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Recirculation factor as a key parameter in continuous-flow biomass selection for polyhydroxyalkanoates production

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ABSTRACT

The effectiveness of polyhydroxyalkanoates (PHA) production with mixed microbial cultures (MMC) largely depends on the selection of PHA-storing microorganisms, conventionally performed in sequencing batch reactors (SBR). These, although easily allow the establishment of the required feast and famine (FF) regime, can represent a factor of cost increase when the process is scaled up. Here, a novel continuous-flow process for MMC selection under FF conditions has been developed by using two sequentially operated reactors. The feast reactor, having a tubular configuration, was continuously fed with a synthetic mixture of acetic and propionic acids (at an organic loading rate of 2.12 gCOD/L d) and the effluent of this reactor was in part sent to the CSTR famine reactor. The recirculation factor (R_C), that is the ratio between the recirculation flow rate and the feeding flow rate to the feast reactor, was the main parameter investigated. Four different runs were performed with the R_C varying from 1 to 8 and the increase in its value caused a decrease of the biomass residence time in each reactor. The intracellular PHA content in the feast reactor almost linearly increased up to R_C 4 (with a value of 34 ± 2 %, wt/wt) and dropped at the R_C 8 condition that, however, showed the maximum PHA content (58 ± 5 %, wt/wt) during the accumulation tests. Indeed, the relative abundance of sequences affiliated with putative PHA-storing bacteria increased up to 90.5 % at R_C 8 and were dominated by members of the *Alphaproteobacteria* class mostly represented by the genus *Meganema* (74 %).

1. Introduction

Nowadays, the world is facing the steady increase of organic and plastic waste due to the human activity. The worldwide plastic production accounted for 359 million tonnes in 2018 and for 368 million tonnes in 2019, with a substantial increase of 9 million tonnes in only one year [1]. The massive use of plastics such as polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), and polyethylene terephthalate (PET), generates huge amounts of plastic wastes that, once disposed in the environment, lead to serious management concerns as well as issues related to climate changes since these plastics are chemically synthesized from fossil raw materials and are not biodegradable. As the world is moving towards the development of the circular economy concept, the use of renewable biological resources and their conversion using innovative technologies in biobased plastics is now extensively encouraged [2]. In that purpose, polyhydroxyalkanoates (PHA) are a family of bioplastics (both biobased and fully biodegradable in the environment) which represent an interesting and promising option to reduce or replace the use of traditional plastic materials for a broad portfolio of applications, including the production of items in the agricultural, textile or medical sectors, the production of packaging materials (that is one of the most demanding sectors), as well as the application as slow-release carbon source for environmental bioremediation [3–6].

PHA are natural polyesters, synthesized by a wide variety of microorganisms in particular unbalanced growth conditions. Generally, when

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those microorganisms are exposed to an excess of external carbon sources, PHA are stored in the form of intracellular granules to be subsequently used as an internal reserve of carbon and energy for the growth [7–10]. Presently, the industrial PHA production is performed by using pure microbial cultures (e.g., *Cupriavidus necator,* previously known as *Ralstonia eutropha*, *Alcaligenes latus*, *Pseudomonas putida*, or recombinant *Escherichia coli*) [11,12], which require sterile conditions, and seriously influence the market price of the polymer (ranging from 2.2 to about 5.0 €/kg), that is at least three times more expensive than commonly used plastic (e.g., PP, PE) with the feedstock cost accounting between 30 % and 50 % of the overall operating cost of PHA production [13–17]. Differently from pure cultures, mixed microbial cultures (MMC) are more resilient to contamination and to any perturbation of the systems. Therefore, cheap or no cost substrates such as food waste and agro-industrial wastewaters can be used as feedstock, decreasing the operative cost and in addition not requiring sterile conditions [18–24]. In this context, the integration of the PHA production process into urban waste treatment plants, which could provide a constant supply of the carbon source hence avoiding extra cost for waste collection, has been proven as a viable strategy to drive the transition of the technology from laboratory to pilot scale [21,25,26]. Despite the remarkable efforts of the past years, the low polymer productivity with MMC-based processes, which typically stall within the range 0.24 – 0.41 gPHA/L h [13], remains one of the main factors hindering the economic competitiveness of the process and, in turn, the potential for a large-scale commercial exploitation of the MMC-PHA.

The process that involves MMC for PHA production typically comprises several stages that include the acidogenic fermentation of the waste feedstock to produce a stream rich into volatile fatty acids (VFA), direct precursors for MMC-PHA production. Also, a selection stage is required to enrich a microbial consortium into PHA – storing microorganisms, as well as a following PHA accumulation stage to maximize the intracellular PHA content of the selected culture [27,28]. The selection step plays a key role on the overall process performance and conventionally occurs in an SBR (Sequencing Batch Reactor), where specific growth conditions, typically referred to as the feast and famine regime, are adopted. An SBR is operated through a sequence of cycles that are characterized by different phases, such as the carbon source feeding and consumption (feast phase), the biomass withdrawal, the PHA consumption and biomass growth (famine phase) [19,29]. Depending on the adopted approach, the biomass can be withdrawn either at the end of the feast phase or at the end of the famine phase, with either previous settling or not, in any case to be sent to the accumulation step. Also, the nitrogen feeding can occur along with carbon feeding (coupled strategy) or at the start of the famine phase (uncoupled strategy), which also depends on the feed actual composition [30–32]. Operating conditions triggering microbial culture selection, stability of the enriched culture, as well as the strategy applied for nutrient supplementation are, among the others, critical parameters to be evaluated for scaling up the MMC-PHA production [13]. Furthermore, SBRs having such a sequence of intermittent feeding and harvesting phases, operated on a short time basis, typically employ oversized pumps and oversized liquids/solids separation units, which rise the capital costs at pilot and industrial scale. The possibility of continuously feeding the substrate to the reactor has been investigated and the obtained results indicated that a true famine phase (i.e., the complete absence of substrate) is not strictly necessary but a true feast phase is essential for the enrichment of PHA producing bacteria [33].

Along this line, the establishment of the feast and famine regime in two continuous reactors connected in series may be an option to reduce the investment costs. In this approach, the system is continuously fed, and the feast and famine phases are separated in space rather than in time. In the literature, very few authors have studied this type of process [34,35] and have experimented the use of two reactors in series with a settling phase. In this kind of systems, the hydraulic retention time (HRT) of the two reactors depends on their respective volumes and their ratio also determines the feast and famine ratio, since the C source is only supplied to the first reactor. Albuquerque and co-workers [34] used two Continuous Stirred Tank Reactors (CSTR) in series fed with fermented molasses as carbon source and evaluated the effect of the influent substrate concentration (60 or 120 Cmmol VFA/L) and HRT ratio between the two reactors (0.2 – 0.5 h/h) on the system efficiency towards microbial selection. Indeed, Bengtson and colleagues [35] used paper mill wastewater to produce PHA in a system made of two separate reactors equipped with a clarifier, the first one (called selector) where the polymer was stored and the second one (main reactor) where the produced PHA was consumed, with a feast and famine ratio (based on the volume of the two reactors) of 0.06 h/h and an influent acids concentration (as Chemical Oxygen Demand, COD) of 6.5 g/L. The cited works reached a maximum intracellular PHA content of 61 % (gPHA/gVSS) and 48 % (gPHA/gTSS), respectively, after accumulation batch experiments.

The aim of this work was to develop a novel approach to improve the efficiency of MMC selection for PHA production in a continuous-flow process along with the maximization of PHA storage capability in the final production stage. To that purpose, a continuous process for the selection of PHA-storing microorganisms was performed in two different reactors in which the feast and famine regime was mimicking the SBR systems: the first reactor with a tubular configuration (Plug Flow-like column) where the substrate was continuously fed (feast) and the second one, consisting of a CSTR, in which the famine phase was obtained. This configuration was adopted since the column reactor allowed to completely consume the carbon source, hence ensuring a low or no presence of organic substrates entering the famine CSTR reactor. The main novelty of this study is the use of a reactor with tubular geometry for the feast phase as well as the absence of a settler after the famine reactor for the recirculation of thickened biomass between the two reactors. Moreover, besides of establishing the HRT, the Organic Load Rate (OLR) and the volume ratio between the two reactors, for the first time an additional control parameter such as the recirculation factor (R_C, which represents the ratio between the recirculation flow rate between the two reactors and the feeding flow rate to the feast reactor) has been investigated. A variation in the R_C value corresponds to variations in the frequency and length of periods whereby microorganisms are subjected to feast and famine conditions. Indeed, an increase in the R_C causes a reduction of the hydraulic retention time in both the feast and famine reactors and, correspondingly, an increase of the frequency at which microorganisms are subjected to the alternance of feast and famine conditions in the respective reactors. Moreover, an increase of the R_C causes the dilution of the substrate solution fed to the feast tubular reactor within a larger recycle solution. All these factors have the potential to affect the establishment of the selective pressure required for microbial selection under FF conditions and an optimal trade-off between the applied operating conditions needs to be identified. Based on this rationale, here the R_C value was increased from 1 to 8 and its impact on the continuous-flow process was proven to be a key factor on the selection of PHA storing microorganisms and on the PHA accumulation performance.

