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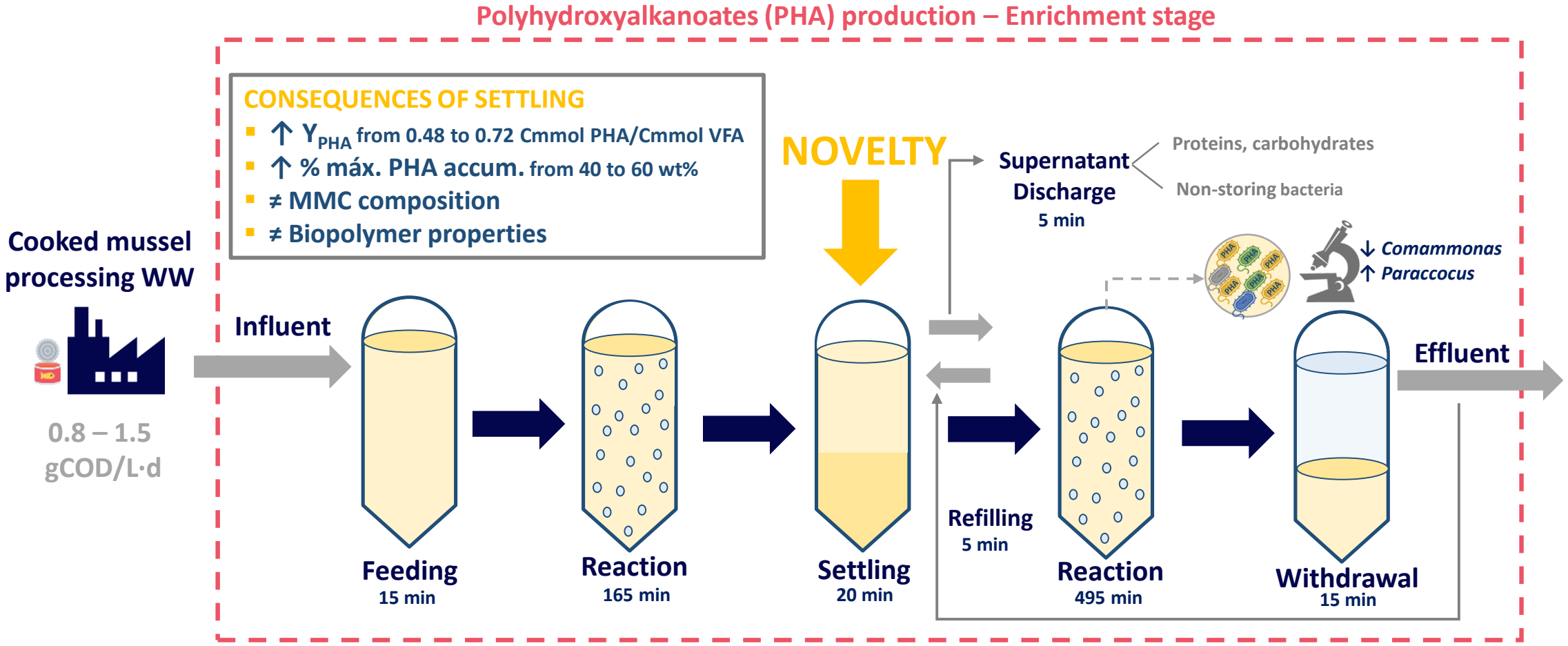
Polyhydroxyalkanoates (PHA) production – Enrichment stage
CONSEQUENCES OF SETTLING
↑ Y_{PHA} from 0.48 to 0.72 Cmmol PHA/Cmmol VFA
↑ % máx. PHA accum. from 40 to 60 wt%
≠ MMC composition
≠ Biopolymer properties
NOVELTY
Cooked mussel processing WW
0.8 – 1.5 gCOD/L·d
Influent
Feeding
15 min
Reaction
165 min
Settling
20 min
Supernatant Discharge
5 min
Proteins, carbohydrates
Non-storing bacteria
↓ Comammonas
↑ Paraccocus
Refilling
5 min
Reaction
495 min
Withdrawal
15 min
Effluent

***Highlights (3 to 5 bullet points (maximum 85 characters including spaces per bullet point)**

Highlights

- Complex saline industrial wastewater as a substrate for PHA production.
- A settling stage was included in the enrichment cycle after VFA consumption.
- Proteins and carbohydrates promoting growth instead of PHA production were removed.
- The PHA production yield increased from 0.48 to 0.72 $Cmmol_{PHA}$ / $Cmmol_{VFA}$.
- The maximum PHA storage capacity of the MMC improved from 40 to 60 wt%.

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ABSTRACT

Polyhydroxyalkanoates (PHA) appear as good candidates to substitute conventional petroleum-based plastics since they have similar properties but with the advantage of being biodegradable. Wastewater streams with high organic content are feasible substrates for PHA production resulting in an opportunity for waste recovery. One of the main challenges is the optimization of the selection of microorganisms with high PHA storage capacity. This microbial selection is performed in sequencing batch reactors (SBR) operated under an aerobic feast/famine (F/F) regime. In the present study, a settling stage was added at the end of the feast phase of the enrichment cycle of a SBR fed with pre-acidified cooked mussel processing wastewater (containing up to 12 g NaCl/L). Settling and subsequent supernatant discharge favoured the wash-out of non-accumulating microorganisms as well as the removal of substances that enhanced their undesired development (proteins and carbohydrates). Microbial analysis performed by fluorescence *in situ* hybridization (FISH) technique showed shifts in the microbial community; the presence of genus *Paracoccus* increased whereas genera *Comamonas* decreased. Moreover, the process efficiency was improved with the increase of the PHA production yield (Y_{PHA}) and the maximum PHA storage capacity (max. PHA) from 0.48 to 0.72 $Cmmol_{PHA}$ / $Cmmol_{VFA}$ and from 40 to 60 wt%, respectively. The polymer composition also changed, its HB:HV ratio varied from 83:17 to 70:30. Results obtained in the present study showed that settling promoted the removal of carbon sources that did not contribute to PHA production and the washout of non-storing bacteria, which favoured the culture enrichment.

Keywords: bioplastics; enrichment; industrial wastewater, mixed microbial culture; polyhydroxyalkanoates; valorization.

1. INTRODUCTION

Plastics are polymers that are present in nearly all aspects of modern life. The great majority are petroleum-based products, durable in use but no biodegradable (Zhu et al., 2013). This fact, in addition to petroleum depletion, has derived in a growing interest in the development of more sustainable alternatives. Polyhydroxyalkanoates (PHA) are a family of biobased, biodegradable and biocompatible linear polyesters (Keshavarz and Roy, 2010) that have been recognized as good candidates to substitute conventional plastics due to their similar properties (Dias et al., 2006). These polymers are produced by certain microorganisms and accumulated as intracellular carbon sources under stress conditions (Reddy et al., 2003). Nowadays, large scale PHA production is based on the use of pure cultures or genetically modified microorganisms that require sterile conditions and highly costly specific substrates (Jiang et al., 2016). Consequently, PHA are not yet competitive in bulk materials markets (Albuquerque et al., 2010). Its current price ranges from 2.2 – 5.0 €/kg, which is more than one-third of the cost reported from the beginning of the last decade but still high in comparison to petroleum-based polymers, which typically cost less than 1.0 €/kg (Valentino et al., 2017). In recent years, research has been focused on the development of cost-effective processes, which involve the use of low-value substrates and mixed microbial cultures that do not need sterile conditions (Albuquerque et al., 2011) and allow the production of high quality biopolymers with a purity (> 98 %) comparable to those obtained using pure cultures (Samorì et al., 2015).

Wastewater streams produced in the agro-alimentary sector are among the most suitable substrates for PHA production due to their high organic matter content (Nikodinovic-Runic et al., 2013). This is the case of fish-canning industries, which generate large volumes of effluents with high pollutant load. In this way, wastewater valorization for

PHA production contributes to the transition to a circular economy model: on one hand, organic matter is recovered and converted into resources and on the other hand, pollutants are removed and treated wastewater can be discharged to natural water bodies. In this way, the concept of wastewater treatment plant (WWTP) moves to a water resource recovery facility (WRRF).

The PHA production system with a mixed microbial culture (MMC) using industrial wastewater as substrate generally consists on a three-stage process: (1) substrate pre-acidification (if necessary) to obtain a suitable carbon source for PHA production, primarily volatile fatty acids (VFA); (2) selection and enrichment of the MMC in PHA-accumulating organisms; and (3) maximization of the PHA storage before biopolymer extraction and purification. The main challenge of this PHA production system is the selection of a MMC with high PHA-storage capacity (Albuquerque et al., 2010). One of the most common strategies to achieve the microbial enrichment is the application of cycles of presence/absence of substrate known as feast/famine regime (F/F) or aerobic dynamic feeding (ADF), operated in sequencing batch reactors (SBR) (Albuquerque et al., 2007). In the last decade, research has been focused on the development of new strategies for the optimization of the culture enrichment. It has been implemented variations such as nitrogen limitation in the feast phase and/or the integration of an intermediate settling phase in the SBR cycle (Kourmentza et al., 2017).

Nitrogen deficiency during the feast phase restricts the growth of non-PHA accumulating bacteria while providing nitrogen during the famine enables their growth, which allows faster selection of a more efficient PHA-storing culture (Ahmadi et al., 2018; Oliveira et al., 2017). For example, Marang et al. (2014) observed that the presence of methanol and acetate promoted the development of non-accumulating microorganisms in the MMC. To solve this problem, Korkakaki et al. (2016) proposed

the addition of a settling phase after the acetate depletion in order to eliminate the methanol with the supernatant and promote the enrichment of the MMC by limiting the growth of non-accumulating bacteria. As a result, they observed a considerable increase in the maximum PHA storage capacity of the biomass from 48 wt% to 70 wt%. Supernatant discharge just after acetate depletion avoided the consumption of the remaining organic matter (methanol) and the consequent growth of side-populations increasing the proportion of PHA-producers in the mixed culture (Kourmentza et al., 2017). Therefore, this modification of the SBR enrichment cycle can be considered as a good solution to improve the microbial selection when there is more than one carbon source in the substrate. However, until now few studies have explored this alternative with industrial complex wastewater.

The objective of the present study was to improve the selection of PHA-accumulating bacteria in a MMC using pre-acidified cooked mussel processing wastewater (with 4 - 12 g NaCl/L and high VFA content) as feedstock. To achieve this goal, a settling stage was implemented in the operational cycle of an enrichment SBR working under F/F regime with the aim of removing undesired substances (mainly proteins and carbohydrates) that promoted the growth of non-accumulating bacteria. The enrichment of the system was evaluated in terms of maximum PHA production and shifts in the microbial community comprising the mixed culture.

2. MATERIALS AND METHODS

2.1 Experimental set-up

For the optimization of the enrichment unit and the consequent evaluation of maximum PHA accumulation of the system, two lab-scale reactors were operated: an enrichment reactor (SBR type) and an accumulation fed-batch reactor (FBR), respectively.

