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PRODUCTION OF BIOPOLYMERS FROM CITRUS INDUSTRY WASTE BY ADVANCED BIOLOGICAL TREATMENTS

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Infine, ma non per importanza, alla mia famiglia, ai miei genitori e a mio fratello per il loro incondizionato sostegno.

Ma che bello il silenzio dopo tutto questo rumore.

Abstract:

The water sector has recently modified the traditional management framework to contribute to the achievement of global sustainable development goals and move towards a circular economy concept (Yadav et al., 2022). To this end, recent European regulations are adopting measures to close the water cycle through a circular concept. Among these, EU Regulation 2020/741 (Regulation EU 2020/741, 2020) establishes minimum requirements for the safe use of reclaimed water intended for agricultural irrigation, which each Member State must transpose and implement before June 2023. Additionally, the management of sewage sludge is addressed by Directive 2018/851, in which the minimization and valorisation of biowaste are considered a priority (Kircher et al., 2023).

As a result, the reuse of domestic and industrial wastewater and the recovery of valuable resources from waste sludge, such as bioplastics, have been explored by researchers for different scenarios (Kehrein et al., 2020). Accordingly, wastewater treatment plants (WWTPs) are nowadays turning into water resource recovery facilities (WRRFs) with the aim of going beyond pollution removal by performing the recovery of energy (i.e., biomethane, hydrogen, heat), materials (i.e., cellulose, bioplastics, fertilizers), and water (Larriba et al., 2020).

Among these materials, microbial-origin polymers have gained increasing attention in recent years due to their biodegradability, non-toxicity, and eco-friendly production, which could partially replace the non-circular production model of petroleum-based plastics (Amorim de Carvalho et al., 2021). Biopolymers are synthesized by different types of bacteria capable of converting organic substrates into polymers outside the cells, such as extracellular polymeric substances (EPS) and alginate-like polymers, or in the form of intracellular granules such as polyhydroxyalkanoates (PHA) (Burniol-Figols et al., 2020).

PHA are biological polyesters used as a carbon source and energy storage by an extensive range of microbial genera when metabolic stress or nutrient limitation occurs (Liu et al., 2021). To date, the production of PHA at an industrial level is based on the use of pure bacterial cultures, which, although enabling high production yields, entails very high operating costs, making the overall production cost still not competitive compared to that of

petroleum-based plastics (5 €/kg vs. 0.5 €/kg) (Chen et al., 2020; Conca et al., 2020).

An environmentally sustainable process for PHA production involves the use of mixed microbial culture (MMC) and low-value feedstocks such as wastewater (Colombo et al., 2019). In addition, the use of MMCs does not require sterile conditions as in the case of pure microbial cultures. These factors allow for a significant reduction in costs during cultivation and enable better competition with production costs of oil-based plastics (Zhou et al., 2022).

The most common process for PHA production from wastewater by MMC involves a three-stage process: 1) acidogenic fermentation, in which complex substrates are transformed into volatile fatty acids (VFAs), 2) selection of PHA-accumulating bacteria, and 3) PHA accumulation by the selected cultures (Matos et al., 2021). The strategy typically employed for the selection of an MMC with high PHA accumulation potential involves alternating phases of abundance (feast) and scarcity (famine) of organic substrates, which induce microorganisms to drive organic matter towards metabolic pathways for storage rather than directly utilizing it for synthesis processes (Valentino et al., 2017).

PHA production is generally implemented as a side-stream process, thereby resulting in the necessity to implement new facilities within the WWTP (Estévez-Alonso et al., 2021). This clearly impacts the system's footprint and complexity. However, this route is often limited by the low carbon concentration and high nutrients (nitrogen and phosphorous) availability in domestic wastewater (Conca et al., 2020), as well as by process complexity in general. Wastewaters produced in the agri-food sector (e.g., olive mill, fish canning, citrus processing, etc.) are considered eligible low-cost feedstocks for PHA production because of the high organic matter content in soluble form and a relatively high carbon to nutrients ratio due to the lack of nitrogen and phosphorous (Estévez-Alonso et al., 2021). In this case, since feedstock fermentation is not necessarily required because the bacteria can directly utilize the soluble organic matter present in the wastewater, a two-stage process can be implemented (Argiz et al., 2022).

Consequently, this would limit the necessity to perform expensive feedstock pretreatments and reduce the entire process complexity. In this framework, citrus processing wastewaters could represent an eligible wastewater to be

used as organic feedstock for PHA production because of the high carbon/nitrogen ratio and the high concentration of soluble organic carbon.
Nevertheless, so far, another challenge remains in implementing PHA production in the mainstream of a WWTP, which is related to the enrichment stage. Indeed, the implementation of biomass enrichment in the mainstream of a WWTP would require that this process should occur simultaneously with the wastewater treatment process.
Thus, the enrichment stage should allow for obtaining an effective selection of PHA storing bacteria, while the effluent of this reactor must comply with the discharge limits imposed by environmental regulations. In recent years, researchers have made significant efforts to develop new strategies to optimize MMC enrichment to increase PHA production rates such as double growth limitation (Argiz et al., 2022) or the introduction of a settling phase following the feast phase (Argiz et al., 2020).
In all studies reported in the literature, these strategies were applied in sequencing batch reactors (SBR) operating with activated sludge (AS). However, conventional processes using AS are susceptible to process dysfunctions (e.g., bulking, foaming), especially under unbalanced growth conditions (e.g., high carbon-to-nitrogen ratio, high organic loadings), which are required for a successful enrichment of the MMC. Furthermore, the low biomass retention capacity of this system reduces achievable PHA productivity (Burniol-Figols et al., 2020).
The use of different biotechnologies such as membrane bioreactors (MBR) technology or aerobic granular sludge (AGS) could eliminate the problems associated with using SBR systems for PHA production while ensuring compliance with regulatory limits for water purification. Both AGS and MBR systems are well-suited for operating under more challenging process conditions than conventional activated sludge systems, as they overcome issues related to sludge settling and can tolerate higher organic loads. However, a comparison of these technologies for PHA production purposes is currently lacking in the literature.
In light of the above, the main objective of this thesis was to evaluate the feasibility of biopolymer production from citrus process wastewater treatment. After a review of the state of art on PHA production process and an explanation of materials and methods used, this thesis focuses on three

different experimentations with SBR, MBR, and AGS technologies to perform the enrichment stage of PHA production.

A fully aerobic feast/famine strategy was adopted to obtain enrichment of biomass with PHA-storing bacteria. All the systems were operated at different OLR equal to 1-2-3 kgCOD $m^{-3}d^{-1}$ in three respective experimental periods. These periods corresponded to the real average load conditions of a citrus wastewater treatment plant (potential of 12,500 PE) located in Palermo (Italy), representing the summer period (low load - Period 1), autumn/spring (medium load - Period 2), and winter (high load -Period 3), respectively. The first experiment, described in Chapter 3, focuses on the possibility of integrating the enrichment phase into the mainstream of a citrus industrial wastewater treatment plant using a conventional SBR process so that the results obtained could be used as a comparison for subsequent experiments. In addition, interesting aspects of the process were analysed such as the effect on sludge settling, the variation of the molecular weight of the polymer produced as a function of the OLR parameter of the enrichment reactor and the resulting shift and change of the microbial community and diversity. The second experiment, described in Chapter 4, aims to assess the possibility of using MBR technology in the mainstream process of a WWTP to recover PHA instead of a conventional process. MBR is a technology that allows for water recovery so, in addition to PHA recovery, the possibility of reusing reclaimed water has also been evaluated. In addition, the evolution of the membrane fouling phenomenon simultaneously with the biomass enrichment with PHA-storing bacteria was studied throughout the experiment.

The experiment in Chapter 5, on the other hand, focuses on the possibility of using aerobic granular sludge (AGS) for PHA production.

Chapter 6 discusses the experiment done in collaboration with Universidade NOVA de Lisboa, which was based on the study of the variations in the characteristics of the produced biopolymer as a result of changes in operating parameters of the SBR-type enrichment reactor. In Chapter 7, challenges and perspectives on the implementation of PHA production in the mainstream of an industrial WWTP are reported.

Finally, the main results obtained in the thesis work with the potentials and limitations of the applied technologies are summarized in Chapter 8. In particular, the enrichment MBR showed the better and stable carbon

removal performance, whereas the effluent quality of the enrichment SBR and enrichment AGS deteriorated at high OLR. Biomass enrichment with PHA-storing bacteria was successfully obtained in all the systems. The enrichment MBR improved the PHA productivity with increasing OLR (max 35% w/w), whereas the enrichment SBR reduced the PHA content (max 20% w/w) above an OLR threshold of 2 kgCOD $m^{-1}d^{-1}$. In contrast, in the enrichment AGS the increase of OLR resulted in a significant decrease in PHA productivity (max 14% w/w) and a concomitant increase of extracellular polymers (EPS) production (max 75% w/w). Results demonstrated that organic carbon was mainly driven towards the intracellular storage pathway in the enrichment SBR (max yield 51%) and enrichment MBR (max yield 61%), whereas additional stressors in enrichment AGS (e.g., hydraulic selection pressure, shear forces) induced bacteria to channel the COD into extracellular storage compounds (max yield 50%) necessary to maintain the granule structure. The results of the thesis indicated that full-aerobic feast/famine strategy was more suitable for flocculent sludge-based technologies, although biofilm-like systems could open new scenarios for other biopolymers recovery (e.g., EPS). Moreover, the enrichment MBR was the most suitable technology for the integration of PHA production in a mainstream industrial wastewater treatment plant, considering the greater process stability and the potential reclamation of the treated wastewater.

Keywords: agro-food wastewater, mixed microbial cultures, polyhydroxyalkanoate, SBR reactor, membrane bioreactor, aerobic granular sludge.

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Symbol	Unit	Description
BOD	[mg O_2 L^{-1}]	Biochemical Oxygen Demand
C	[mg L^{-1}]	Carbon
c	[mg L^{-1}]	Concentration of the polymer solution
COD	[mg O_2 L^{-1}]	Chemical Oxygen Demand
COD_d	[g]	Total mass of COD
COD_r	[g]	Mass of residual COD
DO	[mg L^{-1}]	Dissolved Oxygen
EPS_p	[g]	Mass of EPS produced
FR	[kg $m^{-2}s^{-1}$]	Fouling rate
f_{xH}	[%]	Active fraction of biomass
F/M	[kgCOD $kgTSS^{-1}$ d^{-1}]	Food to microorganisms
HRT	[d]	Hydraulic retention time
J_1	[m^3 m^{-2} s^{-1}]	Permeate flux
k	[gPHA $L^{-1}h^{-1}$]	Rate of PHA production
K	[m $s^{-1}Pa^{-1}$]	Membrane permeability
K	[dL g^{-1}]	Mark-Houwink constant for PHB-$CHCl_3$
M_v	[kDa]	Viscosimetric molecular weight
M_w	[Da]	Molecular weight
MLTSS	[gTSS L^{-1}]	Mixed Liquor Total Suspended Solid
N	[g L^{-1}]	Nitrogen
NH3-N	[mg NH_4 L^{-1}]	Ammonium nitrogen
OLR	[KgCOD $m^{-3}d^{-1}$]	Organic Loading Rate
P	[g L^{-1}]	Phosphorus
PHA_{tot}	[mgPHA $mgSST^{-1}$]	Last value of PHA content at the end of the accumulation assay
PHA_p	[g]	Mass of PHA produced
PHA(t)	[mgPHA $mgSST^{-1}$]	PHA content at a generic time
PN	[g L^{-1}]	Concentrations of proteins
PS	[g L^{-1}]	Concentrations of carbohydrates
$-q_{Horg}$	[CmolVFA $CmolX_A^{-1}$ h^{-1}]	Specific substrate uptake rates
q_{PHA}	[gPHA $L^{-1}h^{-1}$]	Maximum PHA production
q_{PHA}	[CmolPHA $CmolX_A^{-1}h^{-1}$]	Specific PHA accumulation rate
$-qPHA_{famine}$	[CmolPHA $CmolX_A^{-1}h^{-1}$]	Specific substrate consumption rate
R	[-]	Side chain

Symbol	Unit	Description
R_C	[m^{-1}]	Resistance due to cake deposition
R_m	[m^{-1}]	Resistance to filtration due to the membrane
R_{PB}	[m^{-1}]	Resistance due to pore blocking
R_T	[1 m^{-1}]	Total resistance to filtration
SRT	[d]	Sludge Retention Time
SVI	[ml mg^{-1}]	Sludge volume index
TMP	[Pa]	Transmembrane pressure
TN	[mgTN L^{-1}]	Total nitrogen
TP	[mgTP L^{-1}]	Total phosphorus
TSS	[g L^{-1}]	Total suspended solids
VSS	[g L^{-1}]	Volatile suspended solids
v_H	[mgCOD $gTSS^{-1}h^{-1}$]	Removal rate of organic carbon
$v_{H,max}$	[mgCOD $gTSS^{-1}h^{-1}$]	Maximum COD depletion rate
W_{poly}	[g]	Weight of the extracted polymer
X_A	[g L^{-1}]	Active biomass concentrations
X_A	[g] [Cmmol]	Active biomass
Y	[-]	Number of monomers that make up the chain
Y_H	[kgTSS $kgCOD^{-1}$]	Yield coefficient of the heterotrophic biomass
Y_H	[kgCOD $kgCOD^{-1}$]	Maximum growth rate
Y_{obs}	[gTSS $gCOD^{-1}$]	Jobs growth rate
$Y_{PHA/VFA}$	[CmolPHA $CmolVFA^{-1}$]	Storage yields
$Y_{X/PHA}$	[$CmolX_A$ $CmolPHA^{-1}$]	Growth yields on PHA during the famine phase
α	[-]	Mark-Houwink constant for PHB-$CHCl_3$
ΔH^0	[J g^{-1}]	Melting enthalpy
ΔH_m	[J g^{-1}]	Melting enthalpy of the samples
μ	[Pa s]	Permeate viscosity
η	[dL g^{-1}]	Intrinsic viscosity
η_{rel}	[Pa s]	Relative viscosity
η_{sp}	[Pa s]	Specific viscosity
χ	[%]	Degree of crystallinity

List of acronyms

Acronym	Description
ADF	Aerobic Dynamic Feeding
AS	Activated Sludge
MBR	Activated Sludge with Ultrafiltration Membrane /membrane bioreactor
SBR	Conventional Activated Sludge
AGS	Aerobic Granular Sludge
AN/AE	Anaerobic/Aerobic Enrichment
AUSB	Aerobic Upflow Sludge Blanket
C/N	Carbon to Nitrogen ratio
CAS	Conventional Activated Sludge
CDW	Cell dry weight CDW
CPW	Citrus PeelWaste
CW1	Concentrated Citrus Wastewater
CW1*	Concentrated Citrus Wastewater
CW2	Citrus Wastewater with nitrogen and phosphorous
CW2*	Citrus Wastewater with nitrogen and phosphorous
CW3	Citrus Fermented Wastewater
CW3*	Citrus Wastewater
CPWW	Citrus Processing Wastewater
DSC	Differential Scanning Calorimeter
EOs	Essential Oils
EPS	Extracellular Polymeric Substance
F/F	Feast/Famine ratio
FBR	Side-stream Fed-Batch reactor
FTIR-ATR	Fourier Transform Infrared Spectroscopy Attenuated Total Reflection
GAO	Glycogen-Accumulating Organisms
HDPE	High-Density PolyEthylene
HHx	3-hydroxyhexanoate
HPLC	High performance liquid chromatography
iMMB	Immersed Membrane BioReactor
LCL-PHA	Long Chain Length
MBR	Membrane BioReactors
MCL-PHA	Medium Chain Length

Acronym	Description
MCL-PHA	Medium Chain Length
OTUs	Operational Taxonomic Units
OUR	Oxygen Uptake Rate
PAO	Phosphate-Accumulating Organisms
PE	Polyethylene
PHA	Polyhydroxyalkanoates
PHB	Poly-3-hydroxybutyrate
PHMB	Polyhydroxymethylbutyrate /poly(3-hydroxybutyrate-co-3-hydroxy-2-methylbutyrate)
PHMV	Polyhydroxymethylvalerate / poly(3-hydroxybutyrate-co-3-hydroxy-4-methylvalerate)
Poly-P	Polyphosphate
PP	Polypropylene
PSD	Particle Size Distribution
P(3HB)	Poly-3-hydroxybutyrate
P(3HV)	Poly-3-hydroxy valerate
P(3HB-co-4HB)	Copolymers Poly(3-Hydroxybutyrate-co 3 Hydroxyhexanoate)
P(3HB-co-3HV)	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
P(3HB-co-3Hx)	Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)
SCEPPHAR	ShortCut Enhanced Phosphorous and PHA Recovery
SBR	Sequencing Batch Reactors
SBMBR	Sequencing Batch Membrane Bioreactor
SCL-PHA	Short Chain Length
SEC	Size exclusion chromatography
RIS	Resistance-in-series model
PB	Particulate Biodegradable carbon
PI	Particulate Inert carbon
SB	Soluble Biodegradable carbon
SI	Soluble Inert carbon
UASB	Upflow Anaerobic Sludge Blanket
UWWTD	Urban WasteWater Treatment Directive
VFA	Volatile Fatty Acids
WRRFs	Water Resource Recovery Facilities
WWBR	WasteWater BioRefinery
WWTPs	WasteWater Treatment Plants

Chapter 1: Introduction

Summary

Citrus process wastewater (CPWW) is generated from citrus transformation processes such as fruit washing, cleaning of equipment and machinery, as well as cooling, extraction of essential oils, and drying of peels. CPWW is characterized by a high variability of water flow rates (inter-annual, seasonal and weekly), significant qualitative variation in chemical and physical parameters.
Worldwide annual production of CPWW is estimated at 700 million cubic meters (Lucia et al., 2022). COD of citrus wastewater is generally high and ranges between 1,000 mg L^{-1} and 75,000 mg L^{-1} depending on the type of citrus processed (Zema et al., 2018; Garcia et al., 2019). In addition, 70% of the total COD in these effluents is from a readily biodegradable fraction (Corsino et al., 2018). At the same time, citrus wastewaters have a low concentration of nutrients (nitrogen and phosphorus) relative to the organic load, leading to an unbalanced COD/N/P ratio (C/N > 1000).
European regulations (Directive 91/271/EEC of 1991 and Regulation (EU) 2020/741 of 2020) are adopting measures to close the water cycle through a circular concept. Consequently, wastewater treatment plants (WWTPs) are evolving into resource recovery facilities (WRRFs) with the aim of surpassing pollution removal by recovering energy, materials, and water (Larriba et al., 2020).
Polyhydroxyalkanoates (PHA) represent potential products derived from the treatment of material streams with high organic matter content, such as the organic fraction of municipal solid waste and wastewaters. Microbial PHA production is based on the ability of particular bacterial strains to transform low molecular weight organic molecules into PHA in the form of intracellular granules (Coats et al., 2007). A prerequisite for the accumulation of these storage products is an abundance of organic matter and a deficiency of macronutrients such as nitrogen and phosphorous. In this context, CPWW lends itself to microbial valorisation for the recovery of biopolymers such as PHA.
The implementation of the right selection principles and the use of innovative biotechnologies are key aspects for successfully integrating the PHA production process with the concurrent treatment of wastewater produced by the citrus industry.

1.1 Citrus industry

Citrus fruits are among the most widespread crops in the world (Schimmenti et al., 2013), mainly in tropical and sub-tropical regions (Panwar et al., 2021). The global citrus fruit production was about 158.49 million tonnes in 2020. Asia accounted for 47.7% of this value, followed by Africa (43.7%), America (8.1%), Europe (0.4%) and Oceania (0.1%) (Suri et al., 2022). Orange, mandarin, grapefruit and lemon constitute about 98% of the industrialized citrus harvest (Satari & Karimi, 2018; Zheng et al., 2016; Mama et al., 2014). Oranges are the most cultivated fruit in the world and accounts for about 50-60% of the total citrus production (Satari and Karimi, 2018). On average, it is estimated that about 75% of citrus fruit is consumed as fresh fruit while the remaining 20% is used for industrial processing to produce commercial products such as juices, jams, and dehydrated products (Mahato et al., 2019). The main citrus processing countries worldwide are Brazil, United States of America, Mexico, Argentina, and Mainland China. Oranges are the most processed citrus fruits (accounting for about 80%), followed by tangerines, lemon/lime and grapefruit with values of 7.26%, 9.74% and 3.00%, respectively (FAO, 2017). Citrus processing processes primarily involve juice extraction and the production of value-added products like jams, candied fruit (Marín et al., 2007; Torquato et al., 2017; Ferreira-Leitão et al., 2010).

As a result of these processes, approximately 50-70% of the citrus mass ends up in waste streams. This percentage varies depending on the technology used and the type of treated citrus fruit. The waste generated is mostly composed of peels, pulps and seeds (Wikandari et al., 2014; Zema et al., 2019). Moreover, citrus fruits can be processed by chemical and food industries for the production of essential oils, flavouring compounds, dietary fibres, pectin and biofuels (Pourbafrani et al., 2010). Citrus processing includes various stages such as fruit harvesting, short-term storage, washing, sorting and grading, juice extraction and product preparation, heat treatment, packaging and storage (Zema et al., 2019). In general, processing technologies can be different and with variable levels of automation and their selection depends on many factors such as the type of citrus fruit or the desired product. An example of a citrus production chain diagram is shown in Fig. 1.1.

