Integrated production of biopolymers with industrial wastewater treatment: effects of OLR on process yields, biopolymers characteristics and mixed microbial community enrichment

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Abstract

The production of polyhydroxyalkanoates (PHA) using industrial wastewaters as feedstocks is a current and challenging topic. This study investigated the production of biopolymers by a mixed microbial culture under different OLRs equal to 1 kgCOD m\(^{-3}\)d\(^{-1}\) (Period 1), 2 kgCOD m\(^{-3}\)d\(^{-1}\) (Period 2) and 3 kgCOD m\(^{-3}\)d\(^{-1}\) (Period 3). The maximum PHA content was achieved in Period 2 (0.38 gPHA gTSS\(^{-1}\)), whereas lower values were obtained in Period 1 (0.13 gPHA gTSS\(^{-1}\)) and Period 3 (0.26 gPHA gTSS\(^{-1}\)). Overall, the maximum PHA productivity resulted equal to 0.08 gPHA L\(^{-1}\)h\(^{-1}\) (P2), 0.05 gPHA L\(^{-1}\)h\(^{-1}\) (P1) and 0.04 gPHA L\(^{-1}\)h\(^{-1}\) (P3), respectively.

The molecular weight of the PHA increased from Period 1 (250 kDa) to Period 2 (417 KDa) and Period 3 (463 KDa), although resulting in a slight decrease of crystallinity degree. Microbial community analysis, revealed a reduction in bacterial diversity and a progressive shift of the microbial community with the increasing OLR. Alpha-diversity indexes based on Operational Taxonomic Units (OTUs) at 99% identity revealed higher species richness (Taxa (S) 280) and diversity (Shannon (H) 4.06) in Period 1, whereas Period 3 was characterized by reduced richness and diversity and higher dominance (Taxa (S) 133, Shannon (H) 2.40).

Based on the results obtained, it was pointed out that the OLR variation determined significant effects on the process performances, as well as on the productivity and quality of the biopolymers. This means that OLR is a key control parameter to maximize the PHA production and control the physical-chemical characteristics of the polymers.

Keyword: industrial wastewater; mixed microbial cultures; organic loading rate; polyhydroxybutyrate; SBR reactors.

1. Introduction

Material and energy recovery from wastewater treatment plants has become increasingly topical during last years [1]. In this scenario, wastewaters, both of municipal and industrial origin, are
considered a renewable resource from which energy and material could be derived during their
treatment [2,3]. An interesting pathway to recover material from wastewater treatment is the
production of biopolymers, such as polyhydroxyalkanoate (PHA) or polyhydroxybutyrate (PHB).
These are naturally occurring polyesters that are intracellularly accumulated by several bacteria and
might be used for the production of bio-based plastics [4]. During the last decade, the bioplastic’s
market is expanding and is involving various industries in the packaging, automotive and biomedical
sectors, and is expanding towards application within the framework of contaminated soils remediation
[5,6]. Therefore, there is a real interest by the manufacturing sector in developing sustainable
production processes for these biomaterials.
The microbial production of PHA is based on the ability of different bacterial strains to convert low
molecular weight organic molecules into PHA [7]. To date, the production of PHA at industrial level
is based on the use of pure bacterial cultures which, although enabling high production yields, entails
very high operating costs which makes the overall production cost still not competitive compared to
that of petroleum-based plastics (5 €/kg vs 0.5 €/kg) [2,8]. According to recent literature studies, the
use of mixed microbial consortia (MMC) allows to a significant reduction in costs during the
cultivation and enables to better compete with production-costs of oil-based plastics [9]. The selective
pressure to achieve a high enrichment of PHA-producing microorganisms typically involves
alternating excess (feast) and limitation (famine) of carbon substrate availability [10], which is
generally achieved in a sequencing batch reactor (SBR) configuration [11,12]. Previous literature
studies reported that waste-containing sugar and/or fatty acids may be the best feedstocks for PHA
production [13]. Moreover, to maximize PHA accumulation rather than cell synthesis, nutrients
imbalance should be ensured [14]. For this reason, recent researches have focused on wastewater
generated by the agro-food industries, as these are characterized by high concentration of organic
carbon and low nitrogen and phosphorus content [15]. Several applications concerning wastewaters
from the oil-mill, winery and dairy industries are reported in literature, but very few are those referred
to wastewater from citrus processing industries [16,17]. Citrus wastewater is generally characterized
by high concentration of chemical oxygen demand (COD), low nitrogen and phosphorus content [18].

Citrus wastewater is characterized by seasonal fluctuations depending on the type of fruits to be processed [19]. These affect the volume of wastewater produced as well as its organic content (as COD), which varies according to the specificities of the production processes. Certainly, this aspect could affect the bacterial community involved in the biological processes, which could evolve according to the variable process conditions [20]. On the other hand, this might have remarkable effects on the purification performances of the plant, since the selection of the PHA-accumulating biomass occurs simultaneously with the wastewater treatment, but also on the physical characteristics of the PHA produced. In this sense, one of the greatest challenge that could be encountered if PHA production is to be integrated in existing plants would be to apply operating conditions to maximize the PHA production yield without compromising the purification performances and obtain PHA having physical characteristics suitable for downstream processes.

In this light, the present study focused on the possibility to use industrial wastewater characterized by seasonal fluctuations as a feedstock for PHA production. Specifically, the aim of this study was to evaluate the production and the physical characteristics of PHA obtainable from the treatment of wastewater deriving from a citrus industry in a laboratory-scale plant, using a real mixed microbial consortium (MMC) derived from a wastewater plant as inoculum. In more detail, the novelty of the study was to assess the relationship between different OLR, ranging between 1-3 kgCOD m$^{-3}$d$^{-1}$, with the performances and the productivity of PHA, their physical-chemical and mechanical properties and the shift in the composition of the MMC.

2. Materials and methods

2.1 Characterization of citrus wastewater

The citrus wastewater was sampled from an industry that processes citrus fruits located in Palermo (Italy). The wastewater collected from the industry, from now called concentrated citrus wastewater (CW1), was characterized by very high COD concentration (>25,000 mgCOD L$^{-1}$). A stock of this
wastewater was stored at 4 °C and a pH of 3.5 for the entire duration of experiments. Daily, a fraction of this wastewater was diluted with tap water until a COD close to 4,500 mgCOD L⁻¹ was obtained. Then, it was fed to a fermentation reactor, after adjusting the pH to a value close to 7 by adding NaOH. Subsequently, the wastewater was fed to a lab-scale SBR plant after the supply of a concentrated solution (0.1 L d⁻¹) containing nitrogen and phosphorus (2gCH₄N₂O/L, 1gK₂HPO₄/L) to obtain a ratio between carbon (as COD)/nitrogen/phosphorous (CW2) equal to 100: 5: 1.