2.1.1 Enrichment SBR for culture selection

A tubular glass SBR with a working volume of 2 L (SBR-S) was inoculated with PHA-accumulating enriched biomass (0.80 g VSS/L) adapted to saline conditions from other SBR (SBR-I). The SBR-I was operated under the F/F regime treating pre-acidified wastewater collected from a cooked mussel processing industry. This reactor operated in cycles of 12 hours consisting of 15 min of feeding, 690 min of aerobic reaction and 15 min of effluent withdrawal and was completely aerated (Figure S1.A of Supplementary Material). The SBR-S operation was modified with respect to SBR-I by means of including a settling stage at the end of the feast phase. Therefore, the operational cycles of the SBR-S comprised the following stages: 1) 0.8 L of feeding (15 min), 2) aerobic reaction (165 min), 3) settling (20 min), 4) 1 L of supernatant discharge (5 min), 5) reactor refilling with 0.8 L of the previous cycle effluent (recirculation) and 0.2 L of tap water (5 min), 6) aerobic reaction, (495 min) and 7) 0.8 L of effluent withdrawal, (15 min). Specific details about the cycle distribution can be consulted in the Figure S1. B.

Aeration was supplied during all stages, except for settling and supernatant discharge, through a diffuser located at the bottom of the reactor that granted the complete mixture of the system. The temperature was controlled at 30 ˚C by a thermostatic bath (Techne Inc., USA) and the pH was not controlled but periodically measured at the end of the famine phase. A detailed scheme of the SBR-S can be seen in Figure S2 of Supplementary Material.

The length of the aerobic reaction before the settling phase was established by the duration of the feast phase, and stopped when the VFA had just been depleted (Korkakaki et al., 2016). Since during the operation of the SBR-I, reactor where the inoculum was obtained, the feast phase lasted from 2.5 to 3.0 h, the settling stage in the

SBR-S was imposed after 2.75 h (165 min) of aerobic reaction. In addition, to enable the maintenance of a comparable hydraulic and sludge retention time (HRT and SRT, respectively) of 24 h, and ensure biomass duplication (Korkakaki et al., 2016), the effluent volume was established at 0.8 L.

The influent consisted of centrifuged pre-acidified cooked mussel processing wastewater that was five-times diluted with tap water. It was supplemented with an amount of 1.5 mL/L of 33.0 g/L allylthiourea (ATU) solution to inhibit the nitrification activity, and 0.25 mL/L of antifoam agent (Y-30 Emulsion, Sigma Aldrich) to avoid problems of foam during aerated phases. The organic loading rate (OLR) varied between 0.8 and 1.5 g COD/(L·d). The pH of the influent was 6.5 ± 0.5 and its total nitrogen (TN), dissolved organic carbon (DOC) and soluble chemical oxygen demand (CODs) concentrations were 0.14 – 0.31 g /L, 0.41 – 0.93 g /L and 0.97 – 1.86 g /L, respectively. The organic fraction mainly contained VFA (47.45 – 61.15 % g COD_{VFA}/g sCOD) and proteins (34.08 – 39.58 % g $COD_{proteins}$/g sCOD), and to a lesser extent, carbohydrates (2.10 – 4.45 % g $COD_{carbohydrates}$/g sCOD). In addition, this wastewater was characterized by high NaCl concentrations (4.34 – 12.10 g/L). Variations observed in the composition of the influent are a consequence of changes in the factory process.

The characteristics of the SBR-S influent, the supernatant discharged after settling, and the effluent were analysed weekly (typically from two to three days) during the complete operational period (70 days). Additionally, two enrichment operational cycles (days 24 and 62) were monitored. Several parameters were determined in both cases: dissolved oxygen (DO), pH, solids, organic matter constituents, nitrogen species and salinity. PHA content was also analysed during the complete monitoring of the enrichment cycles.

2.1.2 Accumulation FBR experiments to determine maximum PHA accumulation capacity

Two accumulation assays were carried out in a 2.0 L FBR with biomass collected from the SBR-S (days 35 and 64 of operation) in order to evaluate the maximum storage capacity of the MMC. The system was continuously aerated a completely mixed by means of a diffuser located at the bottom of the reactor. The temperature was controlled at 30 ˚C by a thermostatic bath (Techne Inc., USA) and the pH was not controlled. A detailed scheme of the FBR operation can be seen in Figure S3 (Supplementary Material).

To maximize PHA accumulation, the FBR was operated under complete aeration and excess of organic carbon. Bacterial growth was prevented by the absence of nutrients in the influent. The carbon source was added in pulses of 50 mL with a content of 30 Cmmol of a mixture of VFA mimicking the pre-acidified cooked mussel wastewater composition (11.42 g/L of acetic, 2.76 g/L of propionic, 2.48 g/L of butyric and 0.24 g/L of valeric acids). Pulses were added once the carbon compounds fed in the previous pulse were consumed, which was indicated by changes in the DO concentration profile. The maximum accumulation capacity was reached once the DO concentration remained at saturation values, even after the addition of a substrate pulse.

2.2 Analytical methods

The DO concentration and the temperature were monitored on-line with a probe (model HQ40d, Hach-Lange, USA), and the pH was measured with a pH & Ion-Meter (model GLP 22, Crison, Spain).

Total and Volatile Suspended Solids (TSS and VSS, respectively) and total COD (tCOD) were analyzed in raw samples according to Standard Methods for the

Examination of Water and Wastewater (APHA-AWA-WEF, 2017). Filtered liquid samples (0.45 µm pore size, cellulose-ester membrane, Advantec, Japan) were taken for the determination of: soluble COD (sCOD), VFA, carbohydrates, proteins, DOC, total nitrogen (TN), ammonium (NH_4^+) and other ions (Na, Cl^-). sCOD was determined by the open reflux method (APHA-AWWA-WEF, 2017). VFA were determined in a gas chromatograph (Hewlett Packard 5890A, USA) equipped with a flame ionization detector (FID) and an automatic injector (Hewlett Packard 7673A, USA). Carbohydrates were measured by the Loewus method (Loewus, 1952) and expressed in equivalent glucose (Glu) (balances were performed considering that 1 g of Glu contains 1.07 g of COD). Proteins were analyzed by the Lowry method (Lowry et al., 1951) and expressed in equivalent bovine serum albumin (BSA) (balances were calculated taking into account that 1 g of BSA contains 1.32 g of COD and 0.15 g of N). DOC and TN were measured by catalytic combustion in the TOC-L CNS analyzer with the TNM-1 module (Shimadzu, Japan). NH_4^+ was determined by the Bower/Holm Hansen method (Bower and Holm-Hansen, 1980) and ions by ion chromatography (861 Advanced Compact IC, Metrohm, Switzerland).

PHA analysis was performed in the solid phase. For that purpose, fresh biomass samples were centrifuged, frozen and freeze-dried. The PHA content (as g PHA/g VSS) was measured following the method described by Smolders et al., (1994) for the quantification of the monomer propyl esters present in a lyophilized sample. A commercial PHA standard (Sigma-Aldrich, USA) containing 88 % of hydroxybutyrate (HB) and 12 % of hydroxyvalerate (HV) and benzoic acid as internal standard was used. The propyl esters were analyzed by means of gas chromatography in a HP innovax column equipped with a FID (Agilent, USA).

2.3 Calculations

Carbon and nitrogen balances were calculated to evaluate the effects of settling implementation and effluent recirculation for the replacement of the supernatant discharge.

The PHA content of the biomass samples was determined on a mass basis and expressed in dry weight (wt%) as a percentage of the measured VSS (g VSS), according to Eq. 1.

$$PHA\ (wt\%) = g\ PHA/g\ VSS \times 100 \qquad Eq.1$$

The elemental composition of the active biomass (X) was assumed to be $CH_{1.8}O_{0.5}N_{0.2}$ and its amount (as $Cmmol_X$) was determined by the subtraction of the accumulated PHA from the mass of VSS.

Maximum specific conversion rates of VFA consumption ($q_{VFA,}$ $Cmmol_{VFA}/(Cmmol_X \cdot h)$) and PHA production ($q_{PHA,}$ $Cmmol_{VFA}/(Cmmol_X \cdot h)$) (for both HB and HV) were determined from the maximum slopes of the curves obtained from the corresponding experimental data, divided by the active biomass. Yields for HB (Y_{HB}), HV (Y_{HV}) and biomass growth (Y_X) were obtained dividing the production rate ($Cmmol_{PHA}/h$ and $Cmmol_X/h$) by the VFA consumption rate ($Cmmol_{VFA}/h$). HB:HV ratio was calculated as the amount of each compound ($Cmmol_{HB}$ or $Cmmol_{HV}$) divided by the total amount of PHA ($Cmmol_{PHA}$).

2.4 Identification of microbial populations

For the identification of the different populations of the enriched MMC, biomass samples were analyzed by the fluorescence *in situ* hybridization (FISH) following the procedure described by Amann et al. (1990). The probes used for the hybridization of the bacterial cells are listed in Table S1, more detailed information is available at

probeBase (Loy et al., 2007). All probes were commercially synthesized and 5' labeled with either fluorochromes FITC (Fluorescein-5-isocyanate) or Cy3 (Carbocyanine 3). The DNA was detected by using DAPI (4, 6-diamidino-2-phenylindole) as a universal dye. Samples were viewed using an epifluorescence microscope (Axioskop 2 Plus, Zeiss, Germany) coupled with an acquisition system (Coolsnap, Roper Scientific Photometrics).

3. RESULTS AND DISCUSSION

3.1 Origin of the substrate

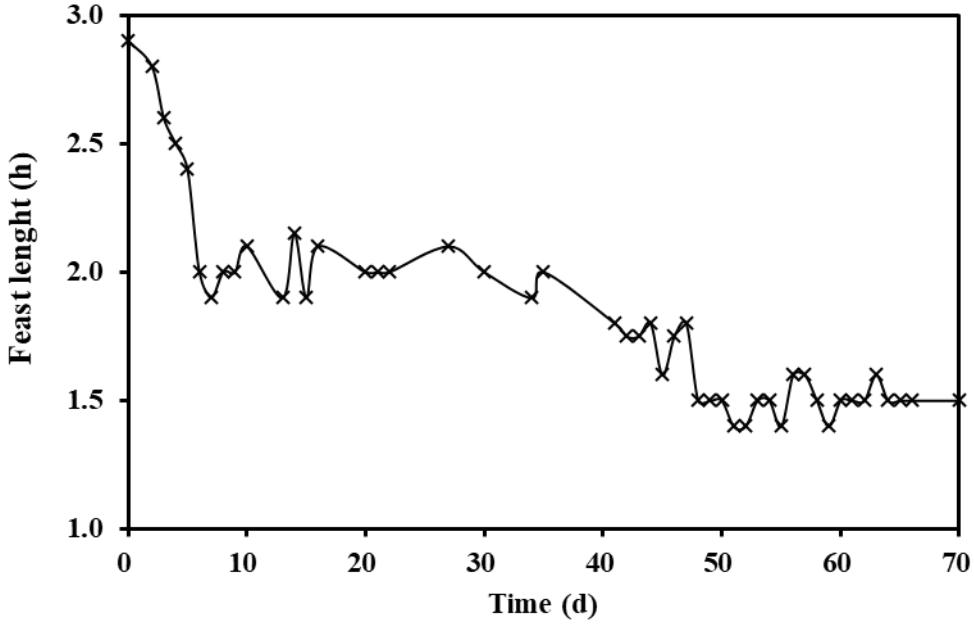
Mussel cooked wastewater was taken from a 5 L acidogenic reactor in which complex organic compounds (carbohydrates, proteins and lipids) were transformed in order to obtain a VFA-rich stream suitable for PHA production.