2. Materials and methods

2.1. PHA-storing biomass selection through feast and famine alternance in two continuous-flow reactors

The continuous process for the selection of PHA-storing biomass was performed in two separated aerobic reactors hydraulically connected through an internal recirculation stream (Fig. 1). Both reactors were initially inoculated with activated sludge from a full-scale municipal wastewater treatment plant. More in detail, in the first reactor (1 L working volume) the feast phase was performed. This reactor presented a geometrical PF (plug flow)-like column configuration but with a partial mixing of the liquid phase only provided by aeration through air

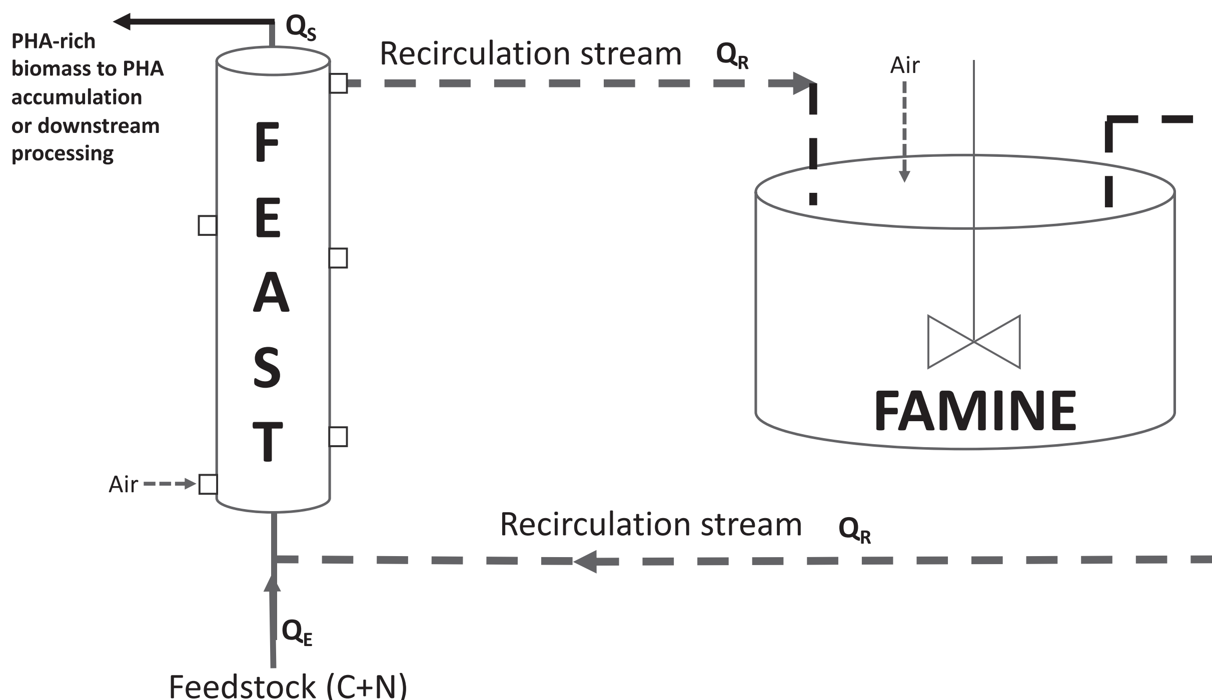

Fig. 1. Scheme of the continuous process for the selection of PHA-storing microorganisms from MMC in two interconnected reactors without settling.

pumps connected to a needle inserted in one of the lateral exits of the reactor. Whereas, in the second reactor, having a 5 L working volume and a CSTR-configuration consisting of a tank totally mixed with a stirring bar and aerated through an air pump connected to glass diffusers, the famine phase was applied. Indeed, the inlet feeding stream to the system (with a flow rate referred to as Q_E) was supplied at the bottom of the feast reactor, at a fixed organic loading rate (OLR) of 2.12 gCOD/L d, along with the recirculation stream coming from the famine reactor. More in detail, the effluent from the feast reactor was in part discharged (at a flow rate corresponding to $Q_S = Q_E$) and in part sent to the CSTR famine reactor by means of an internal recirculation flow rate (Q_{R1}), without any additional feeding supply to allow the establishment of the feast and famine regime. The effluent of the famine reactor was continuously recirculated to the feast reactor via the recirculation flow rate ($Q_{R2} = Q_{R1} = Q_R$). Therefore, since at the given OLR the carbon source contained in the feeding solution was removed in the first reactor and nearly no residual carbon source entered the second reactor through Q_{R1}, a feast and famine regime was achieved, with the volume ratio of the two reactors fixed at 1:5.

A coupled feeding strategy of carbon (C) and nitrogen (N) sources was adopted, with the first one consisting of a synthetic mixture of VFA (2.12 gCOD/L) made of acetic and propionic acids (65 % and 35 % of the overall Chemical Oxygen Demand, COD; respectively), whereas ammonium sulphate (0.28 g/L) was used as *N*-source. In all runs, the ratio between the mass flow of C- and *N*-sources was set at 35 gCOD/gN. The feeding solution was diluted in a mineral medium (containing thiourea to inhibit nitrification) as reported elsewhere [31], and its pH was adjusted (to values between 6.0 and 7.0) by additions of an alkaline solution (NaOH, 3 M). The process was operated at room temperature (ranging between 24 °C and 28 °C). The inlet flow rate Q_E, and both recirculation flow rates Q_{R1} and Q_{R2} (feast-famine and famine-feast) were maintained by peristaltic pumps, whereas Q_s was naturally overflowing from the feast reactor at $Q_S = Q_E$. In particular, Q_E was set at 4.2 mL/min in all runs (Table 1) in order to establish an overall hydraulic retention time (HRT) of 1 day (referred to the overall volume of the system, 6 L). The inlet and outlet flow rates were measured three times a week and, if necessary, adjusted to the setpoint by regulating the speed velocity of the peristaltic pumps. Having no biomass settling, the sludge retention time (SRT) was the same as the HRT. On the other hand, the recirculation flow rates (Q_{R1} and Q_{R2}, referred to as Q_R) were changed in the range between 4.5 and 36 mL/min, corresponding to a recirculation factor ($R_C = Q_R/Q_E$) from around 1 to around 8 (accounting for four runs with R_C equal to 1, 2, 4, or 8). To create an analogy with the conventional approach of obtaining the feast and famine regime consisting of a Sequencing Batch Reactor (SBR), the variation of R_C would correspond to changing the SBR cycle length; i.e. having a "cycling time" (L_C) in the recirculation system of 20.42 h, 10.52 h, 5.38 h, and 2.72 h, respectively; with a corresponding feast phase lasting for 1.91 h (9.40 % of L_C), 1.26 h (12.0 %), 0.75 h (13.9 %), and 0.41 h (15.1 %). PHA, volatile fatty acids, volatile suspended solids, nitrogen concentrations, and pH were daily monitored in both reactors. Sampling for the feast reactor was carried out after collecting Q_S in a beaker (at least 200 mL), whereas sampling for the CSTR famine reactor was directly performed inside the reactor.

Table 1
Operative parameters of the continuous-flow process.

PARAMETER	VALUE			
OLR (gCOD/Ld)	2.12	2.12	2.12	2.12
R_C	1	2	4	8
Time (d)	61	39	43	49
Q_E (mL/min)	4.2	4.2	4.2	4.2
Q_R (mL/min)	4.5	9.0	18	36
Q_S (mL/min)	4.2	4.2	4.2	4.2
FEAST Cycling time (h)	1.91	1.26	0.75	*0.41*
(Percentage respect to L_C)	*(9.40 %)*	*(12.0 %)*	*(13.9 %)*	*(15.1 %)*
FAMINE Cycling time (h)	18.51	9.26	4.63	2.31
(Percentage respect to L_C)	*(90.6 %)*	*(88.0 %)*	*(86.1 %)*	*(84.9 %)*
Cycling time L_C (h)	20.42	10.52	≈ 5.38	≈ 2.72

2.2. PHA batch accumulation trials

Accumulation trials were carried out for each run after at least 15 days of operation of the selection system in the same conditions. In total, three accumulation tests, each one having a duration of 6 h, were performed for each condition in an aerobic fed batch reactor (1 L working

volume). The reactor was maintained at room temperature (∼25° C) and the pH was corrected to values between 7.0 and 8.0 after each C-feeding pulse by adding a NaOH (3 M) solution. A magnetic stirring bar was used to mix the liquid phase, in addition to air blowing through an air pump. In order to monitor substrate depletion, measurements of the Oxygen Uptake Rate (OUR) were performed at the beginning and during each accumulation test, as described elsewhere [36]. The accumulation trials were conducted under *N*-limiting conditions, to favor the PHA-storing mechanism with respect to the biomass growth. Indeed, to ensure these initial *N*-limiting conditions, the biomass for the accumulation trials was withdrawn from the famine reactor in which no or a low residual quantity of N- and C- substrates was present. A defined volume of a high-concentrated synthetic solution of VFA (425 gCOD/L, at the same composition in terms of acetic and propionic acids as for the feeding solution to the selection system) was added to the accumulation reactor in order to have the desired acids concentration (between 0.425 and 1.70 gCOD/L) after each pulse, yet not too high to prevent biomass inhibition. Therefore, when the OUR indicated that the substrate was depleted, to avoid the absence of organic acids inside the reactor, a new pulse of the synthetic VFA solution was immediately supplied. These feeding pulses were repeated until the end of the tests (lasting 6 h). During each trial, samples for monitoring PHA and VFA concentration were taken every hour, whereas the volatile suspended solids (VSS) concentration was determined twice, in correspondence to the beginning and the end of the tests.