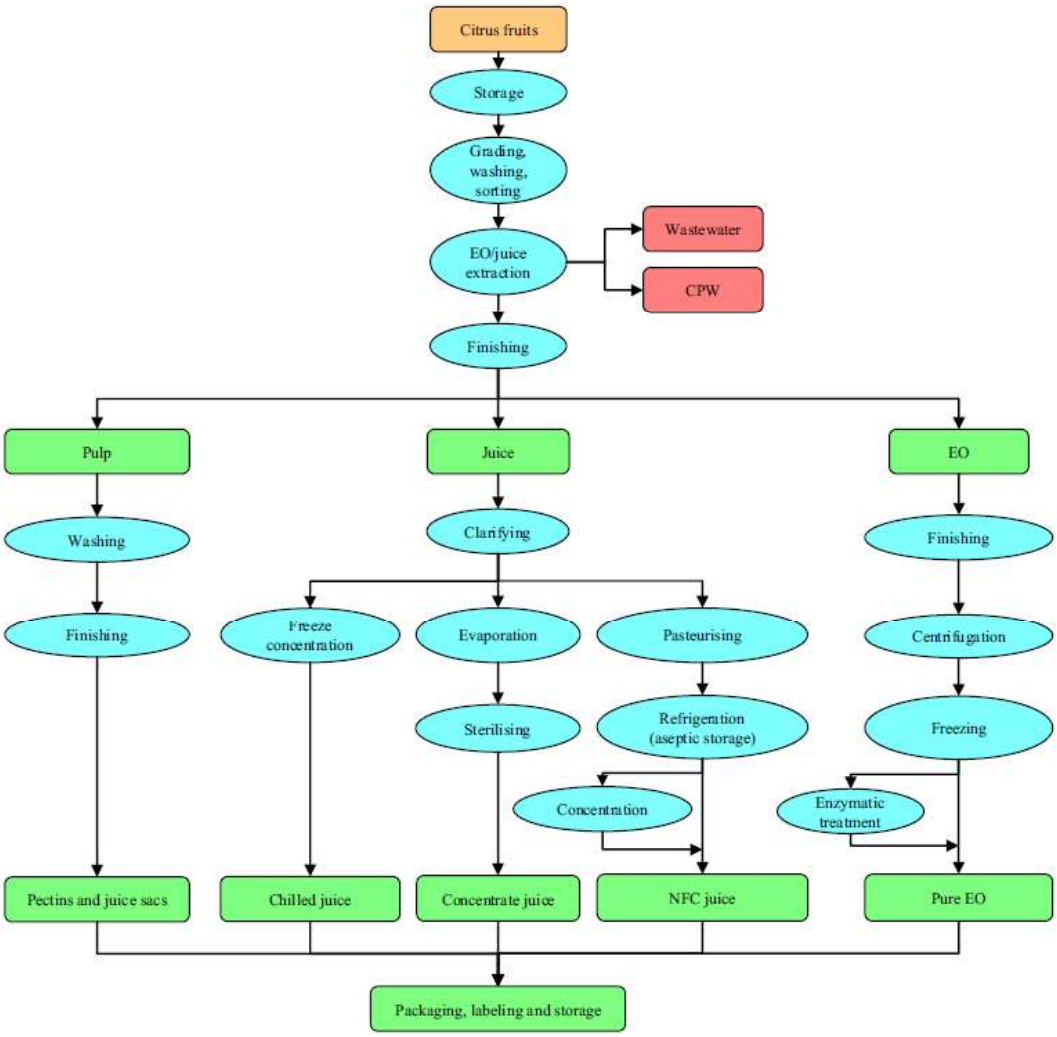


Figure 1.1: Scheme of the citrus processing chain (Zema et al., 2018)

Once citrus fruits are processed, they generate (Zema et al., 2019):

- Citrus-processing wastewater (CPWW): this results from fruit washing, cleaning of equipment and machinery, as well as cooling, extraction of essential oils, and drying of peels. This category also includes effluents from the production of citric acid and pectin, citrus molasses, and peel oil.
- Citrus peel waste (CPW): this refers to solid or semi-solid residues from industrial processing, including rotten fruit, peels, seeds, and portions of pulp.

Currently, the worldwide annual production of CPWW is estimated at 700 million cubic meters (Lucia et al., 2022).

1.2 Characteristics of citrus wastewater

Unlike urban wastewater, citrus wastewater is characterized by a high variability of water flow rates (inter-annual, seasonal and weekly), significant qualitative variation in chemical and physical parameters, as well as high acidity, high electrical conductivity, nutrient scarcity (nitrogen and phosphorus) and a high concentration of essential oils (Corsino et al., 2021; Calabrò et al., 2016; Tamburino et al., 2007). Consequently, it experiences considerable quali-quantitative variability.

1.2.1 Quantitative variability of citrus wastewater

Water consumption per unit of processed product varies widely depending on the type of processing used, the amount of citrus fruit processed and the overall water management in the plant (Zema et al., 2019; IASM-Breda, 1991; Di Giacomo and Calvarano, 1987). Consequently, the amount of wastewater that is produced increases with the use of freshwater flows in fruit processing. The quantity of CPWWs generated is highly variable as it depends on the characteristics of the fruit processing plant, such as the implemented technologies and the number of active processing lines at the same time and the type of fruit (Bozzano et al., 2021). However, some authors have estimated that the amount of CPWWs accounts for approximately 1-17 m^3 for each ton of fruits processed (Di Trapani et al., 2019; Calabrò et al., 2018;). Water volumes involved are considerable, as illustrated in Fig. 1.2.

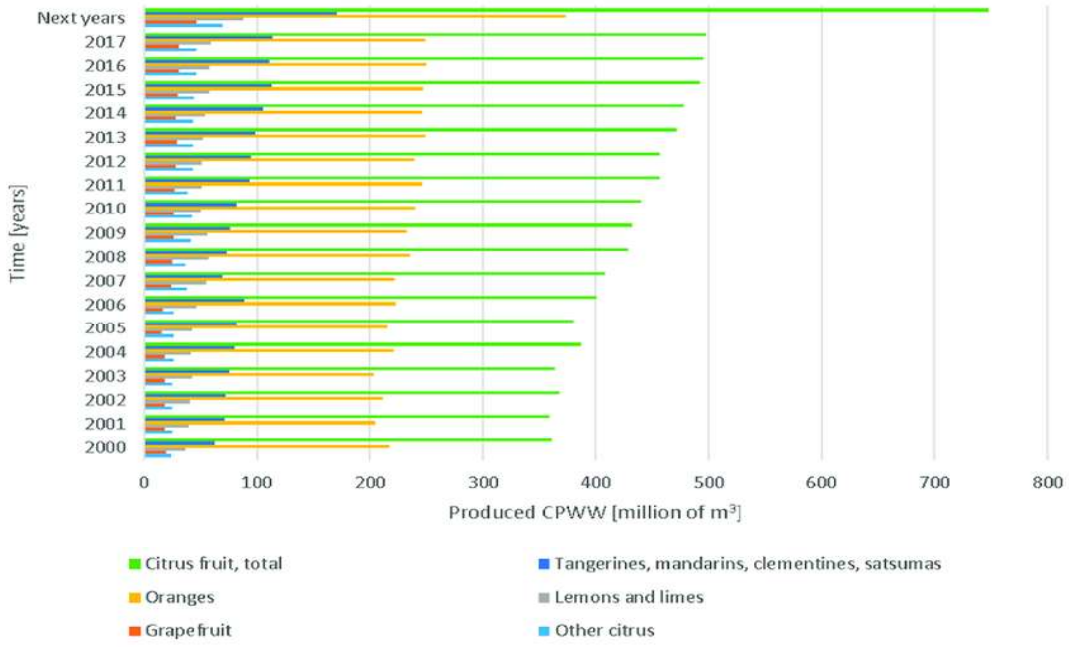


Figure 1.2: World's annual amounts of citrus processing wastewater (Zema et al., 2019)

As mentioned previously, the amount of processed citrus fruit is characterized by considerable inter-annual and intra-annual variability. Inter-annual variability of citrus fruit processing is linked to variations in fruit production from one year to the next, as the amount of citrus fruit processed depends each year on both agricultural production and the market trend for fresh products. Intra-annual variability can be differentiated into seasonal, monthly, weekly, and daily variations. Seasonal variability depends mainly on the operation of the plant and the production time of the product itself. In Mediterranean areas, more than 70% of citrus production for processing is concentrated between February and April with a peak generally in March, so processing industries mainly operate during this period (Zema et al., 2019; Zema et al., 2012).

Monthly variability depends not only on the quantity of citrus fruits processed, but also on the variability of water unit consumption, which in turn is significantly influenced by other factors (e.g. citrus quality and plant management) (Zema et al, 2019). Figure 1.3 shows a typical monthly trend of wastewater production in a Sicilian citrus fruit farm with a capacity of 40,000 tonnes per year of processed citrus fruit (Tamburino et al., 2007).

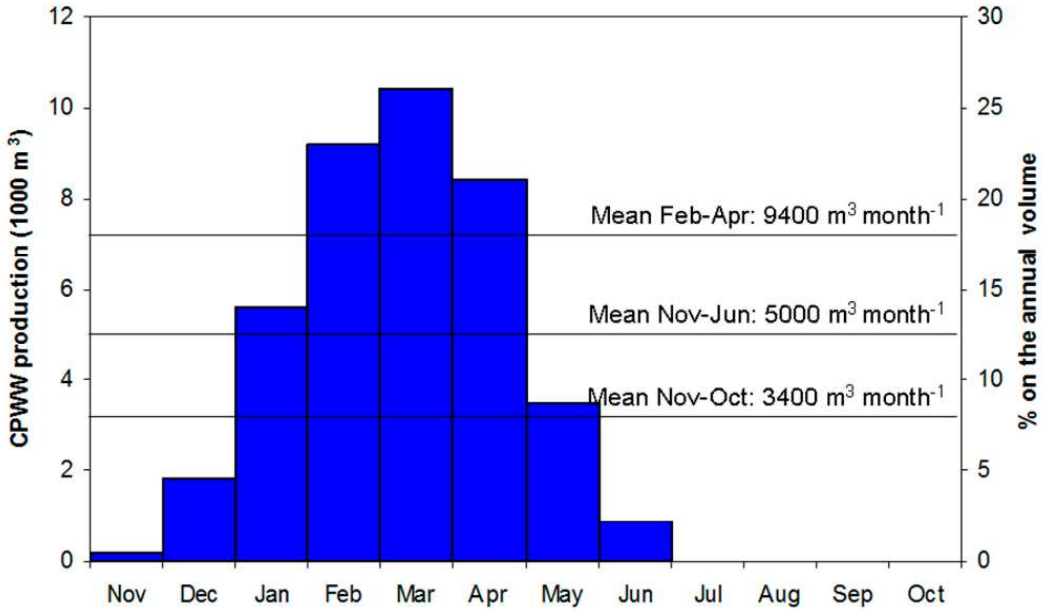


Figure 1.3: Typical monthly distribution of citrus processing wastewater (Tamburino et al., 2007)

Finally, wastewater production is also influenced by weekly and daily variability due to plant inactivity during the night and weekends (Fig. 1.4). The weekly variability of flow rates depends not only on the quantity of citrus fruits processed, but also on the variability of unit consumption, which has a significant degree of uncertainty due to the quality of the raw material processed, the operating mode of the plants and other factors related to plant management (Zema et al., 2019). This variability can be assessed with a weekly peak coefficient, given by the ratio between the average flow rate during the 12 working hours of the peak production day and the average

weekly flow rate, which generally fluctuates in the range of 3-4 (Tamburino et al., 2007).

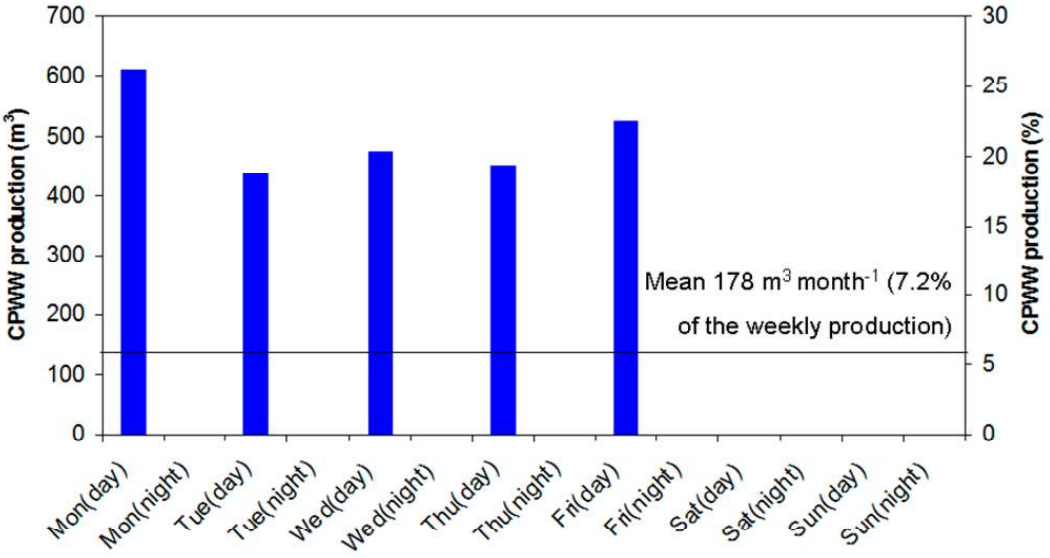


Figure 1.4: Typical daily distribution of citrus processing wastewater (Tamburino et al., 2007)

1.2.2 Qualitative variability of citrus wastewater

The citrus wastewater is also characterized by great variability in its physical-chemical properties even within the same working day. The causes of this variability are represented not only by the type and stage of ripeness of the processed fruit but also by the characteristics and mode of operation of the processing plants (Poiana et al., 2004). Factors affecting the variability of these characteristics (Zema et al., 2019; Torquato et al., 2017; Santos et al., 2010) include:

- the regular emptying schedule of tanks;
- the presence of essential oil extraction lines;
- the technological specifications of centrifuges, extractors, and filters employed in the transformation processes;
- the plant washing system utilizing alkaline solutions (sodium hydroxide and calcium hydroxide);
- the type of water cooling system;
- the degree of recirculation in essential oil extractors;
- the presence or absence of a peel dehydration system;

- the quantity of peel generated and designated for drying processes.

Citrus wastewater is characterized by a high content of organic matter, suspended solids, essential oils and a generally low pH (less than 3), as well as low nitrogen and phosphorus (Zema et al., 2012). The organic load of CPWW is generally high and shows great variability according to the different stages of the production process (Corsino et al., 2018). The COD of citrus wastewater is generally high and ranges between 1,000 mg L^{-1} and 75,000 mg L^{-1} depending on the type of citrus processed (Zema et al., 2018; Garcia et al., 2019). In addition, 70% of the total COD in these effluents is from a readily biodegradable fraction (Corsino et al., 2018). At the same time, citrus wastewaters have a low concentration of nutrients (nitrogen and phosphorus) relative to the organic load, leading to an unbalanced COD/N/P ratio (C/N > 1000). Normally, the bacterial biomass develops adequately for COD/N/P ratios in the order of 200/5/1 (Masotti, 2002), but the low nutrient concentration can cause dysfunctions in biological purification processes due to the lack of nutrients for the microorganisms responsible for organic matter biodegradation processes. This deficiency can be compensated for by the addition of external nutrients, generally supplied in the form of chemicals; however, the abundant use of these substances, dictated by the high COD concentrations in citrus wastewater effluents, can lead to the risk of exceeding the regulatory limits for the discharge of wastewater into water bodies.

Another characteristic of citrus wastewater is its low and highly variable pH values. Furthermore, in oxygen deficiency, fermentation of carbohydrates by acid-resistant bacterial strains takes place, with the production of organic acids that induce further reductions in pH (Indelicato et al., 1997). This leads to interferences with biological treatment processes, which in turn require pHs falling in the range of 6.0-8.5 with very gradual variations over time (Masotti, 2002; Metcalf and Eddy, 2006). Finally, very high concentrations of essential oils (of which d-limonene is the primary constituent) may disturb biological processes, which may even stop if their concentration exceeds 50 mg L^{-1} due to its bacteriostatic action (Ratcliff et al., 1900, Lane et., 1984).

Table 1.1: Main quality characteristics of citrus processing industry wastewater (adapted from Lucia et al., 2019)

Reference	pH	TSS	COD	Total N	Total P	Essential oils
	-	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}
Parish et al. (1986)	4.8	-	9000	0.30	-	2.80
Tamburino et al. (2007)	4.2-4.4	-	-	-	-	-
Koppar & Pullammanappallil (2013)	4.6-4.8	-	-	60	-	-
Zema et al., (2016)	5.1-5.5	-	6600	-	-	-
Torquato et al. (2017)	-	1000	19470	-	-	-
Corsino et al. (2018)	5.2	-	5464	12	3.5	-
Garcia et al. (2019)	-	-	75620	-	-	-
Ioppolo et al. (2020)	2.8	-	-	-	290	0.73
Rosas-Mendoza et al. (2020)	-	12162	38780	-	-	-

1.2.3 The current fate of citrus wastewater and the circular economy model

Intensive biological treatments are the most widely used for the treatment of citrus wastewater, including conventional activated sludge, trickling filters, and biofilters. Additionally, extensive natural treatments, such as aerated lagoons, or the direct use of citrus wastewater in agriculture are often considered (Zema et al., 2019). However, these types of systems are frequently ineffective as they are typically designed based on empirical criteria derived from municipal wastewater treatment, which differs significantly from citrus wastewater (low pH, high organic load, imbalance between C and N, presence of toxic compounds such as essential oils).
For instance, in the case of the activated sludge process, there are disadvantages mainly related to the high management costs and the achievement of steady-state conditions resulting from the seasonal variability that characterizes agro-food wastewater production (Milani, 2020). As mentioned in the previous section, citrus processing industries generate a substantial amount of by-products such as peels, seeds, pomace, and wastewater, whose improper handling or direct disposal is considered a

threat to the environment, human health and the economy (Panwar et al., 2019).

To date, the prevailing approach used in citrus wastewater management follows a linear economic model (Lucia et al., 2022). Nevertheless, in recent decades, environmental legislations have promoted the implementation of practices for the recovery of matter and energy from waste and wastewater (Esteban- Gutiérrez et al., 2018) introducing the concept of Wastewater BioRefinery - WWBR) (Pott et al., 2018). Consequently, WWTPs are turning into water resource recovery facilities (WRRFs) with the aim to go beyond pollution removal by performing recover of energy (i.e., biomethane, hydrogen, heat), materials (i.e., cellulose, bioplastics, fertilizers), and water (Larriba et al., 2020).

Therefore, today's trend is to move from a concept of linear economy, now considered obsolete and inefficient, to the new concepts of "bioeconomy" and "circular economy" (Panwar et al., 2021). Therefore, a shift from a "take-make-dispose-use-dispose" economy model, typically used in citrus wastewater management, to a circular economy model, must be encouraged. Indeed, in most cases, CPWW is discharged into the environment once it has been treated (Lucia et al., 2022). The food and agricultural industries generate an overwhelming amount of waste that can be used as raw materials for high-value-added products, promoting various possibilities for sustainable production (Ng et al., 2020). To ensure compliance with current environmental regulations and move closer to the circular economy model, it is essential to avoid unnecessary use of high-grade water while reuse of treated water and recovery of value-added resources from wastewater must be encouraged (Shrivastava et al., 2022). In this context, the agri-food industry is shifting to a circular economy model, minimizing waste of raw materials and co-products (Mak et al., 2020; Yadav et al., 2022). Agro-food wastes and wastewaters have high potential in terms of energy and nutrient recovery (Vaish et al., 2020), promoting process sustainability and facilitating the closing of environmental nutrient cycles in line with the circular bioeconomy perspective (Mak et al., 2020; Vaish et al., 2020). In this scenario, wastewater, whether of municipal or industrial origin, is considered a renewable resource from which energy, in the form of biogas, and matter, such as biopolymers, can be obtained as part of their treatment (Conca et al., 2020).

1.2.4 Legislative aspects of citrus wastewater

In general, wastewater is regulated by national and regional legislation within the laws of the European Community. The primary European laws concerning the treatment and reuse of industrial waste and wastewaters are Directive 91/271/EEC of 1991 (Urban Wastewater Treatment Directive - UWWTD) and Regulation (EU) 2020/741 of 2020. The first regulation addresses not only the collection, treatment, and discharge of urban wastewaters, but also the treatment and discharge of wastewaters from specific industrial sectors. This directive stipulates, on the one hand, that biodegradable industrial wastewaters from certain industrial sectors (listed in Annex III of this legislation) must be subject to appropriate requirements and special permission, while on the other hand, it establishes minimum requirements and necessary treatment levels for CPWW. The latter are identified in the list of Annex III of this legislation as "Manufacture of fruit and vegetable products". In particular, the competent authority or organism must issue a specific prior authorization allowing the discharge of industrial wastewaters into collecting systems or urban wastewaters treatment plants after specific pre-treatment, if necessary (Article 11). Although Article 11 must be taken as a reference, it is up to each Member State to define how to meet the requirements for CPWW. Every citrus processing industry must have a wastewater treatment plant that ensures safe disposal of the produced sludge and reducing the pollutant load in the wastewater by referring to specific limit values set by national or regional authorities (Lucia et al., 2022). Regulation (EU) 2020/741 promotes the reuse of wastewater in a circular economy perspective to ensure its safety for agricultural irrigation to guarantee a high level of protection for the environment, humans and animals. This regulation can only apply to biodegradable industrial wastewaters if it is discharged into the collecting system and treated in a municipal wastewaters plant. Therefore, it is necessary to refer to local regulations for the reuse of industrial wastewater.

1.2.5 Legislation in Italy

The changes in physical-chemical characteristics and the significant variability in volumes of CPWW impose considerable limitations on their disposal, driven by economic and environmental factors.
Many citrus industries, particularly those in urban areas, still discharge the effluents from their processing operation into the sewage system after treatments. Direct discharge into the sewerage system necessitates preliminary lamination of the effluents in storage tanks and adjustments to municipal treatment plants. Article 101 of Legislative Decree 152/2006, paragraph 7, letter c, addresses the assimilation of wastewater from businesses to domestic wastewater. Specifically, this applies to industrial wastewaters from companies engaged in agronomic utilization (letter b) and involved in activities related to the transformation or valorisation of agricultural production, included with a character of normality and functional complementarity in the company's production cycle and with processed raw materials coming prevalently from the cultivation activity of the land that is available for any reason. Consequently, citrus wastewater must comply with the limits outlined in table 3 of Annex 5 to Legislative Decree 152/2006 in the case of discharge into surface water bodies (Milani, 2020).
Article 74 of Legislative Decree 152/06 defines agronomic utilization as the management of livestock manure, vegetation residues from olives processing, and wastewaters from farms and small agri-food companies. This aims to utilize the nutrients and soil improvers for irrigation or fertirrigation of the land. Agronomic utilization of the aforementioned effluents can only be carried out in the cases and according to the procedures set out in Article 112 of Legislative Decree 152/2006, as amended, and Ministerial Decree of 25 February 2016, in which small agri-food companies are defined as those operating in the dairy, wine and fruit and vegetable sectors that produce quantities of wastewater not exceeding 4,000 m^3 $year^{-1}$ and quantities of nitrogen, contained in said water upstream of the storage phase, not exceeding 1,000 kg $year^{-1}$ (Milani, 2020).