Furthermore, a fraction of the CW1 was fed to a second fermentation reactor (pH = 7). After the fermentation, this wastewater (CW3), without any supply of nitrogen and phosphorous, was fed to an accumulation reactor aimed at producing PHA.

All the above mentioned streams, CW1, CW2 and CW3, were characterized in terms of pH, electrical conductivity, total COD concentration (COD,t), nutrients (nitrogen and phosphorus) and acetate as the main fermentation product.

The average values of the main qualitative parameters of the wastewater used are reported in Table 1:

| Tab. 1 |

2.2 Experimental setup

The experimental activity lasted 180 days and was carried out in a lab-scale plant. It consisted of three main units: a fermenter, an enrichment reactor (SBR₁), in which the treatment of citrus wastewater and the selection of the PHA-accumulating biomass were carried out simultaneously and, finally, a PHA-accumulation reactor (SBR₂) (Figure 1).

The fermenter was a completely mixed reactor, with a volume of 100 L. The operating conditions in the fermentation reactor were chosen based on the results obtained in another study, to which the reader is referred for further information [21].

The SBR₁ (operating volume of 22 L) operated according to cycles lasting 12 hours, divided as follows: 30 minutes of feeding under static conditions, maintaining the aeration and mixing devices
inactive, 9 hours of aeration, 2 hours of settling under static conditions and, finally, 30 minutes of effluent discharge. The reactor was equipped with two porous stone diffusers placed at the bottom of the reactor that were connected to an air blower providing an airflow rate of 15 L min\(^{-1}\). All the equipment was connected to a programmable logic controller that handled the phases’ alternation.

The SBR\(_2\) (volume of 1.5 L) was fed with the excess sludge discharged from the SBR\(_1\) and with the CW3. The SBR\(_2\) was equipped with an air compressor and a dissolved oxygen (DO) sensor connected to a hardware-software system for data acquisition and handling of the aeration.

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**Fig. 1**

2.3 Operating conditions and monitoring activities of the enrichment reactor

Based on both qualitative and quantitative seasonal variations of citrus wastewaters, it was decided to operate the SBR\(_1\) in three different periods, called Period 1, Period 2 and Period 3, characterized by increasing OLRs. In Period 1 (duration 78 days), the SBR\(_1\) operated with a flow rate equal to 5 L d\(^{-1}\), corresponding to an OLR equal to 1 kgCOD m\(^{-3}\)d\(^{-1}\). In Period 2 (duration 32 days), the flow rate was doubled (10 L d\(^{-1}\)) and consequently the OLR increased to 2 kgCOD m\(^{-3}\)d\(^{-1}\). Finally, in Period 3 (duration 62 days), the plant operated with a flow rate of 15 L d\(^{-1}\), corresponding to an OLR of 3 kgCOD m\(^{-3}\)d\(^{-1}\). The above periods corresponded respectively to the real average load conditions of a citrus wastewater treatment plant (potential of 12,500 PE) located in Palermo (Italy), which were referred to the summer period (low load - Period 1), the autumn / spring (medium load - Period 2) and the winter (high load - Period 3), respectively.

The operating volume of the SBR\(_1\) was set to 22 L, increasing the volumetric exchange ratio in each period. The SBR\(_1\) was seeded with activated sludge taken from the treatment plant serving the industry from which the citrus wastewater was collected. The biomass concentration in the seeded sludge was equal to 4.5 gTSS L\(^{-1}\) and it was kept constant in the SBR\(_1\) in all three periods, by purging daily a known volume of mixed liquor variable according to the bacterial growth rate observed.
Consequently, the sludge retention time (SRT) was variable in the three periods and calculated according to a mass balance [22]. The duration of each period was set in order to ensure a minimum of three times the SRT, as this time is generally suggested for the achievement of steady-state conditions in biological systems [23]. The dissolved oxygen profile in the SBR$_1$ was checked once a week in order to assess the duration of the feast and famine phases [24].

2.4 PHA accumulation reactor

The PHA accumulation assays were carried out in the SBR$_2$ using the enriched biomass derived from the SBR$_1$ and the fermented CW3. The assays were carried out at the end of each experimental period, after a time equal to three times the SRT had elapsed. To obtain reproducible and comparable results dependent only on the microbiological composition of the activated sludge, the operating conditions in each of the assays performed were the same, in terms of TSS concentration equal to 4.5±0.23 gTSS L$^{-1}$, reactor volume (1.5 L), concentration of COD in the CW3 (27,000 mgCOD L$^{-1}$) and temperature (20 °C).

The operating conditions were such as to obtain an OLR close to 5 kgCOD m$^{-3}$d$^{-1}$. Moreover, nitrogen and phosphorus were not added to the fermented wastewater to have nutrient imbalance (C: N: P = 300: 1: 0.8). The fermented CW3 was fed according to the "feed on demand" strategy, which consisted in dosing of different small volumes every time the biomass present in the reactor had completely degraded the readily biodegradable organic substrate fed with the previous sample [25].

The consumption of the readily biodegradable organic substrate was monitored indirectly by continuously measuring the rate of oxygen consumption (OUR - Oxygen Uptake Rate). The rapid decrease of OUR indicated the total disappearance of the readily biodegradable organic substrate within the system.

Before carrying out a new dosage of wastewater, a sludge sample was withdrawn from the system and was subjected to the extraction procedure for the quantification and characterization of the intracellular biopolymers. Overall, 8 dosages of wastewater were performed in each test, for a total
of 320 mL. Consequently, the organic load was approximately 5.5 kgCOD m$^{-3}$d$^{-1}$. At the same time, the pH was also monitored, and it ranged between 7.6 and 8.4, showing an increasing trend during the test. Each assays had a duration of 9 hours.

For each accumulation assays, mass balances on the COD were carried out to evaluate the conversion yield of the organic substance into PHA, extracellular polymeric substances (EPS) and new biomass (X).

2.5 Analytical methods

The analyses of total suspended solids (TSS), COD, sludge volume index (SVI), total nitrogen (TN) and total phosphorus (TP) were carried out according to Standard Methods [26]. The SVI was determined by dividing the volume of the settled sludge inside a graduated cylinder of 1 L after 30 minutes of static settling by the concentration of TSS in the sample.

Measurements of pH, DO and electrical conductivity were carried out using electrochemical (pH and conductivity) and optical (dissolved oxygen) sensors. The concentration of acetate in the fermented wastewater was determined by ion chromatography, using sodium acetate (99.9% purity) as the standard for the assessment of the calibration curve.

The calculation of the observed growth rate ($Y_{obs}$) was carried out through mass balances, considering the daily variation of TSS present in the system and those withdrawn as excess sludge, according to literature [22].

The biokinetic parameters, including the maximum removal rate of organic carbon ($v_H$), the active fraction of the heterotrophic biomass ($f_{ch}$) and the maximum growth rate ($Y_H$) were determined by means of respirometric techniques [27].

