Effect of the organic loading rate on the PHA-storing microbiome in sequencing batch reactors operated with uncoupled carbon and nitrogen feeding

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HIGHLIGHTS

- OLR affected PHA production and microbial selection in a SBR with uncoupled C/N feeding.
- The increase of OLR increased PHA production; the process failed at 18 g COD L^{-1}d^{-1}.
- *Rhodobacter*, *Thauera* and *Paracoccus* were associated with the highest PHA production.
- *Nitrinicola* were associated with the highest applied OLR and lowest storage yield.

GRAPHICAL ABSTRACT
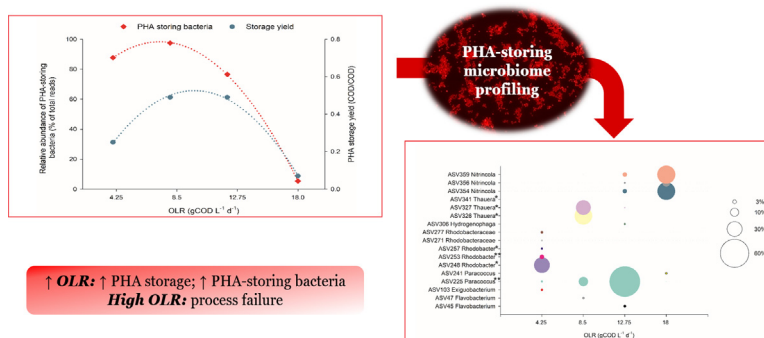
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ABSTRACT

Over the last years, in a search for sustainable and biodegradable alternatives to petrol-based plastics, biotechnological applications turned to the potentialities of mixed microbial cultures (MMC) for producing polyhydroxyalkanoates (PHAs). Under a feast and famine regime, an uncoupled carbon (C) and nitrogen (N)-feeding strategy may be adopted by dosing the C-source at the beginning of the feast and the N-source at the beginning of the famine in order to stimulate a PHA storage response and microbial growth. Even though this strategy has been already successfully applied for the PHA production, very few information is to date available regarding the MMC operating in these systems and the influence of Organic Loading Rate (OLR) on their selection and enrichment. To fill the gap, this study investigated the effect of the OLR on the selection of PHA-accumulating microorganisms in a sequencing batch reactor (SBR) operated with an uncoupled C and N feeding strategy. The SBR cycle length was set at 12 h and four OLRs values (4.25, 8.50, 12.75 and 18 gCOD L^{-1} d^{-1}) were tested by changing the concentration of the feeding solution, made of a synthetic mixture of acetic (85% of the overall COD) and propionic (15%) acids. The PHA-storage yield increased by increasing the OLR (up to 0.69 COD/COD at 12.75 gCOD L^{-1} d^{-1}) but significantly decreased (0.27 COD/COD) at 18 gCOD L^{-1} d^{-1} concomitantly with a longer feast phase and a lower PHA content in the biomass at the end of the feast phase. The selective pressure induced by the applied OLRs strongly influenced the microbiome composition

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revealing a high content of putative PHA-storing bacteria, such as *Rhodobacter*, *Thauera* and *Paracoccus,* in the SBR operated at OLRs 4.25, 8.50 and 12.75 g COD L^{-1} d^{-1} (up to 97.4% of total reads) and a low content (5.4%) in the SBR at 18 g COD L^{-1} $d^{-1}$where the predominance of genus *Nitrinicola* was instead observed.

1. Introduction

Polyhydroxyalkanoates (PHAs) are completely biodegradable polymers of particular interest due to their chemomechanical and thermal properties and the possibility to be produced from renewable resources (Laycock et al., 2014). Currently, their industrial scale production is not cost effective being performed with pure cultures under sterile operating conditions and with the use of synthetic substrates. Over the last years, research interest is increased toward the use of inexpensive waste carbon sources and mixed microbial cultures (MMC) allowing to reduce the cost of production (Albuquerque et al., 2010; Campanari et al., 2014; Valentino et al., 2015, 2017). It has been largely demonstrated that the fermentation of renewable feedstock generates volatile fatty acids (VFAs), suitable and direct substrates for the PHA production (Basak et al., 2011; Ince et al., 2012; Morgan-Sagastume et al., 2015; Nikodinovic-Runic et al., 2013; L. S. Serafim et al., 2008a; Valentino et al., 2019a, 2019b).

In the search of principal operating parameters affecting PHA production, few investigations on the effects of organic loading rate (OLR) on the selection of MMC were performed to date by using a coupled carbon and nitrogen feeding strategy (Marianna Villano et al., 2010a, 2010b; Wen et al., 2018). Despite of these studies revealed a PHA-accumulating MMC mainly colonized by *Lampropedia hyaline*, *Paracoccus* and *Thauera*, general information on the effect of OLR on the PHA-accumulating microorganisms remains limited (Morgan-Sagastume, 2016; Sruamsiri et al., 2020). Previous findings hypothesized an effect of OLR in the selection of PHA-storing bacteria due to the impact of the feast/famine ratios (Albuquerque et al., 2011; Carvalho et al., 2014; Serafim et al., 2008a, 2008b). The large majority of information regarding the diversity of PHA-storing microbial communities was mainly reported in systems fed by synthetic mixture of VFA with OLR in the range 1.50–12.75 g COD L^{-1} d^{-1} (Huang et al., 2018; Morgan-Sagastume, 2016; Queirós et al., 2015; Sruamsiri et al., 2020). Little is known regarding microbial diversity in systems operated at very high OLRs (Dionisi et al., 2007; Villano et al., 2010a, 2010b) and in systems operating with uncoupled nitrogen and carbon feeding (Matos et al., 2021). The uncoupled C and N-feeding strategy may be applied in order to stimulate a PHA storage response during the feast phase (in the presence of C and absence of N) and microbial growth in the famine phase (in the absence of C and presence of N) (Burniol-Figols et al., 2018; Oliveira et al., 2017; Silva et al., 2016). The impact of applied OLR on the PHA-storage performance was previously investigated by using the uncoupled feeding strategy in a laboratory-scale SBR (Lorini et al., 2020), but no information regarding the PHA-accumulating MMC is available. For this purpose, the main objective of the present study was to evaluate the impact of the applied OLR in the range 4.25–18 g COD L^{-1} d^{-1}on the microbial selection of a SBR operated with an uncoupled C and N feeding strategy using a feeding solution made of a synthetic mixture of acetic and propionic acids. Along this line, this study combined advanced biomolecular techniques with traditional Fluorescence in situ hybridization technique (FISH) and fluorescence staining methodologies in order to identify the PHA-storing microorganisms and their dynamics in the SBR in response to the different operating conditions.