2.3. Analytical methods

Liquid samples for ammonium and VFA analysis were filtered through 0.45 μm porosity cellulose acetate filters, and then put in the freezer (-20 °C) until further analysis. The ammonium concentration was determined with the Nessler spectrophotometric method whereby the absorbance of coloured samples was measured at 420 nm wavelength (SHIMADZU Spectrophotometer UV-1800) [37]. For VFA analysis, samples (1 μL) were injected into a gas-chromatograph (GC 8860, Agilent Technologies, USA) equipped with a flame ionization detector (FID) and a capillary column (DB-FFAP). The acids concentration was converted into COD using the conversion factor based on the oxidation stoichiometry (1.07 gCOD/$g_{acetic\ acid}$ and 1.51 gCOD/$g_{propionic\ acid}$). For PHA determination, 5 mL of mixed liquor from biological reactors was sampled and immediately treated with 1 mL of a sodium hypochlorite solution (5 % active Cl_2) to stop the polymer degradation. Samples were then stored in the freezer (-20 °C) for following PHA analysis, which was determined by gas chromatography (GC-FID Perkin Elmer 8410) after hydrolysis and esterification into 3-hydroxyacyl methyl esters [38]. The relative abundance of 3-hydroxybutyrate (HB) and 3-hydroxyvalerate (HV) monomers was determined using as reference standard a commercial P(HB/HV) copolymer with 5 % (wt) HV content (Sigma–Aldrich, Milan, Italy). Also for PHA, concentrations measured by GC were converted into a COD basis according to the stoichiometry conversion factors of 1.67 gCOD/gHB and 1.92 gCOD/gHV.

Regarding suspended solids determination, analyses were performed by using GF/C filters (47 mm in diameter and 1.2 μm in porosity), which were previously placed in an oven at 100 °C for 24 h, to eliminate moisture traces, cooled in a desiccator, and weighted (P_1). Samples of biomass (10 mL) were taken from the biological reactors with a graduated pipette and vacuum filtered onto the previously weighted filters, which were brought back to the oven at 100 °C for at least 4 h, then left at least 15 min in the desiccator and weighted again (P_2). After that, filters were placed in a muffle furnace at 550 °C for 30 min, cooled in the desiccator, and weighted (P_3) [37].

2.4. Calculations

The determination of total, fixed, and volatile suspended solids (TSS, FSS, and VSS; respectively) was obtained by taking into account P_1, P_2 and P_3 (mentioned on section 2.3), according to the following eqs (1), (2), (3):

$$TSS\left(\frac{\mathrm{mg}}{\mathrm{L}}\right) = \frac{(P_2 - P_1)(mg)}{V_{sampled}(L)} \tag{1}$$

$$FSS\left(\frac{mg}{L}\right) = \frac{(P_3 - P_1)(mg)}{V_{sampled}(L)} \tag{2}$$

$$VSS\left(\frac{mg}{L}\right) = TSS - FSS \tag{3}$$

The overall PHA concentration was obtained by summing the concentration of HB and HV monomers as reported in eq (4), and the intracellular PHA content was determined as the ratio between the stored PHA and the biomass (i.e., VSS), according to eq (5):

$$PHA\left(\frac{mg}{L}\right) = HB\left(\frac{mg}{L}\right) + HV\left(\frac{mg}{L}\right) \tag{4}$$

$$PHA\left(\%,\frac{wt}{wt}\right) = \frac{PHA\left(\frac{mg}{L}\right)}{VSS\left(\frac{mg}{L}\right)}.100 \tag{5}$$

The HV content in the stored copolymer was calculated according to eq (6):

$$HV\left(\%,\frac{wt}{wt}\right) = \frac{HV\,(mg)}{PHA\,(mg)}.100 \tag{6}$$

Active biomass (X_A) was defined as the non polymer biomass obtained from the difference between VSS and PHA, according to eq (7):

$$X_A\left(\frac{mg}{L}\right) = VSS\left(\frac{mg}{L}\right) - PHA\left(\frac{mg}{L}\right) \tag{7}$$

The conversion of X_A and VSS into COD basis was determined by using the conversion factor of 1.42 gCOD/g X_A for the active biomass, as reported in the following eqs (8) and (9):

$$X_A\left(\frac{mgCOD}{L}\right) = X_A\left(\frac{mg}{L}\right).1.42\left(\frac{mgCOD}{mg}\right) \tag{8}$$

$$VSS\left(\frac{mgCOD}{L}\right) = X_A\left(\frac{mgCOD}{L}\right) + PHA\left(\frac{mgCOD}{L}\right) \tag{9}$$

The substrate uptake rate (-q_S), growth rate (μX), and storage rate (q_P) referred to the overall system were determined from the concentration of the relative parameters in the feast reactor (wherein acids were mainly consumed and PHA was stored), the inlet and outlet flow rates (Q_E and Q_S), and the volume of the overall system (V_T, 6L), according to eqs. (10), (11), and (12):

$$(-q_S)\left(\frac{mgCOD_{VFA}}{Lh}\right) = \frac{\left[S_E\left(\frac{mgCOD}{L}\right)Q_E\left(\frac{L}{h}\right)\right]}{V_T(L)} - \frac{\left[S_S\left(\frac{mgCOD}{L}\right)Q_S\left(\frac{L}{h}\right)\right]}{V_T(L)} \tag{10}$$

$$(\mu X)\left(\frac{mgCOD_{XA}}{Lh}\right) = \frac{\left[X_A\left(\frac{mgCOD}{L}\right)\right].Q_S\left(\frac{L}{h}\right)}{V_T(L)} \tag{11}$$

$$(q_P)\left(\frac{mgCOD_{PHA}}{Lh}\right) = \frac{HB\left(\frac{mgCOD}{L}\right).Q_S\left(\frac{L}{h}\right)}{V_T(L)} + \frac{HV\left(\frac{mgCOD}{L}\right).Q_S\left(\frac{L}{h}\right)}{V_T(L)} \tag{12}$$

In which S_E and S_S represent the substrate concentration in the influent and effluent stream of the feast reactor, respectively.

Storage yield (Y_{STO}), growth yield (Y_{GRO}), and observed yield (Y_{OBS}) were determined by applying the eqs (13), (14), and (15). In particular, the storage yield represents the fraction of the consumed substrate converted into PHA, and the growth yield is the biomass produced with respect to the consumed substrate. The observed yield was espressed as the sum of the storage and growth yields.

$$Y_{STO}\left(\frac{COD}{COD}\right) = \frac{q_P}{(-q_S)} \tag{13}$$

$$Y_{GRO}\left(\frac{COD}{COD}\right) = \frac{\mu X}{(-q_S)} \quad (14)$$

$$Y_{OBS} = Y_{STO} + Y_{GRO} \quad (15)$$

In batch trials, the specific storage rate (q_P^{batch}, $mgCOD_{PHA}/gCOD_{Xa}$ h) was calculated by linear regression of concentration data versus time, considering the period at constant production rate and the initial active biomass concentration. This period lasted for a maximum of 6 h. The maximum polymer content in the biomass was determined based on the PHA profile and by taking into account the initial VSS concentration, since the growth response was considered negligible due to the absence of nitrogen during the tests. The storage yield (Y_{STO}^{batch}) was calculated at the specific time that the maximum PHA content was achieved.

2.5. Microbiological analysis

Aerobic sludge samples (10 mL) were taken from the feast reactor at the end of operation at R_C 2, R_C 4, and R_C 8, once the steady state condition was reached. For the *in situ* hybridization analysis, 4.5 mL were immediately fixed in formaldehyde and ethanol (5 % and 50 % vol/vol final concentration, respectively) and stored at − 20 °C. A small aliquot (2 mL) was centrifuged at 15,000 rpm for 2 min and the resulting pellet was immediately stored at − 20 °C until DNA extraction. The fixed samples were disaggregated by vortexing with glass beads for 3 min. Fluorescence *in situ* hybridization (FISH) analysis was performed using several oligonucleotide probes following the conditions reported in probe Base (https://www.microbial-ecology.net/probebase/) and according to the protocol described elsewhere [39]. In detail, the probes used in this study were: ALF968 for Alphaproteobacteria, BET42a and GAM42a for the Gammaproteobacteria class. The probes were labelled with sulfoindocyanine dye Cy3 or fluorescein isothiocyanate (FITC) (MWG AG Biotech, Germany). After hybridization, total cells were stained with VectashieldMountingMedium® with DAPI (Vector Labs, Italy) and viewed using epifluorescence microscope, Olympus BX51, equipped with an Olympus XM10 camera (Cell-Sense software).