1.3 Citrus wastewater as a substrate for PHA production: potentiality and challenges

Polyhydroxyalkanoates (PHA) represent potential products derived from treating material streams with high organic matter content, such as the organic fraction of municipal solid waste and wastewaters. These polymers have received increasing attention given their potential use as bio-based plastics.

Achieving environmental and economic sustainability involves using mixed microbial cultures, in other words, bacterial populations capable of utilizing plentiful sources of low-cost carbon waste for producing of biocompatible and biodegradable plastics (Nguyenhuynh et al., 2021).

However, the bioplastics market is expanding, including several industrial sectors, such as packaging, automotive and biomedical sectors, as well as application in contaminated soil remediation (Adeleye et al., 2020; Pérez et al., 2020).

Microbial PHA production is based on specific bacterial strains' ability to transform low molecular weight organic molecules into PHA in the form of intracellular granules (Coats et al., 2007).

A prerequisite for the accumulation of these storage products is an abundance of organic matter and a deficiency of macronutrients such as nitrogen and phosphorous.

Presently, PHA production on an industrial level relies on the use of pure cultures that are not economically competitive with petroleum-based plastics (Conca et al., 2020). An alternative to lower PHA production costs involves utilizing mixed microbial cultures (MMC), such as the biomass commonly found in activated sludge treatment plants, and wastewater as a low-cost carbon source (Adeleye et al., 2020; Sabapathy et al., 2020). Experiences reported in the literature pointed out that the production yields of PHAs achievable in municipal wastewater treatment plants are modest, due to the relatively low availability of organic carbon. The same studies suggested that waste-containing sugar and/or fatty acids might be the best feedstocks for PHA production (Vigneswari et al., 2021). Wastewaters produced in the agri-food sector (e.g., olive mill, fish-canning, citrus processing, etc.) are considered the eligible low-cost feedstocks for PHA production because of the high organic matter content in soluble form and a

relatively high carbon to nutrients ratio due to the lack of nitrogen and phosphorus (Estévez-Alonso et al., 2021; Elain et al., 2016). In this case, since feedstock fermentation is not necessarily required because the bacteria can directly utilize the soluble organic matter present in the wastewater, a two-stage process can be implemented (Argiz et al., 2022). Consequently, this would limit the need for expensive feedstock pretreatments and reduces the overall process complexity. While several applications of wastewaters from the oil, wine, and dairy industry are reported in the literature, citrus wastewaters have been less studied (Waller et al., 2012; Colombo et al., 2019). As mentioned in paragraph 1.2.3, such wastewaters are characterized by a high concentration of organic matter, a low nitrogen and phosphorus content and the presence of some substances, such as essential oils, which makes their treatment by biological means complex (Zema et al., 2018). However, their high availability of easily biodegradable carbon substrates and the imbalance in the nutrient ratio make these wastewaters suitable as low-cost substrate for PHA production. The seasonal variation in the treated flow rate, as well as in the concentration of chemical oxygen demand (COD) that differs according to the specificities of the production process, certainly has effects on the bacterial community that treats it, which evolves as a function of time-varying process conditions (Carvalho et al., 2014). As a result, the productivity of PHA and its chemical and mechanical properties may change over time.

1.4 Production of polyhydroxyalkanoates from microbial cultures

Polyhydroxyalkanoates (PHAs) were initially identified in 1888 by the microbiologist Martinus Willen Beijerinck. Several years later, Maurice Lemoigne discovered the mechanisms of production and accumulation of poly-3-hydroxybutyrate (PHB) in Bacillus megaterium under specific feeding conditions and external stress (Lee S.Y.,1996). The microbial species that synthesize PHAs are approximately 300 and can be grouped into more than 75 genera of Gram-positive and Gram-negative bacteria, such as Bacillus, Rhodococcus, Rhodospirillum, Pseudomonas, Alcaligenes/Ralstonia, Azotobacter, Rhizobium (Rehm, 2003; Reddy et al., 2003). To date, more than 150 different types of PHA or monomer units have been discovered (Mozejko-Ciesielska et al., 2016; Parlane et al., 2016;

Steinbüchel et al., 1995). All these monomers represent a highly promising alternative to conventional fossil-based plastics due to their biodegradability and wide range of physical and mechanical properties depending on their chemical composition. PHAs belong to the bioplastics family as they not only have the characteristic of being bio-based, but they are also biodegradable under aerobic and anaerobic conditions into compounds such as water and carbon dioxide (Shen et al., 2009). These characteristics make them particularly appealing from an environmental point of view since, unlike traditional plastics, they do not contribute to increasing landfill volumes and are not persistent if abandoned in the environment (Koller et al., 2009). According to European Bioplastics, a plastic material is defined as a bioplastic if it is bio-based, biodegradable or has both properties (European Bioplastics, 2018). The term "bio-based" indicates that the material has been produced totally or partially from biological sources. The term "biodegradable" indicates that the material is easily attacked by micro-organisms (usually bacteria, fungi or algae) normally present in the environment that can reduce the organic macromolecules constituting the compound into simpler molecules, to an inorganic stage. Examples of biomass used to produce bioplastics include maize, sugar cane or cellulose (Leal Filho et al., 2021). The main applications associated with these environmentally friendly microbial polymers range from disposable items to packaging for food, agricultural, construction, pharmaceutical, and biomedical uses (Reddy et al., 2003; Ivanov et al., 2014).

1.4.1 Characteristics of PHAs

Polyhydroxyalkanoates (PHAs) are aliphatic thermoplastic polyesters synthesized by both prokaryotic and eukaryotic microorganisms under specific growth conditions, such as the absence of micronutrients and an excess carbon, and serve as energy storage compounds (Raza et al., 2018). PHAs act as an energy and intracellular carbon storage in microorganisms under specific growth conditions. These conditions include the temporary absence of one or more micronutrients (nitrogen, sulphur, phosphorus, magnesium or oxygen) and an excess carbon. Under these conditions, cell metabolism is disrupted, and bacterial cells enter a state of stress.

Consequently, phosphorous in the form of polyphosphate (poly-P) and carbon in the form of PHA or glycogen are stored intracellularly as a carbon and energy reserve (Lee, 1996).
Indeed, in the absence of an extracellular carbon source, microorganisms can depolymerize PHA and use it as a new carbon source (Anderson et al., 1990). Specifically, PHA is stored in the form of granules, ranging in size from 0.2 - 0.7 μm, within the cytoplasm of bacterial cells, up to an amount that can reach 90% of the dry weight of the bacterial mass (Reddy et al., 2003). Furthermore, the number and size of these granules depend on the PHA-accumulating bacterial species. The composition of the granules consists of 97% PHA, 1-2% protein and 0.5% lipids (Koller et al., 2009). PHA are linear macromolecules whose chemical structure is depicted in Figure 1.5.

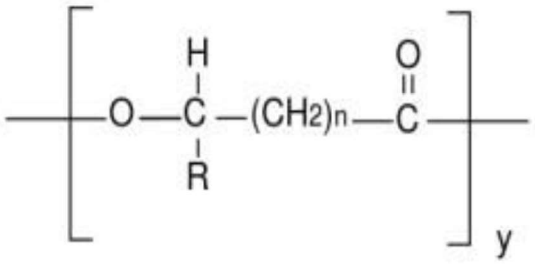
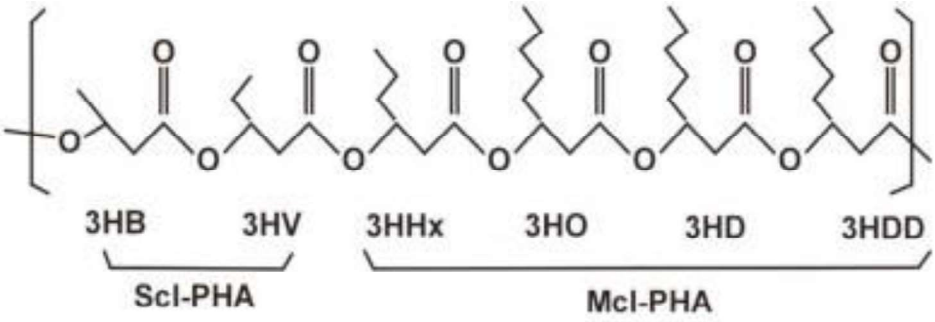


Figure 1.5: Monomeric units of polyhydroxyalkanoates (n= n° of CH_2 in the linear chain, y= n° of repetitive units, typically 100÷30000) (Raza et al.,2018)

In the monomer structure, the letter "n" indicates the number of CH_2 groups, "y" is the number of monomers that make up the chain (varying between 100 and 30.000), and "R" represent the side chain. The R-side group is an alkyl with between 1 and 13 carbon atoms. It can be branched or linear, saturated or unsaturated, and may contain various substituents. The monomeric composition of the polymer is influenced by several factors, including the microbial strain, the composition of the culture medium, the methods of polymer storage and warehousing, and the process parameters during biosynthesis (Lu et al., 2009, Keshavarz et al., 2010). The molecular weight M_w of the polymer increases with the degree of polymerization. In turn, the latter increases with the number of monomer units contained in the macromolecules of the polymer material (Li, 2017). The molecular weight of PHAs is comparable to that of plastics of petroleum origin. The latter

varies between 500,000 and over 1,000,000 Da depending on the degree of polymerization, which ranges between 105 and 107 (Kourmentza et al., 2009; Tan et al., 2017). In general, PHAs are classified into three classes based on the number of carbon atoms in the side alkyl group (R): SCL-PHA (short-chain-length), comprising fewer than 5 carbon atoms; MCL-PHA (medium-chain-length) which are made up of 6-14 C atoms; LCL-PHA (long-chain-length) consisting of 6-14 C atoms. The latter are, to date, less studied (Raza et al, 2018; Keshavarz et al., 2010; Valera, 2001). Although there are LCL-PHAs with more than 15 carbon atoms, but they have not yet been detected in nature (Koller et al., 2009). Figure 1.6 illustrates the general structure of PHAs and their classification.

Figure 1.6: Short- and medium-chain PHA structures (Raza et al., 2018)



The physicochemical and mechanical properties of PHAs are very similar to those of conventional petrochemical plastics that cannot be biodegraded naturally in the environment such as polypropylene (PP) and polyethylene (PE) (Madkour et al., 2013). The biodegradation of such polymers depends on their composition, degree of crystallinity, molecular weight, and environmental conditions (pH, temperature, microbial activity, moisture, colonized surface) (Bugnicourt et al., 2014). PHAs are more resistant to degradation from light and ultraviolet radiation compared to conventional plastics. Furthermore, the latter are insoluble in water, non-phytotoxic, possess a variable degree of crystallinity, present a high degree of polymerization, have antioxidant characteristics and are resistant to high temperatures (Prados & Maicas, 2016; Bugnicourt et al., 2014; Volova et al, 2013). SCL-PHA exhibit thermoplastic properties and they are rigid and brittle with a high degree of crystallinity in the range of 40-80% (Anjum et

al., 2016). These characteristics make them very similar to polypropylene (PP) and polyethylene (PE). MCL-PHAs are generally elastomers and exhibit low melting points, low glass transition temperatures, low crystallinity, and often have the consistency and structure of resins or latex (Koller et al., 2009). PHAs are categorized into homopolymers, such as P(3HB), P(3HV), PHMB, and PHMV, and copolymers like P(3HB-co-3HV), P(3HB-co-3Hx) or P(3HB-co-4HB). Poly-3-hydroxybutyrate P(3HB) is not very stress-resistant and has poor mechanical properties compared to traditional polymers (Sudesh et al., 2000; Anjum et al., 2016). Instead, the copolymers mentioned above are less rigid and brittle, thus improving the mechanical properties of P(3HB). Consequently, the latter could replace polypropylene, polystyrene, polyethylene terephthalate and high-density polyethylene (HDPE) (Anjum et al., 2016; Muhammadi et al., 2015; Sudesh et al., 2000). However, P(3HB) and the copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) P(3HB-co-3HV) are the most common due to their favorable properties and ease of production. This copolymer contains a monomeric hydroxy valerate (3HV) unit that improves mechanical properties such as flexibility, strength and toughness (Khatami et al., 2021; Nguyenhuynh et al., 2021). Furthermore, an increase in the 3HV fraction lowers the melting temperature of the polymer, facilitating the processing of the product (Volova et al., 2019; Reis et al., 2003; Dobroth et al., 2011) The copolymer (3PHB-co-3HV), due to its properties, is identified as a possible biodegradable substitute for polyolefins (Salehizadeh et al., 2004).

1.4.2 PHA biosynthesis

PHA synthesis can be achieved via biological pathways or chemical routes. In this context, PHAs with a higher molecular weight can easily be produced by a biological approach compared to chemical methods (Chen, 2010a). PHAs are produced through a series of enzymatic reactions using bacterial cultures. As mentioned in the previous section, the cultivation technique for PHA-accumulating bacteria involves the presence of high amounts of carbon and an external limitation, such as a lack of nitrogen (N), or an internal limitation of growth enzyme levels (Reddy et al., 2003; Repaske et

al., 1976). The presence of a carbon source is a prerequisite for the synthesis of microbial PHA (Behera et al., 2022). The amount of PHA that is consequently stored within the cell membrane is certainly influenced by the type of substrate used, the macronutrient missing, and the time of application of the restriction (Sudesh et al., 2000; Choi and Lee, 1997). As of now, eight main pathways for PHA biosynthesis have been identified (Mozejko-Ciesielska and Kiewisz, 2016). Metabolic pathway for PHA production depends significantly on the type of bacteria used and the substrate provided such as sugars or volatile fatty acids. Figure 1.7 illustrates the three main metabolic pathways for PHA production: fatty acid β-oxidation, de novo fatty acid synthesis, and carbohydrates biosynthesis (Luengo et al, 2003).

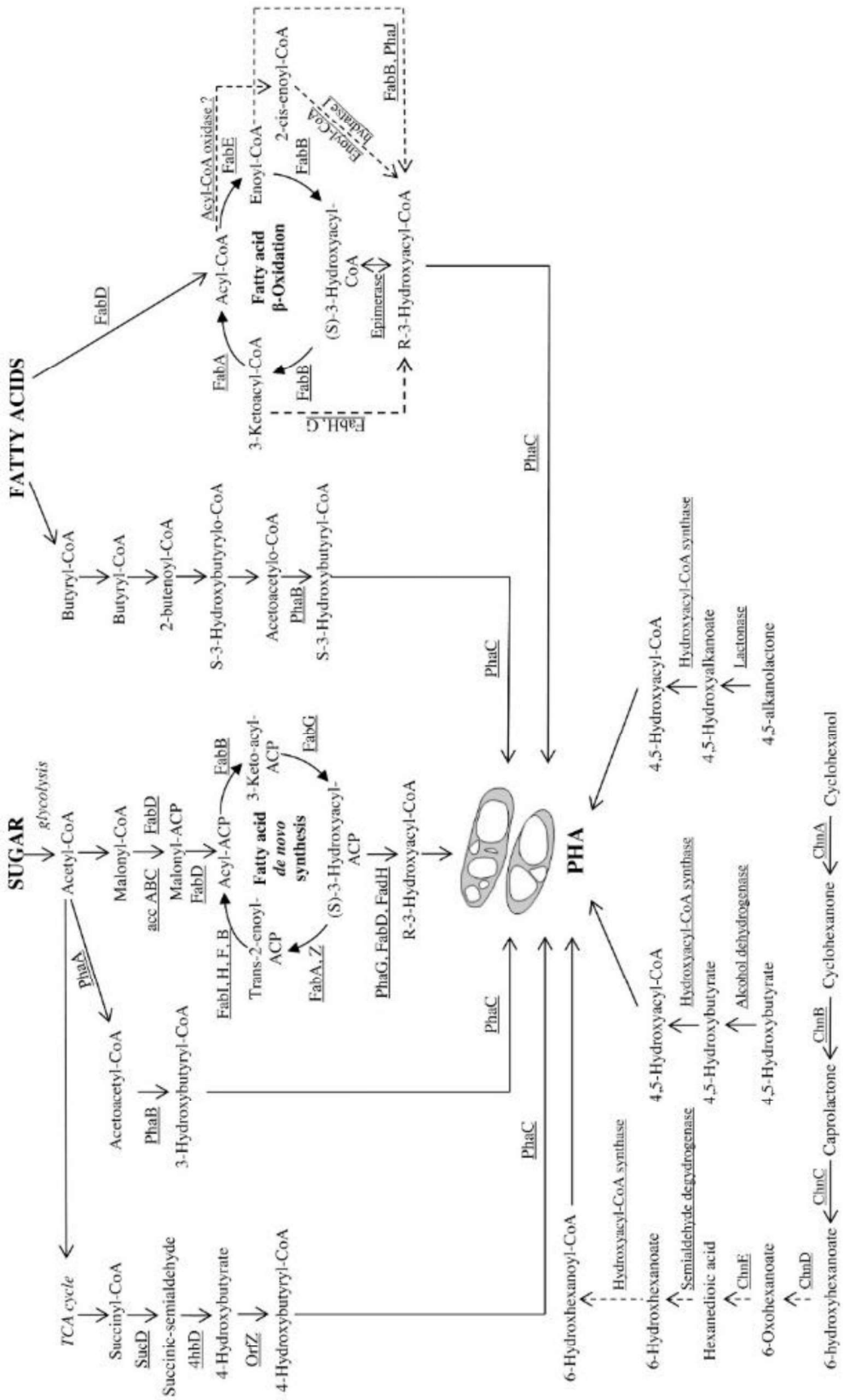


Figure 1.7: Metabolic pathways of PHA synthesis (Pradhan et al., 2020)

In the Novo fatty acid synthesis, the substrates used are glucose, gluconate, or acetate, whereas in the β-oxidation pathway, volatile fatty acids are used as carbon substrates (Huijberts et al., 1992). For each pathway, different enzymes and their respective genes come into play for the different pathways. For example, PhaA, PhaB and PhaC are some essential coding genes that synthesize β-keto thiolase, acetoacetyl-CoA reductase, and PHA synthase, respectively. Most microorganisms, such as *C. necator, Aeromonas hydrophila*, or *Pseudomonas stutzeri*, follow the common three-step pathway for PHA biosynthesis using acetyl CoA as the precursor. Acetyl CoA, the end product of carbohydrate metabolism, is further converted into acetoacetyl CoA, which is then converted into 3-hydroxybutyryl CoA, the substrate for PHA synthesis. Fatty acid β-oxidation and de novo fatty acid synthesis are the two other pathways for PHA synthesis apart from the above-mentioned pathway (Pradhan et al., 2020). Studies in the literature report that the highest amount of PHA produced was obtained using volatile fatty acids as substrates, and that SCL-PHA are the most suitable for PHA production by the bacterial pathway (Luengo et al., 2003; Inoue et al., 2016). Productivity using pure cultures has reached levels of 80-90% PHA per dry weight of bacterial cells. On the other hand, this process requires a sterile environment (reactors, incubators, fermenters, etc.) and a high economic burden to meet oxygen requirements (Villano et al., 2014; Ivanov et al., 2014). To reduce these costs, the scientific community is focusing its efforts on research into mixed microbial cultures (MMC).

1.5 PHA production from mixed microbial cultures (MMC)

Nowadays, the most common way to produce PHA involves the use of pure cultures of natural microbial strains, i.e. populations of microorganisms derived from a single microbial species. Although the PHA productivity achievable in such processes is very high (80-90% PHA per dry weight of bacterial cells), there are several problems associated with using such cultures (Volova et al., 2019; Pakalapati et al., 2018; Akaraonye et al., 2010; Villano et al., 2014; Ivanov et al., 2014) as reported below:

- the need to use pure substrates;

- the need for sterile environments and materials to avoid any form of contamination (reactors, incubators, fermenters, etc.);
- high oxygen requirements.

All these factors represent the main limits to the large-scale diffusion of PHA, because they entail a high cost of production of these materials, making PHA a product that is not competitive with non-biodegradable plastics synthesized from non-renewable sources (5 €/kg vs 0.5 €/kg) (Chen at al., 2020; Conca et al., 2020). In particular, the cost item with the highest weight is related to the pure carbon raw material used (30- 50 % of the total production cost), followed by the costs of extracting and purifying of the polymer (Mohandas et al., 2018; Hassan et al., 2013; Akaraonye et al., 2010). Due to high production costs, the use of mixed cultures (MMC), i.e. microbial populations of indefinite composition and composed of different types of bacterial species, is gaining popularity (Koller, 2020). The latter, in contrast to pure cultures, are not able to accumulate a large amount of PHA but have the advantage of being able to grow in non-sterile environments and the possibility of using a very wide range of complex and inexpensive organic substrates, including waste/sewage streams, without prior sterilization of the substrate. Based on this experimental evidence, many authors have focused their studies on the possibility of integrating PHA production into biological wastewaters and organic waste treatments (Kourmentza et al., 2017; Morgan-Sagastume, 2016a). This could indeed simplify the production process, reducing energy requirements and simplifying its control (Dias et al., 2006; Carvalho et al., 2014; Serafim et al., 2008). Furthermore, PHA production employing MMCs represents an opportunity for the biological treatment to recover organic carbon from raw wastewaters (Valentino et al., 2015a).

MMCs can allow the production of different types of copolymers using the different substrates probably due to the presence of different types of PHA-accumulating microorganisms that can use different production routes (Bengtsson et al., 2008, Dias et al., 2006; Laycock et al., 2013).