2. Materials and methods

2.1. Sequencing batch reactors with uncoupled carbon and nitrogen feeding

The selection and enrichment of PHA-accumulating microorganisms was performed in a fully aerobic SBR (1.0 L working volume), inoculated with an activated sludge from the Treviso full-scale wastewater treatment plant (Treviso, Italy). A mechanical impeller was used for mixing of the culture medium with O_2 provided through air pumps connected to ceramic diffusors. Four runs were performed in sequence by increasing the OLRs for approximately 3.5 total months (Run A 25 days; Run B 41 days; Run C 20 days; Run D 15 days). After the inoculation and each change of operating conditions, the reactor was operated for a period of time long enough to reach biomass adaptation (1-week maximum) and to characterize the process performance under a steady state condition. The operating cycle length was set at 12 h, in all four SBR runs. The cycle structure was composed as follows: initial phase of carbon (C) source feeding (10 min; 0.42 L), a first reaction phase in which the C-source was consumed (substrates depletion was determined by continuously monitoring the dissolved oxygen (DO) concentration; average feast phase lengths for all the runs are reported in Table 1), a withdrawal phase for the discharge of the culture medium (3 min; 0.50 L), a nitrogen (N) source feeding phase (5 min; 0.08 L), and a second reaction phase (with a duration corresponding to the remainder of the cycle) where the PHA was consumed as the only C-source of the medium. The temperature was maintained at 25 °C using a thermostatic jacket. The pH was monitored but not controlled, since it remained stable at around 8.5–9.0 during the whole operative period. The hydraulic retention time (HRT) was 1.0 day, equal to the sludge retention time (SRT) since no settling phase was provided. A synthetic mixture of acetic acid (85% on a COD basis) and propionic acid (15%) was used as C-source. Based on a C-source flow rate of 0.84 L d^{-1} in each run, the applied organic loading rates (OLR) were 4.25 g COD L^{-1} d^{-1} (Run A), 8.5 g COD L^{-1} d^{-1} (Run B), 12.75 g COD L^{-1} d^{-1} (Run C) and 18 g COD L^{-1} d^{-1}, respectively. The C-source was maintained at 4 °C in a refrigerated container for the whole period of operation. In the four runs, mass flows of both C- and N-sources were set in order to establish a C/N ratio of 33.4 g COD g^{-1} N (or 14.3C-mol/N-mol) as the preferable value favoring of an increased PHA storage response, as previously reported (Lorini et al., 2020). The mineral medium had the following composition (mg L^{-1}): K_2HPO_4 (334), KH_2PO_4 (259), $MgSO_4$•$7H_2O$ (100), $CaCl_2$•$2H_2O$ (50), thiourea (20), Na_2EDTA (3), $FeCl_3$•$6H_2O$ (2), H_3BO_3 (0.3), $CoCl_2$•$6H_2O$ (0.2), $ZnSO_4$•$7H_2O$ (0.1), $MnCl_2$•$4H_2O$ (0.03), $NaMoO_4$•$2H_2O$ (0.03), $NiCl_2$•$6H_2O$ (0.02) and $CuCl_2$•$2H_2O$ (0.01). The pH of the C feeding solution was adjusted to a fixed value (6.0–6.5) by adding NaOH. The reactor was controlled by digital timers connected to each peristaltic pump for flow rate management according to the cycle structure. Computer software was used to record the dissolved oxygen (DO) concentration and to detect the time required for C-source consumption (end of the feast phase) (Lorini et al., 2020). Volatile fatty acids (VFA), ammonia, PHA and suspended solids (SS) concentrations were monitored as previously described (Lorini et al., 2020; Silva et al., 2016).

2.2. Analytical methods

Ammonia and VFA quantifications were carried out after the filtration of the liquid samples through 0.45 μm porosity filters. Ammonia was quantified by the Nessler spectrophotometric method: the absorbance of reacted samples was measured at 420 nm wavelength (SHIMADZU Spectrophotometer UV-1800) (APHA, 1995). The VFAs were measured after injection of 1.0 μL of filtered sample into a gas-chromatograph (Dani-Master, Milan, Italy) equipped with packed column (stationary phase Carbowax 20 M 4% on CarboPack B-DA) and a flame ionization detector (FID) (Valentino et al., 2014). The concentrations of the single organic acids were converted into COD based on the oxidation stoichiometry as 1.067 g COD g^{-1} acetic acid and 1.51 g COD g^{-1} propionic acid.

Analytical determination of PHA was made on 5.0 mL of mixed liquor (without filtration). Each sample was immediately treated with 1.0 mL of a NaClO solution (5% active Cl_2) in order to stop possible PHA microbial

Table 1
Main parameters with average data and standard deviations monitored and quantified in the SBR runs.