DNA extraction was performed by using DNeasy PowerSoil Pro Kit (QIAGEN, Germantown, MD) and utilized as template for the amplification of the V1-V3 region of 16S rRNA gene of Bacteria (27F 5′-AGAGTTTGATCCTGGCTCAG-3′; 534R 5′-ATTACCGCGGCTGCTGG-3′). Samples were paired end sequenced (2 × 301 bp) on a MiSeq platform (Illumina) using a MiSeq Reagent kit v3, 600 cycles (Illumina, San Diego, CA, USA) following the procedure for library preparation and sequencing described in [40]. Bioinformatics analyses were carried out using QIIME2 v. 2018.2 [41], following the procedure previously reported [42]. High-throughput sequencing yielded a total of 37,179 sequence reads after quality control and bioinformatics processing that resolved into 461 ASVs. Based on the taxonomical classification, the relative percentage of bacterial genera described in the literature for their capability to accumulate PHA was calculated for each sample and is reported in the text as “putative PHA-storing bacteria abundance”. Dataset are available through the Sequence Read Archive (SRA) under accession PRJNA839563.

3. Results and discussion

3.1. Performance of the continuous-flow MMC-PHA selection process

The microbial selection process, as previously mentioned, was operated for 192 days at a fixed OLR (2.12 gCOD/L d) but at several R_C to identify the optimal working condition for the selection of PHA-storing microorganisms. Hence, R_C 1 can be considered as the starting point and was applied for 61 days, followed by R_C 2 (39 days), then R_C 4 (43 days), and R_C 8 (49 days). All runs were carried out consecutively without interruption, and the main parameters (VFA and nitrogen consumption, volatile suspended solids and PHA production, as well as growth and storage yields) used to evaluate the process performance were daily monitored.

In particular, the process herein developed mimicks the FF (Feast and Famine) conditions typically applied in an SBR. The carbon source, continuously fed to the feast reactor, consisted of a mixture of acetic (65 %) and propionic (35 %) acids at an overall concentration of 2.12 gCOD/L for an HRT and SRT equal to 1 day. As for VFA consumption, Fig. 2A shows the profile of residual organic acids measured both in the outlet stream of the feast reactor and in the famine reactor. During the first 7 working days of system operation, with an applied R_C equal to 1, VFA consumption was not complete likely due to the need of microbial adaptation to the substrate. However, after this initial period and till to the end of the operation period, the average residual concentration of acids accounted for 43 ± 16 mgCOD/L (corresponding to 97 % of VFA consumption). This highlights the fact that the supplied organic acids were almost completely removed in the feast reactor with a negligible concentration in the feast outflow, and this led to practically no organic acids in the famine reactor. This was observed at all R_C values investigated and thus allowed the establishment of the feast and famine conditions in all runs (Fig. 2A). Considering each R_C separately, the R_C 8 condition was the one in which the percentage of acids consumption was the highest with an average value of 100 ± 1 %, compared to 99 ± 1 % for both R_C 2 and R_C 4. Overall, these high VFA consumptions in the feast reactor positively affected the establishment of the FF conditions whereby all organic acids were consumed during the feast phase to produce PHA, which were used for microbial growth during the famine phase. Moreover, if a high amount of substrate is available in a short period of time, PHA-storing microorganisms can have a competitive advantage over the non-storing ones by accumulating PHA during this period and consuming it as an internal carbon source in the famine phase [22]. The VFA uptake rate in the FF process mainly depends on the OLR and the HRT applied to the system. These parameters were fixed and maintained unchanged in all the runs investigated in this work and, interestingly, even if the increasing of R_C decreased the “cycling time” of the biomass in each reactor, the VFA consumption was not significantly affected by the applied R_C. However, a similar high VFA degradation (97 % of OLR) has been also reported in a multistage laboratory scale process for PHA production from fermented paper mill wastewater, made of an acidogenic fermentation reactor connected to a continuous flow activated sludge system [35], whereby the HRT in the selector was adjusted to be long enough for an almost complete carbon depletion. Also, as widely reported in the literature [19], the selective pressure towards an efficient PHA storage is dependent on both the feast to famine (F/F) length ratio (less than 0.20 for optimal physiological biomass adaptation) and the applied OLR, that are strictly correlated between them. Indeed, a high OLR typically means a high F/F ratio and, consequently, a decrease in the process efficiency. In a continuous process for PHA production from sugar molasses with mixed cultures, in which the microbial selection occurred in two sequentially disposed continuous reactors (functioning as the feast and famine reactor, respectively), the F/F ratio was regulated by controlling the HRT of both reactors [34].

Here, a different approach has been adopted whereby the feast reactor consisted of a tubular configuration and the selective pressure (stimulated by the establishment of the FF conditions) was manipulated through the regulation of the recirculation factor. As mentioned earlier, the goal of this study was to obtain an enriched population of PHA-storing microorganisms, and the efficiency of the process can be assessed by monitoring the amount of PHA produced during the feast phase and then consumed during the famine phase. Fig. 2B summarizes the daily profile of PHA concentration in the feast and famine reactors throughout the overall experimental period. Notably, although almost all VFA were consumed in all runs during the feast phase, the amount of PHA produced (in the feast reactor) and consumed (in the famine reactor) was significantly different from one run to another. It can be clearly noticed that the maximum PHA concentration increased from the

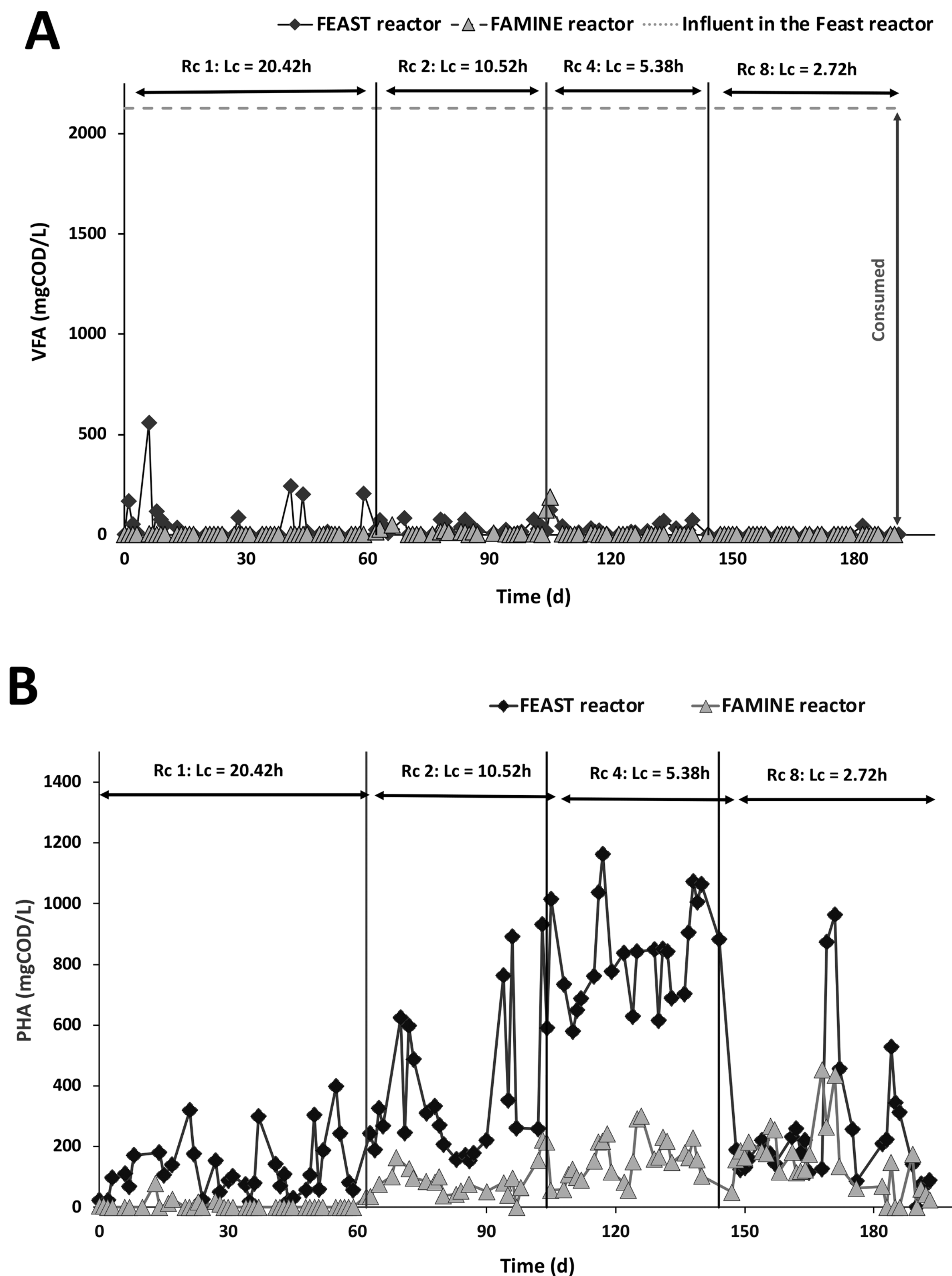

Fig. 2. Concentration of VFA and PHA during the whole selection process at different R_C values: trend of daily monitored VFA (A) and PHA (B) in the effluent stream of the feast reactor and in the famine reactor.