MMCs have a strong and complex extracellular biomass matrix, are resistant to cell hydrolysis, low cell fragility and are also resistant to chlorinated compounds (Patel et al., 2009, Samorì et al., 2015, Majone et al., 2017). All

these characteristics make the extraction process of PHAs in MMCs more difficult than in pure cultures (Mannina et al., 2020).
Furthermore, in MMC processes, unstable PHA structures and inconsistent PHA molecular weights are often obtained due to bacterial heterogeneity and the presence of different absorbed carbon components, resulting in the traversal of multiple metabolic pathways of PHA synthesis during discontinuous batch processes (Chen et al., 2017; Mozejko-Ciesielska et al., 2016; Chen et al., 2015). Recent experience shows that the production yields of PHAs achievable in municipal wastewaters treatment plants are modest, with values of around 0.32 gCOD PHA $gCOD^{-1}$ (Conca et al., 2020). Mainly, this is due to the low availability of organic carbon, in the order of a few hundred milligrams per liter, and the concomitant presence of nitrogen and phosphorus, which limits the ability of bacterial biomass to synthesize biopolymers (Burniol-Figols et al., 2020). However, PHA production via MMC is promising PHA contents of 74% by weight have already been reported with synthetic substrate (Lo Presti et al., 2019) and 75% by weight with real substrate, such as fermented molasses (Albuquerque et al., 2010a). Although the yield of MMCs in terms of production and PHA content is comparable with that of pure cultures, such systems lack PHA productivity. This parameter is very important to make PHA production in full-scale systems economically viable; however, the values for MMC are in the range of 0.236 to 0.41 g PHA L^{-1} h versus 1.38 g L^{-1} h^{-1} for pure cultures (Fauzi et al., 2019; Volova et al., 2019; Burniol-Figols et al., 2018).

1.6 PHA production process

The process layouts that can be used to produce PHA from MMC depend essentially on the type of substrate being processed. If a complex organic carbon source is present, pre-treatment is required to obtain simpler products such as volatile fatty acids (VFAs) with shorter carbon chains and consequently the three-step process is used. The latter consists of three independent steps: acidogenic fermentation, PHA-accumulating biomass enrichment and the accumulation step (Kourmentza et al., 2017). These steps must be separated from each other because the optimal conditions differ from step to step in the process (Reis et al., 2011). Multiple studies have been based on a three-step process for PHA production from MMC

from complex carbon sources such as waste oils, cheese whey, fruit-canned juices, oil mill effluents, and lignocellulose-based hydrolysate (Fauzi et al., 2018; Oliveira et al., 2017; Amulya et al., 2015; Gobi et al., 2014). Specifically, it has been shown that agricultural wastes, wastewater effluents discharged from crude oil, food, pulp and paper industries are particularly suitable for PHA production (Jiang et al., 2012). The main products of the acidogenic fermentation of the organic substrate are H_2, CO_2 and VFA, which are the precursors of PHA (Dahiya et al., 2015). Due to the various biochemical reactions resulting from the simultaneous presence of different microorganisms, several products such as butanol, acetate, propionate, ethanol, and butyrate can be obtained (Zhou et al., 2018; Ghimire et al., 2015).

To optimize the production of VFAs and minimize the phenomena of methanogenesis, and thus the formation of biogas during the fermentation process, it is essential to manipulate operational parameters such as pH, temperature, holding time or organic loading rate (Zhou et al., 2018; Khan et al., 2016; Ghimire et al., 2015; Wang et al., 2014; Silva et al., 2013). In the case of agricultural wastes such as sugar bagasse, wheat/rice straw, corn stover, oil palm empty fruit bunches which are rich in cellulosic material, the anaerobic fermentation process can be replaced by a hydrolysis treatment. In this way, the starting substrate can be broken down into sugars such as glucose, fructose, xylose, mannose, and arabinose (Nguyenhuynh et al., 2021). PHA production using MMC is based on the natural principles of selection and competition of microorganisms with a PHA storage capacity against microorganisms that are not able to store PHA (Kosseva and Rusbandi, 2018). The biomass enrichment phase consists of selecting bacteria capable of accumulating a large amount of PHA in a stable mode and with a high growth rate (Reis et al., 2011). Finally, the PHA accumulation phase aims to maximize the intracellular accumulation of the biopolymer to ensure a high PHA content in microbial cells. When the microbial cells reach maximum PHA content, they are collected and sent to the final extraction processes (Serafim et al., 2008; Kourmentza et al., 2017). Figure 1.8 shows the PHA production process in three steps.

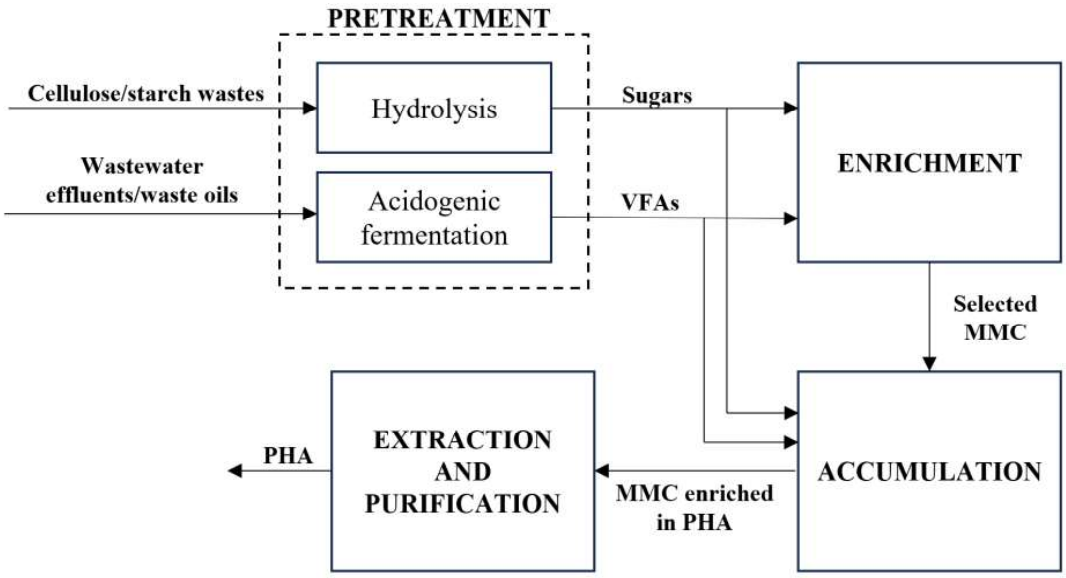


Figure 1.8: PHA production process in three steps.

The two-step process includes only the enrichment and accumulation steps and is generally used when employing glycerol-based substrates or synthetic mixtures of PHAs, which do not require pretreatment (Nguyenhuynh et al., 2021). The advantage of using this process lies in the simplification of the production and a reduction in the cost of PHA production.

1.6.1 Fermentation

In the literature, several studies have utilized various cost-free carbon sources to produce PHAs from mixed microbial cultures. Examples include tomato canning wastewaters (Liu et al., 2008), paper mill wastewater (Bengtsson et al., 2008), mill waste (Beccari et al., 2009), sugar cane molasses (Albuquerque et al., 2010b), wastewaters (Pozo et al., 2011), and food waste (Reddy et al., 2012). The preliminary phase of acidogenic fermentation becomes necessary when dealing with complex substrates to convert them into simpler organic substances that are more suitable for PHA production, i.e., VFAs (Reis et al., 2011). The organic acids obtained during acidogenic fermentation, as mentioned above, influence the monomeric composition of the PHAs produced, and this affects the physical and mechanical properties of the polymers produced (Albuquerque et al., 2011; Bengtsson et al., 2008).

1.6.2 Enrichment

The enrichment phase of MMCs takes place by exploiting the natural principles of selection and competition among microorganisms with a PHA-storage capacity and those unable to accumulate PHA (Kosseva & Rusbandi, 2018). Different strategies can be used to select PHA-accumulating biomass; the most widely used are anaerobic/aerobic enrichment (AN/AE) and the Aerobic Dynamic Feeding (ADF) or feast/famine (F/F) regime (Koller, 2020; Fauzi et al., 2019; Chen et al., 2017; Gobi et al., 2014).

In AN/AE, microorganisms are selected through alternating anaerobic and aerobic conditions. The first stage of the AN/AE process takes place under anaerobic conditions and in the presence of a carbon source. Consequently, in the absence of an external electron acceptor such as oxygen, nitrate or nitrite, phosphate-accumulating organisms (PAO) and glycogen-accumulating organisms (GAO) compete and accumulate PHA. In the subsequent aerobic or anoxic phase, however, the consumption of PHA by bacteria for growth or maintenance is observed (Koller, 2020). In the ADF, the selection of microorganisms is performed by alternating short periods of substrate excess (feast) and long periods of deficiency (famine) of a carbon source (Mannina et al, 2020; Kourmentza et al., 2017; Reis et al., 2011). PHA-accumulating bacteria store excess substrate available as a carbon and energy reserve during the feast phase and use the accumulated PHA to survive during the famine phase. In this way, selective pressure is applied to the MMCs that are enriched by PHA-accumulating bacteria. The latter have a competitive advantage over the rest of the microbial population that instead goes into bacterial death (Albuquerque et al., 2010; Dias et al., 2006; Salehizadeh & Van Loosdrecht, 2004; Majone et al., 1996). ADF is commonly conducted in intermittently fed sequencing batch reactors (SBRs). The operation of such reactors is divided into feeding, reaction, settling and discharge. The feast/famine ratio (F/F) is a crucial parameter influencing the performance of microbial selection and it depends on the OLR, the COD of the influent substrate, and the type of substrate used (Paul et al., 2012, Hao et al., 2018). The F/F ratio is identified as the main parameter to be controlled during the enrichment process (Albuquerque et al., 2010a). Maintaining a low value of F/F ratio allows to select

microorganisms that are most capable of storing PHA and it ensures their physiological adaptation towards PHA synthesis in the feast phase (Villano et al., 2014). In particular, the duration of the feast must be sufficient for the complete depletion of the substrate, while the duration of the famine phase must be long enough to allow significant consumption of the previously stored PHA. Otherwise, the microorganisms will be better fit to grow when supplied with an external carbon source and will accumulate less PHA (Reis et al, 2011). In general, to obtain a good selection of PHA-accumulating biomass, F/F ratio should be at approximately 1/4. After several cycles, a mixed culture rich in PHA-accumulating bacteria is obtained, ready to be sent to the following accumulation step (Fauzi et al., 2019; Serafim et al., 2008; Dionisi et al., 2005).

However, when dealing with complex substrates, limiting the duration of the feast phase may be challenging due to the potentially low carbon source uptake rate (Albuquerque et al. 2010b). If the value of the F/F ratio is below 0.3-0.4, the culture will show stable performance in terms of yield of PHA to carbon substrate, volumetric productivity, PHA content, polymer composition and culture stability (Nguyenhuynh et al., 2021; Reis et al.,2011).

The maximum content of PHA achievable reported in the literature is around 50% with the AN/AE selection process, which is lower than that attainable with ADF, which reaches up to 90% on cell dry weight (Coats et al., 2007, Rhu et al., 2003; Johnson et al., 2009; Jiang et al., 2011). However, the biomass selected with these enrichment strategies is characterized by low PHA productivity, effectively hindering the industrial production of this polymer.

The F/F strategy can be implemented in two different ways depending on when certain nutrients, such as nitrogen and phosphorous, are made available. Specifically, if the supply of nitrogen occurs simultaneously with the supply of carbon, it is called “coupled C and N supply”. In this scenario, PHA-accumulating bacteria utilise part of the available external carbon for bacterial growth, and they reserve another part in the form of PHA. The disadvantage of this strategy is that low productivity is achieved as cell growth is limited. Generally, OLR values above 2 gCOD L^{-1} d^{-1}, or even lower, cause problems in the efficiency and stability of the enrichment process (Nguyenhuynh et al., 2021).

Additional selection strategies, such as double growth limitation, extended cultivation and withdrawal strategy can be used to increase the overall productivity of PHA.

The second viable way is called “uncoupled C and N supply” strategy or “double-growth limitation”, that aims to optimize both MMC selection and cell growth (Oliveira et al., 2017; Silva et al., 2017). In this case, only carbon is made available at the beginning of the feast phase, resulting in its complete storage in the form of PHA within the bacterial cells. This creates a more adverse environment for bacteria that do not accumulate PHA, resulting in improved enrichment. In a comparative study by Silva, et al. (2017), the “uncoupled C and N supply” strategy showed several advantages, such as higher biomass concentration due to increased cell growth, higher PHA yield, and increased productivity. Furthermore, this strategy allowed operating with higher OLR values (8.5 gCOD $L^{-1}d^{-1}$) without causing instability in the system (Oliveira et al., 2017). The "uncoupled C and N supply" strategy can be used in the case of nutrient-free raw materials, such as some industrial wastewaters (Fermented cheese whey) (Oliveira et al., 2017; Pakalapati et al., 2018).

Alternatively, “extended cultivation” is another strategy to achieve higher cell growth, as shown by Huang et al. (2017). This strategy consists of including between the enrichment phase and the accumulation phase a period of bacterial growth (cultivation) in the same enrichment reactor. In this study, cultivation lasted 10 days. Cultivation is achieved by supplying C during the feast phase and then N during the famine phase in the same SBR enrichment reactor. Even with this strategy, improvements were observed in terms of PHA production (Huang et al., 2017). The withdrawal strategy consists of removing excess biomass from the enrichment reactor at the end of the feast phase (Lorini et. al., 2022; Silva et. al, 2017; Campanari et al., 2017). This approach reduces the length of the accumulation assay, and thus operational costs since a part of PHA has already been accumulated within the bacterial cells. However, this strategy could lead to a decrease in the biomass concentration in the enrichment reactor since, compared to the other strategies, a part of the biomass no longer participates in bacterial growth (Cruz et al., 2022).

1.6.3 Accumulation

In the accumulation phase, selected biomass is collected from the enrichment reactor and subjected to prolonged aerobic feast conditions in feed-batch mode with pulsed or continuous substrate feeding (Mannina et al., 2020). Accumulation is typically carried out under nutrient-deficient conditions to promote biochemical reactions favouring PHA storage. Consequently, under nutrient-rich conditions, the storage response will be lower due to cell growth, resulting in a PHA content that is lower than the maximum storage capacity of the microorganisms (Johnson et al., 2010). The feeding strategy used to carry out the accumulation assays plays an important role. The fed-batch modality is widely utilized as it allows for high PHA productivity values; however, there is a risk that the microorganisms will start to degrade the stored polymer at the end of the time between pulses. In addition, providing pulses with high substrate concentrations could cause bacterial inhibition, resulting in reduced PHA productivity (Albuquerque et al., 2007; Serafim et al., 2004). In contrast, using substrates with low carbon concentration can lead to a rapid increase in the working volume (Kourmentza et al., 2017). Finally, this feeding strategy is characterized by a fluctuation in productivity rates throughout the process. Initially, high values are observed, followed by a decrease over time as the substrate is consumed. In contrast, the continuous feed mode can overcome this problem (Albuquerque et al., 2011; Chen et al., 2013a, Montiel-Jarillo et al., 2017). In fact, while productivity is lower compared to accumulation assays performed in fed-batch, on the other hand, it assumes a constant value ensuring the stability of the accumulation assay (Koller, 2020). pH decreases in the presence of VFAs and increases vice versa, making it useful as an indicator to adjust substrate dosing (Johnson et al., 2009; Albuquerque et al., 2011; Chen et al., 2013b; Jiang et al., 2012). Another parameter of reference is DO as this tends to decrease in the presence of substrate due to the increase in biomass respiration rate at constant mixing and aeration (Valentino et al., 2015a; Valentino et al., 2015b).

1.7 Operational parameters governing culture selection in MMC

A crucial factor in ensuring high PHA production lies in the biomass enrichment process independently of the process configuration. Achieving a good selection of the PHA-accumulating microbial community requires selective pressures by manipulating the process parameters. The manipulation of operating conditions for effective selection of PHA-accumulating biomass has been extensively studied in the literature. In addition to the F/F ratio and the different feeding strategies already discussed, the main operating parameters that influence PHA production are: OLR, carbon to nitrogen ratio (C/N), pH, sludge retention time (SRT), cycle length, substrate characteristics and concentration.

1.7.1 Organic Loading Rate (OLR)

Several studies in the literature confirmed that the OLR is a parameter that affects the selection of PHA-accumulating biomass (Carvalho et al., 2014; Morgan-Sagastume et al., 2020; Dionisi et al., 2006). In particular, it has been observed that PHA production increases with the OLR, as greater substrate availability stimulates the selection of bacteria with a high capacity of intracellular storage (Lorini et al., 2020). Additionally, excessively high values of this parameter can lead to negative outcomes, such as the extension of the feast phase, which leads to a reduction in selective pressure, and the subsequent reduction in PHA production (de Oliveira et al., 2019; Dionisi et al., 2005).

For example, Lorini et al. (2020) tested different OLR values for the enrichment step in a SBR operating with an uncoupled C and N feeding strategy. Crognale et al. tested the effect of OLR values between 4.25 g COD $L^{-1}d^{-1}$ and 18 g COD $L^{-1}d^{-1}$ using a mixture of acetic and propionic acids (85:15 in COD content) concluding that the optimum for selection was 12.75 g COD $L^{-1}d^{-1}$ (Crognale et al., 2022). Fang et al., using rice winery wastewater as a substrate, evaluated the effect of the applied OLR with values of 1.2, 2.4 and 3.6 gCOD $L^{-1}d^{-1}$. The highest PHA yield was obtained with the intermediate OLR (2.4 gCOD $L^{-1}d^{-1}$) in which Zoogloea was the most dominant PHA-accumulating microorganism (Fang et al., 2019). Finally, Fauzi et al. (2019), using crude glycerol, found worsening in the

selection phase and instability for values above 1 gC $L^{-1}d^{-1}$ (Fauzi et al., 2019).
Mineo et al. (2023) investigated the relationship between the OLR and the intracellular PHA content using a pilot-scale SBR reactor. In particular, biomass was selected using increasing OLR values (0.8, 1.3 and 1.8 gCOD L^{-1} d^{-1}). The maximum PHA content achieved in the accumulation assays of 60% w/w was obtained using the biomass selected with the highest OLR.
Analysis of the several studies suggests that there is no single optimal OLR value for achieving maximum PHA production, but it is characterized by a great variability. This means that the optimal value of OLR used in the enrichment phase depends on the type of effluent and the technology used.

1.7.2 Carbon to nitrogen ratio (C/N)

A low value of the carbon to nitrogen ratio (C/N) promotes cell growth, while a high value encourages PHA accumulation (Khatami et al, 2021). Nitrogen limitation inhibits microbial protein synthesis, directing biochemical reactions towards biopolymer production (Wen et al., 2010). To overcome this problem, one could consider increasing the duration of the famine phase and thus the cycle time. However, this option could lead to a decrease in OLR (Oliveira et al., 2017).
In the case of industrial byproduct sources such as cheese whey, crude glycerol, and wastewater that are devoid of macronutrients the inorganic source of nitrogen nutrients is generally dosed in the form of ammonia salts (NH_4^+) such as ammonia chloride (NH_4Cl) or ammonium sulfate ($(NH_4)_2SO_4$), so it is important to control the dosage for good bacterial selection (Lee et al., 2015; Korkakaki et al., 2016; Silva et al., 2017; Huang et al., 2017). While N values should not be too high to favor PHA accumulation, C values that are too high can lead to bacterial inhibition with a consequent reduction in PHA accumulation capacity (Valencia et al., 2021). At the same time, using too low values of the C/N ratio, thus dosing too little nitrogen, could be disadvantageous in terms of PHA production. Indeed, in the study by Basak et al., (2010), it was observed that polymer storage ability of biomass was improved more under dynamic conditions with nitrogen deficiency when compared to that without nitrogen

deficiency. The highest PHA production yield was observed with biomass under nitrogen deficiency conditions (C:N 100:2), which was 0.69 g COD PHA g^{-1} COD S with a corresponding polymer content of biomasses were 43.3% (g COD PHA g^{-1} COD X). In contrast, the value obtained without nitrogen deficiency (C:N 100:12) was 0.51 g COD PHA g^{-1} COD S with a corresponding polymer content of biomasses being 38.3% (g COD PHA g^{-1} COD X). Gowda & Shivakumar, (2014) tested the effect of C:N ratio with Bacillus thuringiensis IAM 12077 using agrowastes as the substrate over a fixed range of 2:1, 4:1, 8:1, 10:1 and 20:1 to determine maximum PHA production. Maximum growth (3.6 g L^{-1}), P(3HB) yield (2.6 g L^{-1}), and PHA accumulation (72.8%) were obtained with C:N ratio of 8:1 using starch as the carbon source (10 g/L). Cui et al. (2017) using a culture of *Mediterranean archaeon Haloferax* studied different C/N ratio values of 5, 15, 35 and 65, respectively. The study showed that the highest PHA cell contents, about 47%, were obtained with an intermediate ratio value of 35. In contrast, the highest EPS (Extracellular polymeric substance) productivity was observed at a C/N ratio of 5. Silva et al. (2017) studied the effect of coupled and uncoupled nitrogen feeding and concluded that the C/N ratio alters both the production and the composition of the PHAs produced. The experiment was conducted in two reactors operating with the two different feeding strategies and using a synthetic substrate consisting of a mixture of acetic acid and propionic acid. In particular, three different C/N ratio values of 14.3, 17.9 and 22.3 C mol N mol^{-1} respectively were studied. On the one hand, it was observed that PHA production was similar for the two lowest C/N ratio values, while on the other hand, it decreased for the highest C/N value. At the same time, the hydroxyvalerate (HV) content in the produced polymers decreased from 20% to 12%, increasing the C/N ratio from 14.3 to 17.9 C-mol N-mol^{-1}. The phosphorus content also influences the production and composition of PHAs. Indeed, Montiel-Jarillo et al. (2017) performed batch experiments varying the C/N/P molar ratios at 100/8.8/1.3 and 100/8.8/90.5, respectively. The results showed that a limitation of phosphorus favored the accumulation of 3-HV over 3-HB, while a limitation of P resulted in a higher production of PHA of 42% gPHA $gVSS^{-1}$ of PHA.

In conclusion, by slightly varying the C/N ratio and manipulating the P concentration, it is possible to manipulate the properties and composition of the polymer produced (Khatami et al., 2012).