	Run A	Run B	Run C	Run D
OLR (gCOD L^{-1} d^{-1})	4.25	8.5	12.75	18
C-source	85% acetic acid, 15% propionic acid[a]			
C-N feeding	Uncoupled			
Cycle length (h)	12			
Days of operation	25	41	20	15
Feast phase/cycle length ratio (h/h, %)	29.2 ± 2.9	28.7 ± 1.5	29.4 ± 1.6	39.4 ± 2.3
Active biomass (end of feast; mgX_A L^{-1})	1167 ± 114	1679 ± 113	2033 ± 169	3816 ± 160
PHA concentration (end of cycle; mg L^{-1})	76 ± 8	505 ± 40	1076 ± 121	1168 ± 256
PHA concentration (end of feast; mg L^{-1})	601 ± 50	1780 ± 80	3080 ± 121	2049 ± 110
PHA content (end of feast; gPHA g^{-1} VSS)	0.34 ± 0.03	0.52 ± 0.03	0.62 ± 0.02	0.36 ± 0.05
Storage Yield ($Y_{P/S}^{feast}$; COD COD^{-1})	0.45 ± 0.04	0.69 ± 0.02	0.69 ± 0.04	0.27 ± 0.03
Observed Yield (Y_{OBS}^{SBR}; COD COD^{-1})	0.60 ± 0.02	0.60 ± 0.03	0.62 ± 0.01	0.48 ± 0.01
HV content (end of feast; gHV g^{-1} PHA)	0.15 ± 0.02	0.21 ± 0.01	0.25 ± 0.01	0.06 ± 0.02
Nitrogen concentration (end of the cycle; mgN L^{-1})	20 ± 3	34 ± 3	10 ± 3	101 ± 14

[a] Synthetic VFA mixture.

consumption, and then stored at −20 °C for the following analysis. Esterification into 3-hydroxyacyl methyl esters was necessary for the PHA determination by gas-chromatography (GC-FID Perkin Elmer 8410) (Braunegg et al., 1978). The abundance of 3-hydroxybutyrate (HB) and 3-hydroxyvalerate (HV) monomers was obtained using a commercial P(HB/HV) copolymer at 5% *w*/w HV content (Sigma–Aldrich, Milan, Italy). The stoichiometry conversion numbers used in order to express PHA concentration in terms of COD were 1.67 g COD g^{-1} HB and 1.92 g COD g^{-1} HV.

2.3. Calculations

In the SBR, the amount of stored PHA (ΔPHA) was calculated as the difference between the maximum (end of feast) and minimum (end of cycle, corresponding to the beginning of the following cycle) PHA concentration. The non-polymer biomass or active biomass (X_A) was the difference between VSS and PHA (at the same cycle time): X_A = VSS - PHA. The specific PHA production rate was the ratio of the stored PHA to the feast phase length (t) per unit of X_A: $qP^{feast} = ΔPHA/(t \cdot X_A)$; both PHA and X_A were expressed on a COD basis. The specific substrate uptake rate was the ratio of VFA fed per cycle (ΔS) to the time required for its depletion (t, feast phase length) multiplied by X_A: $(-qS^{feast}) = ΔS/(t \cdot X_A)$; both VFA and X_A were expressed on a COD basis. The storage yield in the feast phase was the ratio between ΔPHA and ΔS: $Y_{P/S}^{feast} = ΔPHA/ΔS$ (COD basis). The overall observed yield, referred to the entire SBR cycle, was quantified as the ratio between the VSS (at the end of the feast phase) and the ΔS, as given by: Y_{OBS}^{SBR} = VSS/ΔS (COD basis). The polymer content in the biomass was the ratio between PHA and VSS concentration (at the same cycle time): % PHA = PHA/VSS = PHA/(X_A + PHA).

2.4. Sample treatment and DNA extraction

Inoculum and aerobic sludge samples (10 mL) were taken at the end of SBR operation at different OLRs, 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. DNA extraction was performed by using PowerSoil® DNA Isolation Kit (MoBio - Carlsbad, CA) following the manufacturer's instructions. Purified DNA from each sample was eluted in 50 μL sterile Milli-Q water. Extracted DNA was checked for quality (1.6 <

A260/280 < 1.8 and A260/230 > 2) with a Nanodrop 3300 (Thermo Scientific, Italy) and stored at −20 °C.

2.5. Fluorescence in situ hybridization (FISH) and Nile blue staining

The biopolymer storage accumulating capacity in the SBR was checked by applying the Nile blue staining on dry sludge samples (Ostle and Holt, 1982). The fixed samples were disaggregated by vortexing with glass beads for 3 min. Fluorescence in situ hybridization analysis was performed using several oligonucleotide probes following the conditions reported in probeBase (http://www.microbial-ecology.net/probebase/) and according to the protocol describe by Nielsen et al. (2009). In detail the probes used in this study were: EUBmix (equimolar concentrations of EUB338, EUB338-II, and EUB338-III) for total *Bacteria*, BET42a and GAM42a for the *Gammaproteobacteria* phylum, ALF968 for *Alphaproteobacteria*, LGC354abc for *Firmicutes*, CF319a for *Bacteroidetes* and CTE specific for *Comamonadaceae* family. Furthermore, genus specific probes were applied, such as THAU646 and THAU832 for *Thauera* spp.; PAR1244, PAR1457, PAR651 for *Paracoccus* spp.; G-Rb and E2d21002 for *Rhodobacter* spp. The probes were labelled with sulfoindocyanine dye Cy3 or fluorescein isothiocyanate (FITC) (MWG AG Biotech, Germany). After hybridization, total cells were stained with Vectashield Mounting Medium® with DAPI (Vector Labs, Italy). Both FISH and Nile blue samples were viewed using epifluorescence microscope, Olympus BX51, equipped with an Olympus XM10 camera (Cell-Sense software).

2.6. High-throughput 16S rRNA gene sequencing and bioinformatic processing

The extracted DNA was utilized as template for the amplification of the V1-V3 region of bacterial 16S rRNA gene (27F 5'-AGAGTTTGATCCTGGC TCAG-3'; 534R 5'-ATTACCGCGGCTGCTGG-3') following the procedure described in Crognale et al. (2019a, 2019b). All PCR reactions were carried out with Phusion High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Waltham, MA USA). The Agencourt® AMpure XP bead protocol (Beckmann Coulter, USA) was used for the purification of amplicon libraries. The purified libraries were quantified with Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA USA), pooled in equimolar concentrations, and diluted to 4 nM. The samples were paired end sequenced (2x301bp) on a MiSeq platform (Illumina) using a MiSeq Reagent kit v3, 600 cycles (Illumina, USA) following the standard guidelines for preparing and loading samples. 15% Phix control library was spiked in to overcome low complexity issue often observed with amplicon samples.