The average size of activated sludge flocs was measured using an optical granulometer. The extracellular polymeric substance (EPS) content and characterization were determined by a first extraction step according to the literature [28] and subsequently by measuring the protein [29] and carbohydrates concentration [30]. The microscopic observations and abundance of filamentous
bacteria were carried out according to the procedures developed by Eikelboom [31] and Jenkins [32], using a phase contrast optical microscope.

2.5.1 Extraction of intracellular biopolymers

The intracellular biopolymers were extracted following the procedure developed by Fiorese et al. [33] using 1-2 propylene-carbonate as a solvent. The extracted polymer was subsequently subjected to a three washing cycles with methanol. The separation of the polymer from the methanol was obtained by suspension centrifuging at 4,000 rpm for 10 minutes, at the end of which the supernatant was recovered using a "Pasteur" pipette. The polymer thus obtained on the bottom of the test tube was dried in an oven at 60 °C for 4 h.

2.5.2 Chemical and thermal properties of biopolymers

The chemical properties of the extracted polymers samples were assessed by spectroscopic analysis. Fourier Transform Infrared Spectroscopy Attenuated Total Reflection (FTIR-ATR) analysis was carried out by using a Perkin-Elmer FTIR-NIR Spectrum 400 spectrophotometer. The spectra were recorded in the range 4000–400 cm$^{-1}$. The calorimetric properties of the biopolymers were studied by using a Differential Scanning Calorimeter (DSC), (Setaram, model DSC131). The samples with approximately the same weight (~7 mg) were sealed in aluminum pans. The analysis was carried out with one cycle of heating from –20 °C up to 200 °C at 5 °C/min under nitrogen flow.

The degree of crystallinity ($\chi$) of PHA composites was calculated according to the following equation (eq. 1):

$$\chi(\%) = \frac{\Delta H_m}{\Delta H^0} \times 100 \quad [1]$$

where $\Delta H_m$ is the melting enthalpy of the samples and $\Delta H^0$ is the melting enthalpy of 100% crystalline, which is assumed to be 146.6 J g$^{-1}$ [34].
The intrinsic viscosity ($\eta$) was measured by means of aniVisc Capillary Viscometer LMV 830 (Lauda Proline PV 15, Lauda-Königshofen, Germany) instrument equipped with a Ubbelohde ($K = 0.009676$) capillary viscometer in thermostatic oil bath set at 30 °C. The polymer was dried and then dissolved in CHCl$_3$ under stirring for 3 h to prepare a polymeric solution at the 0.1 wt%. Flow time measurements were performed in triplicate for each sample until the standard deviation was below 0.5 s. The intrinsic viscosity ($\eta$) values was calculated according to Solomon-Ciuta [35] (eq. 2):

$$\eta = \frac{\sqrt{2}}{c} \sqrt{\eta_{sp} - \ln \eta_{rel}} \quad [2]$$

where $c$ is the concentration of the polymer solution, $\eta_{sp}$ and $\eta_{rel}$ are the specific and relative viscosity, respectively. The solution viscosity of each sample was obtained by averaging 5 flow measurements. The viscosimetric molecular weight ($M_v$) was calculated using the Mark-Houwink's equation (eq. 3):

$$[\eta] = K M_v^\alpha \quad [3]$$

The parameter values of the Mark-Houwink constants, $\alpha$ and $K$, depend upon the specific polymer-solvent system. For PHA-CHCl$_3$, $K = 1.18 \times 10^{-4}$dL/g and $\alpha = 0.78$ [36].

2.5.3 Total DNA extraction and 16S rRNA gene sequencing

The mixed microbial consortia of the sludge samples collected from the SBR$_1$ at the end of each experimental period were analyzed, using a molecular approach based on high throughput 16S rRNA gene amplicon sequencing. About 20 mL of every sludge sample were filtered using a Corning® 150 mL Vacuum Filter/Storage Bottle System, 0.45 µm Pore, CA membrane to retrieve the microbial biomass. Total DNA was extracted from the membranes using the QIAamp Fast DNA™ Stool Mini Kit (QIAGEN) according to manufacturer’s instructions. Purity and concentration of total DNA were assessed using a NanoDROP ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and DNAs were stored at −20°C until further analysis.
Extracted DNA was used as template to amplify bacterial and archaeal V3-V4 hypervariable region of the 16S rRNA gene using primers Pro341F (5’-CCTACGGGNGGCASCAG-3’) and Pro805R (Rev 5’-GACTACNVGGGTATCTAATCC-3’) [37].

PCR conditions are the following: an initial denaturation at 94 °C for 1 min; 25 cycles of 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 45 s; and a final extension at 68°C for 7 min.

PCR products were purified with AMPure XP beads and indexed using the Nextera XT Index Kit in a second PCR step. The amplicons were sequenced on an Illumina MiSeq Sequencer at BMR Genomics (Padova, Italy) with 300 bp paired-end reads. The sequences’ quality was checked using the FASTQC software. Reads denoising and feature filtering (0.005%) were performed using Qiime2 tools version 2019.4. Taxonomy was assigned using trained Operational Taxonomic Units (OTUs) at 99% from GreenGenes database version 13-8.

2.5.4 Calculations

The PHA content in the extracted intracellular biopolymer was calculated using the following equation (eq. 4):

\[
PHA = \frac{W_{poly} \cdot \%PHA}{TSS} \left( \frac{gPHA}{gTSS} \right)
\]

where \( W_{poly} \) is the weight of the extracted polymer and \( \%PHA \) the percentage of PHA, evaluated by spectrophotometric analysis. The percentage of PHA was calculated as the ratio between the absorbance (235 nm) of a sulfuric acid solution having the same concentration of the extracted biopolymer and a standard PHA (HB–HV 88/12%, Sigma-Aldrich, Germany).

The mass balances for the COD in the accumulation tests were evaluated by applying the following equation (eq. 5):
\[ COD_{d(gCOD)} = PHA_{p(gCOD)} + EPS_{p(gCOD)} + X_{p(gCOD)} + COD_{r(gCOD)} \]  \[ 5 \]

where:

- COD\(_d\): the total mass of COD dosed during the test, obtained by multiplying the total volume dosed by the concentration of COD in the sample;

- PHA\(_p\): the mass of PHA produced, obtained by multiplying the difference between the PHA concentrations at the end and the beginning of the test, by the volume of the reactor and by the stoichiometric coefficient (equal to 1.67 gCOD gPHB\(^{-1}\) and 1.92 gCOD gPHV\(^{-1}\)) as reported in the literature [2];

- EPS\(_p\): the mass of EPS produced, obtained by multiplying the difference between the sum of concentrations of proteins (PN) and carbohydrates (PS) at the end and at the beginning of the test, by the reactor volume and by the stoichiometric coefficients respectively equal to 1.36\(\pm\)0.03 gCOD gPS\(^{-1}\) and 1.40\(\pm\)0.04 gCOD gPN\(^{-1}\) obtained experimentally. Glucose [30] and bovine serum albumin [29] were used as standards for carbohydrates and proteins, respectively.