3.2 Improvement of the enrichment due to settling implementation

3.2.1 Evolution of the enrichment step

The SBR-S was operated for 70 days and the biomass was considered adapted to the new operation after 10 days, when the feast phase length was stabilized as shown in Figure 1.A. The downward evolution of the feast phase length caused a decrease of the feast/cycle length ratio from 0.18 (day 10) to 0.13 (day 65). Considering that values lower than 0.2 - 0.3 were associated to a MMC capable of biopolymer storage (Oliveira et al., 2017), the simple monitoring of the feast/cycle length ratio evidenced the improvement of the selection of accumulating bacteria in the MMC after the implementation of the settling stage.

A)



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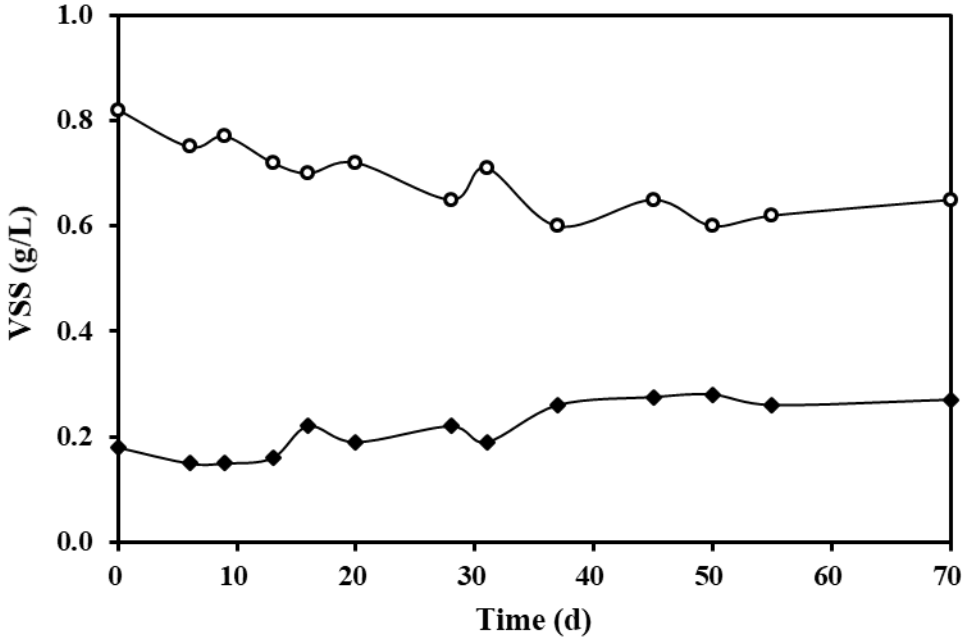
B)



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256 **Figure 1.** Evolution in the SBR-S operational period of: A) the feast phase length (x) and B) the
257 VSS concentrations inside the SBR-S at the end of the cycle (the same as in the effluent) at min 705 (○)
258 and in the supernatant discharged after the settling stage (◆) at min 200.

259

260

During the first operational days, the VSS concentration inside the SBR-S and end of the cycle (min 705) decreased while its concentration increased in the supernatant discharge (min 200) (Figure 1.B). Then, when the feast length was stabilized, the VSS concentrations were maintained at values of 0.66 ± 0.05 g/L and 0.23 ± 0.04 g/L in the reactor (measurements performed at the end of the cycle) and in the supernatant, respectively. Therefore, not only non-accumulating microorganisms were removed from the system at the end of the cycle at min 705 (wash-out at the end of the famine phase), but also part of them were discharged with the supernatant at min 200 (wash-out at the end of the feast phase).

Consequently, apart from the removal of non-desired substances in the supernatant, which favoured the development of non-accumulating microorganisms (proteins and carbohydrates) during the famine phase, the implementation of the settling stage at the end of the feast phase favoured the growth of PHA-accumulating microorganisms in the MMC that could use the accumulated PHA as carbon source for growth (Korkakaki et al., 2016; Kourmentza et al., 2017).

The settling stage was implemented after the feast phase, when accumulating bacteria had more intracellular PHA. It is known that the cell weight of these microorganisms is proportional to their cell size and PHA content, which increases the cell density and therefore their settling velocity (Chen et al., 2016). Thus, non-accumulating bacteria presented a lower settleability and they were washed-out with the discharged supernatant after settling. This was in accordance with the results obtained by Chen et al., (2015), who only used acetate as a carbon source (there was no residual COD present in the experiments) in such a way that the improvement of the selection capacity could only be attributed to differences in the cell density that led to physical selection.

3.2.2 Enrichment cycle profile and kinetics with (SBR-S) and without (SBR-I) settling phase

The differences in the cycle profile between SBR-I and SBR-S can be seen in Figure 2. In the case of SBR-I, the DO concentration after feeding decreased, corresponding to VFA depletion during the feast phase, and then increased during the famine phase due to exhaustion of external carbon source (Figure 2.A).

When the settling stage was implemented in the SBR-S, the aeration was stopped to settle the biomass and then, the supernatant was discharged in the absence of oxygen. For this reason, the DO concentration profile showed a strong decrease between the feast and the famine phases (Figure 2.B). Furthermore, it can be observed that the period of VFA consumption was reduced from 3 h in the SBR-I (Figure 2.A) to less than 1 h at day 62 of operation in the SBR-S (Figure 2B), which implied a faster PHA accumulation.

To quantify this effect Table 1.A compares the kinetics of SBR-I and SBR-S at day 62. Despite both MMC showed similar specific substrate uptake rates (q_{VFA} = 0.26-0.27 $Cmmol_{VFA}/(Cmmol_X \cdot h)$), an increase on the q_{PHA} from 0.07 to 0.22 $Cmmol_{VFA}/(Cmmol_X \cdot h)$ was observed. Consequently, a higher PHA production yield (Y_{PHA} = 0.80 $Cmmol_{PHA}/h$) and a higher quantity of PHA accumulated (18.32 wt%) were obtained.

These results correlate with the downward trend of the feast phase length (Figure 1) and indicate a progressive enhancement on PHA-accumulating microorganisms selection with the implementation of the settling phase.

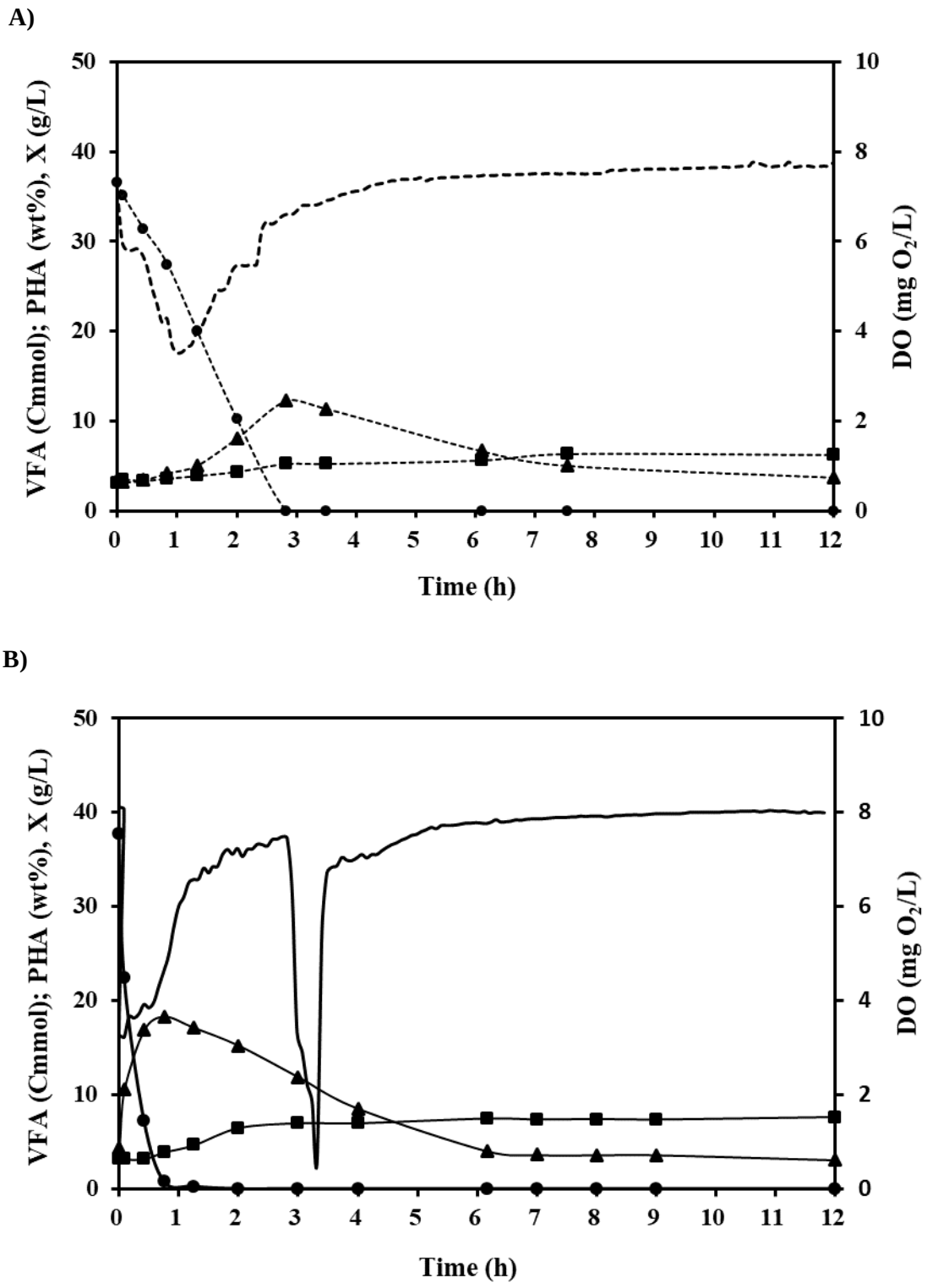


Figure 2. Evolution of the parameters monitored in representative enrichment cycles: DO (-), VFA (●), X (■) and percentage of PHA accumulated (wt%) (▲). A) Reactor operated without settling stage (SBR-I, - - -) at the moment of inoculum collection. B) Reactor operated with settling stage (SBR-S, ──) at day 62 of operation.

Table 1. Comparison of experimental kinetic parameters and yields of: A) enrichment reactors operated with (SBR-S) and without settling stage (SBR-I). B) accumulation essays with enriched biomass from the reactors operated with (FBR-S) and without settling stage (FBR-I).