The reads were quality assessed with Fastqc software, then analyzed using QIIME2 v. 2018.2 (Bolyen et al., 2019) as described in Crognale et al. (2019b). The total reads were subsampled and rarefied at the same value for each sample by using the feature-table rarefy plugin (Weiss et al., 2017). Amplicon sequence variants (ASVs) were identified by applying DADA2 pipeline (Callahan et al., 2016, 2017) (Callahan et al., 2016). Taxonomy was assigned to ASVs using a pre-trained naïve-bayes classifier based on the 16S rRNA gene database at 99% similarity of the Silva132 release (Quast et al., 2013). High-throughput sequencing of the V1-V3 region of the bacterial 16S rRNA gene yielded a total of 101·166 sequence reads after quality control and bioinformatic processing that resolved into 407 ASVs. The taxonomic assignment of 23 major ASVs was additionally carried out by BLASTn algorithm (Madden, 2002). Based on the taxonomical classification, the relative percentage of bacterial genera described in literature for their capability to accumulate PHA was calculated for each sample and is reported in the text as "putative PHA-storing bacteria abundance". The Dataset is available through the Sequence Read Archive (SRA) under accession PRJNA721895.

2.7. Statistical analysis

The process data were incorporated into a Non-metric MultiDimensional Scaling ordination plot (NMDS) in order to graphically synthesize the Euclidean dissimilarity between samples. Process parameters and microbial data were then projected onto the NMDS ordination using a vector-fitting procedure, in which the length of the arrow is proportional to the correlation between NMDS-axes and each variable. NMDS analysis was performed by using PAST software (PALAEONTOLOGICAL STATISTICS, ver. 2.17) (Hammer et al., 2001). This method allowed determining the variation pattern of each projected variable discriminating the samples taken at different OLRs (Amalfitano et al., 2014; Foulquier et al., 2013). Process data and values of major ASVs revealed by 16S rRNA high-throughput sequencing (only ASVs $\geq 3\%$ of total reads were considered) were normalized by $\log(X + 1)$. Venn diagram was generated with VENNY v.2.1 (Oliveros, 2007).

3. Results and discussion

3.1. Sequencing batch reactors with uncoupled carbon and nitrogen feeding

Four runs were carried out applying the uncoupled C- and N-sources feeding strategy and progressively increasing the applied OLR. Each run was operated for a long enough time to reach biomass adaptation and to characterize the process performance under a steady state. The C-source was fed at the beginning of the cycle without N addition in order to maximize the storage yield in the feast phase and optimize the selective pressure on the culture. Therefore, the N-source was fed at the beginning of the famine phase (i.e., after the complete VFA depletion) and the ammonia was used only for the growth of PHA-storing microorganisms. As previously reported, for all the investigated OLRs, the pH of the feeding was adjusted at a value between 6.0 and 6.5 by adding a NaOH solution. Despite this, even if it was not controlled during the experimentation, the operative pH of the biomass remained stable between 8.5 and 9.0 during the whole period. These are crucial aspects for a complete evaluation of biomass behaviour as a response to the applied OLR changes. Indeed, pH, temperature and C/N ratio were unchanged parameters and the progressive increase of the OLR was the effective change applied on the biomass. However, other possible effects on the microbial selection and biomass response caused by other factors (e.g. salinity variation) could not be excluded for a complete evaluation of the process performance. Table 1 summarizes the main process parameters and performance of the PHA storage process. During the Run A (OLR 4.25 g COD L^{-1} d^{-1}), PHA concentration at the end of the feast phase was 601 ± 50 mg PHA L^{-1}, while the one measured at the end of the famine phase (corresponding to the end of the cycle) was 76 ± 8 mg PHA L^{-1}, as it should be in a typical feast-famine regime (Valentino et al., 2017). Indeed, the produced PHA was consumed together

with the ammonia fed at the beginning of the famine phase, clearly suggesting the biomass growth. Similar trends were observed in the following Runs B and C, where the applied OLR was increased up to 12.75 g COD L^{-1} d^{-1}. The feast phase length was maintained in the same range (29.2 ± 2.9, 28.7 ± 1.5, $29.4 \pm 1.6\%$ of the cycle length, for Runs A, B and C, respectively), meaning that the selective pressure was efficiently maintained during the three runs. Compared to the values obtained in previous studies (Lorini et al., 2020) the feast phase length to cycle length ratios resulted slightly higher, but still in the range for a good biomass selection in the case of uncoupled feeding strategy (Silva et al., 2016). The PHA concentration at the end of the feast phase achieved higher values, 1780 ± 80 and 3080 ± 121 mg PHA L^{-1} for Runs B and C, respectively, according to the higher applied OLRs and also the ammonia was almost completely depleted at the end of cycle, in line with previous evidences (Lorini et al., 2020). Subsequently, the further increase of the OLR (18 g COD L^{-1} d^{-1}) in Run D led to an increase of the feast phase length ($39.4 \pm 2.3\%$ of the cycle length). As a consequence, a substantial decrease of the PHA concentration at the end of feast phase (2049 ± 110 mg PHA L^{-1}) with respect to Run C, was observed. Moreover, a residual ammonia concentration of 101 ± 14 mg N L^{-1} was found at the end of the cycle. In this way, at the beginning of the following feast phases, an N-source was still present into the SBR, limiting and affecting the selective pressure on PHA-producing biomass. This aspect led to an increase of the active biomass (X_a) concentration, at the end of the feast phase, that almost doubled respect to that measured in Run C (2033 ± 169 mg X_a L^{-1}), up to 3816 ± 160 mg X_a L^{-1}. In combination with the lower PHA concentration (2049 ± 110 mg PHA L^{-1}), a lower intracellular PHA content was obtained (0.36 ± 0.05 g PHA g^{-1} VSS), almost the half of that obtained in Run C (0.62 ± 0.02 g PHA g^{-1} VSS). In the first three runs, the storage yield ($Y_{P/S}^{feast}$) was high enough to consider the applied OLR range technically feasible for the culture selection. Runs B and C exhibited the highest $Y_{P/S}^{feast}$ values (0.69 ± 0.02 and 0.69 ± 0.04 COD/COD respectively); in Run D (18 g COD L^{-1} d^{-1}), the $Y_{P/S}^{feast}$ dramatically decreased to 0.27 ± 0.03 COD COD^{-1} in accordance with previous studies using a coupled CN feeding strategy (Villano et al., 2010a, 2010b). The storage response was observed in the first three runs, while the MMC selection process suffered the further increase of OLR from 12.75 to
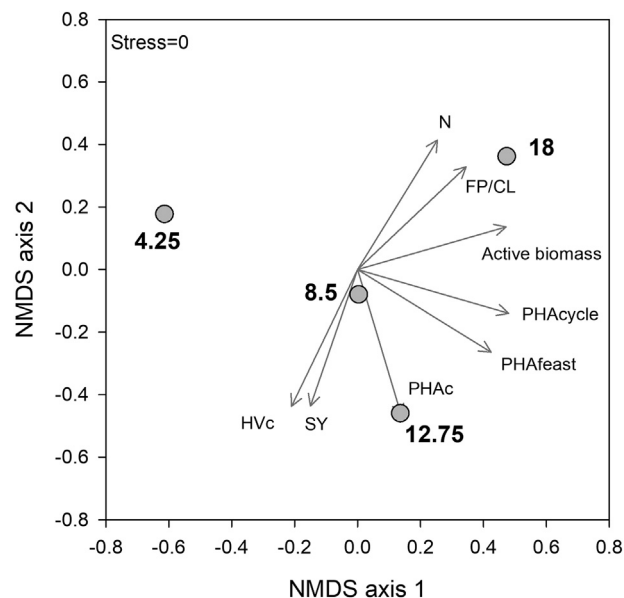