- X\(_p\): the mass of new bacterial cells, measured by multiplying the difference between the concentrations of volatile suspended solids (VSS) at the end and at the beginning of the test, net of the concentrations of EPS and PHA, by the volume of the reactor and by the stoichiometric coefficient equal to 1.42\(\pm\)0.07 gCOD gVSS\(^{-1}\) determined by direct measurements;

- COD\(_r\): the mass of residual COD was calculated by multiplying the concentration of COD of the supernatant at the end of the test by the volume of the reactor.

The data of PHA production obtained from accumulation assays were interpolated using an exponential equation (eq. 6):

\[ PHA(t) = PHA_{tot} \cdot (1 - e^{-k t}) \]  \[ 6 \]
where PHA(t) is the PHA content at a generic time, PHA\text{tot} is the last value of PHA content at the end of the accumulation assay, k is the rate of PHA production and t is the time. PHA\text{tot} and k were estimated by minimizing the sum square of errors between the experimental data obtained from the accumulation assays and the model. Therefore, the maximum PHA production (q_{\text{PHA}}, \text{gPHA L}^{-1}\text{h}^{-1}) was calculated as the product between PHA\text{tot} and k.

3. Results and discussion

3.1 Performances of the enrichment reactor (SBR\textsubscript{1})

The performances of SBR\textsubscript{1} were assessed in terms of COD removal and settling properties of the activated sludge through the SVI parameter. Furthermore, the production of the excess sludge was evaluated by calculating the $Y_{\text{obs}}$.

Figure 2 shows the trends of the COD concentrations in the inlet and outlet of the SBR\textsubscript{1} and the related removal performance (Fig. 2a), as well as the trend of the SVI (Fig. 2b) and the average values of $Y_{\text{obs}}$ and SRT (Fig. 2c) in the three periods.

Overall, the COD removal was always higher than 90%, showing a decreasing trend as the OLR increased. Specifically, in Period 1, the steady value of the COD removal was approximately 97%, with an average concentration of COD in the effluent equal to 40 mg L\textsuperscript{-1}. In Period 2, the COD removal was like that observed in the previous period and the COD concentration in the effluent was on average equal to 50 mg L\textsuperscript{-1}. Finally, in Period 3, a decrease in the COD removal was observed in the early stages to about 93%, whereas it remained constant and almost equal to 95% in the following days. However, a decrease in COD removal was observed in the long term. Specifically, at the end of the observed period, the concentration of COD in the effluent was on average equal to 225 mg L\textsuperscript{-1}, thus resulting in a removal close to 91%. The above results confirmed what reported in previous studies concerning the biological treatment of citrus wastewater, where it was found that the COD
removal efficiencies decreased for OLR values higher than 3 kg COD m\(^{-3}\)d\(^{-1}\) [19,38]. Nevertheless, the effluent COD concentration in Period 3 was below the regulatory limit imposed for the discharge of production activities in the sewer (500 mg L\(^{-1}\)), although it is reasonable that higher OLR may determine the failure in complying the discharge limits by conventional activated sludge systems. Because of the lack of studies referred to citrus processing wastewater with aerobic biological processes, it was not possible to carry out a comprehensive comparison with other studies. Another possible explanation could be due to the accumulation of essential oils (EOs) as the OLR increased. Indeed, in recent studies, it was reported that aerobic degradation of the organic matter decreased as the EOs concentration increased because of their toxic effect exerted on the biomass [18,39]. Nevertheless, the COD removal obtained in this study was in good agreement with previous applications of aerobic activated sludge systems for the treatment of high strength wastewater from food industry [40,41]. These results confirmed that aerobic granular sludge is a suitable technology for the treatment of high strength wastewater.

Regarding the sludge settling properties, a significant SVI decrease from 250 mL gTSS\(^{-1}\) to about 50 mL gTSS\(^{-1}\) was observed in Period 1, probably due to the transition from a continuous feeding system to a SBR type. Indeed, the intermittent feeding conditions generate substrate concentration gradients that are more favorable to the growth of floc-forming bacteria than filamentous ones, with an important benefit on the settling properties of the activated sludge [42]. In Period 2, a slight worsening of the sludge settling properties was observed especially in the early stage when the SVI increased to about 100 mL gTSS\(^{-1}\), although it decreased to about 40 mL gTSS\(^{-1}\) in the following days, indicating excellent settling characteristics of the activated sludge, comparable with granular biomass systems [43]. Finally, in Period 3, a gradual worsening of the sludge settling properties was observed. Indeed, the SVI progressively increased during the entire period, at the end of which it was equal to about 150 mL gTSS\(^{-1}\), indicating the onset of the filamentous bulking dysfunction. The results obtained were consistent with those reported in other studies. In particular, Corsino et al. [38] observed that as the OLR increased there was a proportional increase in the abundance of filamentous bacteria. Similar
results were also obtained by Zema et al. [44], confirming that high OLR determined the onset of process instability, especially in terms of worsening of the sludge settling characteristics due to the overgrowth of filamentous bacteria as will be better discussed in the following paragraphs.

The $Y_{obs}$ increased proportionally to the OLR. In Period 1, the average value of the $Y_{obs}$ was equal to 0.26 gTSS gCOD$^{-1}$ and the same increased to 0.53 gTSS gCOD$^{-1}$ and 0.72 gTSS gCOD$^{-1}$ in Period 2 and Period 3, respectively. The increasing value of $Y_{obs}$ observed in the three periods was due to the increase of the F/M from 0.22 kgCOD kgTSS$^{-1}$d$^{-1}$ (Period 1) to 0.68 kgCOD kgTSS$^{-1}$d$^{-1}$ (Period 3), which led to a significant increase in cell synthesis phenomena. As a result, the SRT decreased from about 22 days in the Period 1 to 8.8 days and 5 days in the Period 2 and Period 3, respectively.

Overall, it was observed that as the OLR increased, there was a worsening tendency in the overall performances of the system, in terms of COD removal, sludge settling properties and excess sludge production. Consequently, higher values of the OLR might reduce the stability of the biological system, which could make unsustainable the biopolymers recovering from waste sludge in the long term given the simultaneous reduction of the process purification performances. Besides, high OLR represent a critical condition for conventional activated sludge systems [45], thus this parameter should be properly managed to avoid process dysfunctions. Otherwise, the application of advanced technologies for dealing higher load pollutants should be considered [46].