1.7.3 pH

Generally, the enrichment process is carried out with a neutral or slightly alkaline pH. Padan et al. (2005) demonstrated that the majority of non-extremophilic bacteria thrive in a pH range of 5.5 and 9 during the enrichment phase. However, other studies showed that PHA accumulation was significantly inhibited at pH values below 7 probably due to the lack of acid dissociation in the substrate (Chua et al., 2003; Dias et al., 2006). Moreover, maintaining the same or slightly higher pH during the accumulation phase enhances PHA productivity, as each culture performs optimally near its ideal pH value (Reis et al., 2011). The study by Chua et al. (2003) on the influence of pH on the selection of microbial cultures using acetate showed that the same performance in terms of PHA content and storage rate was displayed by cultures acclimatized to pH values of 7 and 8, respectively. However, in the study by Villano et al. (2010), it was observed that raising the pH from 7.5 to 9.5 resulted in decreased storage rate and PHA content. They concluded that the optimal pH for the accumulation phase lies between 7.5 and 8.5 (Villano et al., 2010). However, Khor et al., (2023) showed that the enrichment of PHA accumulators is feasible when pH is not controlled under uncoupled C and N feeding strategies using raw glycerol. Something similar was observed in the study by Montiel-Jarillo et al. (2017). In particular, the influence of pH was evaluated in the accumulation phase using activated sludge and acetate as a carbon source. Several tests were carried out at a pH between 4 and 8.5 and one at uncontrolled pH. The highest yields in terms of biopolymer accumulation (44% gPHA $gVSS^{-1}$) were obtained without pH control. In contrast, values ranging between 17-23% gPHA $gVSS^{-1}$ were obtained under acidic conditions. Tamis et al., (2018), however, conducted accumulation assays with pH values ranging from 4.5 to 10.5, obtaining the optimal performance at a pH of 8.

1.7.4 Sludge retention time (SRT)

In general, the storage yield of PHA decreases as the SRT decreases. The reciprocal of this parameter corresponds to the effective specific growth rate. Therefore, lower SRT values lead to a higher substrate utilization for microbial growth (Reis et al., 2011). A time of 2 days can be used as a reference value for SRT. In fact, following the study by Beun et al. (2002), it was observed that the PHA yield for SRT longer than 2 days remained constant while it decreased drastically using lower SRT values. In the first case, this implies that the specific growth rate did not depend on SRT, while in the second case, it increased as SRT decreased. However, the selected culture must have not only a high PHA storage capacity but also a high growth rate to achieve a high cell density in the accumulation phase. This can be done by manipulating the operating conditions of the enrichment reactor. Many studies have utilized an SRT parameter value of 1 day to select the PHA-accumulating biomass (Korkakaki et al., 2016; Marang et al., 2013; Beccari, et al 2009; Johnson et al., 2009).

1.7.5 Cycle length

There are several studies in the literature on the effect of cycle length on biomass selection for PHA production. Dionisi et al., (2007) observed that cycle length influenced the biomass response to excess substrate. They evaluated PHA storage using lactate and propionate at the same OLR, varying the cycle length between 1 and 8 hours. The best storage performance was obtained for lengths between 2 and 4 hours, whereas for length values too low or too high the biomass tended to grow rather than accumulate PHA. Several subsequent studies have confirmed that increasing the cycle length results in increased PHA content in the selected biomass. However, this does not necessarily lead to the selection of a culture with the highest storage capacity and productivity (Marang et al. 2016; Valentino et al. 2014; Jiang et al. 2011). Pereira et al. (2020) observed that using long cycles and low OLR values in MMCs promotes PHA storage rather than cell growth. Cycle length may also influence the metabolic response of the biomass. Moralejo-Gàrate et al. (2013) produced poly glucose and PHA from Crude glycerol using two sequencing batch reactors with cycles of 6

and 24 hours, respectively. Although the dominant microorganism was the same in both reactors, a shorter cycle promoted poly glucose production over PHA production, while a longer cycle encouraged PHA production. Similar observations were made by Freches and Lemos (2017), who used cycles with lengths of 6, 12, and 24 hours, respectively.

1.7.6 Substrate characteristics and concentration

The composition and concentration of the substrate used influence the total yield of PHA production, the type and composition of polymers produced, the kinetics of substrate consumption and polymer storage as well as the composition of the microbial community. For instance, acetic acid is associated with the exclusive production of 3-HB while propionic acid allows the production of both monomers 3-HB and 3-HV, depending on the ratio of acids in the feed (Janarthanan et al., 2016). In general, the best substrates for PHA production are those consisting of simple molecules that are easily assimilated by the biomass. Three different types of substrates, such as acetate, glucose, and starch, were used during the biomass enrichment stage in the study conducted by Cui et al. (2016). The results in terms of PHA content for acetate, glucose and starch were 64.7%, 60.5% and 27.3%, respectively. The PHA content decreased as the substrate complexity increased. While the first two substrates can be used directly, starch must first be converted into simpler molecules to be assimilated. Jiang et al. (2011b), in contrast, demonstrated, using different mixtures of acetate and propionate, that the composition of the final polymer obtained depends on the composition of the substrate used during the biomass enrichment step. Specifically, the ratios used were on a Cmol basis, with acetate/propinate ratios of 100/0, 75/25, 50/50, and 0/100. In the absence of propionate, the polymer produced was entirely PHB, while in the absence of acetate, a copolymer with the composition of 11 Cmol% 3HB and 89 Cmol% 3HV was obtained. Grousseau et al. (2014) administered propionic acid alone and a mixture of propionic and butyric acid to *Cupriavidus necator*, resulting in different proportions of 3HV to PHA. In the former case, 50% mol 3HV/ mol PHA was obtained, while in the latter case only 32% mol 3HV/ mol PHA was obtained. Carvalho et al., (2018) observed that using different

substrates results in changes in the selected microbial community. Specifically, the dominant bacterial species was Actinobacteria using sugarcane molasses, while in the case of whey, Firmicutes dominated. Substrate concentration assumes a key role in the biomass selection process and PHA production yields. In general, the rate of substrate uptake and PHA production increases as the substrate concentration fed increases until it reaches a maximum value. The use of higher substrate concentrations leads to a slowdown and/or inhibition of bacterial activity (Reis et al, 2011; Serafim et al., 2004). At the same time, however, too low substrate concentrations are inconvenient for a good selection of PHA accumulating biomass (Albuquerque et al., 2010).

1.8 Integration of enrichment in the mainstream of WWTP

The implementation of the biomass enrichment in the mainstream of a WWTP would require that this should occur concurrently with the wastewater treatment process. Thus, the enrichment stage should allow for obtaining an effective selection of PHA storing bacteria, while the effluent of this reactor must comply with the discharge limits imposed by environmental regulations. Implementation of the PHA production process could potentially reduce sludge production, leading to lower costs associated with sludge management such as transportation, treatment, and disposal. In addition, it could enhance process efficiency through competition for substrate between PHA-accumulating bacteria and other microorganisms present, resulting in a potential reduction of organic pollutants in wastewater and promoting environmental sustainability.
In the vast majority of cases, the type of reactor used to select the MMC is the SBR operating with activated sludge (AS). The main advantage of SBR technology over conventional activated sludge (CAS) lies in the fact that biological reactions and biomass settling all take place within the same reactor, thus reducing the required surface area. Such reactors operate following cycles divided into the filling, reaction, sedimentation, discharge, and idle phases (Vives Fàbregas, 2004). However, conventional processes using AS are susceptible to process dysfunctions (e.g., bulking, foaming), especially under unbalanced growth conditions (e.g., high carbon-to-nitrogen ratio, high organic loads), which are required for a successful

enrichment of the MMC. Therefore, because of the worsening of the sludge settling properties, purification performances decreased, and the effluent quality exceeded limit regulations. At the same time, such microorganisms although having high PHA storing capability, are gradually washed-out from the system, resulting in a significant decrease of PHA productivity. Another drawback of this process is the low volumetric productivity of PHA achieved by the low biomass concentration in operation in the enrichment and accumulation reactors. In this context, the application of a complete cell retention strategy was suggested as a feasible approach to tackle this issue (Burniol-Figols et al., 2020). In several studies reported in the literature the ideal operating conditions for the enrichment stage, in terms of OLR (> 5-10 kgCOD $m^{-3}d^{-1}$) and food to microorganism ratio (F/M) (> 2-5 kgCOD $kgSS^{-1}d^{-1}$), are hardly replicable in a mainstream biological unit without compromising its purification performances (Lorini et al., 2020; Valentino et al., 2019). The operational conditions imposed in the enrichment stage do not ensure the necessary purification performance to meet the regulatory discharge limits into receiving water bodies. Due to these issues the integration of a conventional mainstream SBR process might be inconvenient.

For this reason, the enrichment process is carried out in reactors placed in the side-stream to the main wastewater treatment line (Morgan-Sagastume et al., 2015). This clearly impacts the system's footprint and complexity.

The innovative aspect of the thesis consists in the integration of enrichment into the mainstream. Moreover, considering the problems associated with using SBR systems for PHA production while ensuring compliance with regulatory limits for water purification, the use of different biotechnologies such as membrane bioreactors (MBR) technology or aerobic granular sludge (AGS) were analyzed.

The use of MBR technology or AGS could eliminate the problems associated with using SBR systems for PHA production while ensuring compliance with regulatory limits for water purification. AGS was successfully applied for PHA production, thanks to its higher biomass retention capacity, ensuring higher productivity compared to conventional flocculent sludge-based systems (Amorim de Carvalho et al., 2021). Additionally, some preliminary experiences reported promising results using MBR technology (Burniol-Figols et al., 2020; Kumar et al., 2017).

In MBR system bacterial selection is strictly based on the ability of microorganisms to adapt in a specific environment (high/low substrate or oxygen availability), or under precise operating conditions, independently from their capability to aggregate in dense and settleable flocs. For these reasons, MBRs are characterized by a greater variety of the microbial community compared to a conventional system (Baek and Pagilla, 2009), thus potentially resulting in a better enrichment of the MMC. According to what above stated, the use of MBR could be implemented as a treatment/enrichment stage in the mainstream of a WWTP to produce high-quality effluent eligible for reuse and the selection of PHA-storing bacteria, simultaneously.
Both AGS and MBR systems are well-suited for operating under more challenging process conditions than conventional activated sludge systems, as they overcome issues related to sludge settling and can tolerate higher organic loads.

1.9 Innovative biotechnology for enrichment of MMC

1.9.1 MBR Process

MBRs are units that combine an activated sludge biological system with a membrane filtration system for biomass separation.
These systems have garnered significant interest in the scientific community due to their high depuration performance with small planimetric and volumetric footprints (Gkotsis et al, 2014). Despite their widespread use in wastewater treatment, there are relatively few studies on their application in PHA production (Judd, 2010). The submerged MBR configuration (sMBR), in which the filtration modules are immersed in the mixed liquor and aeration, allows for better control of fouling, thereby reducing operating costs (Carstensen et al., 2012). The MBR system can be combined with SBR technology resulting in a sequencing batch membrane bioreactor system. Due to its high operational flexibility, this reactor offers improved hydraulic conditions for membrane filtration process, enhanced effluent quality, complete retention of microorganisms, and the possibility to operate at high biomass concentrations (Zonoozi et al., 2015; Dong and Jiang 2009; Vargas et al. 2008; McAdam et al. 2005). However, membrane fouling is the main

drawback of MBR systems. These systems are designed to handle larger volumes of wastewater and encounter higher biomass concentrations during the filtration phase (Zonoozi et al., 2015; Lobos et al. 2007; Kiso et al. 2005). Few studies in the literature explore sMBR systems for PHA production. Kumar et al, (2017) investigated the co-production of PHA and carotenoids during the glycerol fermentation process by the microorganism Paracoccus sp. LL1. The installation of submerged ceramic filters was able to double the cell concentration within the reactor. This configuration resulted in a total dry cell weight of 24.2 g L^{-1} containing 39.3 wt% PHA. However, the PHA productivity was less than 0.1 gPHA L^{-1} h^{-1} due to the low PHA yield. Burniol-Figols et al. (2020) performed several accumulation assays using classical pressure-driven MBRs (hollow fibers and ceramic filters) and a new diffusion-driven MBR for the selection of PHA accumulating bacteria. The first type of reactors provided yielding productivity values between 0.87-1.44 g L^{-1} h^{-1} despite the use of a diluted substrate feed (10 g L^{-1} of VFA) while observing no reduction in process flux. However, hollow fiber MBRs may be subject to cell deposition, resulting in decreased metabolic activity and PHA production rate.

1.9.2 Granular sludge process

Granular sludge technology operates under either anaerobic conditions in UASB (Upflow Anaerobic Sludge Blanket) systems or aerobic conditions with AUSB (Aerobic Upflow Sludge Blanket) reactors. Aerobic technology has been refined with Granular Sequencing Batch Reactor (GSBR) reactors in which the simultaneous removal of ammonia nitrogen and phosphorus has led to the use of sequential batch reactors. AGS technology is a potential replacement for CAS, offering intensified wastewater treatment in limited space, high effluent quality, and enabling resource recovery (Derlon et al., 2016; Luiz de Sousa Rollemberg et al., 2020). Specifically, compared to conventional activated sludge systems, AGS systems allow simultaneous removal of organic fractions and nutrients in the same reactor, achieve high biomass concentrations with high settling ability, reduce footprint by about 75 % and reduce power consumption by about 30-50 % (Thwaites et al.,

2018). In such systems, slow-growing bacteria are developed so that even if the biomass concentration is high, sludge production is limited.
The number of bacterial cells in aerobic granules surpasses that found in activated sludge when considering a similar volume of aerobic granules and activated sludge (Tay et al., 2001). Consequently, AGSs should be able to produce a higher amount of PHA than activated sludge (Gobi & Vadivelu, 2014).
AGS was successfully applied for PHA production, thanks to its higher biomass retention capacity, which ensures higher productivity compared to conventional flocculent sludge-based systems (Amorim de Carvalho et al., 2021). Granular biomass systems are also suitable to produce exopolymers, given that one of the basic mechanisms of the granulation process is precisely the secretion of Extracellular Polymeric Substances (EPS) that promote microbial adhesion (Schambeck et al., 2020). EPS are functional substances that protect cells from external environmental agents, such as toxic substances and, similarly to PHAs, they can be used as a source of carbon and energy under nutrient-lacking conditions (Liu et al., 2004). EPS represent a potential recoverable resource that can be used as a substitute for alginate in various industries, including pharmaceuticals, food, drugs, and textiles (Hamza et al., 2022; Licciardello et al., 2019).
Gobi & Vadivelu (2014) used AGS to promote the conversion of VFAs present in palm oil mill effluent (POME) to PHAs within the granules by the ADF strategy. The aerobic granules removed 90% of COD (51.000 mg L^{-1}) from POME and VFAs (propionic and butyric acids) were converted entirely to PHA. The highest PHA content, specifically P (HB-co-HV), was 0.6833 mgPHA mg^{-1} cell dry weight (CDW). Gobi & Vadivelu (2015) used different SBR inoculated with aerobic granules, observing that PHA content changed according to granule size at different stages of development. In addition, it was seen that PHA content ranged from 0.66 to 0.87 g PHA $gCDW^{-1}$, as a result of the increase in OLR from 0.91 to 3.64 kgCOD m^{-3} d^{-1}. Morgan-Sagastume et al. (2015) utilized an AGS reactor for the enrichment phase of PHA-accumulating MMCs under ADF conditions using plug-flow filtered municipal wastewater as substrate (290-570mgCOD L^{-1}, 35-60 mg N L^{-1}, 4.3-7.6 mg P L^{-1}). The system operated with an OLR of 3.0 ± 0.8 gCOD L^{-1} d^{-1}, a HRT of about 3 hours, and a variable SRT to regulate the TSS within the reactor. Such a system allowed

a removal of COD, nitrogen and phosphorus by 70%, 24%, and 46%, respectively. The PHA content in the granules was 0.14 gPHA $gVSS^{-1}$. Wang et al. (2014) selected PHA-enriched aerobic granular sludge in under nitrogen deficient conditions using synthetic wastewater. The granular sludge obtained had a PHA content of 40 ± 4.6% and high settling ability. The authors observed that EPS and PHA yield followed similar profile and they were influenced by reactor operating conditions (HRT, C/N and shear force). Similarly, Ghosh & Chakraborty (2020), treated oily wastewater with two AGS systems, operating with mixed sludge granules and *Micrococcus aloeverae* strain SG002 originated granules, respectively. They observed that PHA production increased proportionally with EPS concentrations. Pronk et al. (2017) demonstrated that different types of biopolymers could be extracted from AGS depending on the operating conditions applied. Indeed, considering that the key element leading to the synthesis of PHA and EPS is the carbon, it was postulated that a regulatory mechanism in carbon utilization could switch the pathway from PHA polymerization to EPS synthesis and vice versa (Kopperi et al., 2021; Cui et al., 2017).

Recent studies have investigated the simultaneous recovery of EPS and PHA. For example, Kopperi et al. (2021) demonstrated that simultaneous production of PHA and EPS from isolated *Providencia sp.* can be achieved. The authors tested different combinations of pH (6, 7 and 8) and carbon load (20, 30 and 40 g L^{-1}) observing that the maximum yield of both PHA and EPS was obtained for values of 7 and 30 g L^{-1}, respectively. In their study, Kopperi et al. (2021) found that optimization of operating conditions increased the yield of PHA to 2.62 g L^{-1} and the yield of EPS to 3.92 g L^{-1}. Similarly, in a recent study, EPS and PHA were successfully produced from an MMC treating municipal wastewater (Karakas et al., 2020). More specifically, a PHA content of 10.8% was obtained when AGS operated under OLR conditions (3.3 ± 0.3 kgCOD m^{-3} d^{-1}) while EPS recovered from the AGS showed potential to be used for several technical uses because they had the capacity to form hydrogels.

1.10 Integration of enrichment in the mainstream of WWTP

The integration of PHA-enrichment into the mainstream of a citrus wastewater treatment can be beneficial in several aspects. This approach certainly contributes to a circular economy by promoting the recovery of materials from industrial wastewater and the production of a sustainable polymer that can be used for various industrial applications. PHA constitutes a valuable product that can be resold on the market, potentially generating additional revenue, and consequently decreasing overall treatment costs. Furthermore, the production of PHA also can help reduce the plant's overall carbon footprint.

However, before the integration of enrichment into the mainstream of WWTP, it is essential to conduct a study that evaluates the advantages and disadvantages based on the plant configuration, needs and local conditions. It should be noted that integrating the enrichment phase into the mainstream could result in high initial costs due to the implementation of the necessary technologies and on the other hand to complicate the operation/maintenance of the plant. Variation in organic pollutant loads could affect process performance. It must always be considered that the selection of PHA-storing biomass occurs simultaneously with wastewaters treatment. A significant increase in OLR in the selection reactor could lead to an inevitable reduction in purification performance (Di Trapani et al., 2019).

Achieving optimal operating conditions that balance PHA production yield and purification performance remains a significant challenge when integrating this process into the mainstream of a wastewater treatment plant. Given that the citrus industry generates high amounts of wastewater, this doctoral thesis aims to integrate the enrichment stage into the mainstream of a citrus wastewater treatment to explore the feasibility of introducing a new valorization pathway coherent with the circular economy approach. Specifically, it aims to compare SBR, MBR and AGS for PHA production integrated into the mainstream of a wastewater treatment plant, as, to the best of the author's knowledge, such a comparison is currently absent in the literature.

1.11 Objectives and thesis structure

In light of the above, the objective of this thesis was to evaluate the feasibility of biopolymer production from citrus process wastewater treatment. Three types of biological reactors were employed for this purpose: SBR (or enrichment SBR), MBR (or enrichment MBR), and AGS (or enrichment AGS). Specifically, their integration into the mainstream of a citrus industrial wastewater treatment plant was assessed to simulate the wastewater treatment process and the MMC enrichment phase for PHA production simultaneously (Figure 1.9).

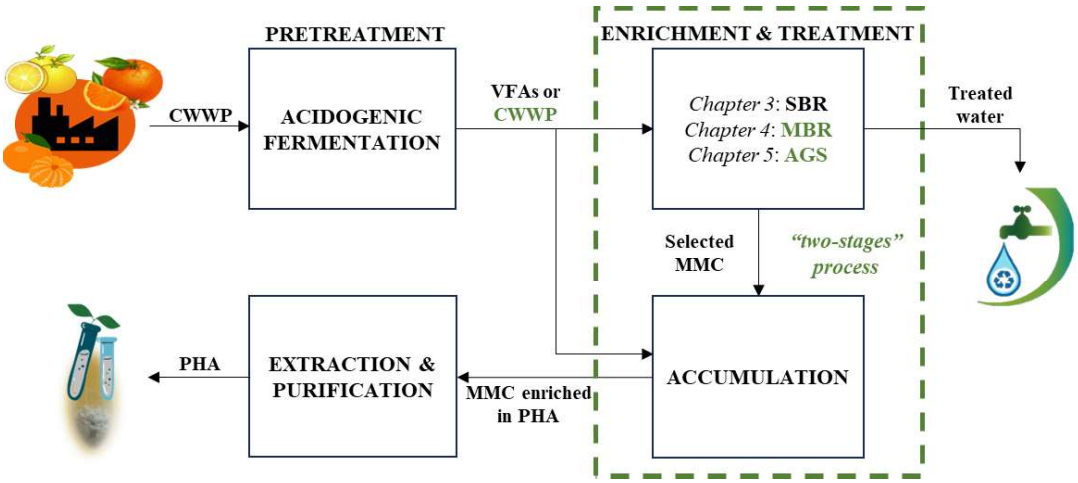


Figure 1.9: Integration of PHA production into the mainstream of a citrus industrial wastewater treatment plant

Given the qualitative and quantitative seasonal variations in citrus wastewaters, each experiment was organized in three different periods characterized by increasing OLRs (1, 2, and 3 kgCOD $m^{-3}d^{-1}$). These periods corresponded to the real average load conditions of a citrus wastewater treatment plant located in Palermo (Italy). Additionally, the performance of the three systems was compared in terms of both water treatment performances and biopolymer production perspectives. An in-depth analysis was conducted on concurrent PHA-accumulation processes related to metabolic pathways of EPS production. Finally, a focus was placed on the variation in the characteristics of the produced biopolymer, such as

molecular weight, as a function of the operating parameters of the enrichment reactor.

The first experimentation, described in detail in Chapter 3, focuses on the possibility of integrating the enrichment phase into the mainstream of a citrus industrial wastewater treatment plant using a conventional SBR process. The results obtained were used as a comparison for subsequent experiments. Additionally, aspects such as the effect on sludge settling, the variation of the molecular weight of the polymer produced in relation to the OLR parameter of the enrichment reactor, and changes in microbial community and diversity were analyzed.