Fig. 1. NMDS ordination plots, based on Bray-Curtis distance matrixes of log-transformed data. The vector length is proportional to the correlation between the NMDS axes and each process parameter. The stress value (i.e., <0.2) suggests for an accurate representation of the dissimilarity among samples. The variation pattern of N, nitrogen content; FP/CL, feast phase/cycle length ratio; Active biomass; PHAcycle, PHA concentration at the end of cycle; PHAfeast, PHA concentration at the end of feast; PHAc, PHA content; HVc, HV content; SY, storage yield.

18 g COD L^{-1} d^{-1}. Accordingly, the positive correlation of the specific storage yield with increasing the OLR was evident in the NMDS ordination plot (Fig. 1).

For the sake of clarity, a comparison in terms of PHA production between the results obtained in this study and those obtained in a previous work (Lorini et al., 2020) is reported in Fig. S1 of Supplementary Materials.

Overall, the biomass productivity, the storage yield ($Y_{P/S}^{feast}$), and the intracellular PHA content herein obtained at 4.25, 8.5 and 12.75 g COD L^{-1} d^{-1} are in agreement with earlier reported findings (Lorini et al., 2020). This is particularly relevant, because highlights the reproducibility of the experimental data and, in turn, the robustness of the MMC-PHA production process, independently from the origin of the activated sludge used as the SBR inoculum. However, at the OLR of 18 gCOD L^{-1} d^{-1}, the significant increase of the feast phase length to cycle length ratio (39.4 ± 2.3%) (Table 1) in combination with the increase of the biomass productivity up to 3.8 g X_a L^{-1} d^{-1} caused the drastic decrease of the storage yield and a lower PHA content at the end of the feast phase.

3.2. PHA-accumulating microbiome dynamics

In line with process data, FISH analysis in combination with high-throughput 16S rRNA gene sequencing revealed successional changes in microbial composition in response to the different applied operating conditions. In particular, the selective pressure established at the different investigated OLRs strongly influenced the microbial communities' composition. Indeed, the biomass selection increased with the increase of OLR up to 12.75 gCOD L^{-1} d^{-1}, revealing the occurrence of PHA-accumulating bacterial classes widely found in previous studies (Morgan-Sagastume, 2016). In particular, FISH analysis (Fig. 2) revealed a mixed microbial community in Run A (4.25 gCOD L^{-1} d^{-1}) mostly composed by *Alphaproteobacteria* and at lower extent by *Gammaproteobacteria*. A further increase in *Alphaproteobacteria* abundance was observed with the increase of the OLR (at 8.50 and 12.75 g COD L^{-1} d^{-1}) mostly belonging to the genus *Paracoccus* as revealed by the use of genus-specific probes (PAR1457 + PAR651 probes). Moreover, gammaproteobacterial cells (BET42a + GAM42a oligonucleotide probes) mainly belonging to *Thauera* (THAU646 and THAU832 probes) were also detected. On the contrary, greater occurrence of *Gammaproteobacteria* and less of *Alphaproteobacteria* was observed in the Run D (18 g COD L^{-1} d^{-1}). This finding is in line with the literature, since the vast majority of PHA-storing bacteria described so far precisely
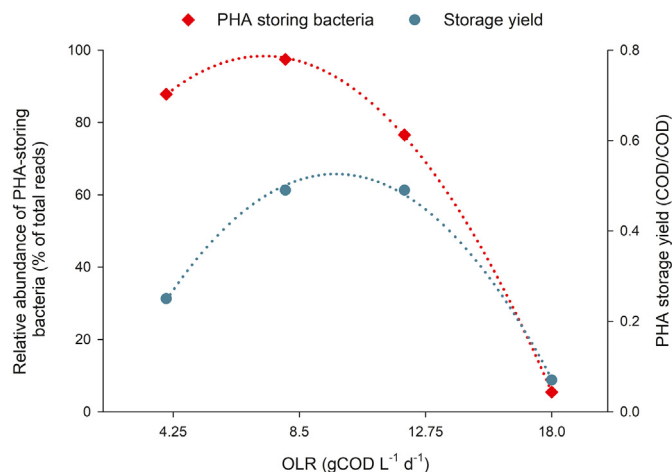

Fig. 3. Relative abundance of sequences affiliated with putative PHA-storing bacteria out of total sequences and storage yield trend in the bioreactors at different OLRs.

belongs to the classes *Alpha-* and *Gammaproteobacteria* (Morgan-Sagastume, 2016; Queirós et al., 2015). In line with FISH data and with the increase of the storage yield, a very high relative abundance of sequences affiliated with known putative PHA-storing bacteria was found in the SBR operated at OLRs 4.25, 8.50 and 12.75 g COD L^{-1} d^{-1} (up to 97.4% of total reads). In contrast, only 5.4% of PHA-storing bacteria was found in Run D (18 g COD L^{-1} d^{-1}) in line with the lowest storage yield observed (0.10 COD COD^{-1}) (Fig. 3).