A)	**SBR-I**	**SBR-S**	
		Day 24	**Day 62**
Feast lenght (h)	2.62 ± 0.10	2.00	1.50
q_{VFA} (Cmmol VFA/Cmmol X·h)	0.26 ± 0.06	0.22	0.27
q_{PHA} (Cmmol PHA/Cmmol X·h)	0.07 ± 0.02	0.14	0.22
q_{HB} (Cmmol HB/Cmmol X·h)	0.06 ± 0.01	0.12	0.15
q_{HV} (Cmmol HV/Cmmol X·h)	0.01 ± 0.01	0.02	0.07
Y_{PHA} (Cmmol PHA/Cmmol VFA)	0.32 ± 0.21	0.61	0.80
max. PHA (wt%)	12.80 ± 0.76	14.77	18.32
HB:HV	88:12 ± 6:6	88:12	70:30

B)	**FBR-I**	**FBR-S**	
		Day 35	**Day 64**
q_{VFA} (Cmmol VFA/Cmmol X·h)	0.20	0.30	0.26
q_{PHA} (Cmmol PHA/Cmmol X·h)	0.10	0.14	0.19
q_{HB} (Cmmol HB/Cmmol X·h)	0.08	0.12	0.14
q_{HV} (Cmmol HV/Cmmol X·h)	0.02	0.02	0.05
Y_{PHA} (Cmmol PHA/Cmmol VFA)	0.48	0.49	0.72
max. PHA (wt%)	40.87	44.28	59.92
HB:HV	83:17	82:18	70:30
q_{VFA} (Cmmol VFA/Cmmol X·h)	0.20	0.30	0.26

3.2.3 Organic matter balance

The pre-acidified cooked mussel processing wastewater used as feedstock contained two different carbon sources: (1) preferred substrates that are readily available and can be efficiently converted into PHA by PHA producers (desirable VFA) and (2) compounds that constitute a carbon source that allows non-accumulating microorganisms growth (non-desirable carbohydrates and proteins) (Valentino et al., 2017).

The carbon balance of these compounds (detailed in Table S2 of Supplementary Material) was calculated (as sCOD) during the operational enrichment cycle of the

SBR-S: between the beginning of the cycle (Initial), the supernatant discharge after settling (Supernatant) and the end of the cycle (Withdrawal). It was observed an effective removal of non-desired carbon sources with the implementation of the settling stage. In the supernatant were discharged proteins and to a lesser extent carbohydrates (40.16 ± 8.15 % and 34.55 ± 10.13 % of the present at the beginning of the cycle, respectively), which were in a much lower concentration.

The concentration of proteins and carbohydrates inside the reactor before settling and after refilling decreased from 120 – 215 mg/L to 60 – 110 mg/L (Figure 3.A) and from 20 – 40 mg/L to 15 – 30 mg/L (Figure 3.B) respectively. It should be pointed out that the refilling stream (from the previous cycle effluent) presented low carbohydrates concentrations (14.57 ± 2.58 mg/L) and normally there were no proteins due to their hydrolysis into NH_4^+, except in isolated cases in which it was measured concentrations of 5 – 10 mg/L. Therefore, the supernatant discharge promoted the removal of substances that could favour the development of non-accumulating microbial populations.

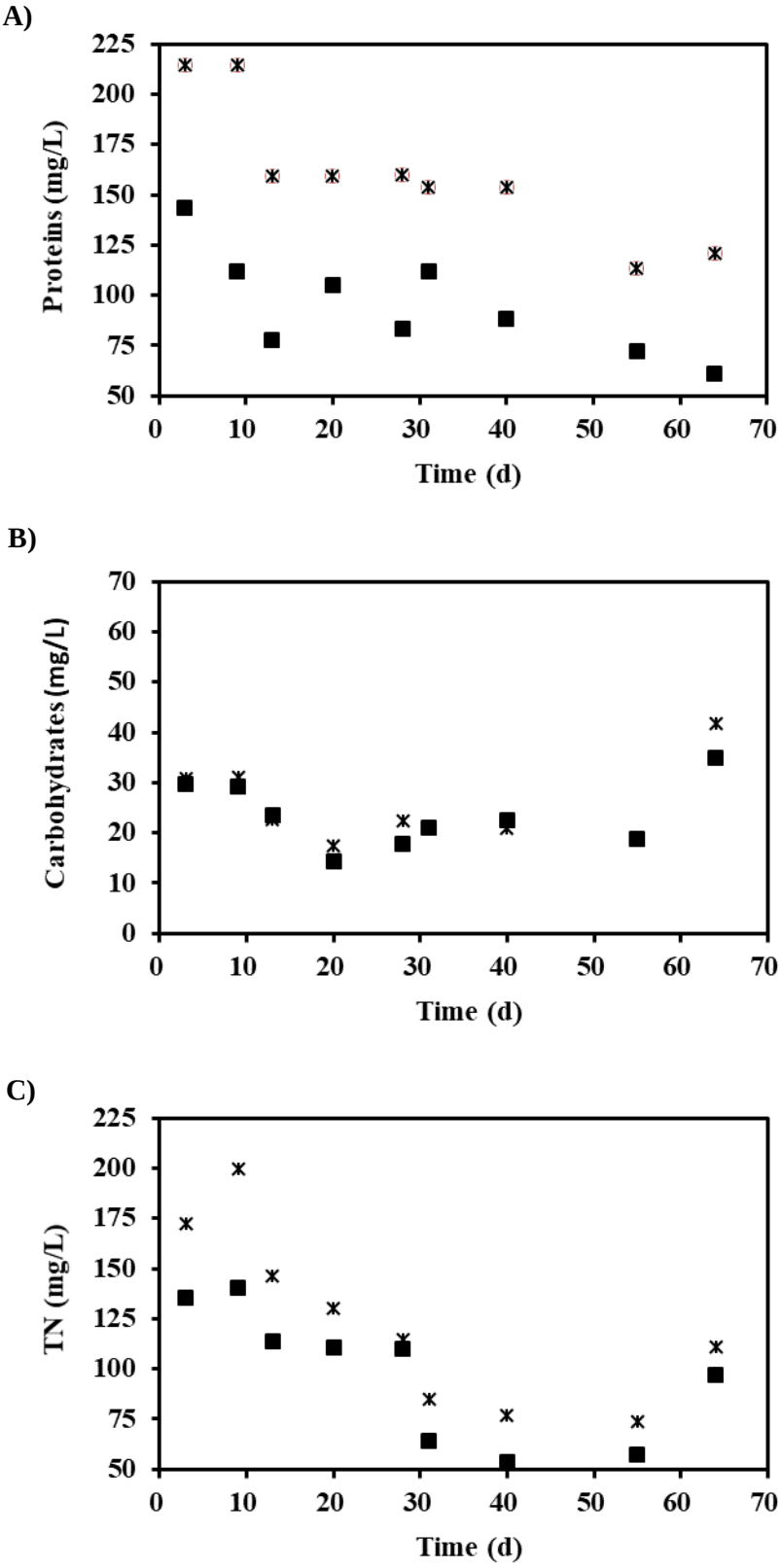


Figure 3. Proteins A), carbohydrates B) and TN C) concentrations before settling (*) and after refilling (■) in the SBR-S reactor.

3.2.4 Nitrogen balance

Nitrogen balances of enrichment were calculated in the SBR-S (more information is detailed in Table S3 and Table S4 of Supplementary Material) showing that 41 – 65 % of the TN present at the beginning of the cycle was discharged with the supernatant after the settling stage. It was also observed that approximately 15 % of the TN present at the beginning of the cycle was consumed during the feast phase, whereas 50 % of the TN present after the reactor refilling was consumed during the famine phase.

TN removal with supernatant discharge could limit nutrients availability during the famine phase (when PHA accumulating microorganisms used the accumulated carbon source (PHA) for growth) whereas it was still present during the feast phase (when non-storing microorganisms could use non-desired carbon sources for growth) negatively affecting the enrichment of the system (Figure 3.C). However, refilling provides the SBR with nutrients that lack in the famine phase. In fact, if comparing the reactors with (SBR-S) and without (SBR-I) settling, there was no evidence of a negative effect on the enrichment of the system due to nitrogen removal with supernatant discharge (Table 1).

These results controvert the advantages of the carbon-nitrogen uncoupled systems, in which the development of non-accumulating microorganisms was observed to be restricted by applying nitrogen deficiency during the feast phase, resulting in higher PHA production yields and productivities compared to conventional ADF systems (Kourmentza et al., 2017). It is presumed that the decoupling of the system could further improve the enrichment of the MMC. However, the complex and changeable characteristics of the feedstock would surely make it difficult to implement a system like this with such a substrate. It would be necessary to add a pre-treatment of the substrate to remove the ammonium content and later, if possible, recover the previously removed ammonium and reintroduce it in the system, to have nutrient availability

374 during the famine. If not, it would be necessary to add an external nitrogen source
375 which, in addition to the needed pre-treatment, could have an important economic
376 impact in the process.

377 **3.2.5 Considerations for the settling stage implementation**

378 It is necessary to point out that the supernatant should be treated before discharge
379 because of the COD and TN content. It could also be considered its possible reuse or
380 valorization as a fertilizer due to the high NH_4^+ concentration. However, this effluent is
381 also characterized by a high salinity because of the nature of the industrial cooked
382 mussel process, which could be an important hindrance. Regarding economic
383 considerations, settling implementation will presumably lead to an increase in the costs
384 due to the need for an extra pump for recirculation. However, 20 % and 25 % increases
385 of PHA accumulation and PHA production yield, respectively, are expected to
386 compensate investment costs. Operational costs mainly concerning energy requirements
387 for pumping, might be compensated with the lack of aeration during the settling stage.