condition with R_C 1 to that with R_C 2, and then reached a maximum value at R_C 4 (with a total "cycling time" of about 5 h), but sharply dropped at R_C 8. Indeed, the profile of PHA concentration in the feast reactor was always clearly higher (from R_C 1 to R_C 4) than the trend in the famine reactor, underscoring the effectiveness of PHA production in the feast reactor and its consumption in the famine reactor. The only exception was the condition at R_C 8, during which the PHA concentration in both reactors was almost the same (except for some operating days). More specifically, average PHA concentrations (mgCOD/L) of 155.7 ± 24.5; 340.8 ± 34.4; 789.7 ± 26.6; 243.5 ± 44.5 in the feast reactor, and 4.3 ± 1.8; 70.5 ± 6.4; 156.5 ± 10.6; 177.1 ± 18.6 in the famine reactor were detected at R_C 1, R_C 2, R_C 4 and R_C 8, respectively. Thus, the difference in the polymer concentration between the two reactors (ΔPHA) increased by increasing the R_C from 1 to 4 and then decreased at R_C 8 (with values of 151.4; 270.3; 633.2; and 66.4 mgCOD/L, respectively). In all the performed runs, the starvation reactor received a low VFA concentration and a limited nitrogen concentration from the feast reactor, so microorganisms in the famine reactor could

only grow using PHA as a carbon source, and therefore the polymer concentration in this reactor was low except for the run at R_C 8.

3.2. Comparison of process performance at different R_C values

The clear effect of varying the R_C of the system on PHA production is also confirmed by the trend of the intracellular polymer content in the feast reactor (Fig. 3A), which was lower at R_C 1 (accounting for 7 ± 1 %, wt/wt) than at the other conditions: 18 ± 1 % (wt/wt); 34 ± 2 % (wt/wt); 9.0 ± 0.1 % (wt/wt) at R_C 2, R_C 4 and R_C 8, respectively. In the famine reactor the intracellular PHA content ranked between 0.31 ± 0.01 % (wt/wt); 5 ± 1 % (wt/wt); 10 ± 1 % (wt/wt) and 11.2 ± 0.1 % (wt/wt) for R_C 1, R_C 2, R_C 4 and R_C 8, respectively; indicating that PHA was truly cycled in both reactors, with the only exception for the run at R_C 8 in which the intracellular PHA content was very similar between the feast and famine phase. Fig. 3A emphasizes again that R_C 4 was the run holding the highest polymer production and intracellular PHA content during selection, thus the one in which the best microbial selection occurred among all the investigated runs. This clearly suggests that the R_C parameter influenced the storage capacity of the selected microorganisms by modifying the residence time of the biomass in each reactor. Likewise, Albuquerque and colleagues [34], working on a similar process but with a different substrate (fermented molasses), observed that by changing the volume ratio of the feast and famine reactors (from 0.25L/0.50L to 0.25L/1.10*L*) the PHA production during the selection process was enhanced from 6.4 ± 0.3 % to 23 ± 2 % (gPHA/gVSS) in the feast reactor, and from 2.0 ± 0.1 % to 8 ± 2 % (gPHA/gVSS) in the famine reactor.

However, in the present study, the volume ratio between the two reactors was fixed (1L/5L) and did not change during the whole experimental period. It should be noted that, by changing the recirculation flow rate, the biomass cycling time (Lc) in each reactor was also changed but the relative ratio was only slightly affected. Indeed, as shown in Table 1, the ratio between the feast biomass cycling time and the total cycling time varied from 9.40 % to 15.1 %. In other words, the feast time and Lc ratio (as well as the F/F ratio) remained very low in all conditions and less than the fixed volumetric ratio between the feast and famine reactors. Thus, this suggests that the feast and famine biomass cycling time is not a major factor to justify the observed behaviour in terms of PHA production. On the other hand, the cycling time changed a lot, varying from 20.42 h (R_C 1) to 2.72 h (R_C 8). As above mentioned, we consider that this parameter is analogous to the cycle length in an SBR, which has been shown to have a strong effect on the selective pressure exerted by FF regime even at the same HRT and OLR [36]. Indeed, by varying the R_C two major effects are expected: a) on one hand by increasing the recirculation flow rate, the feed is more diluted, i.e., the strength of substrate pulse is lower and, consequently, the feast pressure is also lower; b) on the other hand, the frequency by which the microorganisms are exposed to the stress of passing from famine to feast conditions is higher. Hence, both conditions affect the physiological state of microorganisms and eventually the biomass selection.

The relatively low intracellular PHA content observed at R_C 1 and R_C 8 in this study suggests the hypothesis that, under these operating conditions, either the PHA-storing microorganisms were not dominant in the system or were dominant but unable to properly store PHA. This is also in agreement with the trend of Fig. 3B, which illustrates the PHA storage and growth yields. It is evident that higher PHA storage yields (0.15 ± 0.02 and 0.34 ± 0.02 COD/COD) were achieved at R_C 2 and R_C 4, respectively, which also corresponded to lower growth yields (0.53 ± 0.04 and 0.50 ± 0.10 COD/COD, respectively) than the values obtained at the highest investigated R_C condition. This clearly indicates that in these two R_C runs, PHA accumulation was a more predominant phenomenon compared to the other two conditions (R_C 1 and R_C 8). More particularly, the low residence time at R_C 8 did not allow a significant

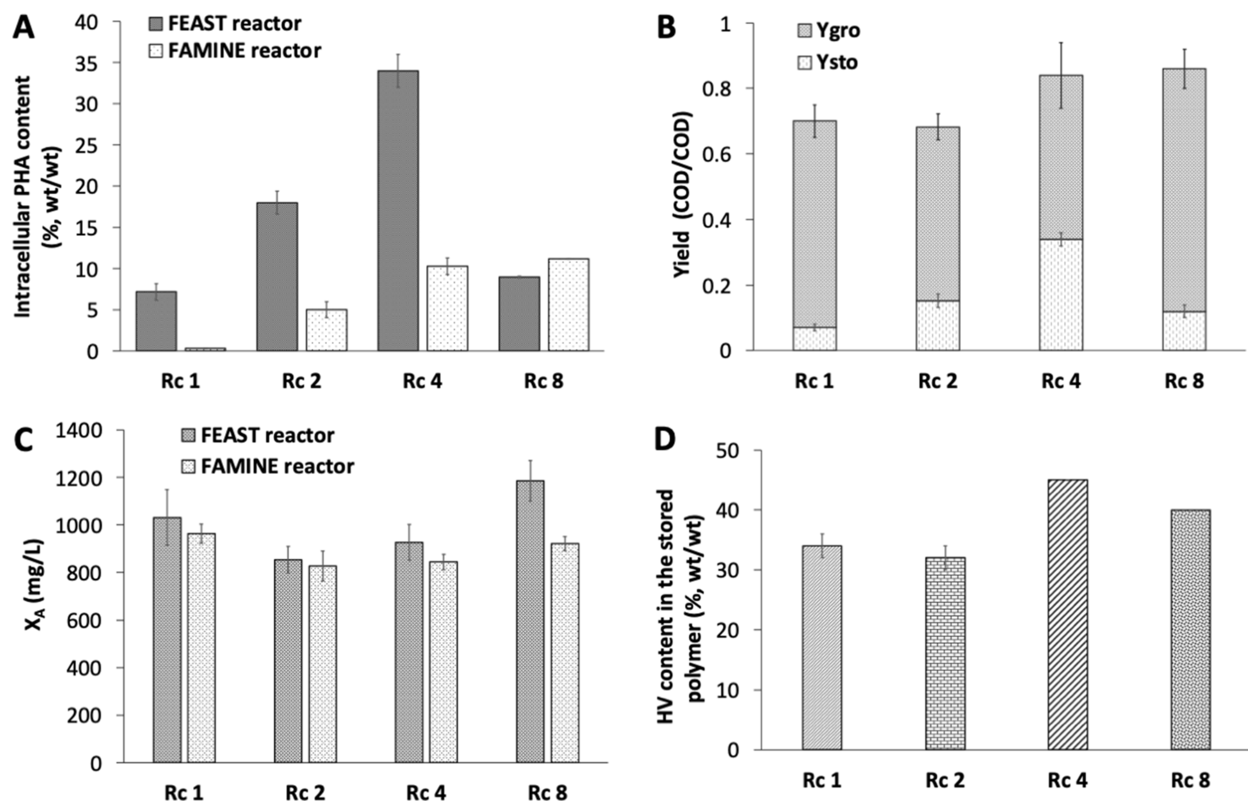

Fig. 3. Process performance at each investigated R_C in terms of: intracellular PHA content (A), storage and growth yield (B), active biomass concentration (C), and HV content in the stored polymer in the feast reactor (D). Error bars represent ± standard error of the average measurements (n > 10) taken over the course of each run.