The 16S rRNA gene high-throughput sequencing revealed *Proteobacteria* as the dominant phylum in all runs, representing on average 91.5% of total reads (Fig. 4a). The phyla *Bacteroidetes* (4.6%) and *Firmicutes* (3.2%) followed to a lesser extent. The inoculum was characterized by the presence of *Thauera* (49.5% of total reads) together with several members of *Alphaproteobacteria* such as *Brevundimonas*, *Paracoccus* and *Rhodobacter* (up to 12.7%) (Fig. 4b). In Run A (4.25 gCOD L^{-1} d^{-1}) the microbiome was mainly composed by the PHA-storing *Rhodobacter* (47.3%), *Exiguobacterium* (12.7%) and at minor extent by *Paracoccus* (4.7%) commonly found in PHA-production systems (Iqbal et al., 2016; Lee et al.,
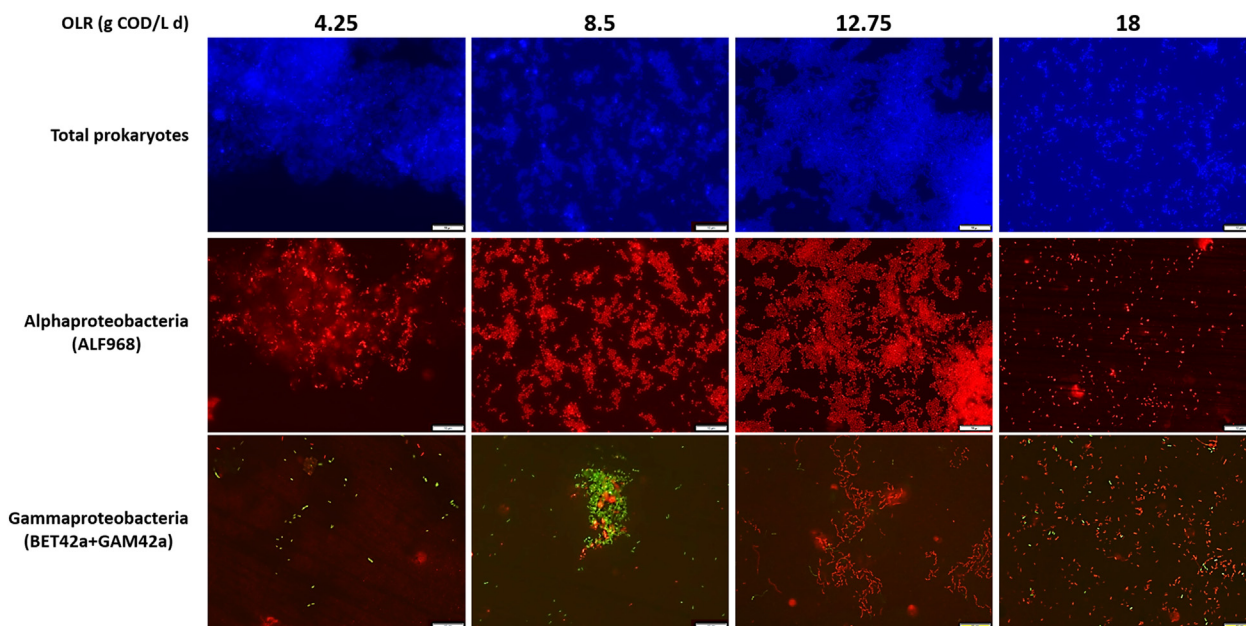

Fig. 2. FISH images of the biomass reactor at different OLRs showing total prokaryotic cells (DAPI staining), members of classes *Alphaproteobacteria* (ALF968 probe) and *Gammaproteobacteria* (BET42a + GAM42a probes). Scale bar = 10 μm.
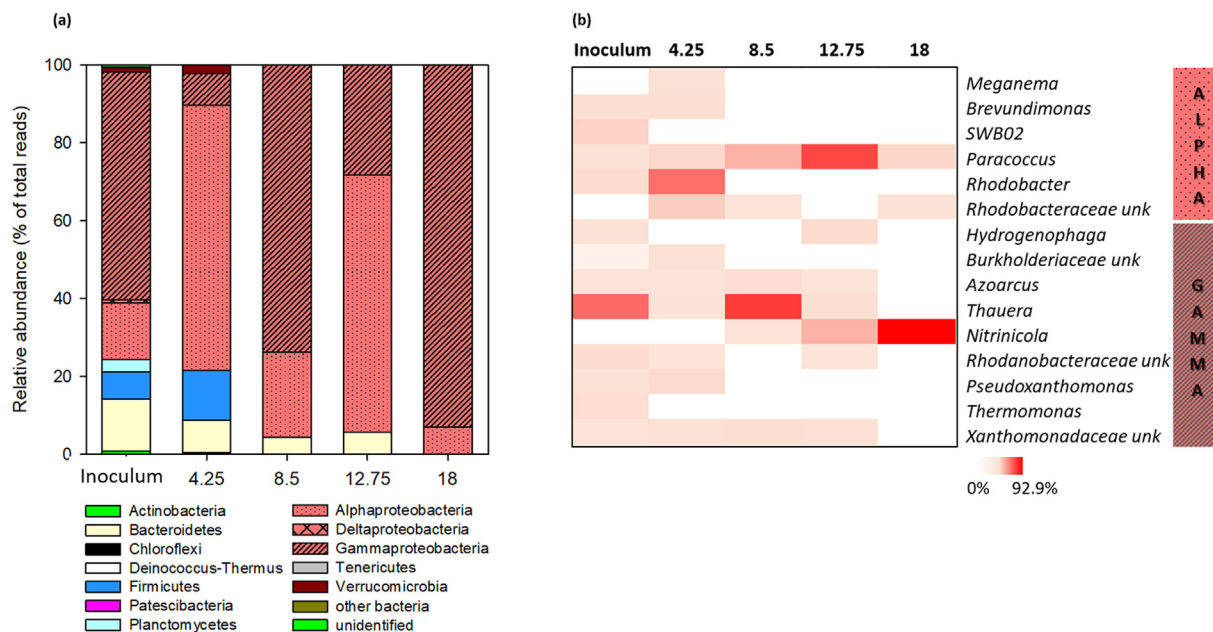
Fig. 4. Microbial community composition in the biomass reactor at different OLRs. (a) Relative abundance (% of total reads) of main phyla and classes within *Proteobacteria* phylum. (b) Frequency heat-map of microbial genera affiliated with *Proteobacteria* phylum (≥1% relative abundance of total reads in at least one sample). The color intensity in each cell shows the relative abundance.