3.2 Metabolic kinetics of the enriched biomass

The kinetic parameters of heterotrophic bacteria performed on the enriched MMC of the SBR$_1$ are summarized in Table 2:

| Tab. 2 |

The maximum yield coefficient of the heterotrophic biomass ($Y_H$) increased proportionally to the OLR, consistently with what was observed in the previous paragraph with reference to the $Y_{obs}$. The minimum value was obtained in the Period 1, in which the $Y_H$ was equal to 0.53 kgTSS kgCOD$^{-1}$. 

whereas the maximum was obtained in the Period 3 where it was equal to 0.60 kgTSS kgCOD\(^{-1}\). This result indicated that high OLR were favorable to the growth of fast growing bacteria as suggested by recent literature studies [47,48].

The active fraction of heterotrophic biomass (f\(_{XH}\)) did not show a significant relationship with the OLR. Indeed, the highest value, equal to 29% of the VSS, was obtained in the Period 2, whereas in Period 1 and Period 3 it resulted equal to 15% and 28%, respectively.

The maximum COD depletion rate (v\(_{H,\text{max}}\)) showed a non-linear trend with the OLR, but consistent with the active fraction values. The lowest v\(_{H,\text{max}}\) value was observed in Period 1, resulting equal to 104 mgCOD gTSS\(^{-1}\) h\(^{-1}\), whereas the maximum was observed in Period 2 (274 mgCOD gTSS\(^{-1}\) h\(^{-1}\)). In Period 3, the v\(_{H,\text{max}}\) value was slightly lower than the previous period, being equal to 189.8 mgCOD gTSS\(^{-1}\) h\(^{-1}\). A recent study observed that the maximum COD depletion rate was not affected by the OLR, whereas it was driven by the SRT [49]. It should be stressed that in contrast to what was observed from Period 1 to Period 2 where there was a significant increase in both the active fraction and the v\(_{H,\text{max}}\), in Period 3, a significant decrease was observed only for the maximum COD depletion rate. The results obtained suggested that from Period 1 to Period 2, the greater availability of substrate determined a greater abundance of active biomass, demonstrating that high availability of rapidly biodegradable organic substrate was favorable to bacterial synthesis. Furthermore, from Period 2 to Period 3, the lower value of v\(_{H,\text{max}}\) while maintaining the same f\(_{XH}\), suggested the development of different bacterial strains characterized by a lower rate of COD removal.

According to the literature, a prominent kinetic parameter for assessing the effectiveness in the selection of the PHA-accumulating biomass is the ratio between the duration of the feast and famine phases [2]. The values obtained in this study were always lower than 0.20, with a minimum value observed in Period 2 (0.10±0.07), a maximum in Period 3 (0.16±0.03) and an intermediate value in Period 1 (0.14±0.05). Such values indicated the effective selection of PHA-accumulating biomass [50].
The observed kinetic parameters were on average higher than those obtained in other studies dealing with citrus wastewater reported in the literature [19]. On the other hand, the results were comparable with those obtained in a SBR type plant with granular biomass treating citrus wastewater [38]. By comparing the above results with those reported in other studies on PHA accumulation biomass, in was noted that the kinetic parameters were similar in these studies [51]. This confirmed the ability of the mixed culture biomass to robustly adapt to a shift of OLR [52]. Therefore, based on the above, it can be stated that the selection of a PHA-accumulating biomass in SBR reactors allows operating with process kinetics even greater than conventional continuous-flow systems.

3.3 Morphological characteristics of the activated sludge

The different operating conditions in the SBR₁ had important implications on the morphology of the activated sludge floc. Obviously, these changes could affect not only the PHA productivity, but also the settling characteristics of the activated sludge and the removal performance of COD. Figure 3 shows the microscopic images of the activated sludge in the three periods carried out on the fresh sample of the SBR₁ (left column) and on the samples of the SBR₁ (central column) and SBR₂ (right column) subjected to Sudan Black staining.

Fig. 3

The seed sludge was characterized by flocs with an average size of 170 µm having a weak, open and poorly thickened structure. Moreover, it was observed an abundant presence of filamentous bacteria (class 5 of abundance) forming inter-bridging connections between the flocs. Previous studies reported the abundance of filamentous bacteria in plants fed with wastewater containing high concentrations of short-chain volatile fatty acids [53,54]. The transition from the continuous flow-feeding regime of the full-scale plant from which the seed sludge was taken, to the discontinuous one of the SBR₁, resulted in a significant improvement of the activated sludge flocs structure in the Period
The flocs appeared denser and more compact, although slightly smaller (160 µm), due to a greater abundance of floc-forming bacteria. Furthermore, a lower abundance of filamentous bacteria (class 3) was observed, which were mostly internal to the flocs. The reduction in the abundance of filamentous bacteria was attributed to the transition from continuous to discontinuous feeding regime, which involved the implementation of a kinetic selection principle of the floc-forming bacteria [55]. In Period 2, the flocs size increased until reaching an average size of about 210 µm. Furthermore, the flocs had a much denser and compact structure than the previous period with a significant prevalence of floc-forming bacteria over the filamentous. Filamentous bacteria were mainly within the floc and did not have any effect on its structure as no bridging neither open-floc structure were observed. Sudan Black stains also showed a greater abundance of biopolymers inside the cells than the previous period.

In Period 3, the floc structure changed significantly. Indeed, the flocs, although characterized by an average size close to 200 µm, had an open, poorly dense structure, with a high abundance of filamentous bacteria (class 5). In this case, the filamentous bacteria caused the formation of flocs with open structure and inter-bridging connections between the flocs, as observed in the seed sludge. The decrease of the SRT and the increase of the F/M from Period 2 to Period 3 probably favored the overgrowth of filamentous bacteria that caused remarkable effects on the flocs morphology. This result was in good agreement with previous literature [56]. Nevertheless, abundant Sudan Black staining positive granules were observed within the cells of filamentous bacteria, indicating the ability of such bacteria in PHA accumulation [32,57]. The results discussed above demonstrated that the different operating conditions in terms of OLR determined significant variations in the morphology of the activated sludge. The results were also consistent with the settling characteristics of the sludge discussed in paragraph 3.1, highlighting how the preponderance of floc-forming bacteria in the Period 1 and Period 2 led to the achievement of very low SVI, whereas the overgrowth of filamentous bacteria in SBR1 in Period 3, caused a significant worsening of the activated sludge settling properties. Finally, the greater abundance of
filamentous bacteria in the Period 3 would be consistent with the metabolic kinetics discussed in section 3.2. Indeed, filamentous bacteria are characterized by a lower rate of organic carbon removal compared with floc-forming bacteria, thereby confirming that their overgrowth from Period 2 to Period 3 caused a decrease of the maximum COD depletion rate [32].