PHA production in the feast reactor and its consumption in the famine reactor, consequently the intracellular PHA content was almost the same in the two reactors (Fig. 3A). In addition, R_C increased the rate at which the biomass was shifted from one reactor to the other and decreased the residence time of biomass in each reactor. Indeed, the biomass spent 1.91 h (9.40 % of the total cycle time) and 0.41 h (15.1 %) in the feast reactor and 18.51 h and 2.31 h in the famine reactor for R_C 1 and R_C 8, respectively. Such condition at R_C 8 (with the lowest cycling time) certainly allowed the microbial selection but was not suitable for a sufficient polymer accumulation.

In other words, the decrease in the cycling time in each reactor at R_C 8 caused the biomass not to have sufficient time to exploit its PHA storage potential due to the very short feast period. Overall, Table 2 summarizes the main parameters used to monitor the process performance. In particular, since acids and nitrogen were mainly consumed in the feast reactor, their residual concentration entering the famine reactor was very low, and in the latter the main carbon source consisted of PHA (internal carbon). However, in order to use PHA as a carbon source for growth under the FF regime, microorganisms need both nutrient availability and sufficient residence time, since PHA consumption by the biomass for growth is a "lengthy" process [43]. Fig. 3C shows the profile of the average value of non-polymer biomass concentration (active biomass, X_A) in the feast and famine reactors, which represents the biomass (VSS) concentration without PHA. Data show that the highest growth among all the investigated runs was observed at R_C 8, and it mainly occurred in the feast reactor. This was possibly due to the coupled C and N feeding strategy [31] applied to the feast reactor in all investigated conditions, whereby the famine reactor was almost completely nitrogen-free and this limited the growth phenomenon using PHA, as expected. Hence, these observations allow hypothesizing that all the polymer produced was used for metabolic functions related to cell maintenance rather than for growth. This, along with the simultaneous biomass self-consumption (the endogenous metabolism) resulted in reduced growth of the storing biomass in the famine reactor. Thus, at R_C 8 the low consumption of PHA in the famine reactor (due to the low residence time) reflected in a stronger decrease of X_A from the feast to the famine reactor compared to the other runs (R_C 1, R_C 2, and R_C 4). Notably, the substrate uptake rates determined at all R_C were very similar but, as previously discussed, the fraction of the removed substrate diverted towards microbial growth or PHA storage was dependent on the applied R_C, with a higher polymer production rate obtained at R_C 4 than in the other conditions.

Furthermore, polymer composition was also analyzed in order to investigate the effect of R_C on this parameter. Fig. 3D shows the HV content in the poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) copolymer produced in this study, which ranged between 32 % and 45 % (wt/wt). Therefore, it can be highlighted that R_C did not substantially affect the polymer composition and the high HV content obtained in this work (more than 20 %, wt/wt) was certainly due to the substrate composition (65 % acetic acid and 35 % propionic acid) since the propionic acid was the only precursor of the HV monomer available in the feeding solution and it was incremented (from 15 % to 35 %) in this study compared to other studies performed with the same acids mixture [44]. Other researchers have also highlighted the importance of the HV fraction in the PHBV copolymer in view of obtaining PHA for several applications, including food packaging [45–47]. In this context, Melendez-Rodriguez and colleagues [47] demonstrated that a copolymer with an HV content of 40 % (molar fraction), similar to the compositions obtained in this study, showed better characteristics for the processing of a biopaper in food packaging.

3.3. Batch accumulation trials

The storage ability of the microbial culture selected in the continuous-flow process was assessed by means of batch PHA production tests performed under limited ammonium concentration. For each R_C, tests were carried out after at least 15 days of operation of the selection reactors in the same conditions. Fig. 4A and 4B report the trend of OUR (Oxygen Uptake Rate), and VFA and PHA concentration, respectively, observed during a typical batch trial (the example refers to the microbial cultures selected at R_C 8). The monitoring of the OUR during the test allowed to indirectly monitor the VFA concentration inside the batch reactor: thus after a few minutes (two minutes) from the first substrate impulse, it was noticed a rapid increase in the OUR up to 115 mgO_2/Lh. This increase revealed a prompt reaction of the microorganisms to the added substrate. Then, the OUR decreased over time down to the endogenous value (15 mgO_2/Lh), meaning that the substrate was completely depleted and, therefore, another impulse was added (as indicated by arrows in the figure) and this process was repeated during all the trial (6 h). Samples for the measurement of PHA and VFA concentrations were taken inside the reactor at regular intervals (one hour). The trend of PHA concentration in this trial (Fig. 4B) reflects the same trend observed in the other trials, thus while the VFA concentration decreased the PHA concentration increased over time and the sequential substrate additions occurred to maximize the PHA production.

Fig. 5A shows the trend of the average values of the intracellular PHA content detected in correspondence to both the beginning and the end of the batch trails for all the studied R_C. In each investigated condition, the accumulation tests caused a substantial improvement in the PHA content and, with the main reference to the final values, this parameter increased from 18 ± 6 % (wt/wt) to 58 ± 5 % (wt/wt) by increasing the R_C in the selection process from1 to 8, respectively. These outcomes are in agreement with those obtained in the feast reactor when the R_C was raised from 1 to 4, which resulted in an increased intracellular PHA content (up to 34 ± 2 %, wt/wt), but are in contrast with the behavior observed for R_C 8. Indeed, during the batch experiments, the maximum PHA content (58 ± 5 %, wt/wt) was observed at this R_C, although the average value detected in the feast reactor during the selection phase was lower than the values obtained at R_C 2 and R_C 4. Accordingly, during accumulation tests with the biomass selected at R_C 8, highest values of the storage yield and specific storage rate (0.80 ± 0.10 COD/

Table 2
Performance of the continuous flow process for microbial selection during the overall operation period.

	R_C 1: L_C 20.42 h		*R_C 2: L_C 10.52 h*		*R_C 4: L_C 5.38 h*		*R_C 8: Lc 2.72 h*	
PARAMETER	**FEAST**	**FAMINE**	**FEAST**	**FAMINE**	**FEAST**	**FAMINE**	**FEAST**	**FAMINE**
Operation period (d)	61		39		43		49	
ORL (gCOD/L d)	2.12		2.12		2.12		2.12	
N (mg/L)	60		60		60		60	
C/N	35		35		35		35	
% PHA ± S.E. (wt/wt)	7 ± 1	0.31 ± 0.01	18 ± 1	5 ± 1	34 ± 2	10 ± 1	9.0 ± 0.1	11 ± 1
N res*(mg/L)	8 ± 1	9 ± 1	11 ± 1	5 ± 1	7 ± 1	3.0 ± 0.3	3.0 ± 0.1	2.9 ± 0.2
q_p ± S.E. ($mgCOD_{PHA}/Lh$)	6 ± 1		13 ± 1		29 ± 2		10 ± 2	
$-q_s$ ± S.E. ($mgCOD_{VFA}/Lh$)	86 ± 1		86 ± 1		86 ± 1		87 ± 1	
μX ± S.E. ($mgCOD_{VSS}/Lh$)	54 ± 4		46 ± 3		47 ± 6		63 ± 11	

S.E.: Standard Error; L_C: total cycling time; R_C: Recirculation factor; N res*: residual nitrogen concentration.