2020; Matos et al., 2021; Oshiki et al., 2013). The genera *Paracoccus* and *Thauera* constituted the core microbiome of biomass in Runs B and C encountering for a total of 89.3% and 65.9% of the reads, respectively. These two genera were often reported as the main component of the PHA producing mixed microbial cultures in systems fed with acetate under feast/famine conditions (Albuquerque et al., 2013; Carvalho et al., 2014; Chen et al., 2019; Çiğgin et al., 2011; Huang et al., 2018; Lemos et al., 2008; Queirós et al., 2014; Sruamsiri et al., 2020; Wen et al., 2018). Nevertheless, although several advancements have been made during last years, the operating conditions that favour the growth of these organisms over the others have yet to be fully elucidated (Carvalho et al., 2014; Morgan-Sagastume, 2016). Most likely the selection of different microbial populations observed in this study can be mainly due to the application of diverse OLRs (Lemos et al., 2008). Indeed, few previous reports suggested that the change in OLR, together with the carbon source composition and origin, had a high impact on the feast/famine ratios and subsequently on microbial community structure of the PHA producing system (Albuquerque et al., 2011; Carvalho et al., 2014; Serafim et al., 2008a, 2008b). Nevertheless, such applied OLRs using a mixture of pure substrates were not so high as that used in this study. The loss of PHA-accumulating capacity of microbial cultures at very high OLR was hypothesized by Dionisi et al. (2006) in terms of biomass competition. As suggested by Dionisi et al. (2006), at OLR in the range 8.50–12.75 gCOD L^{-1} d^{-1} the fast-storing microorganisms were favoured and became predominant. On the contrary, with very high OLR (up to 31.25 g COD L^{-1} d^{-1}) the fast-storing microbes were not enough to remove all the substrate that remained available for non-storing microorganisms that therefore prevailed. Unfortunately, no information regarding microbial diversity in these systems is available. A previous study that reports microbial diversity at high OLR (20 gCOD L^{-1} d^{-1}) was carried out with experimental conditions different from those herein tested (i.e., cycle length in the range 1–8 h, feeding mixture of acetic, lactic and propionic acids in the ratio 2:2:1) and showed a microbial community mainly composed by genera *Alcaligenes*, *Flavobacterium*, *Acinetobacter*, or *Comamonas* (Dionisi et al., 2007).

In agreement with Dionisi et al. (2006), at the highest investigated OLR (18 gCOD L^{-1} d^{-1}) in the present study, even though organic acids were completely depleted by the end of the feast phase, this represented about 40% of the overall cycle length, not allowing for the establishment of an appropriate feast and famine regime. Furthermore, the residual ammonia-

concentration at the end of the cycle in Run D (OLR 18 g COD L^{-1} d^{-1}) favoured the microbial growth over the PHA storage during the feast phase, strongly affecting the microbial community selection. Indeed, in line with the lowest storage yield, the biomass showed a very low content in PHA-storing bacteria (Fig. 3). Moreover, the high presence of sequences affiliated with *Nitrinicola* genus (92.9% of total reads) was most likely due to the ammonia availability that affected the selective pressure on PHA-producing biomass and favoured bacteria able to exploit the accumulation of N-source. This genus, in fact, includes non-fermenting, facultative anaerobic, chemoorganotrophic and alkaliphilic bacteria with the capability to reduce nitrate and nitrite mainly isolated from alkaline lakes (Borsodi et al., 2017; Dimitriu et al., 2005; Joshi et al., 2016).

The microbial succession during reactors operation was also apparent at the level of amplicon sequence variants (ASVs) (Fig. 5a; Table S1). In agreement with the process data, the increase in biomass selection correlated with the performance trend was depicted also in the NMDS ordination plot (Fig. 5b). Overall, a total of 23 ASVs counted between 60.2 and 94.9% of total reads obtained from samples. Among the major bacterial component, genus *Paracoccus* comprised only two ASVs, with ASV225 being the sole one shared by all samples (Fig. 5c). The presence of this ASV was previously observed in a pilot plant producing PHA from the organic fraction of municipal solid waste (Crognale et al., 2019b; Moretto et al., 2020) most likely due to the activated sludge used as inoculum in this study coming from the same wastewater treatment plant. Among the three ASVs classified as *Thauera*, ASV326 and ASV341 were present only in Run A and B, respectively (Fig. 5a). All of the *Rhodobacter* and *Thauera* ASVs herein detected were the same as the previous found in Moretto et al., 2020. Lastly, three ASVs (ASV354, ASV356, ASV359) represented genus *Nitrinicola* in the two bioreactors with highest OLR (Fig. 5a).