Chapter 4 assesses the use of MBR technology in the mainstream process of a WWTP to recover PHA instead of a conventional process. MBR technology allows for water recovery; thus, the possibility of reusing reclaimed water was also evaluated.

Furthermore, the evolution of membrane fouling phenomenon and biomass enrichment with PHA-storing bacteria was studied throughout the experiment.

The use of aerobic granular sludge (AGS) for PHA production is described in Chapter 5. This chapter also focuses on EPS production, as EPSs serve as the basis for granulation mechanisms, constituting additional potentially recoverable products.

Chapter 6 reports a preliminary study on the influence of the OLR parameter on the characteristics of produced PHAs, such as monomeric composition and M_w. Specifically, a laboratory-scale enrichment reactor SBR operated with OLR values of 2, 4, and 6 kgCOD $m^{-3}d^{-1}$ during periods 1, 2, and 3, respectively.

Accumulation assays were performed in both Fed-Batch and continuous modality using synthetic substrate, synthetic substrate with N&P, and agro-industrial wastewater. This experiment was conducted at the Nova School of Science and Technology (Lisbon, Portugal), during a research period abroad.

Chapter 7 discusses the challenges and perspectives on the implementation of PHA production in the mainstream of an industrial WWTP.

Finally, Chapter 8 summarizes the main results obtained in the thesis, highlighting the potentials and limitations of the applied technologies.

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Chapter 2: Materials and methods

Summary

The citrus wastewater was sampled from an industry that processes citrus fruits located in Palermo (Italy). The wastewater was characterized by very high COD concentration (>25,000 mgCOD L^{-1}) and low pH (about 3.5). Because the raw wastewater lacked nitrogen and phosphorous, a concentrated nutrient solution containing urea and potassium phosphate was supplied to obtain a ratio between chemical oxygen demand (COD), total nitrogen (TN) and total phosphorous (TP) equal to 200:5:1. The enrichment reactors were operated with three different OLRs in respective experimental periods: 1 kgCOD $m^{-3}d^{-1}$ in Period 1, 2 kgCOD $m^{-3}d^{-1}$ in Period 2 and 3 kgCOD $m^{-3}d^{-1}$ in Period 3. The above periods corresponded respectively to the real average loading conditions of a citrus wastewater treatment plant (potential of 12,500 PE) located in Palermo (Italy), which were associated with the summer period (low load - Period 1), the autumn/spring (medium load - Period 2) and the winter (high load - Period 3), respectively. A SBR, MBR and AGS operating in batch mode were employed to simulate the wastewater treatment process and the MMC enrichment phase simultaneously. In the first experimentation with conventional activated sludge SBR (Chapter 3), a three-stage process was employed to produce PHA. This was because, due to the Covid-19 pandemic, previously stored effluent containing a substantial percentage of particulate matter was available. In the experimentation described in Chapter 4 (MBR) and Chapter 5 (AGS), fermentation was omitted because the reactors were operational during the citrus processing season, thus providing access to fresh effluent. This raw wastewater was characterized by high concentrations of readily biodegradable organic carbon. Additionally, recent studies have suggested that agro-food wastewaters with high organic matter content in soluble form but deficient in nitrogen and phosphorous could be used for PHA production without fermentation. In this scenario, since feedstock fermentation is not necessarily required, a two-stages process can be implemented (Argiz et al., 2022; Morgan-Sagastume et al., 2015). This chapter explains the methodologies employed for determining all physical-chemical parameters of the wastewater. The biopolymer extraction procedure and the analysis of the physical and chemical characteristics of the produced polymers are also described. Finally, a detailed discussion on the mass balance for COD is provided.

2.1 Characterization of citrus wastewater

The citrus wastewater was sampled from an industry that processes citrus fruits located in Palermo (Italy). CPWW derived from the processing of several citrus fruits, primarily oranges, lemons, and tangerines. The volume of CPWW produced by the industry amounted to approximately 500 m^3 d^{-1}. Prior to treatment, CPWW underwent a physical process for essential oils extraction, thus their content in the wastewater was negligible. CPWW was then stored into an equalization unit before being collected to the pilot plant. 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 throughout the duration of the experiments.
In the first experimentation with conventional activated sludge SBR (Chapter 3), a three-stage process was employed to produce PHA due to the characteristics of the wastewater available at that time. Fermentation occurred in the absence of fermenting biomass inoculum but utilizing the biomass naturally present in the effluent. Daily, a fraction of this wastewater was diluted with tap water until a COD close to 4500 mgCOD L^{-1} was obtained. Subsequently, it was fed to a fermentation reactor, after adjusting the pH to a value close to 7 by adding NaOH. The wastewater was then collected to a lab-scale SBR plant after the supply of a concentrated solution (0.1 L d^{-1}) containing nitrogen and phosphorus (2gCH_4N_2O L^{-1}, 1gK_2HPO_4 L^{-1}) to achieve a ratio between carbon (as COD)/nitrogen/phosphorous (CW2) equal to 200: 5: 1. Furthermore, a fraction of the CW1, diluted with tap water, was fed to a second fermentation reactor (pH = 7). After the fermentation, this wastewater (CW3), without any supply of nitrogen and phosphorous, was directed to an accumulation reactor aimed at producing PHA. All the 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 2.1.

Table 2.1: Characteristics of the citrus wastewater used for the experiments on enrichment SBR

Parameter	Unit	CW1	CW2	CW3
Total COD	[mg L^{-1}]	27,189 ± 436	4158 ± 386	26,187 ± 198
Total nitrogen	[mg L^{-1}]	94.3 ± 12.9	219.1 ± 12.6	89 ± 10.8
Total phosphorus	[mg L^{-1}]	75.8 ± 4.5	42.9 ± 3.5	38.6 ± 2.7
pH	[–]	2.89 ± 0.18	6.86 ± 0.31	7.03 ± 0.04
Acetate	[mg L^{-1}]	1786 ± 354	2286 ± 132	5673 ± 102
Conductivity	[mS / cm]	1.42 ± 0.18	1.56 ± 0.18	1.48 ± 0.15

In the experimentations on MBR and AGS systems, the fermentation phase was omitted because the reactors were operational during the citrus processing season, thus providing access to fresh effluent. This raw wastewater was characterized by high concentrations of readily biodegradable organic carbon. Additionally, in recent years, some studies stated that agro-food wastewaters characterized by high organic matter content in soluble form and the lack of nitrogen and phosphorous could be used to produce PHA without being fermented. In this case, because feedstock fermentation is not necessarily required, a two-stages process can be implemented (Argiz et al., 2022; Morgan-Sagastume et al., 2015). Removing fermentation is surely an advantage because it allows to obtain a substantial process simplification. In the case of MBR and AGS reactors the CW1* was diluted with tap water and mixed with NaOH (0.10 M) to adjust the pH to neutral within equalization tank (200 L).

After the addition of nitrogen and phosphorus, this effluent (from now called CW2*) was sent to the enrichment reactors. Additionally, a fraction of the CW1* was send to another equalization tank where it was diluted to 4500 g L^{-1} with tap water as needed and pH was adjusted to 7 without addition of nutrient. This, wastewater (CW3*) was fed to the accumulation reactor aimed at producing PHA.

The total COD content of CW2* was on average close to 4500 mg L^{-1}. The organic content of CW2* was mainly in the soluble form (>75%), of which about 45% was acetate. The amount of the inert organic matter and that of the slowly biodegradable accounted for approximately 20% of the total COD. Figure 2.1 shows the average results of COD fractionation assays

performed during the experimental phases. The amount of the overall biodegradable fraction, including the soluble readily biodegradable and hydrolysable COD and the particulate slowly biodegradable COD, accounted for over 95%. The above results evidenced the high biodegradability of CW2* and very low content of non-desired carbon sources (inert and slowly biodegradable organic matter). More in detail, the soluble organic substrate, constituted by the readily biodegradable and hydrolysable COD was close to 80%, indicating the presence of easily metabolizable carbon sources (VFAs) (Silva et al., 2017). Analysis of the VFAs in the raw wastewater revealed that acetate was the main organic present in the CW2*, whereas the concentrations of valeric, propionic, butyric, and other acids were negligible.

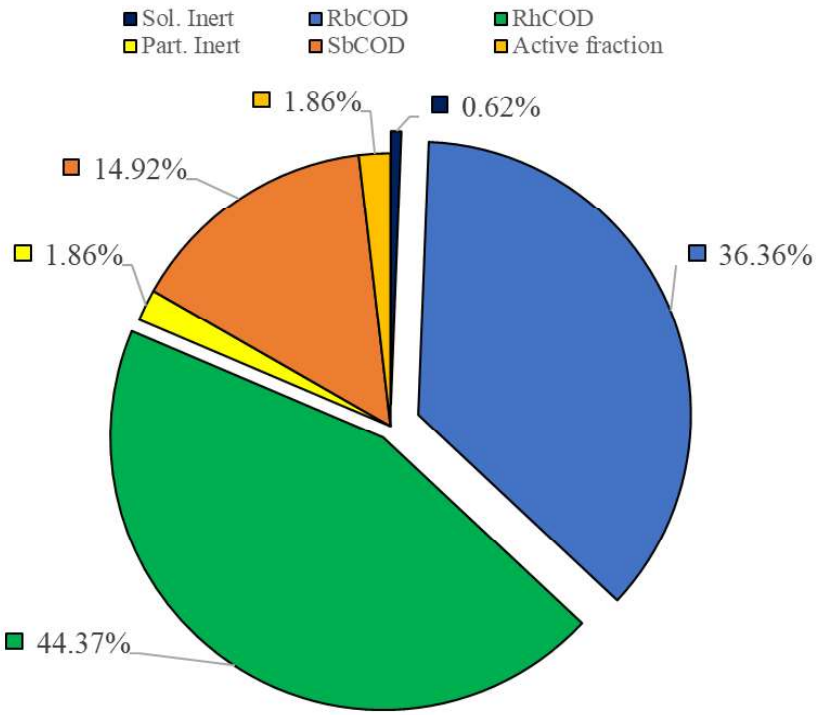

Figure 2.1: Average results of the COD fractionation assays performed during the entire experiment (Legend: Sol. Inert: Soluble inert COD; RbCOD: readily biodegradable COD; RhCOD: readily hydrolysable COD; SbCOD: Slowly biodegradable particulate COD; Part. Inert: Particulate inert COD)

The readily biodegradable COD can be directly used as substrate for PHA biosynthesis, whereas the utilization of the readily hydrolysable fraction requires a preliminary breakdown of more complex molecules. Indeed,

CW2* is a sugar-rich wastewater in which glucose, fructose, and sucrose are the main sugars, hence the readily biodegradable fraction, and carbohydrates (e.g., pectin) constitute the organic compounds from which hydrolysis sugars are produced (Ting and Deszyck, 1961). The above results indicated that CW2* can be directly use as substrate for PHA biosynthesis without any pre-treatment, as the easily metabolizable carbon sources necessary for the MMC enrichment and PHA accumulation are naturally present in the medium. Consequently, as feedstock fermentation is not required when using CW2* for PHA production the possibility to apply a two-stages process instead of the conventional three-stages enable to obtain a considerable simplification of the plant layout.

2.2 Operating conditions and monitoring activities of the enrichment reactors

The enrichment reactors were operated with three different OLRs in respective experimental periods: 1 kgCOD $m^{-3}d^{-1}$ in Period 1, 2 kgCOD $m^{-3}d^{-1}$ in Period 2 and 3 kgCOD $m^{-3}d^{-1}$ in Period 3. 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. Enrichment reactors operated in batch mode according to the aerobic dynamic feeding regime. All enrichment reactors were inoculated with the activated sludge collected from the citrus processing industry. Because the raw wastewater lacked nitrogen and phosphorous, a concentrated nutrient solution containing urea and potassium phosphate was supplied to obtain a ratio between COD, TN and TP equal to 200:5:1. Process conditions were changed only after steady performances were observed in each system, typically after a duration exceeding three times the SRT. The dissolved oxygen concentration, the chemical oxygen demand, the PHA and EPS content during the enrichment cycle were periodically monitored. Operation cycles were periodically monitored by measuring the DO, COD, the content of PHA and EPS in the mixed liquor. The biomass selection process was monitored over time by calculating the ratio between the duration of the feast and famine phases (F/F). The dissolved oxygen

profile in the enrichment reactors was checked once a week in order to assess the duration of the feast and famine phases (Argiz et al., 2020).

2.3 Operating conditions and monitoring activities of the accumulation reactor

The accumulation reactor used to perform the accumulation assays was the same for all experimentations. It consisted of a 2 L jacketed glass reactor (1.5 L of operating volume) and it was fed with the excess sludge discharged from the enrichment reactors (SBR, MBR or AGS) at the end of the famine phase and with raw wastewater without nutrients addition to prevent bacterial growth and promote PHA accumulation. Accumulation reactor operated in full aerobic conditions at the end of each experimental period only. To assess the maximum PHA accumulation, the accumulation reactor was continuously aerated maintaining the DO concentration higher than 4 mg L^{-1} and the temperature of the reactor was maintained at 20 °C by a thermostatic bath. Raw wastewater was fed according to the "feed on demand" strategy, which consisted in the dosage of different small volumes every time the biomass in the reactor had completely degraded the readily biodegradable organic substrate fed with the previous sample (Morgan-Sagastume et al., 2014). The assays were carried out at the end of each experimental period, after three SRT times were elapsed and once the feast/famine ratio was below 0.2. At this point, it was considered that steady state in terms of MMC enrichment was achieved (Conca et al., 2020). 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, reactor volume (1.5 L), concentration of COD of the raw wastewater and temperature (20 ◦C). 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 solid-liquid separation by a laboratory centrifuge and extraction procedure for the quantification and characterization of the

intracellular biopolymers. Each accumulation assay lasted at least 5 hours. The accumulation reactor 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 and pH sensor for online monitoring. 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).

2.1 Analytical methods

All the physical-chemical analyses for the assessment of total suspended solids (TSS), volatile suspended solids (VSS), Chemical Oxygen Demand (COD), biochemical oxygen demand (BOD), ammonium nitrogen (NH3-N), total nitrogen (TN) and total phosphorus (TP) were carried out according to the standard methods (APHA/AWWA/WEF, 2012). The COD fractions, including the soluble biodegradable (SB), particulate biodegradable (PB) and particulate inert (PI) were determined according to the literature (Capodici et al., 2016). Fractionation of the COD was carried out by respirometric techniques according to the literature (Corsino et al., 2020) during each experimental period. As far as concerns the analysis of the water quality for reuse, physical-chemical and biological parameters reported in the EU 2020/741 and in the Italian Ministerial Decree DM 185/03 were considered. The latter is still in force and establishes the standard for reuse of industrial wastewater for irrigation, civil and industrial purposes, while the former refers to agricultural reuse only. The methods for the analytical study of the relevant chemical parameters were based on national and international methodologies, specifically: UNI EN ISO 15587-2:2002; EPA 6020B 2014; APAT CNR IRSA2020/2090/2050/2100/4020/ 4080/4160/5070/5120/5130/50160/7030 Man 29 2003. The SVI of the SBR reactor was determined by dividing the volume of the settled sludge inside a graduated cylinder of 1 L after 30 minutes of static settling for the concentration of TSS in the sample.

The settling properties of the granular sludges in the enrichment reactors were assessed by calculating the SVI_{30} and SVI_5. These were calculated by dividing the volume occupied by the sludge inside a 1 L graduated cylinder after 30 min, or 5 min in the case of SVI_5, of static settling by the

concentration of TSS in the sample. A unit ratio between SVI_5 and SVI_{30} was considered as an indicator to assume the achievement of complete granulation in the enrichment AGS. Moreover, the average particle size and the particle size distribution (PSD) of granular sludge were measured by an optical granulometer (QICPIC-Sympatec). The percentage of granules in the enrichment AGS was assumed to be equal to the percentage of particles with a size greater than 400 mm (de Kreuk, 2006).
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 (Yobs) was carried out through mass balances, considering the daily variation of TSS present in the system and those withdrawn as excess sludge, according to the literature (de Oliveira et al., 2018).
The biokinetic parameters, including the maximum removal rate of organic carbon (vH), the active fraction of the heterotrophic biomass (fxH) and the maximum growth rate (YH) were determined by means of respirometric techniques (Capodici et al., 2016). The average size of activated sludge flocs was measured using an optical granulometer. The microscopic observations, the identification and abundance of filamentous bacteria were carried out according to the procedures developed by Eikelboom (Eikelboom and Van Buijsen, 1981) and Jenkins (Jenkins et al., 2003), using a phase contrast optical microscope (Olympus). Sudan black stain was performed to assess the presence of PHA within the bacterial cells (Jenkins et al., 2003).

2.1.1 Extraction of PHA

The intracellular biopolymers were extracted following the procedure developed by Fiorese and co-authors using 1-2 propylene-carbonate as a solvent (Fiorese et al., 2009). The main extraction steps were as follows. First, 50 mL of sludge samples from the enrichment or the accumulation reactors were centrifuged (4000 rpm for 5 minutes). After freeze-drying the centrifuged samples, this was placed in a borosilicate glass beaker

containing 50 mL of 1-2 propylene-carbonate at 140 °C for 30 minutes, to allow PHA solubilization. Hereafter, the sample was filtered, and the solvent let to evaporate in a controlled environment at room temperature. The PHA content in the sample was first weighted and further analyzed through a spectrophotometric method utilizing a crotonic acid solution as a standard (ranging from 10 to 40 μg). The PHA extracted from the biomass samples was expressed in dry weight as a percentage of the measured TSS (% w/w).

2.1.2 Extraction of EPS

The heating method was used to extract the EPS from sludge samples (Le-Clech et al., 2006). The protein and carbohydrate concentrations were then measured using bovine serum albumin (Lowry et al., 1951) and glucose (DuBois et al., 1956) as standards, respectively. The total EPS contes was calculated as sum of the protein and carbohydrate concentrations and referred to the measured TSS (% w/w).

2.1.3 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 (χ) of PHA composites was calculated according to the following equation (Eq. (1)):

$$\chi\,(\%) = \frac{\Delta H_m}{\Delta H^0} \times 100 \qquad [2.1]$$

where ΔH_m is the melting enthalpy of the samples, respectively and ΔH^0 is the melting enthalpy of 100% crystalline which is assumed to be 146.6 J g^{-1} (Ansari and Fatma, 2016).
The intrinsic viscosity (η) 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 an oil bath thermostated 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 (η), values was calculated according to Solomon-Ciuta (Solomon and Ciută, 1962) (Eq. (2)):

$$\eta = \frac{\sqrt{2}}{c}\sqrt{\eta_{sp} - ln\,\eta_{rel}} \qquad [2.2]$$

where c is the concentration of the polymer solution, η_{sp} and η_{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)):

$$[\eta] = KM_V^{\alpha} \qquad [2.3]$$

The parameter values of the Mark-Houwink constants, α and K, depend upon the specific polymer-solvent system. For PHB-CHCl3, K = 1.18·10-4dL/g and α = 0.78 (Keenan et al., 2004).

2.1.4 Calculations

The PHA content in the extracted intracellular biopolymer was calculated using the following equation (Eq. (2.4)):

$$PHA = \frac{W_{poly} \cdot \%PHA}{TSS}\left(\frac{gPHA}{gTSS}\right) \qquad [2.4]$$

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 assays were evaluated by applying the following equation (Eq. (2.5)):

$$COD_{d(gCOD)} = PHA_{p(gCOD)} + EPS_{p(gCOD)} + X_{p(gCOD)} + COD_{r(gCOD)} \quad [2.5]$$

where:

- COD_d: the total mass of COD dosed during the test until the maximum accumulation capacity was obtained, 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 literature (Conca et al., 2020);
- 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 gCOD gPS^{-1} e 1.40gCOD gPN^{-1}. Glucose (DuBois et al., 1956) and bovine serum albumin (Lowry et al. 1951) 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 gCOD $gVSS^{-1}$ determined by direct measurements;
- COD_r: obtained from the product of the reactor volume by the COD concentration measured in the supernatant at the end of the accumulation assay.

The percentage of EPS produced expressed in dry weight (%wt) was calculated by dividing the mass of EPS by the mass of VSS present in the medium (Eq. (2.6)):

$$EPS\ (\%) = \frac{gEPS}{gTSS} x\ 100 \qquad [2.6]$$

The data of PHA production obtained from accumulation assays were interpolated using an exponential equation (Eq. (2.7)):

$$PHA(t) = PHA_{tot} \cdot \left(1 - e^{-k \cdot t}\right) \qquad [2.7]$$

where PHA(t) is the PHA content at a generic time, PHA_{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_{tot} and k were estimated using the solver function of Excel (MS Office), by minimizing the sum square of errors between the experimental data obtained from BMP assays and the results from the model. Therefore, the maximum PHA production (qPHA, gPHA $L^{-1}h^{-1}$) was calculated as the product between PHA_{tot} and k.

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Chapter 3: Utilization of SBR for PHA recovery from citrus wastewater treatment

Summary

The production of polyhydroxyalkanoates (PHA) using industrial wastewaters as feedstocks is a current and challenging topic. This chapter 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.

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3.1 Introduction

The present experiment 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 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 and 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. In addition, interesting aspects of the process were analyzed such as the effect on sludge settling, the variation of the molecular weight of the polymer produced as a function of the OLR parameter of the enrichment reactor and the resulting shift and change of the microbial community and diversity.

3.2 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 (SBR), 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 (Fig. 3.1). 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 (Corsino et al., 2021). The enrichment SBR (operating volume of 22 L) operated according to cycles lasting 12 h, divided as follows: 30 min of feeding under static conditions, maintaining the aeration and mixing devices inactive, 9 h of aeration, 2 h of settling under static conditions and, finally, 30 min 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. Details of the accumulation reactor can be found in Chapter 2.