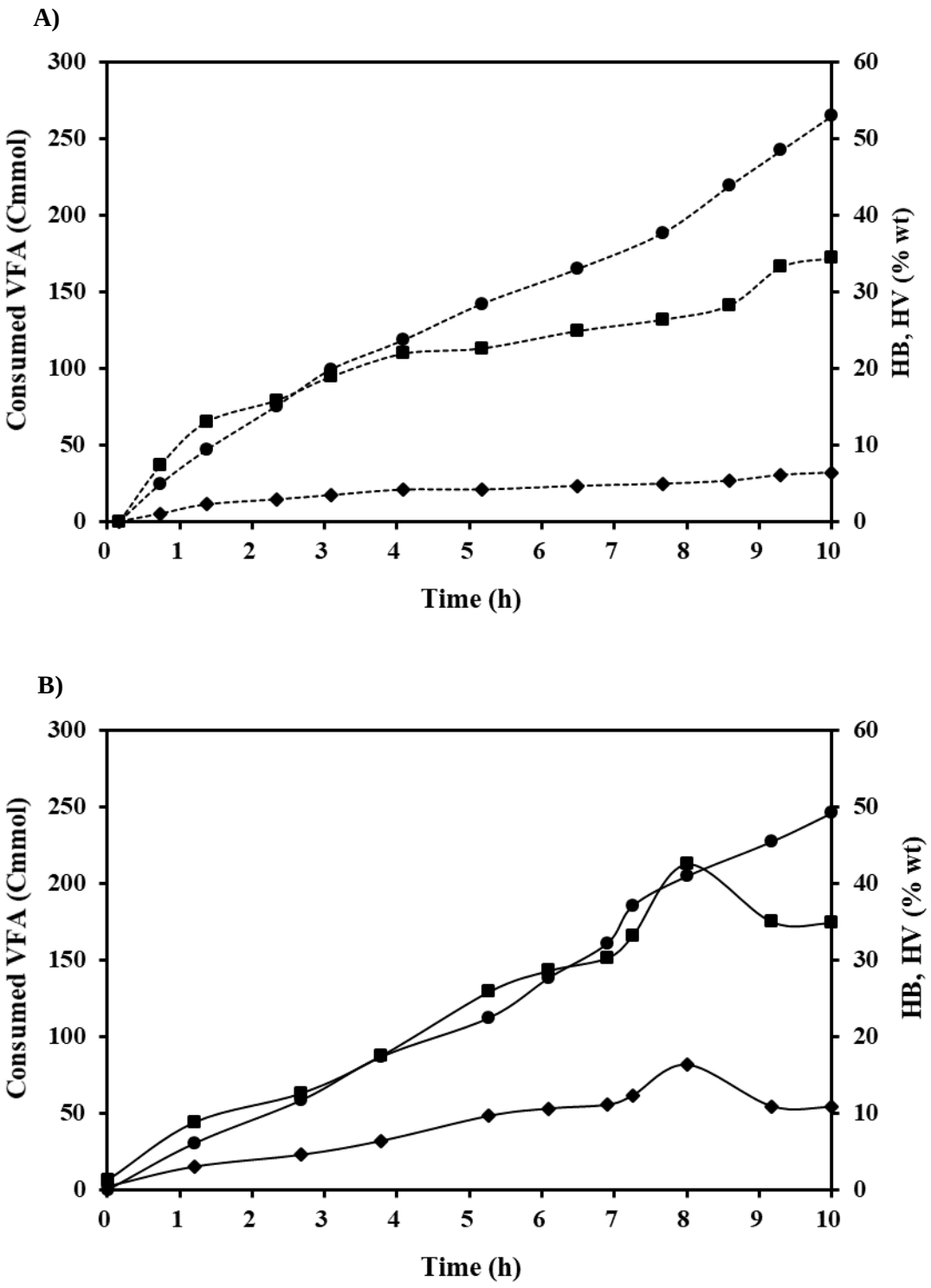
388 The results obtained suggested an enrichment of the MMC in PHA-accumulating
389 bacteria and therefore an optimization of the system due to the implementation of the
390 settling stage. Moreover, it was observed a reduction of the feast phase length from 3.0
391 to 1.5 hours because of the enrichment of the MMC. Therefore, in order to discharge the
392 supernatant just after VFA depletion and avoid the consumption of the remaining
393 carbon sources (proteins and carbohydrates), which are preferably used for growth of
394 non-accumulating bacteria, the pre-settling reaction stage could be reduced from 165
395 min (imposed in the present SBR-S operation) to 90 minutes. It was also observed an
396 improvement of the enriched biomass settleability and therefore a higher settling
397 velocity of the enriched culture, which suggested a possible reduction of the settling
398 stage length. This coincided with the results obtained by (Korkakaki et al., 2016), who

observed compaction of the flocs due to their selection after settling implementation and removal of suspended cells.

3.3 Effect of MMC selection on the maximum PHA accumulation capacity

To determine the effects of settling in the maximum PHA-accumulation capacity of the MMC, discontinuous accumulation assays were carried out with biomass from SBR-S (FBR-S) and compared with previous assays with biomass from SBR-I (FBR-I).

Results of the accumulation assays with biomass from both reactors are considered in Figure 4. Table 1.B shows the experimental kinetic parameters and yields obtained. The maximum PHA-accumulation capacity (max. PHA) of the MMC notably increased in the FBR-S assays (60 %) in comparison with the FBR-I ones (41 %), which correlated with the higher production yield obtained (Y_{PHA} = 0.72 $Cmmol_{PHA}$/h). For similar values of the specific substrate uptake rate (q_{VFA}), the improvement of the enrichment in SBR-S promoted an increase of the specific PHA production rate (q_{PHA}) in the accumulation FBR-S experiments, which indicated the improvement of the efficiency of the global PHA production process. In addition, results showed a positive effect on the accumulation kinetic parameters of the FBR-S between days 35 and 64, which correlates with the progressive enhancement of the enrichment SBR-S selection capacity (Table 1.B).



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419

420 **Figure 4.** Evolution of the parameters monitored in accumulation essays (FBR) performed with
421 enriched biomass: VFA (●) and percentage of accumulated PHB (■) and PHV (♦). A) Reactor operated
422 without settling stage (FBR-I, - - -) at the moment of inoculum collection. B) Reactor operated with
423 settling stage (FBR-S, ——) at day 64.

424

425 **Table 2.** Data from accumulation essays carried out with biomass obtained under different enrichment strategies.

MMC origin & Enrichment strategy	**Feedstock**	**$Y_{PHA/S}$ (Cmmol PHA/ Cmmol S) Max. PHA (wt%) q_{PHA} (CmmolPHA/Cmmol X·h)**	**Reference**
Activated sludge ADF without settling	VFA mixture and lactate	$Y_{PHA/S}$: 0.05 – 0.45	Dionisi et al., 2007
Activated sludge ADF without settling	Sodium acetate	Max. PHA: 69.00 – 89.00	Johnson et al., 2009
Water from a river estuary ADF + settling before withdrawal	Sodium acetate, glucose or starch	$Y_{PHA/S}$: 0.60 sodium acetate / 0.54 glucose / 0.30 starch Max. PHA: 64.70 sodium acetate / 60.50 glucose / 23.70 starch	Cui et al., 2016
Activated sludge ADF without and with settling after feast phase	Mixture of sodium acetate and methanol	Max. PHA: 48.00 without settling / 58.00-70.00 with settling	Korkakaki et al., 2016
Activated sludge ADF + settling after feast phase	Nutrients rich fermented centrate sludge	$Y_{PHA/S}$: 0.25 - 0.27 Max. PHA: 17.00 - 39.00	Morgan-Sagastume et al., 2015
Activated sludge ADF uncoupled C and N feeding strategy + settling after feast phase	Industrial soft drink wastewater	Max. PHA: 13.80	Ahmadi et al., 2018
Activated sludge ADF coupled and uncoupled carbon and nitrogen feeding strategy + settling before withdrawal	Fermented cheese whey	$Y_{PHA/S}$: 0.86 coupled / 0.96 uncoupled q_{PHA}: 0.29 coupled / 0.40 uncoupled	Oliveira et al., 2017
Activated sludge ADD (aerobic dinamic discharge) + two settling stages (after feast phase and before withdrawal)	Mixture of sodium acetate, NH_4Cl and KH_2PO_4	q_{PHA}: 0.47 - 1.89	Chen et al., 2016
Enriched biomass from the C-SBR, which was inoculated with activated sludge ADF + settling stage after feast phase	Synthetic VFA mixture mimicking the composition of pre-acidified cooked mussel wastewater	$Y_{PHA/S}$: 0.72 Max. PHA: 59.92 q_{PHA}: 0.19	This work

Results obtained in FBR-S are compared in Table 2 with others found in the literature using MMC, real and synthetic feedstocks and different enrichment strategies (coupled and uncoupled systems with and without settling). The results obtained in the present research work are comparable to those reached in the literature using synthetic feedstocks (both for the enrichment of the culture and evaluation of the maximum accumulation capacity) (Cui et al., 2016; Johnson et al., 2009; Korkakaki et al., 2016; Dionisy et al., 2007), and higher to the obtained in systems fed with real substrates (Ahmadi et al., 2018; Morgan-Sagastume et al., 2015) in spite of the complexity and high salinity of the pre-acidified cooked mussel wastewater. It has been reported that NaCl concentrations cause a severe effect over the respiration activity, which diminishes with the increase of salt (IC_{50} = 5 g NaCl/L in a MMC non-adapted to salinity) and negatively affects the PHA accumulation (Palmeiro-Sánchez et al., 2016a). Biopolymers obtained in FBR-S assays were composed by HB and HV monomers with a HB:HV ratio of 70:30 whereas in the FBR-I assays HV monomer was in a lower proportion (HB:HV ratio of 87:13). It has been previously reported that the PHA composition is a function of the VFA profile in the feed, where the HV fraction increases with the abundance of VFAs with an odd number of carbon atoms such as propionate and valerate (Albuquerque et al., 2013, 2007). However, in the present study both reactors were fed with the same substrate, which was mainly composed by acetic acid (63.85 ± 5.16 %), but also propionic (21.76 ± 7.82 %), butyric (13.49 ± 2.59 %) and valeric acids (0.89 ± 1.53 %). Small variations observed in the VFA profile during the operation were a consequence of the changeable characteristics of the influent. Therefore, changes could be a consequence of possible variations in the microbial community due to settling implementation since there is a correlation between the microbial community structure and the biopolymer composition (Carvalho et al., 2014).

3.4 Variations in the MMC enhanced the obtention of different biopolymers from the same influent

The MMC was characterized by the presence of heterotrophic bacteria with high PHA accumulation capacity. Results obtained by FISH technique showed that microorganisms present in the inoculum of the SBR-S mainly belonged to phyla *Bacteroidetes* and *Proteobacteria*, and this latter included microorganisms from genera *Comamonas*, *Azoarcus* and *Thauera*. All these identified microorganisms have been previously reported as PHA-storing bacteria in MMC (Morgan-Sagastume et al., 2015)

Changes in the microbial community were observed at day 63 of SBR-S (Figure 5). Genus *Paracoccus* sharply increased whereas the abundance of genera *Comamonas* decreased. Those populations that reduced their abundances, even though they were reported as PHA-storing microorganisms, probably accumulated PHA in less proportion. Therefore, they had slower settling velocity and were washed-out with the supernatant discharged after the settling stage. These changes in the MMC composition presumably modified the substrate uptake preferences, which could explain the variations observed in the HB:HV ratio. Thus, as previously reported, the same substrate could result in PHA with different %HV depending on the microbial populations and their metabolic characteristics (Carvalho et al., 2014). This succession of dominant microbe from *Paraccocus* to *Comamonas* could not be attributed to the high salinity in the wastewater. Thus, NaCl concentrations in the influent of the SBR-I (5 – 13 g NaCl/L), from which was taken the inoculum, were similar to those of the SBR-S (4 – 12 g NaCl/L).

Albuquerque et al., (2013) mimicked the simultaneous presence of different substrates in a bioreactor and observed clear substrate-uptake preferences in a PHA-storing

community. All the populations could take up several substrates simultaneously but there was a substrate preference: acetate was mainly taken up by *Azoarcus* and *Paracoccus*; propionate by *Paracoccus*; butyrate by *Thauera*; and *Paracoccus* and valerate by *Paraccocus* (Albuquerque et al., 2013; Morgan-Sagastume et al., 2016). The generally accepted metabolic pathway for PHA synthesis from organic acids assumes acetate and butyrate as HB precursors and propionate and valerate as HV precursors (Duque et al., 2014). According to this, VFA composition in the influent (63.85 ± 5.16 % acetic acid, 21.76 ± 7.82 % propionic acid, 13.49 ± 2.59 % butyric acid and 0.89 ± 1.53 % valeric acid) explains the high HB proportion in the HB:HV ratio (70:30).