4. Conclusion

In conclusion, this study described the effect of the applied OLR on the microbial selection in a SBR operated with an uncoupled C and N feeding strategy. Imposing an OLR in the range of 4.25–12.75 g COD L^{-1} d^{-1}, the feast/famine regime was easily established, and a strong PHA-storing biomass selection in line with the high storage yield was observed. The specific PHA-content increased despite the detected dynamic fluctuation of the three main microbial components namely referred to *Rhodobacter*, *Thauera*
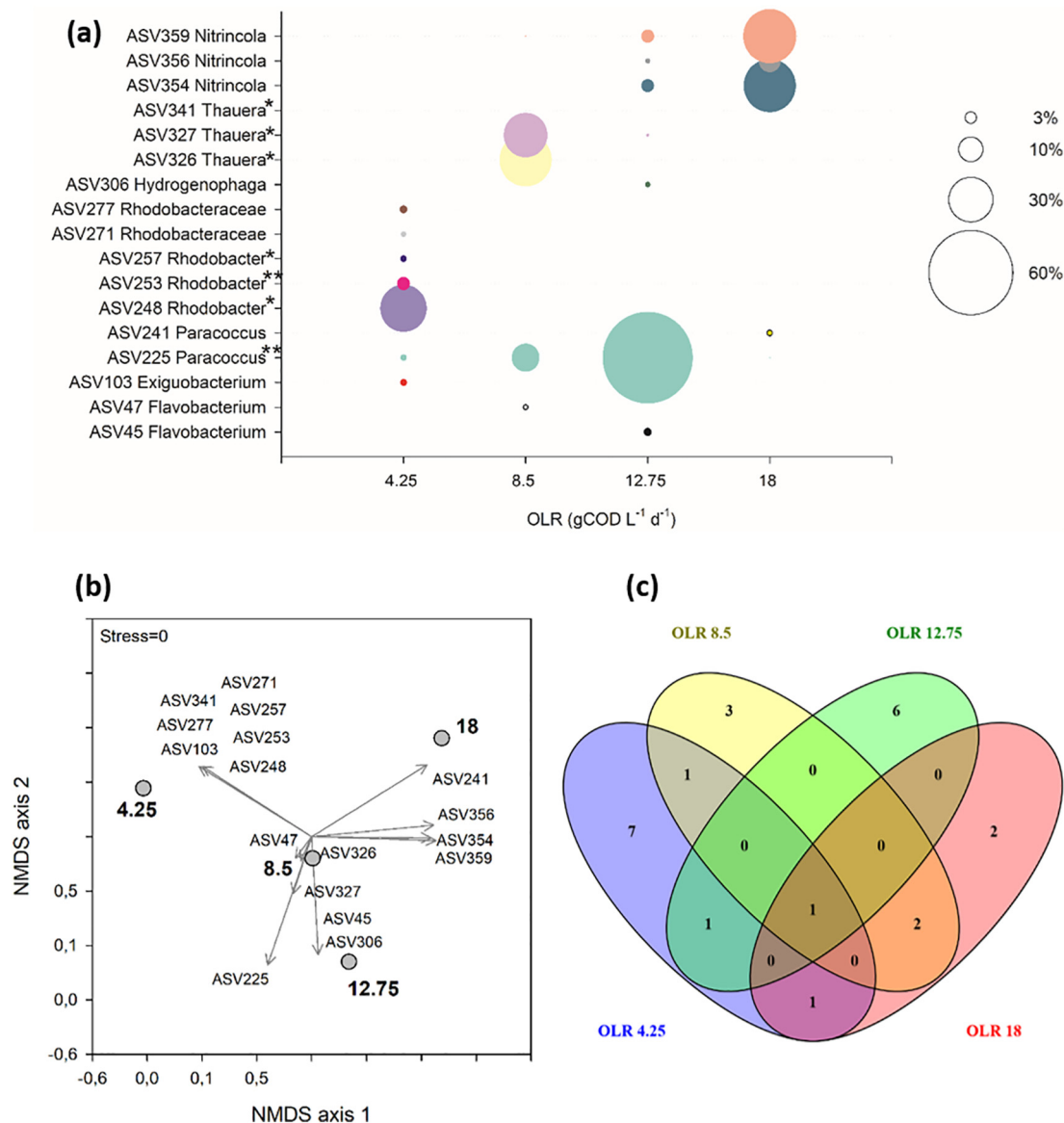
Fig. 5. a) Bubble plot depicting the relative abundance (as % of total reads) of the main ASVs (≥3% in at least one sample) in the biomass reactor at different OLRs. *ASV present also in Moretto et al., 2020; ** ASV present also in Crognale et al., 2019a, 2019b and Moretto et al., 2020. b) The relative abundance of the main ASVs (≥3% in at least one sample) is projected onto the NMDS ordination synthesising the chemical dissimilarity. For ASV affiliation referred to Table S1.c) Venn diagram showing the number of the main microbial ASVs (≥3% in at least one sample) in biomass reactor at different OLRs.

and *Paracoccus* genera. These microorganisms were capable of high PHA production when the operational conditions imposed were appropriate (e.g., an adequate OLR). On the contrary, when the OLR was further increased (18 g COD L^{-1} d^{-1}), the system was unstable and the microbial community strongly affected, resulting in a very low content of PHA-storing microorganisms and the lowest storage yield. In fact, the high OLR imposed represented a detrimental condition for the system in terms of microbial selection and PHA production.

The correlation between the OLR applied to the SBR and the composition of the selected mixed microbial culture are particularly relevant to identify the optimal operating conditions which allow to reach a high intracellular PHA content by the end of the feast phase, that is a crucial parameter to be considered for the process scale-up. As an example, the highest PHA storage yield and intracellular polymer content reached in this study applying an OLR of 12.75 g COD L^{-1} d^{-1} may allow process simplification by directly collecting the biomass at the end of the feast phase from the SBR, at its maximum PHA content, skipping the traditional accumulation step. However, this hypothesis needs to be further confirmed, also at pilot scale, since the SBR operation at high OLR for a long time could be unstable. Moreover, the exploitation of nutrient deficient organic waste (such as paper mill and olive oil mill wastewaters, cheese whey permeate or sugar-cane molasses) may be realized including a nitrogen and phosphorus addition in the famine phase and an OLR modulation.

CRediT authorship contribution statement

Crognale Simona: Investigation, Formal analysis, Data curation, Visualization, Writing – original draft. **Lorini Laura:** Investigation, Formal analysis, Data curation, Writing – original draft. **Valentino Francesco:** Conceptualization, Writing – review & editing. **Villano Marianna:** Conceptualization, Writing – review & editing. **Marzo Gago Cristina:** Investigation, Data curation. **Tonanzi Barbara:** Investigation, Data curation, Visualization. **Majone Mauro:** Supervision, Funding acquisition,

Conceptualization. **Rossetti Simona:** Supervision, Funding acquisition, Conceptualization, Writing – review & editing.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.153995.

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