3.4 Microbial diversity in SBR$_1$

The MMC of the sludge samples taken at the end of each period from the enrichment reactor SBR$_1$ were analyzed through 16S rRNA gene amplicon sequencing (MiSeq) at the end of experimental Period 1 (P1) and Period 3 (P3) to evaluate the microbial community shift during the experiment. In total 20.592 and 29.999 reads were obtained from samples P1 and P3 respectively, which were clustered at 99% identity in 280 and 133 OTUs (Operational Taxonomic Units) respectively. Several attempts to extract high quality DNA from Period 2 samples failed, thus the community of Period 2 is missing. Alpha-diversity indexes based on OTUs at 99% identity reveal higher species richness (Taxa, S) and higher diversity (Shannon, H) in Period 1. The sample of Period 3 was characterized by reduced richness and diversity and higher dominance, suggesting that the microbial community of the enrichment reactor became more specialized over the time of treatment because of the higher OLR, as also confirmed by previous studies [58,59]. A recent study suggested that under high OLR the diversity of the bacterial community decreased because the increased VFA accumulation [60]. This could be the reason of the reduction of biodiversity observed in Period 3.

Diversity indexes values obtained in this study were consistent [58] or higher (Carvalho et al., 2014; Dionisi et al., 2006) if compared to other studies on PHA-producing MMCs. Although this result can be explained by the differences in the analytical methods used. Coats et al. [61] suggested that microbial communities grown on real wastewater, similar to the one used in this study, may be characterized by higher richness and diversity than MMCs cultivated on synthetic or selected substrates [62]. In particular, real wastewater could enrich for different PHA-producing species, making the MMC more adaptive to changes in operational conditions. Indeed, changes in the
operational conditions imposed by the SBR1 seem to have affected the microbial community composition, leading to the enrichment or the decrease of different taxa, and this is particularly evident at genus level.

Tab.3

The dominant phyla in both the P1 and P3 MMC were Proteobacteria and Bacteroidetes (Fig. 4). Few other phyla, Verrucomicrobia, Acidobacteria, Firmicutes and the TM7 division, had relative abundances >1% in at least one of the two samples (Fig. 4). Period 1 MMC was dominated by Betaproteobacteria (41.14%) and Bacteroidetes (27.51%). The most abundant families of Betaproteobacteria were Rhodocyclaceae, dominated by the genus Zoogloea (26.07%), and Comamonadaceae, comprising Pseudorhodoferax (7.88%) and Aquincola (1.11%). Bacteroidetes in P1 community comprised Saprospiraceae (11.13%) and Flavobacteriaceae, entirely made up of the genus Flavobacterium (7.58%). At genus level, Saprospiraceae were mainly unclassified, apart from Haliscomenobacter accounting for 3.17% of the community. Haliscomenobacter, a genus comprising only one isolated species so far, the filamentous H. hydrossis, may be involved in the hydrolysis of polysaccharides to gain energy and carbon for growth, while little is known on the role and ecophysiology of Saprospiraceae in activated sludge [63].

In Period 3, the community was enriched in the phylum Bacteroidetes (54.48%) that became dominant followed by Betaproteobacteria (34.25%). Within Bacteroidetes Flavobacterium (Flavobacteriaceae) and Runella (Cytophagaceae) were the main genera, accounting for 36.73% and 12.93% respectively of the P3 community. Verrucomicrobia were also less abundant in respect to P1. Betaproteobacteria in P3 were dominated by Pseudorhodoferax (25.03%, Comamonadaceae) and Azoarcus (6.58%, Rhodocyclaceae). Betaproteobacteria, which usually include the main denitrifiers in activated sludge systems, represent an abundant group in many wastewater treatment plants (Thomsen et al., 2007). In particular, the genus Azoarcus (Rhodocyclaceae), which was enriched in
P3 community, beyond having a role as denitrifier in wastewater treatment systems (Thomsen et al., 2007) was also previously found to dominate PHA producing communities (Carvalho et al., 2014). Bacteroidetes are frequently found in activated sludge treatment plants and they comprise highly specialized bacteria involved in polysaccharide degradation, protein hydrolysis and aminoacid consumption (Nielsen et al., 2009). Bacteroidetes were the dominant phylum during PHA accumulation in microbial communities fed with acetate and propionate as carbon source (Janarthanan et al., 2016). The genus *Flavobacterium* (Flavobacteriaceae), which is often found in mixed culture under feast/famine conditions for PHA production (Dionisi et al., 2005), and *Runella* (Cytophagaceae) comprise strains isolated from activated sludge performing enhanced biological phosphorus removal (Bernardet and Bowman, 2015; Ryu et al., 2006) although it is also responsible for sludge bulking [63].

Both P1 and P3 communities comprised known Poly (3-hydroxybutyrate) - accumulating genera (Figure 5) such as *Zooglea*, *Pseudorhodoferax*, *Aquincola* (Betaproteobacteria) and *Rhodobacter* (Alphaproteobacteria) (Unz, 2015; Bruland et al., 2009; Chen et al., 2013; Lechner et al., 2007; Monroy & Buitrón, 2020). The genera *Zooglea* and *Pseudorhodoferax* may be among the main PHA-producers in Period 1 and Period 3 communities, respectively. Moreover, *Zooglea*, the most abundant genus in Period 1, is also a known floc-forming microorganism, responsible for exopolymer production in the sludge (Unz, 2015).

Beyond the molecular taxonomic analysis, a polyhydroxybutyrate producing *Bacillus* sp. was isolated by dilution plating method on Nutrient Agar from the enrichment reactor SBR1 at the end of Period 3 (data not shown). This genus was not detected by metagenomics analysis and the phylum it belongs, Firmicutes, was scarcely abundant in the bacterial assemblage. This discrepancy can be explained by
the difficulties in extracting genomic DNA from spore-forming microorganisms but also because only a minor fraction of environmental bacteria can be isolated on laboratory media.

3.5 Results of biopolymers accumulation assays

The accumulation assays and the extraction of biopolymers were carried out at the end of each experimental period, once steady-state conditions were reached.

Figure 6 shows the results of the FTIR-ATR analysis carried out on the samples extracted in each period (Fig. 6a), the maximum biopolymers production at the end of the accumulation assays (Fig. 6b) and the theoretical productivity of PHA referred to the volume of wastewater treated (Fig. 6c).

FTIR-ATR measurements revealed the typical bands of (hydroxybutyrate) HB monomer, and any other co-monomers were observed (Fig. 6a). Therefore, PHB were found as the main PHA polymer in all the periods. This result was consistent with previous studies in which the wastewater fed into the accumulation reactor was characterized by a high concentration of acetate [2,20,64]. Furthermore, other authors observed that the production yield of polyhydroxyvalerate (PHV) was modest in plants operating with a OLR lower than 5 kgCOD m$^{-3}$d$^{-1}$, resulting in a ratio between the feast and famine phases lower than 0.20 [20]. Furthermore, other authors reported that if the pH in the biopolymer accumulation reactor is higher than 7.50, the production of PHV is significantly reduced [65]. These observations can justify the absence of PHV in the biopolymers extracted in this study, given that the conditions unfavorable to the synthesis of PHV were all achieved. FTIR spectra showed prominent peaks at 1726 cm$^{-1}$ and 1279 cm$^{-1}$ denoting carbonyl (C=O) and asymmetric C-O-C stretching vibration, respectively, characteristic for ester bonding found in PHB molecule. Other adsorption
bands obtained at 1383 cm\(^{-1}\), 1462 cm\(^{-1}\), 2959–2854 cm\(^{-1}\), and 3442 cm\(^{-1}\) denoted the -CH\(_3\), -CH\(_2\), -CH, and -OH groups, respectively. The absorption bands at 1138 cm\(^{-1}\) to 829 cm\(^{-1}\) were consigned to C-O and C-C stretching vibration that could be attained by amorphous phase of PHB. The FTIR-ATR peaks, obtained at different extraction period, were found to be almost identical, thus highlighting that the biopolymer chemical structure did not change significantly as a function of the experimental period.