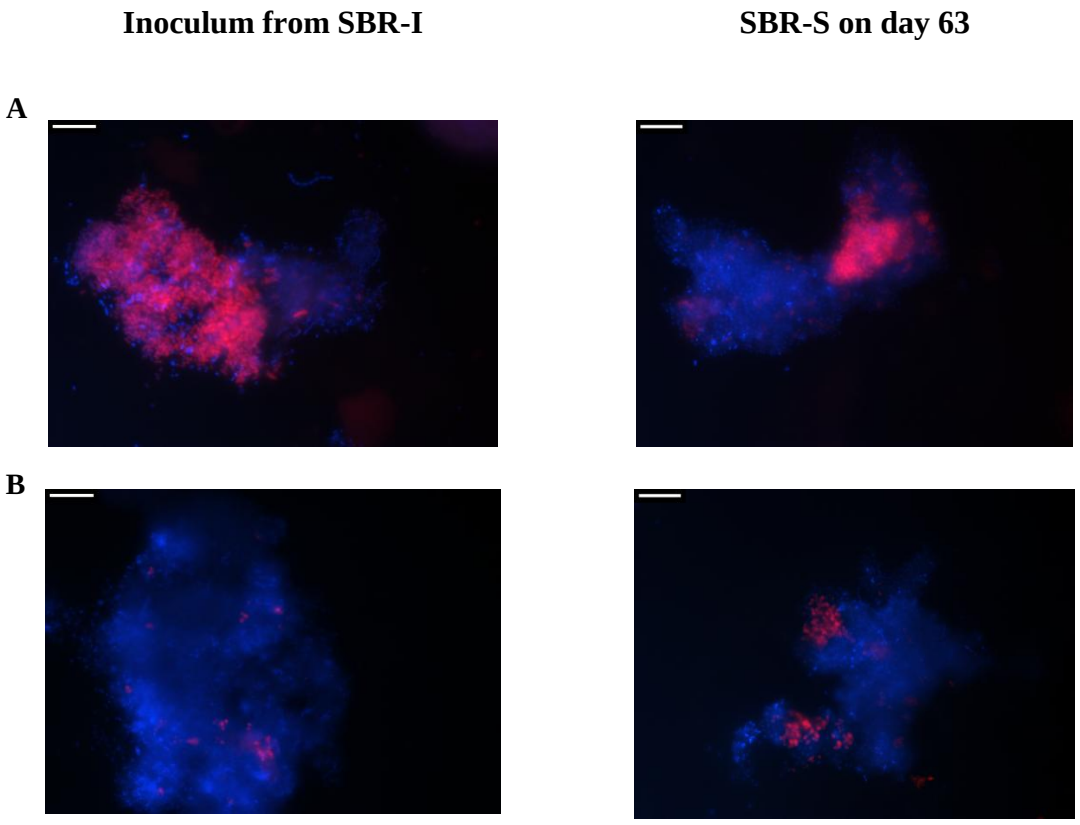

Figure 5. FISH images of the enriched biomass from the SBR-I (inoculum of SBR-S) and SBR-S at day 63. A) *Comamonas* (Cte: Cy3, red) and all DNA (DAPI, blue) and B) *Paraccocus* (Par1244; Cy3, red) and all DNA (DAPI, blue). The bar represents 10 µm.

The pronounced increase observed in the presence of genus *Paracoccus* in the SBR-S could derive on a higher propionate and valerate affinity of the whole microbial community in comparison with the inoculum (from SBR-I). Consequently, this result explains the bigger HV proportion in the HB:HV ratio of SBR-S (70:30) compared with the SBR-I (87:13) (Table 1.A). In addition, *Thauera* abundance was found to play an important role in the HV content of the biopolymer, even more than the propionate + valerate fraction. This might indicate that distinct metabolic pathways are prevalent in each microbial group for PHA production (Carvalho et al., 2014). However, a high HB proportion was still maintained, which correlates with the observed by Albuquerque et al., (2013) and Carvalho et al., (2014). These authors stated that *Paracoccus* enrichments favour the storage of VFA as PHA polymers with a higher HB content.

PHA properties are generally tailored using specific substrates to obtain copolymers with different monomer compositions (Duque et al., 2014; Palmeiro-Sánchez et al., 2016b). In addition, it was also studied the possibility of manipulating the biopolymer composition without changes in the feedstock. Albuquerque et al., (2011) demonstrated that the feeding regime (pulsed versus continuous) of the accumulation reactor affected polymer composition and observed that continuous feeding resulted in a higher HV content using the same feedstock. In the present research work, the implementation of a settling stage generated shifts in the microbial community, which directly correlated with the type and characteristics of the PHA produced, due to the link existent between the microorganisms present in the mixed culture and their carbon substrate-uptake preferences. Therefore, the results of the present study showed the possibility of modifying the biopolymer properties through the imposition of changes in the SBR operational cycle without changing the substrate composition or the feeding strategy in the accumulation reactor. In this particular case, settling implementation caused an

514 increase in the HV fraction. This derived on a reduction of the biopolymer crystallinity
515 and the melting and glass transition temperatures, which ultimately enhances the
516 biopolymer flexibility and processability (Dias et al., 2006).

517

518 **4. CONCLUSIONS**

519 The addition of a settling phase to the operational cycle (at the end of the feast phase) in
520 an enrichment SBR for PHA production, fed with pre-acidified complex saline
521 wastewater generated in mussel cookers, was evaluated. The enrichment of the MMC
522 was improved by the wash-out of microbial populations with lower or no PHA-storage
523 capacity after the feast phase. Moreover, the discharge of the supernatant after the feast
524 phase favoured the removal of substances that enhanced the development of non-
525 accumulating microorganisms. Variations in the microbial community were observed
526 and genus *Paracoccus* notably increased in abundance, whereas genera *Comamonas*
527 decreased. The implementation of the settling stage improved the maximum PHA
528 storage capacity (from 40 up to 60 wt%) and varied the co-polymer composition in
529 terms of HB:HV ratio (HV proportion increased from 17 to 30 %).

530

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667

Table 1. Comparison of experimental kinetic parameters and yields of: A) enrichment reactors operated with (SBR-S) and without settling stage (SBR-I). B) accumulation essays with enriched biomass from the reactors operated with (FBR-S) and without settling stage (FBR-I).

A)	**SBR-I**	**SBR-S**	
		Day 24	**Day 62**
Feast lenght (h)	2.62 ± 0.10	2.00	1.50
q_{VFA} (Cmmol VFA/Cmmol X·h)	0.26 ± 0.06	0.22	0.27
q_{PHA} (Cmmol PHA/Cmmol X·h)	0.07 ± 0.02	0.14	0.22
q_{HB} (Cmmol HB/Cmmol X·h)	0.06 ± 0.01	0.12	0.15
q_{HV} (Cmmol HV/Cmmol X·h)	0.01 ± 0.01	0.02	0.07
Y_{PHA} (Cmmol PHA/Cmmol VFA)	0.32 ± 0.21	0.61	0.80
max. PHA (wt%)	12.80 ± 0.76	14.77	18.32
HB:HV	88:12 ± 6:6	88:12	70:30

B)	**FBR-I**	**FBR-S**	
		Day 35	**Day 64**
q_{VFA} (Cmmol VFA/Cmmol X·h)	0.20	0.30	0.26
q_{PHA} (Cmmol PHA/Cmmol X·h)	0.10	0.14	0.19
q_{HB} (Cmmol HB/Cmmol X·h)	0.08	0.12	0.14
q_{HV} (Cmmol HV/Cmmol X·h)	0.02	0.02	0.05
Y_{PHA} (Cmmol PHA/Cmmol VFA)	0.48	0.49	0.72
max. PHA (wt%)	40.87	44.28	59.92
HB:HV	83:17	82:18	70:30
q_{VFA} (Cmmol VFA/Cmmol X·h)	0.20	0.30	0.26

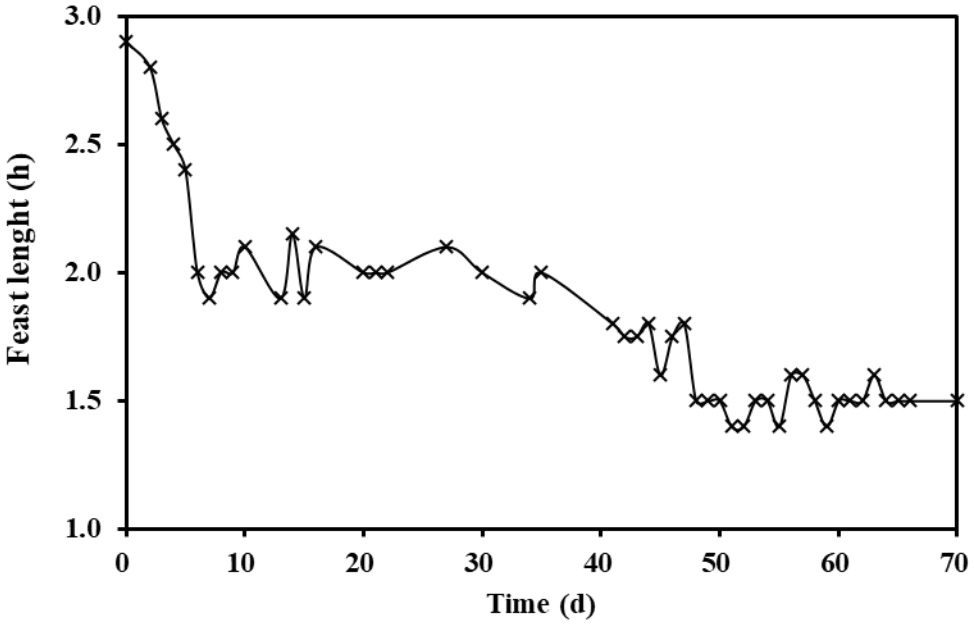
8 **Table 2.** Data from accumulation essays carried out with biomass obtained under different enrichment strategies.

