 0.51
After 3rd spike	82.92 ± 0.04
After 4th spike	88.72 ± 1.19
After 5th spike	86.61 ± 0.61
After 6th spike	87.73 ± 0.06

Accumulation test 24-03-2021 (2nd operational period)	
Parameter	**Value**
Feed stream composition (mg COD L^{-1}):	
Acetic acid (mg COD L^{-1})	1.81
Propionic acid (mg COD L^{-1})	0.77
Butyric acid (mg COD L^{-1})	0.93
Total VFAs after first spike (mg COD L^{-1}):	88.18 ± 0.18
Acetic acid (mg COD L^{-1})	63.72 ± 0.06
Propionic acid (mg COD L^{-1})	4.86 ± 0.02
Butyric acid (mg COD L^{-1})	19.59 ± 0.09
Initial TSS (g L^{-1})	1.44 ± 0.06
Final TSS (g L^{-1})	1.51 ± 0.53
Initial VSS (g L^{-1})	1.42 ± 0.28
Final VSS (g L^{-1})	1.47 ± 0.22
Initial purge volume (L)	0.66 ± 0.01
Pulse-feeding volume (L $spike^{-1}$)	0.08 ± 0.01
Number of spikes	5
PHA content (% on SS basis):	
Initial	17.16 ± 0.85
After 1st spike	22.14 ± 1.28
After 2nd spile	31.10 ± 0.36
After 3rd spike	38.03 ± 0.09
After 4th spike	37.96 ± 1.77
After 5th spike	42.44 ± 0.73
PHB proportion in PHA (%):	
Initial	87.40 ± 0.62
After 1st spike	85.87 ± 1.05
After 2nd spile	82.51 ± 0.20
After 3rd spike	82.33 ± 0.25
After 4th spike	82.24 ± 1.6
After 5th spike	87.09 ± 0.01

Table ii.6 and table ii.7 – Results of the accumulation tests: 05-03-2021 (table ii.6) and 24-03-2021 (table ii.7).