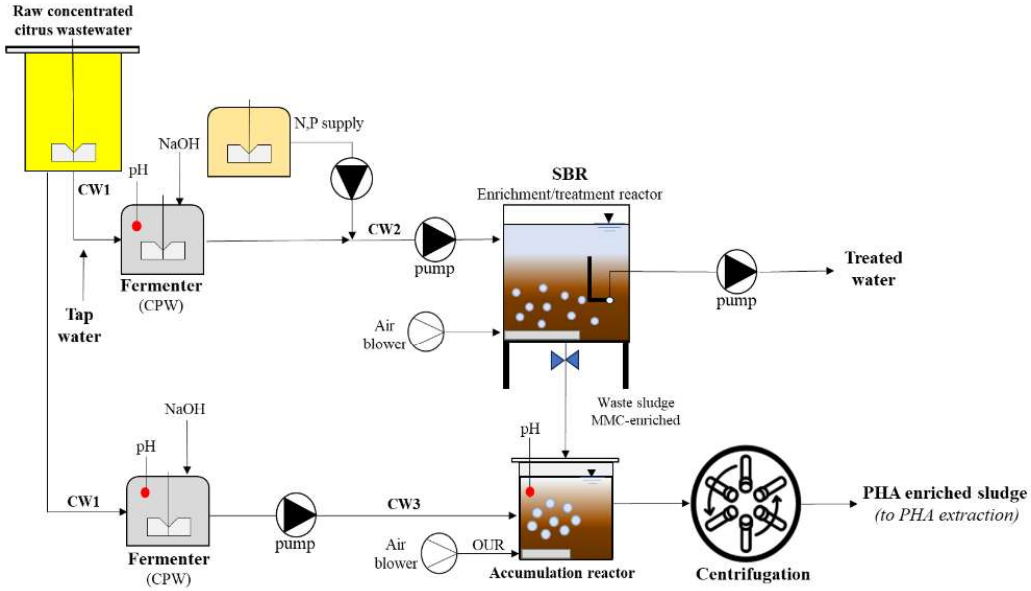


Figure 3.1 - Plant layout

3.3 Operating conditions and monitoring activities of the enrichment SBR

Based on both qualitative and quantitative seasonal variations of citrus wastewaters, it was decided to operate the enrichment SBR in three different periods, called Period 1, Period 2 and Period 3, characterized by increasing OLRs. In Period 1 (duration 78 days), the enrichment SBR 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 operating volume of the enrichment SBR was set to 22 L, increasing the volumetric exchange ratio in each period. The enrichment SBR 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 enrichment SBR in all three periods, by purging daily a known volume of mixed liquor variable according to the bacterial growth rate observed. Consequently, the SRT was variable in the three periods and calculated according to a mass balance (de Oliverira et al, 2018). 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 (Dionisi et al.,2016).

3.4 PHA accumulation reactor

The PHA accumulation assays were carried out in the accumulation reactor using the enriched biomass derived from the enrichment SBR and the fermented CW3 as reported in chapter 2. 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 set 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 as described in chapter 2. 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 h.

3.5 Analytical methods

All the physical-chemical analyses for the assessment, such as TSS, VSS, COD, BOD, NH_3-N, TN, TP, pH, DO, electrical conductivity, concentration of acetate, as well as biokinetic parameters were carried out according to Chapter 2. In addition, DNA sequencing analysis was added for this specific activity according to the method described in the following subsection.

3.5.1 Total DNA extraction and 16S rRNA gene sequencing

The mixed microbial consortia of the sludge samples collected from the enrichment SBR at the end of each experimental period were analyzed using a metagenomics approach based on 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 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′-CCTACGGGNBGCASCAG -3′) and Pro805R (Rev 5′-GACTACNVGGGTATCTAATCC -3′) (Takahashi et al., 2014). 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.

3.6 Results and discussion

3.6.1 Performances of the enrichment reactor

The performances of enrichment SBR 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 Yobs. Fig. 3.2 shows the trends of the COD concentrations in the inlet and outlet of the enrichment SBR and the related removal performance (Fig.3.2a), as well as the trend of the SVI (Fig. 3.2b) and the average values of Yobs and SRT (Fig. 3.2c) 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^{-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^{-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^{-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 $m^{-3}d^{-1}$ (Corsino et al., 2018; Di Trapani et al., 2019). 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 (Ioppolo et al., 2020; Zema et al., 2018). 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 (Shrivastava et al., 2022; Barbera et al., 2018). 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 favourable to the growth of floc forming bacteria than filamentous ones, with an important benefit on the settling properties of the activated sludge (Sheker et al., 1993). 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 (Pronk et al., 2015). 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. (2018) 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. (Zema et al., 2019), 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. Yobs increased proportionally to the OLR. In Period 1, the average value of the Yobs 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 Yobs 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 (Hreiz, et al., 2015), 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 (Banti et al., 2020).

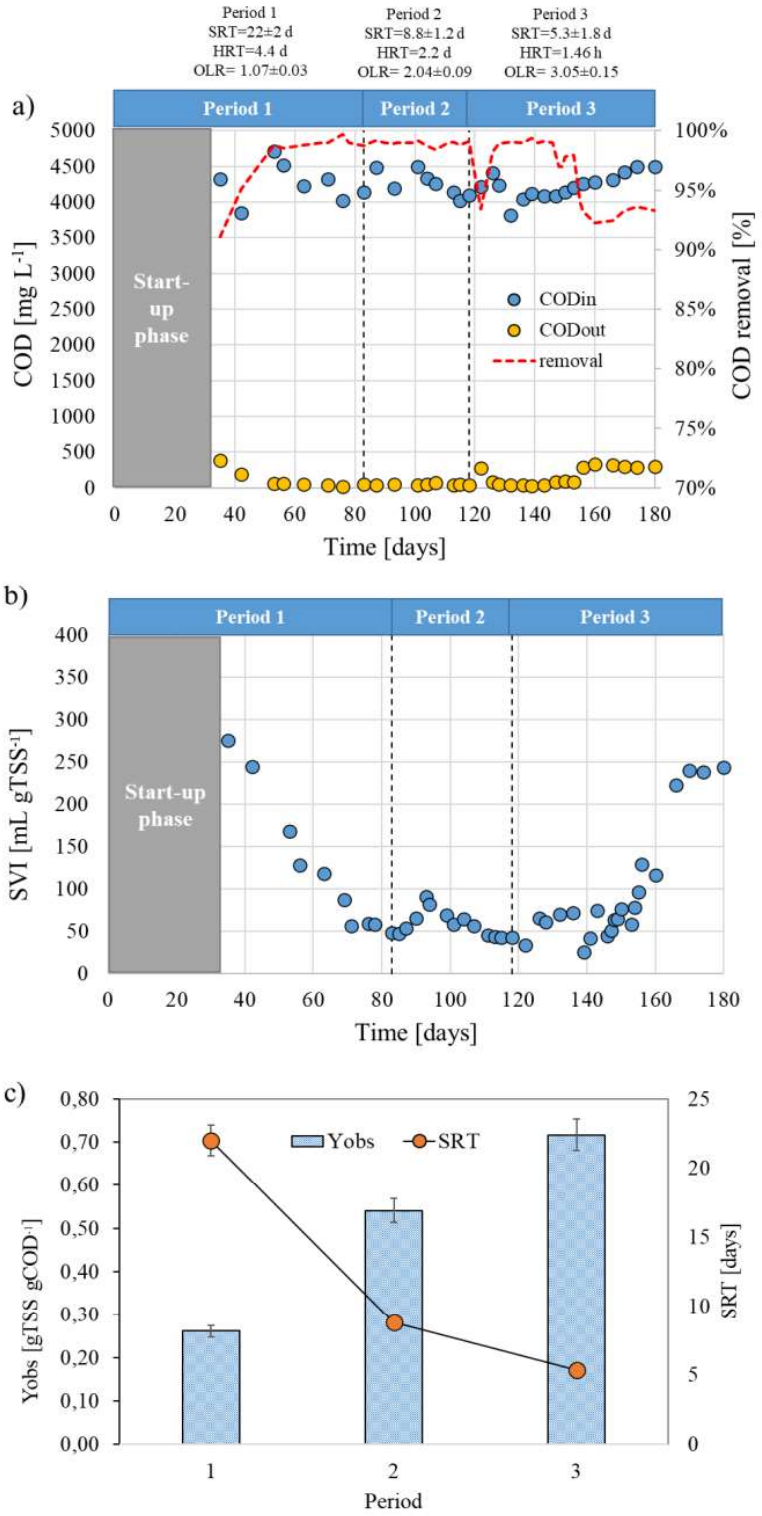


Figure 3.2 - Trends of the COD concentration in the influent and effluent of the enrichment SBR an COD removal efficiency (a); trend of the SVI (b); average values of Yobs and SRT in the three experimental periods (c)

3.6.2 Metabolic kinetics of the enriched biomass

The kinetic parameters of heterotrophic bacteria performed on the enriched MMC of the enrichment SBR are summarized in Table 3.1:

Table 3.1: Summary of the kinetic parameters of the activated sludge in the three experimental periods.

Period	Organic loading rate *OLR* [kgCOD $m^{-3}d^{-1}$]	Food to microorganisms *F/M* [kgCOD $kgTSS^{-1}d^{-1}$]	Maximum yield Y_H [kgCOD $kgCOD^{-1}$]	Active fraction f_{xH} [%]	COD depletion rate $v_{H,max}$ [mgCOD $gTSS^{-1}h^{-1}$]	Feast/famine ratio *F/F* [hours]
1	1	0.22	0.75	15	104.3	0.13
2	2	0.43	0.83	29	274.4	0.19
3	3	0.63	0.86	28	189.8	0.39

Legend: MLTSS (Mixed Liquor Total Suspended Solid); OLR (Organic loading rate); F/M (Food/microorganisms); Y_H (Maximum yield); f_{xH} (Active fraction); $v_{H,max}$ (COD depletion rate), *F/F* (Feast/famine ratio).

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 Yobs. The minimum value was obtained in the Period 1, in which the YH 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 (Paulo et al., 2021; Devlin et a., 2016).

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 ($v_{H,max}$) showed a non-linear trend with the OLR, but consistent with the active fraction values. The lowest $v_{H,max}$ 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 $v_{H,max}$ 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 (Matos et al., 2021). 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 $v_{H,max}$, 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 the high availability of rapidly biodegradable organic substrate was favorable to bacterial synthesis. Furthermore, from Period 2 to Period 3, the lower value of $v_{H,max}$ while maintaining the same f_{xH}, suggested the development of different bacterial strains characterized by a lower rate of COD removal (Table 3.2).

Table 3.2: Alpha-diversity indexes based on Operational Taxonomic Units (OTUs 99%) of P1 and P3 bacterial communities in SBR

Diversity indexes	Enrichment SBR samples	
	P1	P3
Taxa_S	280	133
Dominance_D	0.05798	0.2027
Shannon_H	4.064	2.406
Evenness_e^H/S	0.208	0.08342
Equitability_J	0.7213	0.4921
Fisher_alpha	45.83	17.92

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 (Conca et al., 2020). The values obtained in this experimentation were lower than 0.20 in Period 1 (0.13 ± 0.07) and in Period 2 (0.19 ± 0.08). Such values indicated the effective selection of PHA-accumulating biomass (Hao et al., 2018). The observed kinetic parameters were on average higher than those obtained in other studies dealing with citrus wastewater reported in the literature (Zema et al., 2018). On the other hand, the results were comparable with those obtained in a SBR type plant with granular biomass treating citrus wastewater (Corsino et al., 2018). 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 (Chakravarty et al., 2010). This confirmed the ability of the mixed culture biomass to robustly

adapt to a shift of OLR (De Grazia et al., 2017). 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. In Period 3, residual organic carbon in soluble form was measured in the effluent of enrichment SBR. This suggested that soluble COD was not entirely depleted during the feast phase and it remained available during the famine phase. As a result, the F/F was resulted higher than 0.30.

3.6.3 Morphological characteristics of the activated sludge

The different operating conditions in the enrichment SBR 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.

Fig. 3.3 shows the microscopic images of the activated sludge in the three periods carried out on the fresh sample of the enrichment SBR (left column) and on the samples of the enrichment SBR (central column) and accumulation reactor (right column) subjected to Sudan Black staining.

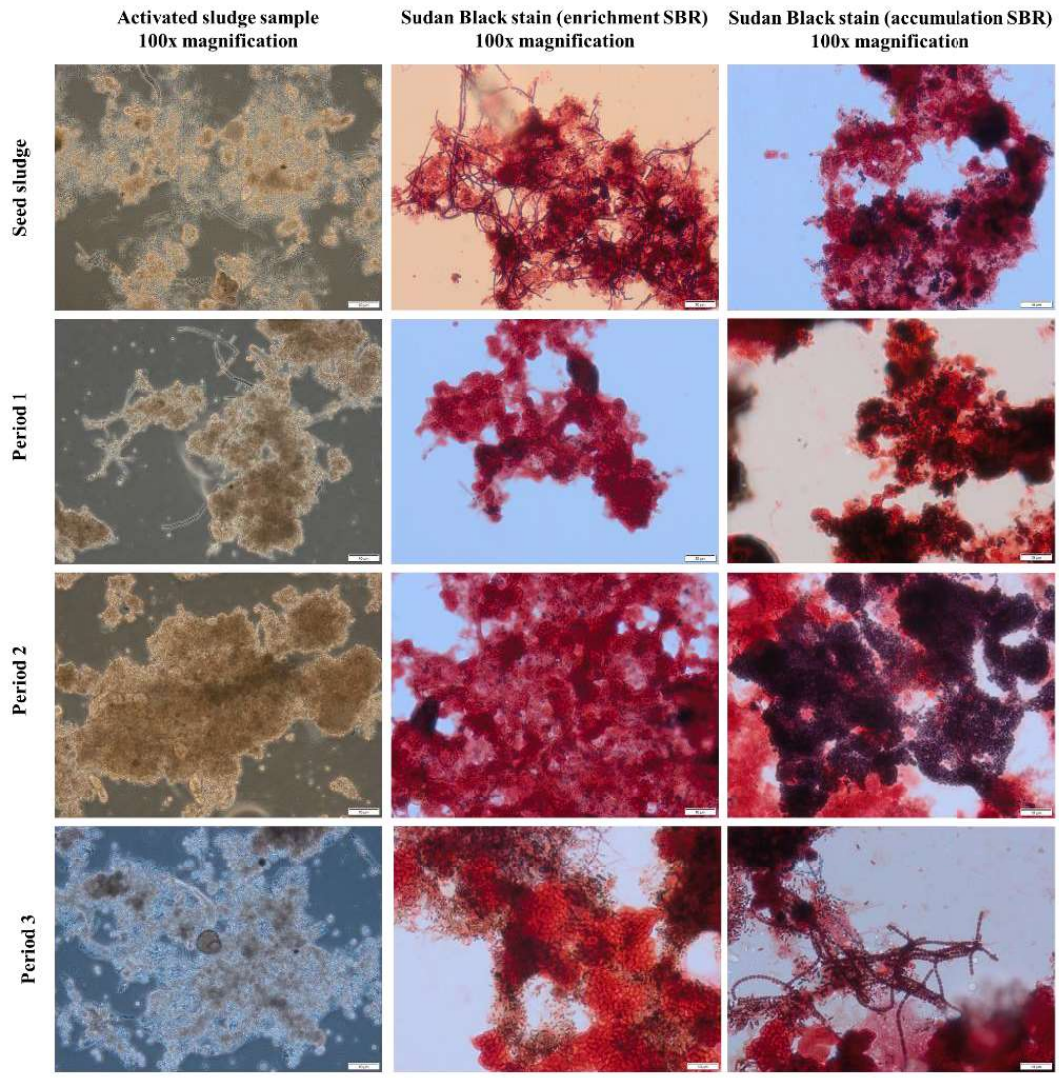


Figure 3.3: Microscope images of the activated sludge of the seed sludge and the three periods: left column - fresh sample from the enrichment SBR; central column - sample of the enrichment SBR stained with Sudan Black; right column - sample of the accumulation reactor stained with Sudan Black. Bars are 10 μm long

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 (Nielsen et al., 2009; Tsang et al., 2008). 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 SBR, resulted in a significant improvement of the activated sludge flocs structure in the Period 1. 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 (Majone et al., 1996).

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 neither bridging nor 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 favoured the overgrowth of filamentous bacteria that caused remarkable effects on the flocs' morphology. This result was in good agreement with previous literature (Jones et al., 2010). Nevertheless, abundant Sudan Black staining positive granules were observed within the cells of filamentous bacteria, indicating the ability of such bacteria in PHA accumulation (Bengtsson et al., 2010; Jenkins et al., 2003).

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 3.6.1, 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 enrichment SBR 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 paragraph 3.6.2. 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 (Jenkins et al., 2003).

3.6.4 Bacterial diversity in the enrichment reactor

The MMC of the sludge samples taken at the end of each period from the enrichment reactor enrichment SBR 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 (Pereira et al., 2020; Dionisi et al., 2006). A recent study suggested that under high OLR the diversity of the bacterial community decreased because the increased VFA accumulation (Chen et al., 2020). This could be the reason of the reduction of biodiversity observed in Period 3.
Diversity indexes values obtained in this experimentation were consistent (Pereira et al., 2020) 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. (Coats et al., 2016) suggested that microbial communities grown on real wastewater, similar to the one used in this experimentation, may be characterized by higher richness and diversity than MMCs cultivated on

synthetic or selected substrates (Chen et al., 2020). 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 enrichment SBR 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.

The dominant phyla in both the P1 and P3 MMC were Proteobacteria and Bacteroidetes (Fig. 3.4). Few other *phyla, Verrucomicrobia, Acidobacteria, Firmicutes* and the *TM7* division, had relative abundances >1% in at least one of the two samples (Fig. 3.4). Period 1 MMC was dominated by *Betaproteobacteria* (41.14%) and *Bacteroidetes* (27.51%). The most abundant families of B*etaproteobacteria* 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 (Zhang et al., 2019).

In Period 3, the community was enriched in the phylum Bacteroidetes (54.48%) that became dominant followed by Betaproteobacteria (34.25%). Within *Bacteroidetes Flavobacterium* (*Flavobacteraceae*) 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).

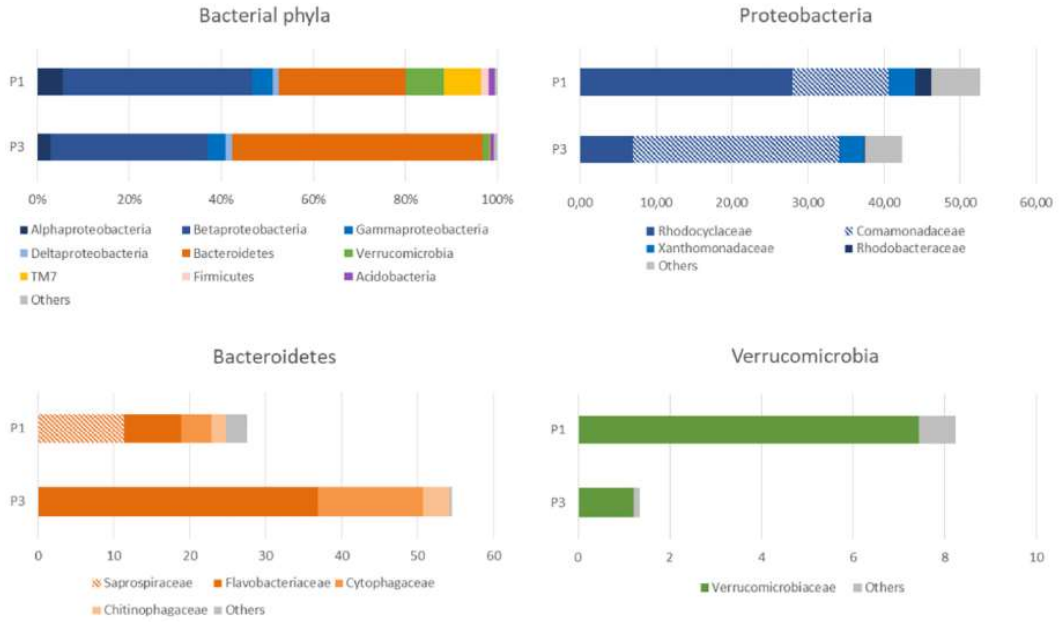


Figure 3.4: Bacterial community composition of the enrichment SBR at the end of Period 1 (P1) and Period 3 (P3). Relative abundance of bacterial phyla and distribution of families (>1%) within the most abundant phyla. The families within the other phyla are not shown because their abundances are <1%. The phylum Proteobacteria is classified as classes.

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* (*Flavobateriaceae*), which is often found in mixed culture under feast/famine conditions for PHA production (Dionisi et al., 2006), and *Runella* (*Cytophagaceae*), comprise strains isolated from activated sludge performing enhanced biological phosphorus removal (Bernardet & Bowman, 2015; Ryu et al., 2006) although it is also responsible for sludge bulking (Zhang et al., 2019). Both P1 and P3 communities comprised known Poly (3-hydroxybutyrate) - accumulating genera (Fig. 3.5) such as *Zooglea, Pseudorhodoferax, Aquincola* (*Betaproteobacteria*) and *Rhodobacter* (*Alphaproteobacteria*) (Monroy & Buitròn, 2020; Chen et al., 2013; Unz, 2015; Bruland et al.,

2009; Lechner et al., 2007). The genera *Zooglea* and *Pseudorhodoferax* may be among the main PHA-producers in Period 1 and Period 3 communities, respectively. Moreover, *Zoogloea*, 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 enrichment SBR 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.