MMC origin & Enrichment strategy	**Feedstock**	**$Y_{PHA/S}$ (Cmmol PHA/ Cmmol S) Max. PHA (wt%) q_{PHA} (CmmolPHA/Cmmol X·h)**	**Reference**
Activated sludge ADF without settling	VFA mixture and lactate	$Y_{PHA/S}$: 0.05 – 0.45	Dionisi et al., 2007
Activated sludge ADF without settling	Sodium acetate	Max. PHA: 69.00 – 89.00	Johnson et al., 2009
Water from a river estuary ADF + settling before withdrawal	Sodium acetate, glucose or starch	$Y_{PHA/S}$: 0.60 sodium acetate / 0.54 glucose / 0.30 starch Max. PHA: 64.70 sodium acetate / 60.50 glucose / 23.70 starch	Cui et al., 2016
Activated sludge ADF without and with settling after feast phase	Mixture of sodium acetate and methanol	Max. PHA: 48.00 without settling / 58.00-70.00 with settling	Korkakaki et al., 2016
Activated sludge ADF + settling after feast phase	Nutrients rich fermented centrate sludge	$Y_{PHA/S}$: 0.25 - 0.27 Max. PHA: 17.00 - 39.00	Morgan-Sagastume et al., 2015
Activated sludge ADF uncoupled C and N feeding strategy + settling after feast phase	Industrial soft drink wastewater	Max. PHA: 13.80	Ahmadi et al., 2018
Activated sludge ADF coupled and uncoupled carbon and nitrogen feeding strategy + settling before withdrawal	Fermented cheese whey	$Y_{PHA/S}$: 0.86 coupled / 0.96 uncoupled q_{PHA}: 0.29 coupled / 0.40 uncoupled	Oliveira et al., 2017
Activated sludge ADD (aerobic dinamic discharge) + two settling stages (after feast phase and before withdrawal)	Mixture of sodium acetate, NH_4Cl and KH_2PO_4	q_{PHA}: 0.47 - 1.89	Chen et al., 2016
Enriched biomass from the C-SBR, which was inoculated with activated sludge ADF + settling stage after feast phase	Synthetic VFA mixture mimicking the composition of pre-acidified cooked mussel wastewater	$Y_{PHA/S}$: 0.72 Max. PHA: 59.92 q_{PHA}: 0.19	This work

9

Figure 1

A)


Feast lenght (h)
3.0
2.5
2.0
1.5
1.0
0
10
20
30
40
50
60
70
Time (d)

B)

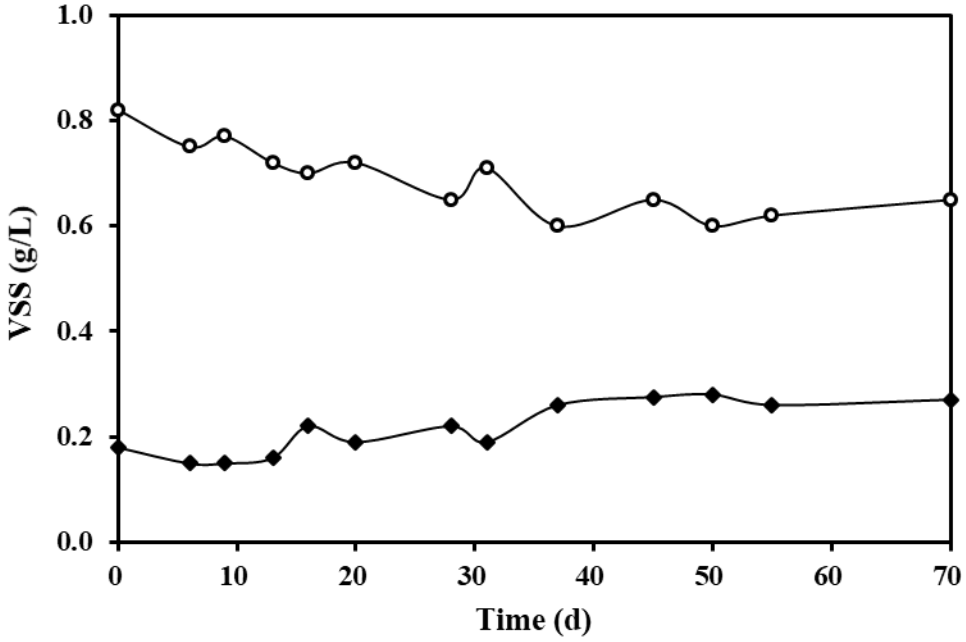
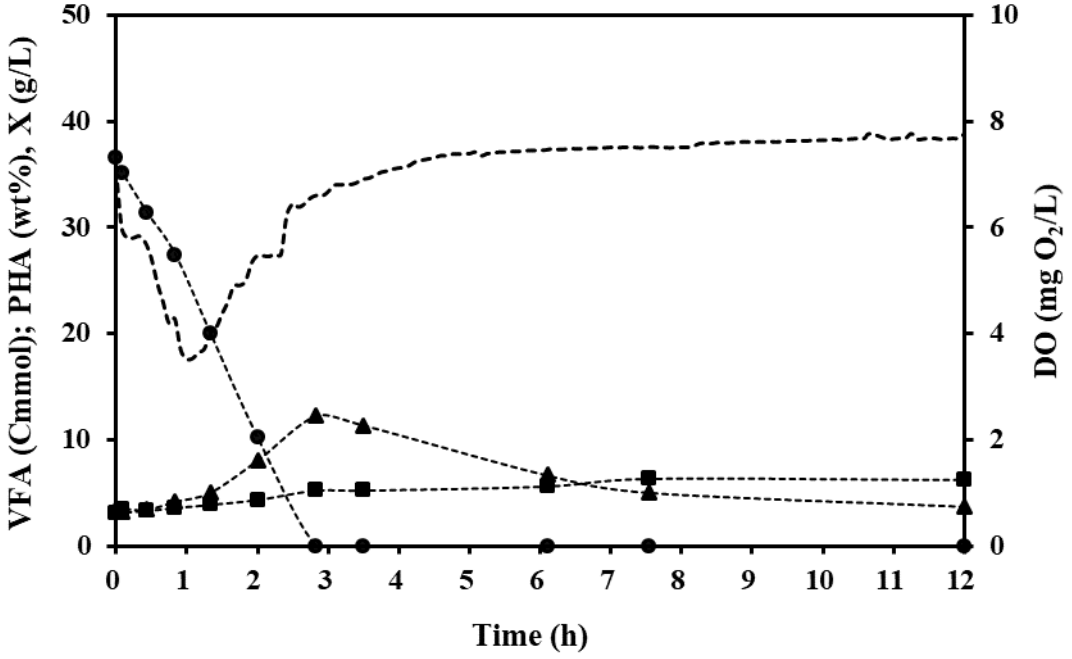
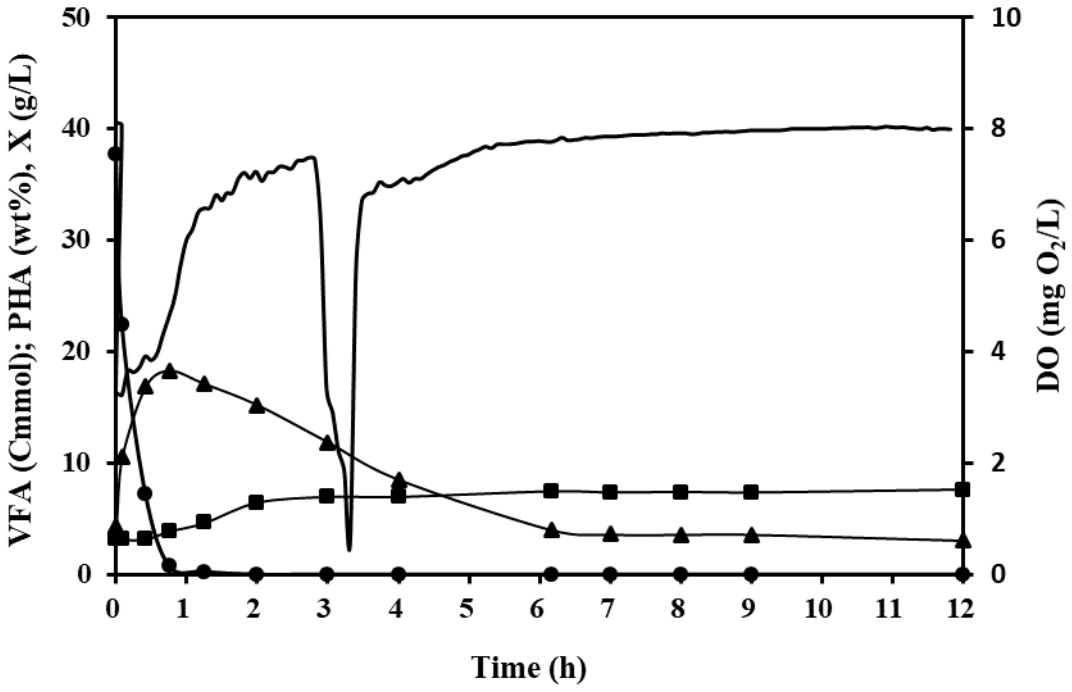

VSS (g/L)
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
70
Time (d)

Figure 2

A)


VFA (Cmmol); PHA (wt%), X (g/L)
DO (mg O2/L)
Time (h)
0
10
20
30
40
50
0
2
4
6
8
10
0
1
2
3
4
5
6
7
8
9
10
11
12

B)


VFA (Cmmol); PHA (wt%), X (g/L)
DO (mg O2/L)
Time (h)
0
10
20
30
40
50
0
2
4
6
8
10
0
1
2
3
4
5
6
7
8
9
10
11
12