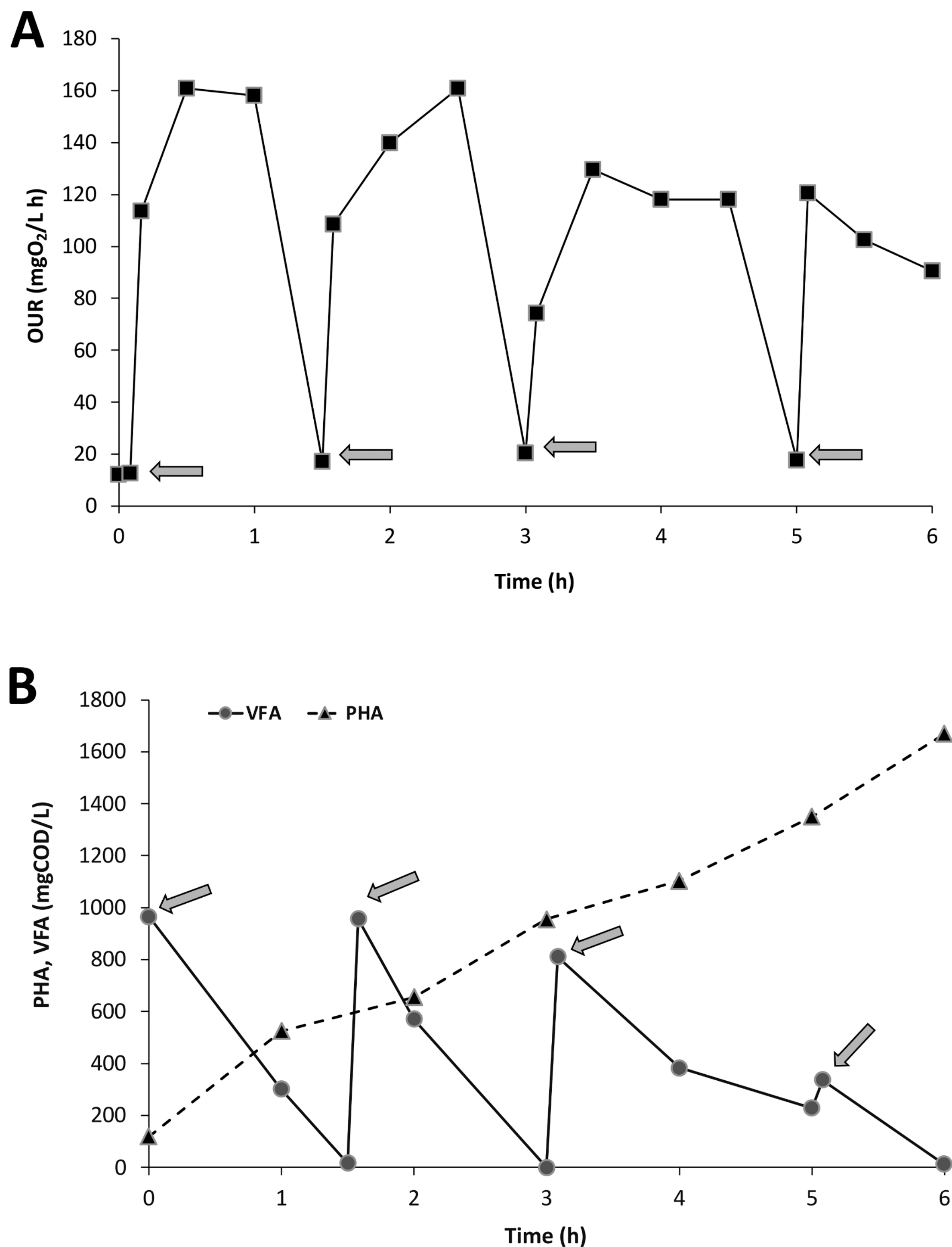

Fig. 4. Operative parameters monitored during a typical batch trial (with the biomass selected at R_C 8): profile of the Oxygen Uptake Rate (A), and VFA and PHA concentration (B) over time. Arrows indicate substrate impulses during the test.

COD and 193 ± 7 $mgCOD_{PHA}/gCOD_{Xa}$ h, respectivey) were obtained with respect to the other conditions (as reported in Table 3). This could be related to the fact that the increase of R_C during the selection process caused a decrease in the cycling time of the biomass in each reactor, which means that the biomass spent more time with substrates at R_C 1 (1.91 h) than at R_C 2, R_C 4, and R_C 8 (i.e., 1.26, 0.75, and 0.41 h, respectively), and at the highest investigated R_C the cycling time in the famine reactor was also very short. Therefore, the biomass at R_C 8 underwent high stress imposed by the feast and famine conditions which reflected in a low PHA storage performance. Notwithstanding these set conditions of feast and famine regime (i.e., a very short feast phase in the presence of nitrogen), which did not allow significant polymer storage at R_C 8, the biomass managed to store to a major extent when was placed in the absence of nitrogen for a prolonged period during the batch tests (6 h). Interestingly, the obtained data indicate that the microbial culture selected at R_C 8 was capable of fully exhibiting the storage ability during the accumulation phase. It follows that in the investigated process, the efficiency of the accumulation experiment depends on both the efficiency of biomass selection under the set L_C and the residual nitrogen concentration in the famine reactor (that was negligible at all R_C, as

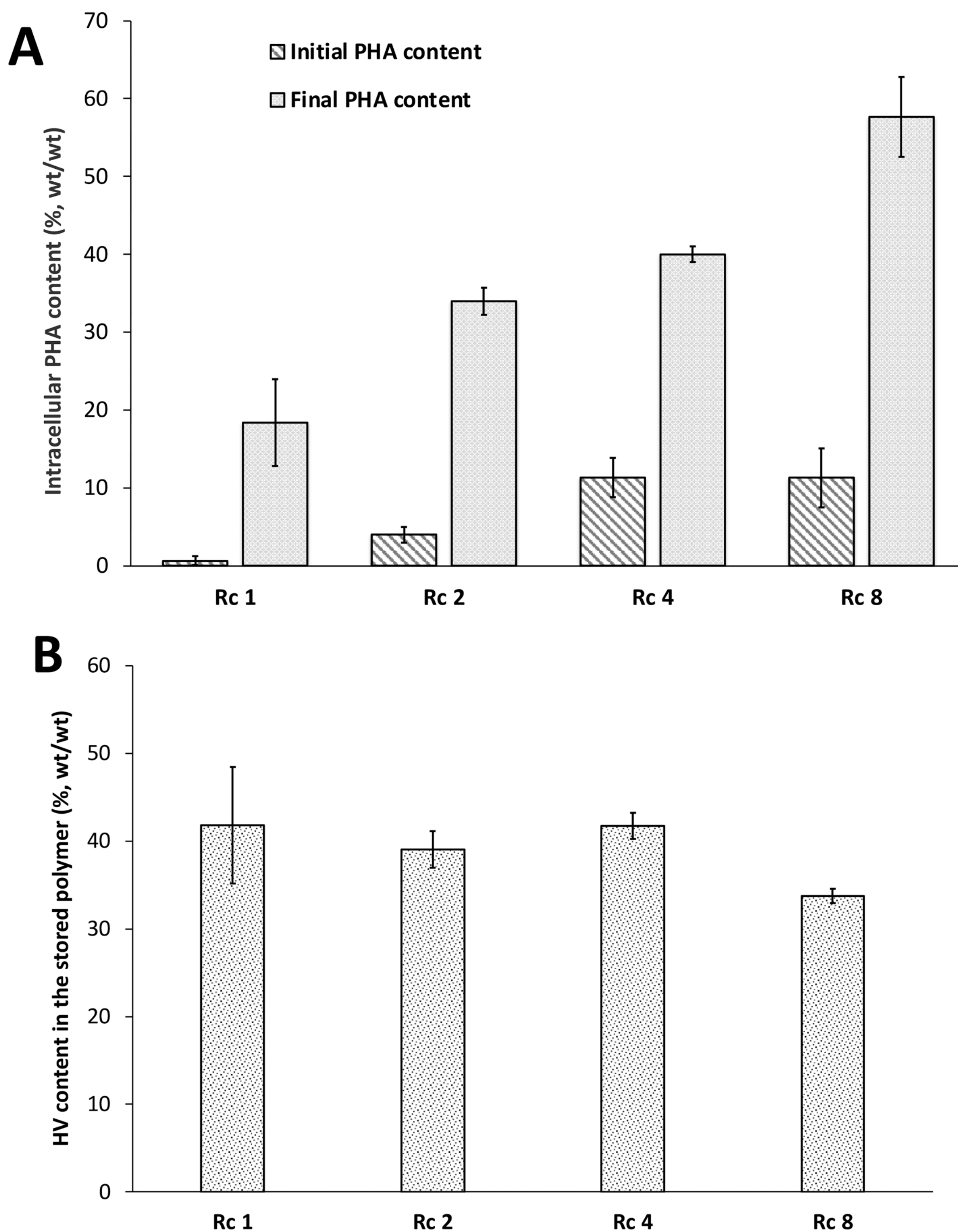

Fig. 5. Performance of the batch accumulation stage in terms of: intracellular PHA content (A), and average HV content in the stored copolymer (B). Error bars represent ± standard error of the average measurements (n = 3) taken over the course of each run.

reported in Table 2). Remarkably, a previous study [32] reported an intracellular PHA content as high as 64 % (wt/wt) with a corresponding high storage yield on the removed substrate (0.66 COD/COD), in batch tests performed with the biomass selected in an SBR fed with a synthetic mixture of acetic (85 %) and propionic (15 %) acids at an OLR of 4.25 gCOD/L d, and removed from the reactor in correspondence to the end of the cycle (nitrogen-free condition). Comparing these results with those of the present study, it can be considered that the microbial cultures selected in the continuous-flow process presented a good polymer storage capacity and the best performance in terms of intracellular PHA content (58 %, wt/wt) reached in batch accumulation tests at R_C 8 was very close to that previously obtained with the biomass selected in an SBR operated at higher OLR. Therefore, the low polymer content observed at R_C 1 suggests a poorer acclimation to the substrate under these conditions and that even though fixing the OLR, the HRT, and the feast/famine regime based on the volumetric ratio of the two reactors is enough to control the effectiveness of biomass selection; the “cycling time” as established by the recirculation factor (R_C) is an additional key parameter to control the process performance.

Regarding the composition of the stored PHA, Fig. 5B shows the

Table 3
Performance of batch accumulation tests with the microbial culture enriched at all Rc investigated.

PARAMETER	R_C 1: L_C 20.42 h	R_C 2: L_C 10.52 h	R_C 4: L_C 5.38 h	R_C 8: L_C 2.72 h
Maximum PHA content (%, wt/wt)	*18 ± 6*	*34 ± 2*	*40 ± 1*	*58 ± 5*
Specific Storage Rate (q_P^{batch}; $mgCOD_{PHA}$/ $gCOD_{Xa}$ h)	*63 ± 22*	*138 ± 9*	*132 ± 44*	*193 ± 7*
Storage Yield (Y_{STO}^{batch}; COD/COD)	*0.40 ± 0.20*	*0.60 ± 0.10*	*0.50 ± 0.20*	*0.80 ± 0.10*
HV content (%, wt/wt)	*42 ± 7*	*39 ± 2*	*42 ± 2*	*34 ± 1*

profile of the HV fraction in the stored copolymer at the end of the batch experiments for each run studied. In particular, this parameter did not significantly change compared to the values measured in the outlet stream from the feast phase in the selection process (Fig. 3D). On average, the HV content was not significantly different at the different R_C investigated and ranged between 34 ± 1 % and 42 ± 7 % (wt/wt).