The maximum PHA content per unit of dry weight is shown in Fig. 6b. The maximum PHA content in Period 1 was equal to 0.12 gPHA gTSS\(^{-1}\), whereas the same increased in Period 2 and Period 3 to 0.34 gPHA gTSS\(^{-1}\) and 0.23 gPHA gTSS\(^{-1}\), respectively. The results obtained were in line with those reported in other studies dealing with wastewater generated by food-industries [66,67]. This confirmed that PHA production by MMC appears consistently achievable using real wastewater from food industries enriched in VFA [67]. By applying the Equation 6, it was obtained that the maximum PHA productivity resulted equal to 0.08 gPHA L\(^{-1}\)h\(^{-1}\) (P2), 0.05 gPHA L\(^{-1}\)h\(^{-1}\) (P1) and 0.04 gPHA L\(^{-1}\)h\(^{-1}\) (P3), respectively. These results indicated that also the kinetics of PHA accumulation were affected by the operating conditions imposed in the enrichment reactor. A low PHA yield was observed both at the lowest and highest OLR. Indeed, the maximum PHA yield was obtained in Period 2 under intermediate OLR. The effect of OLR on PHA accumulation was widely studied. Several studies demonstrated that applying too high OLR increased the biomass production and reduced the selective pressure and hence the biopolymer production. For instance, in recent studies it was observed that the optimum OLR for the achievement of enriched MMC was 4.7 gCOD L\(^{-1}\)d\(^{-1}\) (tested OLR between 2.4 and 8.4 gCOD L\(^{-1}\)d\(^{-1}\)) [68] and 2.4 gCOD L\(^{-1}\)d\(^{-1}\) (tested OLR between 1.2 and 3.6 gCOD L\(^{-1}\)d\(^{-1}\)) [69]. The results obtained in this study confirmed that operating with low OLR increased the selective pressure to enrich the MMC with PHA-accumulating organisms.

Overall, the PHA productivity obtained in this study was lower compared with that reported in other studies. Indeed, in the study conducted by Conca et al. [2], it was observed that the PHA productivity was close to 0.22 gPHA L\(^{-1}\)h\(^{-1}\) and similar results were obtained by Morgan-Sagastume et al. [70]
treating fermented municipal wastewater containing acetic, propionic and valerate acid in different ratios (0.40 gPHA L⁻¹h⁻¹). A possible explanation to the above result could be due to the OLR applied in the accumulation reactor (SBR₂), which was about three times the one applied in the other studies [71]. Indeed, some authors showed that as the OLR applied in the biopolymer accumulation reactor increased, there may be a slowdown in the PHA accumulation kinetics or even a total bacterial inhibition [71]. In this respect, in a recent study it was observed a decrease of the PHA productivity at high OLR as a clear consequence of substrate inhibition rather than the result of culture selection [49]. Nevertheless, the PHA productivity was similar to that achieved in studies carried out with real industrial wastewaters (0.03-0.09 gPHA L⁻¹h⁻¹) [68,72]. This could be related to the presence of complex organic molecules that reduce the metabolic activity of PHA-accumulating organisms. Indeed, the presence of possible inhibiting substances in the citrus wastewater, such as essential oils, which could induce partial inhibition of bacterial biomass, cannot be completely neglected and should be better investigated in future studies [73]. As reported in the literature, to avoid PHA-accumulation inhibition, industrial waste feedstocks required pretreatment to remove recalcitrant or toxic components that could impair the process [24,74].

The theoretical overall PHA productivity was estimated considering the PHA content in the biomass and the daily production of the excess sludge. This value was then referred to the volume of daily wastewater treated (Fig. 5c). Based on the results obtained, it was noted that the minimum production of PHA occurred in Period 1 (0.12 kgPHA m⁻³), whereas the maximum was observed in Period 2 (0.77 kgPHA m⁻³). Lastly, in Period 3, the overall PHA productivity resulted approximately 0.66 kgPHA m⁻³. It should be noted that the lower PHA content observed in Period 3 was offset by a higher production of excess sludge. Indeed, in Period 3 the SRT was lower than the other periods. Thus, a lower SRT favored the selection of populations which are characterized by having higher maximum specific growth rates but lower storage rates [49]. Consequently, the growth rate of MMC that determines the excess of sludge production should be also considered in the overall assessment of the PHA productivity.
3.6 Organic carbon mass balances in accumulation assays

For each of the accumulation assays, COD mass balances were carried out to evaluate the conversion of the organic substrate into three main products: intracellular polymers (PHA), extracellular polymers (EPS) and new biomass. All these products were expressed in terms of COD using the respective conversion coefficients reported in the paragraph 2.5.4. The results obtained are reported in Table 4.

**Tab.4**

The maximum conversion yield of COD into PHA was observed in Period 2, where about 64% of the COD was converted into intracellular polymers, while in the Period 1 and Period 3 the yields were lower and equal to 38% and 56%, respectively. The results obtained were consistent with those obtained by other authors, who reported maximum PHA production yields equal to about 0.60 gCOD\textsubscript{PHA}/gCOD\textsuperscript{-1} [75–77], even in case of using agro-food wastewaters. Indeed, Gouveia et al. [78] obtained a maximum PHA production yield between 0.56-0.68 gCOD\textsubscript{PHA}/gCOD\textsuperscript{-1} using dairy wastewater, while Campanari et al. [79] have obtained similar yields (0.55 gCOD\textsubscript{PHA}/gCOD\textsuperscript{-1}) by treating wastewater from the olive mill industries.