13 **Figure 3**

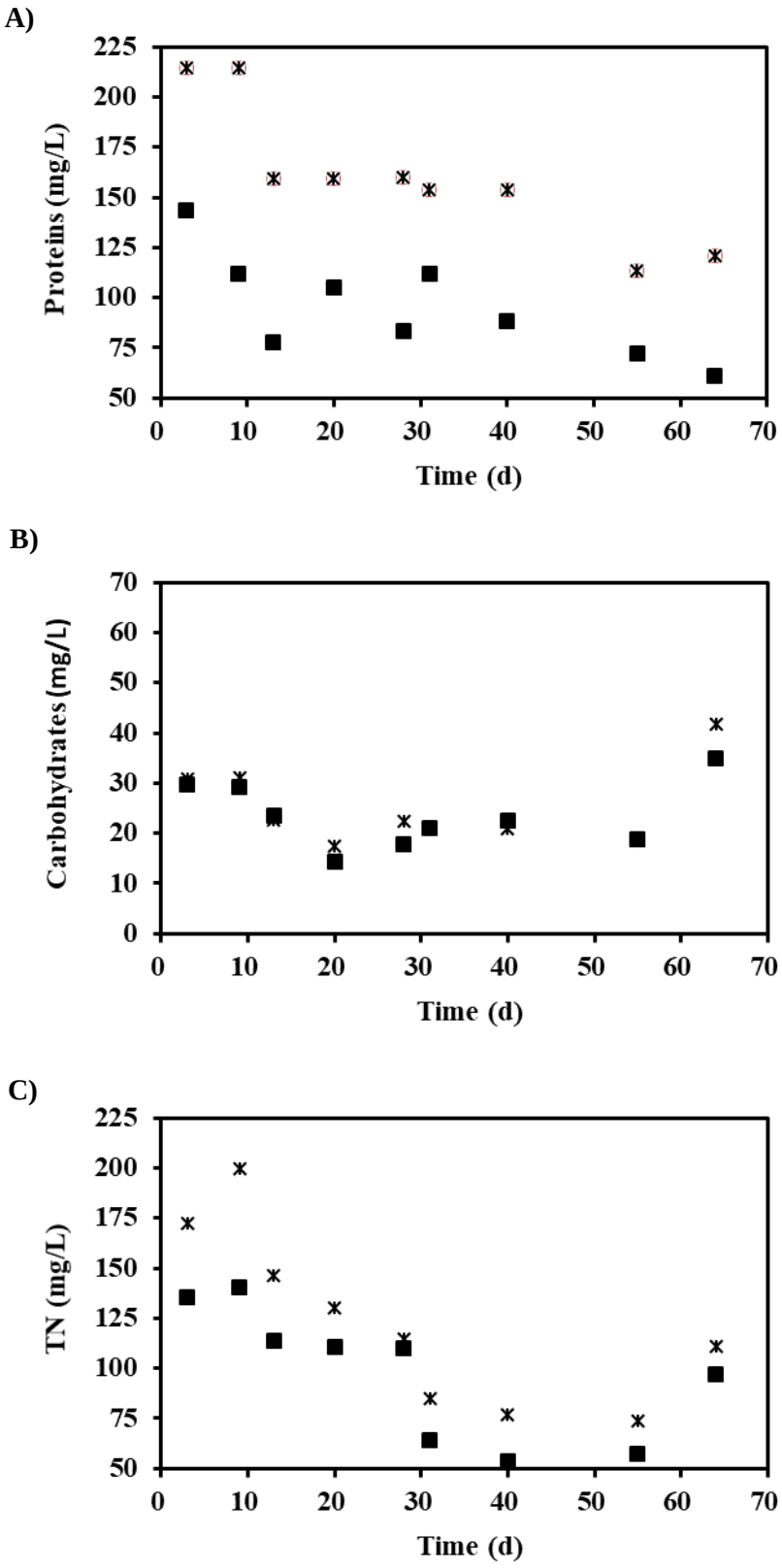


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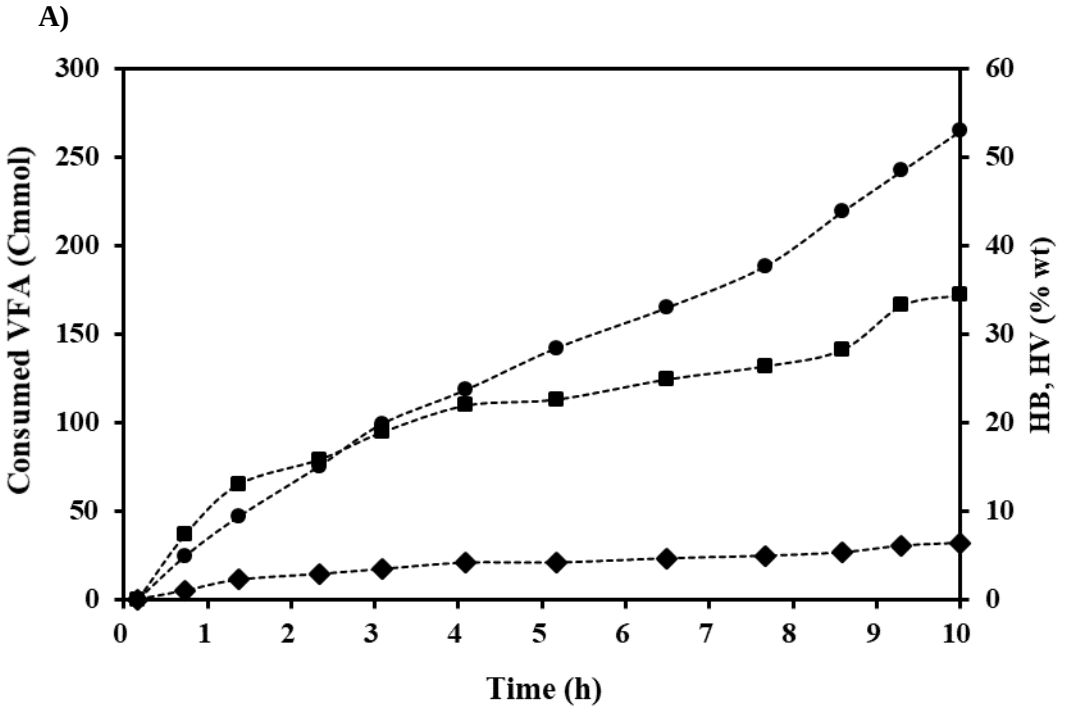
Figure 4


A)
Consumed VFA (Cmmol)
HB, HV (% wt)
Time (h)
0
50
100
150
200
250
300
0
10
20
30
40
50
60
0
1
2
3
4
5
6
7
8
9
10

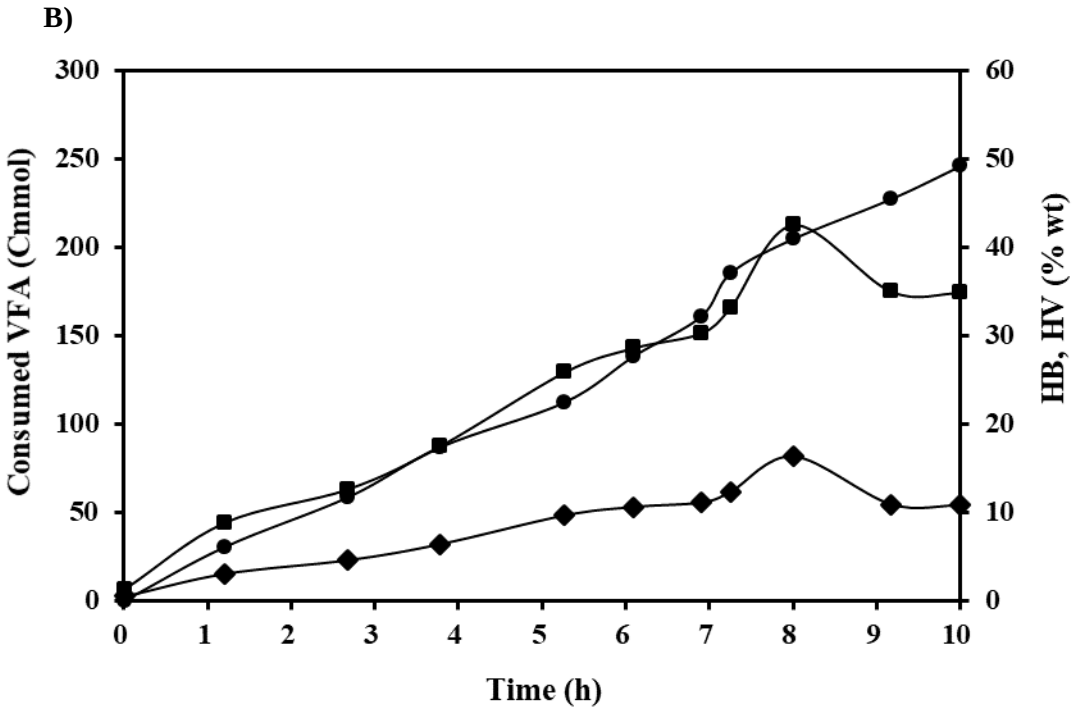

B)
Consumed VFA (Cmmol)
HB, HV (% wt)
Time (h)
0
50
100
150
200
250
300
0
10
20
30
40
50
60
0
1
2
3
4
5
6
7
8
9
10

Figure 5

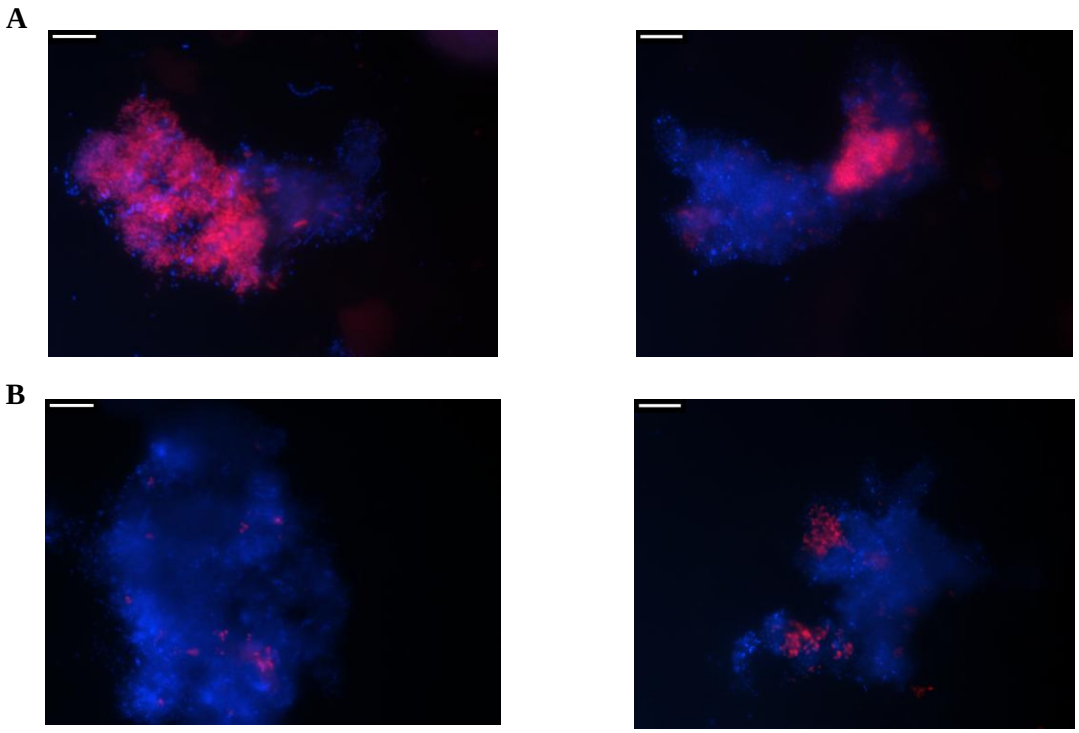


FIGURE CAPTIONS

Figure 1. Evolution in the SBR-S operational period of: A) the feast phase length (x) and B) the VSS concentrations inside the SBR-S at the end of the cycle (the same as in the effluent) at min 705 (○) and in the supernatant discharged after the settling stage (⬥) at min 200.

Figure 2. Evolution of the parameters monitored in representative enrichment cycles: DO (-), VFA (●), X (■) and percentage of PHA accumulated (wt%) (▲). A) Reactor operated without settling stage (SBR-I, - - -) at the moment of inoculum collection. B) Reactor operated with settling stage (SBR-S, ──) at day 62 of operation.

Figure 3. Proteins A), carbohydrates B) and TN C) concentrations before settling (*) and after refilling (■) in the SBR-S reactor.

Figure 4. Evolution of the parameters monitored in accumulation essays (FBR) performed with enriched biomass: VFA (●) and percentage of accumulated PHB (■) and PHV (♦). A) Reactor operated without settling stage (FBR-I, - - -) at the moment of inoculum collection. B) Reactor operated with settling stage (FBR-S, ──) at day 64.

Figure 5. FISH images of the enriched biomass from the SBR-I (inoculum of SBR-S) and SBR-S at day 63. A) *Comamonas* (Cte: Cy3, red) and all DNA (DAPI, blue) and B) *Paraccocus* (Par1244; Cy3, red) and all DNA (DAPI, blue). The bar represents 10 µm.

Supplementary Material

Click here to download Supplementary Material: Supplementary Material.doc

***Declaration of Interest Statement**

1 Declaration of interest none.

Credit Author Statement

Lucia Argiz: Investigation, Writing – Original Draft, Visualization; **Andrea Fra-Vazquez:** Conceptualization, Validation; **Angeles Val del Rio:** Validation, Visualization, Supervision, Funding Acquisition; **Anuska Mosquera:** Validation, Supervision, Project Administration, Funding Acquisition.