3.4. PHA-storing bacterial community composition

The combined use of *in situ* hybridization technique and 16S amplicon sequencing revealed a different microbial community composition in the samples taken from the feast reactor operated at different R_C. In particular, the abundance of *Alphaproteobacteria* increased by increasing R_C representing between 6.3 and 81.3 % of total reads. FISH analysis showed different morphologies of cells hybridized with *Alphaproteobacteria* probe (Fig. 6A). In line with this observation, 16S amplicon sequencing confirmed a shift in the alphaproteobacterial members with reads affiliated with family *Rhodobacteraceae* mainly present in reactor at R_C 2 and R_C 4 (up to 21.8 % of reads). In contrast, *Meganema* appeared to be the most abundant genus at R_C 8; the reads affiliated with this filamentous genus encountered for the 74.0 % of total reads (Fig. 6B). As commonly reported in the literature, all the retrieved *Alphaproteobacteria* in this study are able to accumulate PHA [48,49].

A reduction of *Gammaproteobacteria* was observed by increasing R_C, representing 42.0 % of total reads in the reactor at R_C 2, and 4.7 % at R_C 8. Also in this case, FISH analysis revealed different morphologies of cells hybridized with gammaproteobacterial probes (GAM42a + BET42a) (Fig. 6A). The class *Gammaproteobacteria* was mainly represented by genera *Sphaerotilus* and *Azoarcus* (9.9 and 17.5 % of total reads at R_C 2), followed at minor extent by *Comamonas*, *Undibacterium* and *Acinetobacter* representing up to 7.7 % of total reads at R_C 2 (Fig. 6B). The highly presence of *Alpha*- and *Gammaproteobacteria* in the feast reactor is in line with the process data and previous evidence, since the vast majority of PHA-storing bacteria described so far precisely belong to these proteobacterial classes [50,51]. Furthermore, sequences affiliated with phylum *Actinobacteria* were present in all samples showing range between 11.0 and 50.2 %. This phylum was mainly represented by PHA-accumulating genera *Corynebacterium* 1, *Gordonia*, *Rhodococcus*, and *Microbacterium* (Fig. 6B) [52–55].

Taken as a whole, the high-throughput sequencing evidenced that the relative abundance of sequences affiliated with putative PHA-storing bacteria increased by increasing the R_C, representing 48.3 % of total reads at R_C 2, 78.6 % at R_C 4, and 90.5 % at R_C 8 (Fig. 6C). Although the reactor operated at R_C 8 showed a very low intracellular PHA content (Fig. 3A), the biomass in this reactor was dominated by PHA-storing bacteria. In line with process data, this evidence suggested a selective pressure at R_C 8 that favoured PHA-accumulating bacteria, anyway the low residence time was not suitable for significant PHA production. In agreement, when the same biomass was used in the batch accumulation trials reached the highest intracellular PHA content (Fig. 5A).

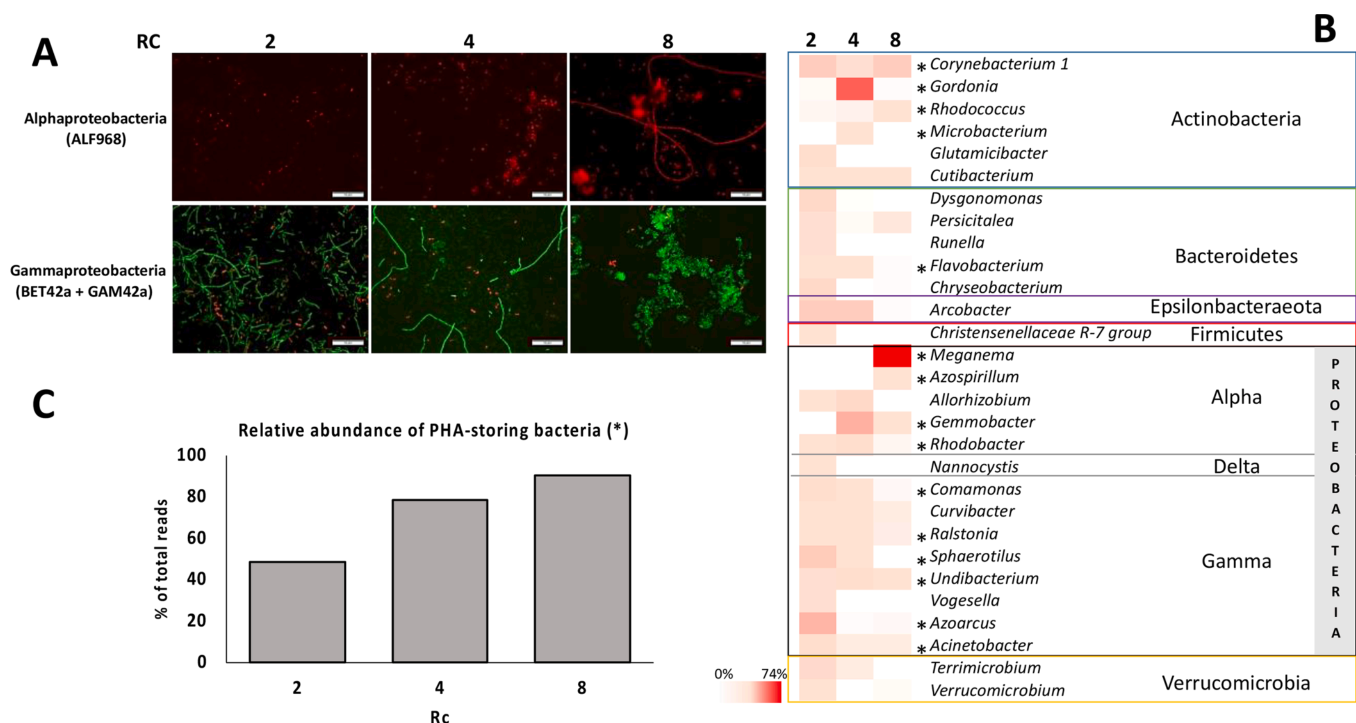

Fig. 6. Microbial community composition in the biomass of feast reactor at different R_C: FISH images members of classes *Alphaproteobacteria* (ALF968 probe) and *Gammaproteobacteria* (BET42a + GAM42a probes in green and red, respectively), scale bar = 10 μm (A); Frequency heat-map of microbial genera (≥1% relative abundance of total reads in at least one sample), the color intensity in each cell shows the relative abundance (B); Relative abundance of sequences affiliated with putative PHA-storing bacteria out of total sequences (*genera comprised in the PHA-storing bacteria relative abundance calculation according to the literature) (C). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Conclusions

The use of mixed microbial communities is unquestionably a good option for lowering PHA prices. Therefore, investigating novel processes to improve the selection of PHA-storing microorganisms from an activated sludge becomes necessary for this purpose, thus opening new perspectives for process application.

Although the continuous process for microbial selection in two separate reactors (instead of the conventional Sequencing Batch Reactor) has previously received some attention in the literature, this work introduces important novel features. These include the use of a reactor with tubular geometry for the feast phase, the absence of a settler for thickened biomass recirculation between the feast and the famine reactors, and the exploitation of the recirculation factor (R_C) to fragment the residence time of the biomass in each reactor and favor the selection of PHA-storing microorganisms. In particular, the volume ratio between the feast and famine reactors was fixed (1L / 5L) and the R_C increase from 1 to 8 caused a decrease in the biomass cycling time in each reactor, but the ratio between the feast biomass cycling time and the total cycling time only slightly varied (from 9.40 % to 15.1 %, respectively), indicating the establishment of the feast and famine conditions. Indeed, the microbiological analysis revealed the presence of PHA-storing microorganisms in the feast reactor and the R_C increase shifted the composition of the microbial community towards an increase in the abundance of *Alphaproteobacteria* and a reduction in the abundance of *Gammaproteobacteria*. Overall, the relative abundance of sequences affiliated with putative PHA-storing bacteria increased with the R_C, up to 90.5 % of total reads at R_C 8. This finding is particularly interesting since at this R_C the PHA production in the selection process dropped, likely due to the short cycling time in the feast and famine reactors, but the microbial culture dominated by PHA-storing microorganisms managed to significantly accumulate the polymer in batch experiments, reaching an intracellular PHA content of 58 ± 5 % (wt/wt). This value is substantially higher than the maximum value (34 ± 2 %, wt/wt) obtained in the feast reactor at R_C 4, which was the condition presenting the highest PHA production rate (29 ± 2 $mgCOD_{PHA}$/L h) in the selection system between those investigated.

Hence, the recirculation factor represents a key factor to control the operation of the adopted continuous-flow configuration functioning at a fixed OLR (2.12 gCOD/L d) for the selection of PHA-storing biomass and, based on this outcome, the effect of other parameters (such as the applied OLR and the use of real feedstocks) on the process performance also requires further investigations.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

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