The conversion of COD into EPS decreased from Period 1 to Period 3. Indeed, the production of EPS was equal to 0.16 gCOD/gCOD\textsuperscript{-1} in Period 1, 0.13 gCOD/gCOD\textsuperscript{-1} in Period 2 and, finally, 0.05 gCOD/gCOD\textsuperscript{-1} in Period 3. This result suggested the lower propensity of biomass to produce exopolymers as the OLR in the SBR\textsubscript{1} increased. It should also be noted that in Period 3 the presence of filamentous bacteria in SBR\textsubscript{2}, which have less capacity to produce extracellular polymers, significantly increased, whereas in Period 1 and Period 2 the greater prevalence of floc-forming bacteria led to a greater production of extracellular polymers [80]. Therefore, it is possible that the microbiological composition of the sludge influenced the conversion of COD in the various fractions mentioned.
above. As the abundance of filamentous bacteria increased, the fraction of COD converted into EPS decreased. Similar results were also obtained in a previous study, in which the authors observed that the organic carbon was mainly converted into EPS rather PHA if fast-growing bacteria prevailed in the MMC [81]. This was a consequence of the higher OLR in Period 3 that promoted the overgrowth of bacteria with a higher growth rate and lower PHA accumulation ability [68].

Regarding the production of new biomass, in all three cases, it was in the order of 5%, suggesting that the process conditions in the accumulation reactor were not favorable to microbial growth because of the lack of nutrients.

During the accumulation assays, a residual COD was observed at the end of each test, since only the most biodegradable fraction of the organic matter was used by bacteria, according to the assays operating conditions. The residual COD fraction observed in each test was also consistent with the COD removal performances observed in SBR1. The lowest residual COD was observed in Period 2 (20%), whereas this was greater in Period 1 (42%) and Period 3 (32%).

Based on the results obtained, it is possible to assess that the best operating condition in terms of PHA production was that of Period 2. Conversion of COD into EPS, on the other hand, was likely dependent on the ratio of floc-forming and filamentous bacteria in the activated sludge and it increased when the abundance of the former was higher. In this sense, previous studies demonstrated that under fully aerated conditions the COD conversion into EPS is a competitive reaction to that of intracellular biopolymers. In fact, the carbon source is divided between EPS and PHA synthesis pathways, thereby reducing the PHA yield [82]. The results obtained in this study demonstrated that the change in the operating conditions and the consequent modification of the MMC led to a different route for COD conversion into EPS or PHA.

3.7 Physical and thermal characteristics of the biopolymers

In Figure S1 the DSC thermograms and the viscosimetric molecular weight of the biopolymers extracted in each experimental period are reported.
DSC thermograms for the first heating scan of the extracted biopolymers are reported in Figure S1a. The melting temperature of the biopolymers was approximately 176°C independently of the experimental period. The relatively high melting enthalpy, of about 106 J g\(^{-1}\) for the sample of Period 1 and around 101 J g\(^{-1}\) for those referred to Period 2 and Period 3, suggested the highly crystalline nature of the extracted polymer which was calculated to be around 72.5 % (Period 1) and 69 % (Period 2 and Period 3). Similar values of melting temperature (170-177°C) and crystallinity (60-80 %) had been determined for other PHA previously [36,83,84]. The only difference between the thermal properties of the extracted polymers can be observed between the sample referred to Period 1 with those referred to Period 2 and Period 3, suggesting that the different operating conditions slightly affects the thermal properties of the polymer.

Molecular weight represents an important parameter, which determines suitability of a biopolymer for specific applications. Regardless of final application, molecular weight of recovered PHA should be sufficiently high. The molecular weight of biopolymers extracted during Period 1 was about 250 kDa (Fig. S1b) and it increased up to 417 KDa and 463 KDa in Period 2 and Period 3, respectively. This result was coherent with the slight decrease of crystallinity observed during the last two extractions since it is well known that generally a decrease of the polymer weight usually led to an increase of the crystallinity of a polymer.

3.8 General remarks and future developments

In this study, it was shown that changes in the operational conditions that can occur in wastewater plants characterized by seasonal fluctuations can cause a significant shift in the bacterial composition of the mixed microbial consortium and some PHA-producing species might prevail over others. Therefore, seasonal load fluctuations could affect both PHA yields and characteristics. Consequently, the amount and the quality of the extracted polymer could be different during the year and this could
limit their use especially for those applications that require a precise and constant standard quality over time. Overall, the use of a real wastewater enabled to obtain a rich and diverse MMC that was monitored by molecular methods throughout the three periods. This allowed enriching the MMC with different species of PHA-producing organisms that improved the process performances and making it more flexible from an operating point of view than a process carried out with a pure microbial culture.

Moreover, in view of integrating the PHA production into existing facilities of a wastewater treatment plant, it should be considered that the enrichment of the MMC occurs simultaneously with the treatment of the wastewater that is used as feedstock. Therefore, the choice of the operating conditions that enable an efficient enrichment of the MMC in the biopolymer-producing fraction should be made taking into consideration the purification performances and the compliance with the discharge limits imposed by regulations.

The existence of an optimal OLR value that allowed to simultaneously maximize the purification performance and the production of biopolymers suggested the need to operate with systems able to adjust the OLR according to seasonal qualitative-quantitative variations of the wastewater. Moreover, this study demonstrated that the operating condition also affected the quality of the biopolymers. In this light, further studies are necessary to enhance PHA production with MMCs, for example by implementing advanced biological processes (e.g., membrane bioreactor, granular sludge). Indeed, these could improve the biomass retention capacity into the biological reactor, increase the overall productivity because of the higher biomass concentration and achieve higher purification performances than conventional activated sludge systems at higher OLR.

4. Conclusions

The effects of OLR on the production of biopolymers obtainable from the simultaneous treatment of wastewater deriving from a citrus industry were evaluated in this study. The optimal OLR in terms of both COD removal performance (98%) and biopolymer production (0.38 gPHA gTSS⁻¹) was equal
to 2 kgCOD m$^{-3}$d$^{-1}$. For higher OLR, a gradual decrease was observed in terms of both purification performances and production of the biopolymers, although its quality increased as indicated by the lower degree of crystallinity. The results obtained demonstrated that the maximum production yields of biopolymers were comparable with those obtained in many other studies, although the productivity was strongly affected by the OLR. Nevertheless, it was demonstrated that microbial diversity of real sludge provides both enough degradation potential and PHA accumulating strains to fulfill integrated wastewater treatment and biopolymers production. The results obtained in this study demonstrated the potential feasibility of using citrus wastewater as a low-cost substrate for the synthesis of biopolymers, although the variability of the quality of this wastewater determined a different production yield of the biopolymers with different mechanical characteristics.

Based on the above considerations, further efforts should be devoted to the optimization of PHA production in WWTP subjected to seasonal fluctuation of the OLR. Monitoring the MMC by using high-throughput DNA sequencing allowed to identify PHA accumulating taxa that are enriched during the treatment. Further studies are needed to understand the effects of the process conditions to reach the possibility to optimize PHA production by modulating the MMC composition. Moreover, application of advanced biological-based technologies aimed at improving the selection of the MMC (e.g., membrane bioreactor, aerobic granular sludge) should be tested. This could be a topic of great interest for the scientific community because these systems operate with higher TSS concentration than CAS, thus the PHA productivity could significantly be increased.

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