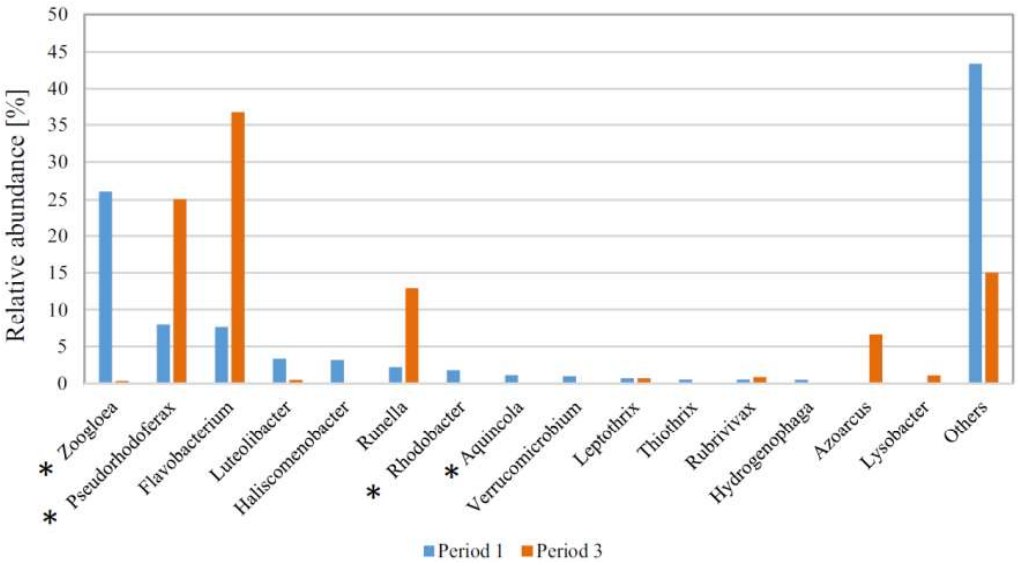


Figure 3.5: Abundance of bacterial genera in enrichment SBR, at Period 1 and Period 3. Only genera with relative abundances >0.5% in at least one sample are shown. Unclassified and less represented (<0.5%) genera are grouped as "Others". The asterisk indicates genera including known PHA producing species as reported in the literature.

3.6.5 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. Fig. 3.6 shows the results of the FTIR-ATR analysis carried out on

the samples extracted in each period (Fig. 3.6a), the maximum biopolymers production at the end of the accumulation assays (Fig. 3.6b) and the theoretical productivity of PHA referred to the volume of wastewater treated (Fig. 3.6c).

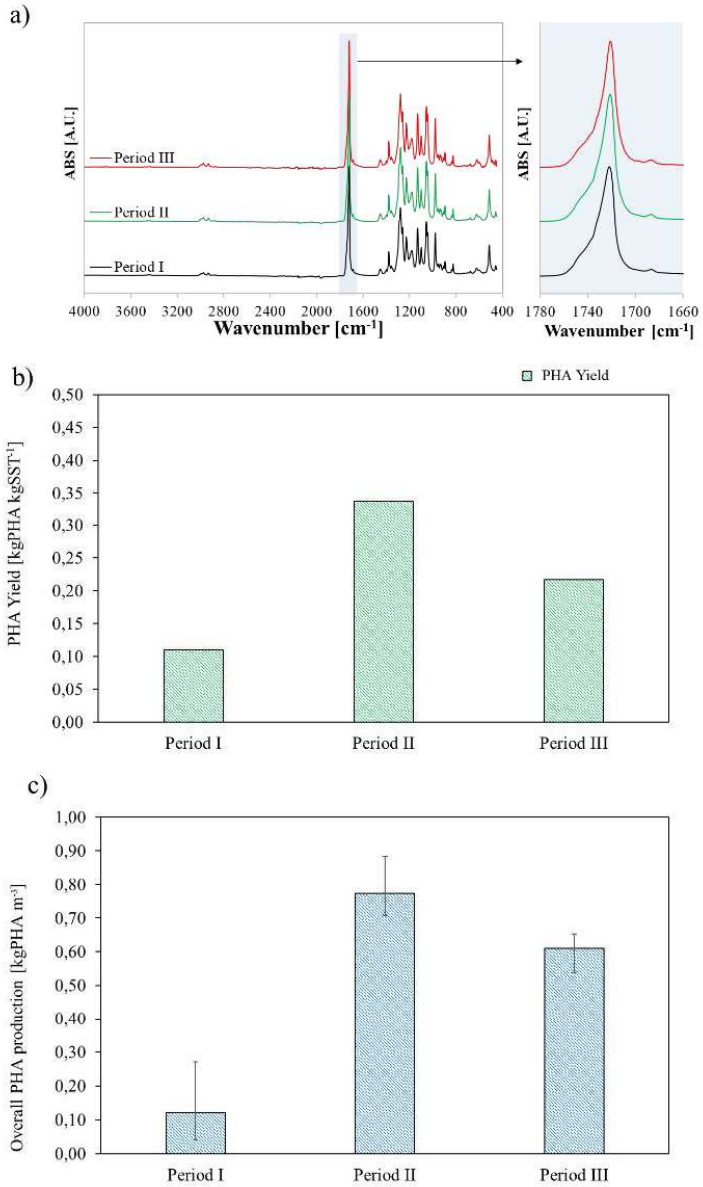


Figure 3.6: FTIR-ATR spectra of the extracted biopolymers (a); maximum biopolymers productivity (b); maximum specific production of PHA obtained in each period (c).

FTIR-ATR measurements revealed the typical bands of (hydroxybutyrate) HB monomer, and any other co-monomers were observed (Fig. 3.6a). 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 (Conca et al., 2020; Morgan-Sagastume et al., 2015; Carvalho et al., 2014). Furthermore, other authors observed that the production yield of polyhydroxyvalerate P(3HV) 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 (Carvalho et al., 2014). Furthermore, other authors reported that if the pH in the biopolymer accumulation reactor is higher than 7.50, the production of P(3HV) is significantly reduced (Kourmentza et al., 2016). These observations can justify the absence of P(3HV) in the biopolymers extracted in this experimentation, given that the conditions unfavourable to the synthesis of P(3HV) 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 -CH3, -CH2, 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. 3.6b. 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 (Morgan-Sagastume et al., 2020; Valentino et al., 2017). This confirmed that PHA production by MMC appears consistently achievable using real wastewater from food industries enriched in VFA (Morgan-Sagastume et al., 2020). By applying the Eq. (2.6), 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 0.04gPHA 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 $L^{-1}d^{-1}$) (Campanari et al., 2014) and 2.4 gCOD $L^{-1}d^{-1}$ (tested OLR between 1.2 and 3.6 gCOD L-1d-1) (Fang e tal., 2020). 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. (Conca et al., 2020), 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., (2020) treating fermented municipal wastewater containing acetic, propionic and valerate acid in different ratios (0.40 gPHA L^{-1} h^{-1}). A possible explanation to the above result could be due to the OLR applied in the accumulation reactor, which was about three times the one applied in the other studies (Valentino et al., 2019). 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 (Valentino et al., 2019). 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 (Matos et al., 2021). Nevertheless, the PHA productivity was similar to that achieved in studies carried out with real industrial wastewaters (0.03-0.09 gPHA L^{-1} h^{-1}) (Campanari et al., 2014; Bengtsson, et al., 2008). 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 (Calabrò et al., 2016). 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 (Jayakrishnan et al., 2021; Argiz et al., 2020).

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. 3.6c). 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 (Matos et al., 2021). 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.

3.6.6 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 2.1.4. The results obtained are reported in Table 3.3.

Table 3.3: COD mass balance results over the three periods

Period	*PHA* [%]	*EPS* [%]	*New cells* [%]	*Residual COD* [%]	*Other [%]*
1	23	18	4	52	*3*
2	51	13	3	31	2
3	36	5	5	51	3

The maximum conversion yield of COD into PHA was observed in Period 2, where about 51% of the COD was converted into intracellular polymers, while in the Period 1 and Period 3 the yields were lower and equal to 23% and 36%, respectively. The results obtained were consistent with those

obtained by other authors, who reported maximum PHA production yields equal to about 0.60 gCOD PHA $gCOD^{-1}$ (Lorini et al., 2020; Sudesh et al., 2011; Albuquerque et al., 2010), even in case of using agro-food wastewaters. Indeed, Gouveia et al. (Gouveia et al., 2017) obtained a maximum PHA production yield between 0.56 and 0.68 gCODPHA $gCOD^{-1}$ using dairy wastewater, while Campanari et al. (Campanari et al., 2017) have obtained similar yields (0.55 gCODPHA $gCOD^{-1}$) 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 0.18 gCOD $gCOD^{-1}$ in Period 1, 0.13 gCOD $gCOD^{-1}$ in Period 2 and, finally, 0.05 gCOD $gCOD^{-1}$ in Period 3. This result suggested the lower propensity of biomass to produce exopolymers as the OLR in the enrichment SBR increased. It should also be noted that in Period 3 the presence of filamentous bacteria in accumulation reactor, 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 (Li et al., 2008). 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 (Cui et al., 2017). 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 (Campanari et al., 2014).

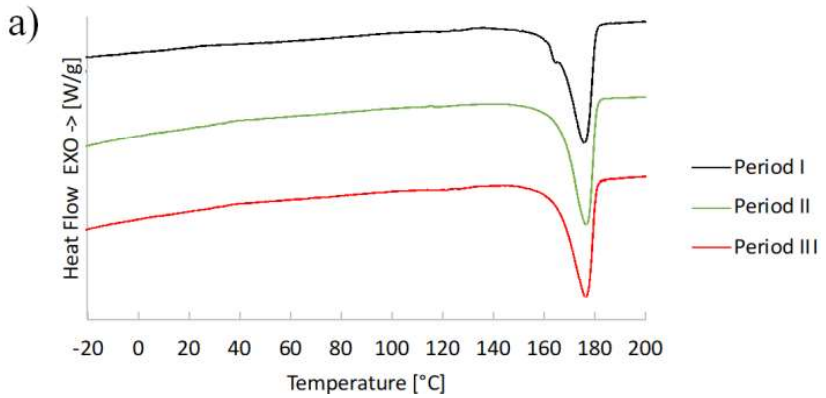
Regarding the production of new biomass, in all three cases, it was in the order of 5%, suggesting that the process conditions in the accumulation reactor were not favorable to microbial growth because of the lack of nutrients.

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 enrichment SBR. The lowest

residual COD was observed in Period 2 (31%), whereas this was greater in Period 1 (52%) and Period 3 (51%).
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 (Mitra et al., 2020). The results obtained in this experimentation 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.

3.6.7 Physical and thermal characteristics of the biopolymers

In Fig. 3.7 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 Fig. 3.7a.



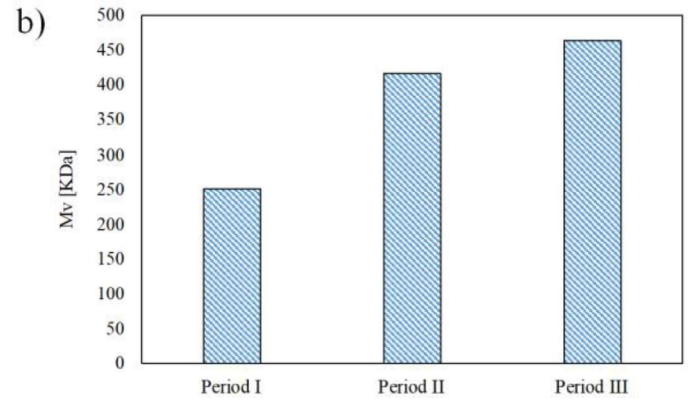


Figure 3.7: DSC thermograms (a) and viscosimetric molecular weight of PHB (b) as a function of the experimental period.

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 (Wellen et al., 2013; Keenan et al., 2004; Lee et al., 2002). 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. 3.7b) 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.

3.6.8 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 experimentation. 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 experimentation 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.

3.6.9 References

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Chapter 4: Utilization of MBR for PHA recovery from citrus wastewater treatment

Summary

The present chapter investigated the combined production of reclaimed water for reuse purposes and polyhydroxyalkanoates (PHA) from an agro-food industrial wastewater. A pilot plant implementing a two-stage process for PHA production was studied. It consisted of a mainstream MBR in which selection of PHA-accumulating organisms and wastewater treatment were carried out in, and a side-stream enrichment reactor where the excess sludge from the MBR was used for PHA accumulation. The performance of the MBR was compared with that of the conventional SBR, as discussed in the previous chapter, under different F/M ratios ranging between 0.125-0.650 kgCOD $kgTSS^{-1}$ d^{-1}. The MBR enabled to obtain very high-quality effluent in compliance with the relevant national (Italy) and European regulations (Italian DM 185/03 and EU 2020/741) in the field of wastewater reclamation, whereas the performances in the SBR collapsed at F/M higher than 0.50 kgCOD $kgTSS^{-1}d^{-1}$. A maximum intracellular storage of 45% (w/w) and a production yield of 0.63 gPHA $L^{-1}h^{-1}$ were achieved when the MBR system was operated with a F/M ratio close to 0.50 kgCOD $kgTSS^{-1}d^{-1}$. This resulted approximately 35% higher than those observed in the SBR, since the ultrafiltration membrane avoided the washout of dispersed and filamentous bacteria capable of storing PHA. Furthermore, while maximizing PHA productivity in conventional SBR systems led to process dysfunctions, in the MBR system it helped mitigate these issues by reducing membrane fouling behaviour. The results of this experimentation supported the possibility to achieve combined recovery of reclaimed water and high-value added bioproducts using membrane technology, leading the way for agro-food industrial wastewater valorization in the frame of a circular economy model.

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4.1 Introduction

The results obtained in the enrichment SBR experiment showed that productivity was strongly influenced by OLR. Higher values of OLR led to a gradual decrease in both purification performance and biopolymer production, although the quality of biopolymer increased, as indicated by the lower degree of crystallinity. 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 take into consideration both purification performance and compliance with discharge limits imposed by regulations. To enhance PHA production with MMCs, the second experiment focused on the possibility of implementing an advanced biological process of the MBR type. Such a system 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. However, to the author's knowledge few studies in the literature explore MBR systems for PHA production. Moreover, MBR is a technology that allows for water recovery so, in addition to PHA recovery, the possibility of reusing reclaimed water has also been evaluated. Additionally, the evolution of the membrane fouling phenomenon simultaneously with biomass enrichment with PHA-storing bacteria was studied throughout the experiment.

4.2 Experimental set-up

For this experiment a sequencing batch MBR was used as MMC selector and wastewater treatment unit simultaneously. Specifically, the enrichment MBR was operated according to the aerobic dynamic feeding regime to enrich the MMC with PHA-storing microorganisms and obtain effluent wastewater for reuse purposes. A schematic layout of the pilot plant is depicted in Figure 4.1.

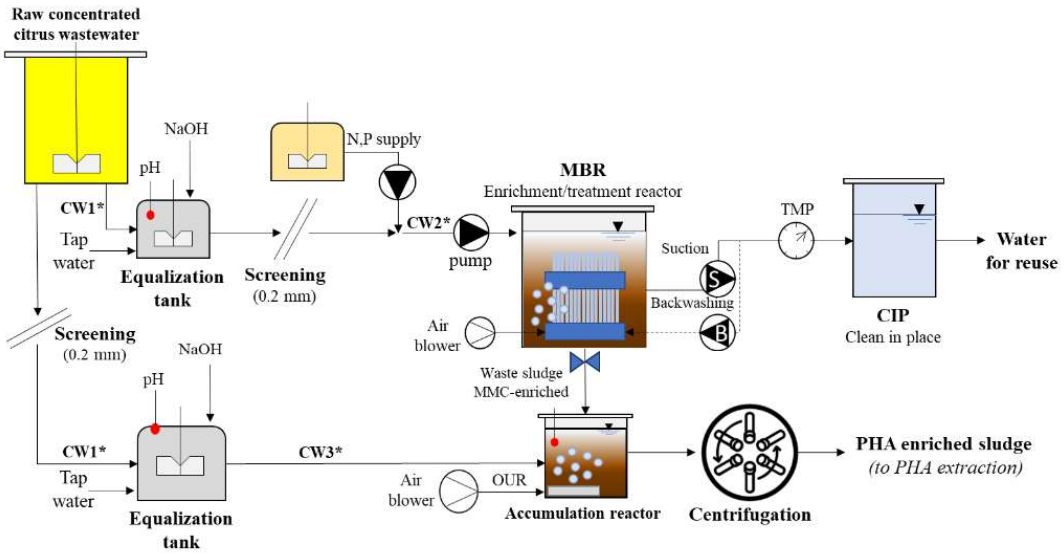

Figure 4.1: Pilot plant layout MBR

The enrichment MBR consisted of a fiberglass tank with a working volume of 45 L equipped with an ultrafiltration hollow-fibers membrane in submerged configuration (0.03 µm of porosity, 1.4 m^2 of surface, PURON® triple bundle Demo). The MBR system operated by alternating periods of carbon source availability and absence according to the feast-famine regime. More precisely, operational cycles were of 12 hours consisting of 30 minutes of feeding under static conditions, maintaining the aeration, and mixing devices inactive, 640 minutes of aeration, 60 minutes of filtration and finally 30 minutes of idle to allow membrane relaxation. The filtration cycle had a duration equal to 6 minutes, divided into 5 minutes of permeate extraction and 1 minute of backwashing. The membrane backwashing was carried out by pumping a volume of permeate back through the membrane fibers from a clean in place (CIP) tank in which the permeate was stored. The reactor was equipped with a pair of porous stone diffusers placed at the bottom of the reactor that were connected to an air compressor providing a flow of 25 L min^{-1}. Aeration was supplied during all stages excepting for the feeding and the idle phases. All the equipment were connected to a programmable logic controller that handled the phases' alternation. The accumulation reactor was fed with the excess sludge withdrawn from the MBR and CW3*. The characteristics of the wastewater and accumulation reactor are reported in Chapter 2.

4.3 Plant operations

The enrichment MBR was inoculated with activated sludge collected from a conventional activated sludge plant that treated the same CPWW. The experiment lasted 210 days divided into four periods, named Period 1 (P1), Period 2 (P2), Period 3 (P3) and Period 4 (P4), characterized by different food to microorganism's ratio (F/M). In more detail, the F/M was increased from 0.125 kgCOD $kgTSS^{-1}$ d^{-1} (P1) to 0.25 kgCOD $kgTSS^{-1}$ d^{-1}, 0.50 kgCOD $kgTSS^{-1}$ d^{-1} and 0.65 kgCOD $kgTSS^{-1}$ d^{-1} in P2, P3 and P4, respectively. The different F/M were obtained by increasing the influent flow rate, hence the volumetric exchange ratio of the MBR, and by adjusting the TSS concentration in the reactor. More precisely, the volume of CPWW treated in a day was equal to 10 L in Period 1 and Period 2, whereas it was increased to 20 L d^{-1} and 30 L d^{-1} in Period 3, and Period 4, respectively. Overall, the HRT (Hydraulic Retention Time) resulted equal to 4.5 d, 2.25 d and 1.5 d in Period 2, Period 3, and Period 4, respectively. The membrane flux was increased in all the experimental periods by increasing volume of permeate extracted during each period, while maintaining it below the critical flux suggested by the membrane manufacturer (20 L $m^{-2}h^{-1}$). The TSS concentration in the SBMBR was decreased from 8 gTSS L^{-1} (P1 and P2) to 6 gTSS L^{-1} (P3) and 6.3 gTSS L^{-1} (P4), to achieve the desired F/M. The sludge retention time was calculated according to a mass balance between the overall biomass present in the reactor and the one withdrawn as waste sludge. The results of the MBR were compared with that of the experiment carried out with the conventional SBR in Chapter 3, which was operated for 3 experimental phases only, corresponding to Period 2, Period 3 and Period 4 of this Chapter in terms of F/M. Table 4.1 summarized the operating conditions of the enrichment MBR and enrichment SBR.

Table 4.1: Summary of the main operating conditions of the MBR and SBR enrichment reactors

	Period 1		Period 2		Period 3		Period 4	
	SBR*	MBR	SBR	MBR	SBR	MBR	SBR	MBR
Duration [days]	*	60	78	65	32	42	62	43
Influent total COD [g L-1]	*	4.45±0.2	4.53±0.1	4.51±0.3	4.45±0.1	4.46±0.4	4.30±0.2	4.48±0.1
Daily flow [L d^{-1}]	*	10	5	10	10	20	15	30
Biomass [gTSS L^{-1}]	*	8.1± 0.1	4.5± 0.1	8.0± 0.2	4.42±0.1	6.3± 0.1	4.46±0.2	6.3± 0.2
F/M [kgCOD $kgTSS^{-1}d^{-1}$]	*	0.125±0.1	0.23±0.1	0.24±0.2	0.48±0.1	0.51±0.3	0.64±0.1	0.65±0.2
SRT [days]	*	17	14	14	11	11	5	9

*: *not operated*

4.4 Analytical methods

All the physical-chemical analyses for the assessment, such as TSS, VSS, COD, BOD, NH_3-N, TN, TP, pH, DO, electrical conductivity, concentration of acetate, as well as biokinetic parameters were carried out according to Chapter 2. In addition, membrane fouling analysis was added for this specific activity according to the method described in the following subsection.

4.4.1 Membrane Fouling Analysis

The membrane fouling was investigated by assessing the total resistance to filtration (R_T) and the fouling rate (FR), the latter calculated as the daily increase of the R_T. Specifically, the permeate flux and TMP were measured during normal plant operations, thus the R_T was calculated as follows (Eq. 4.1):

$$R_{t0} = \frac{TMP}{J_1 \cdot \mu} \qquad [4.1]$$

where J_1 [m^3 m^{-2} s^{-1}] and TMP_1 [Pa] are the permeate flux and the transmembrane pressure measured at the end of the filtration cycle, respectively, while μ is the permeate viscosity [Pa s] at the operating temperature (Di Bella et al., 2018).

In addition, the membrane permeability (K) was calculated with the following equation (Eq. 4.2):

$$K = \frac{J_1}{TMP} \qquad [4.2]$$

The resistance-in-series (RIS) model was used to assess the membrane fouling mechanisms. For further details, the reader is referred to the literature (Di Bella et al., 2018).
The settling properties of the SBR sludge were assessed by performing the sludge volume index test.

4.5 Results and discussion

4.5.1 Performances of the enrichment MBR

Performances of the enrichment MBR in terms of BOD and COD removal efficiency were compared with the average obtained in the enrichment SBR, as reported in the previous chapter. Figure 4.3 shows the trends the influent BOD (a) and COD (b) and relative values of the effluent from the MBR and SBR enrichment/treatment reactors. The effluent values were compared with the limit values reported in the main European and Italian directives in force: the EU 2020/741 concerning the reuse of treated wastewater for agricultural irrigation (BOD < 10 mg L^{-1}; COD not reported), the Italian Ministerial Decree DM 185/03 (COD < 100 mg L^{-1}; BOD < 20 mg L^{-1}) and the Italian Legislative Decree 2006/152 implementing the CEE 91/271 for the discharge into the public sewer system (COD < 500 mg L^{-1}; BOD < 250 mg L^{-1}).

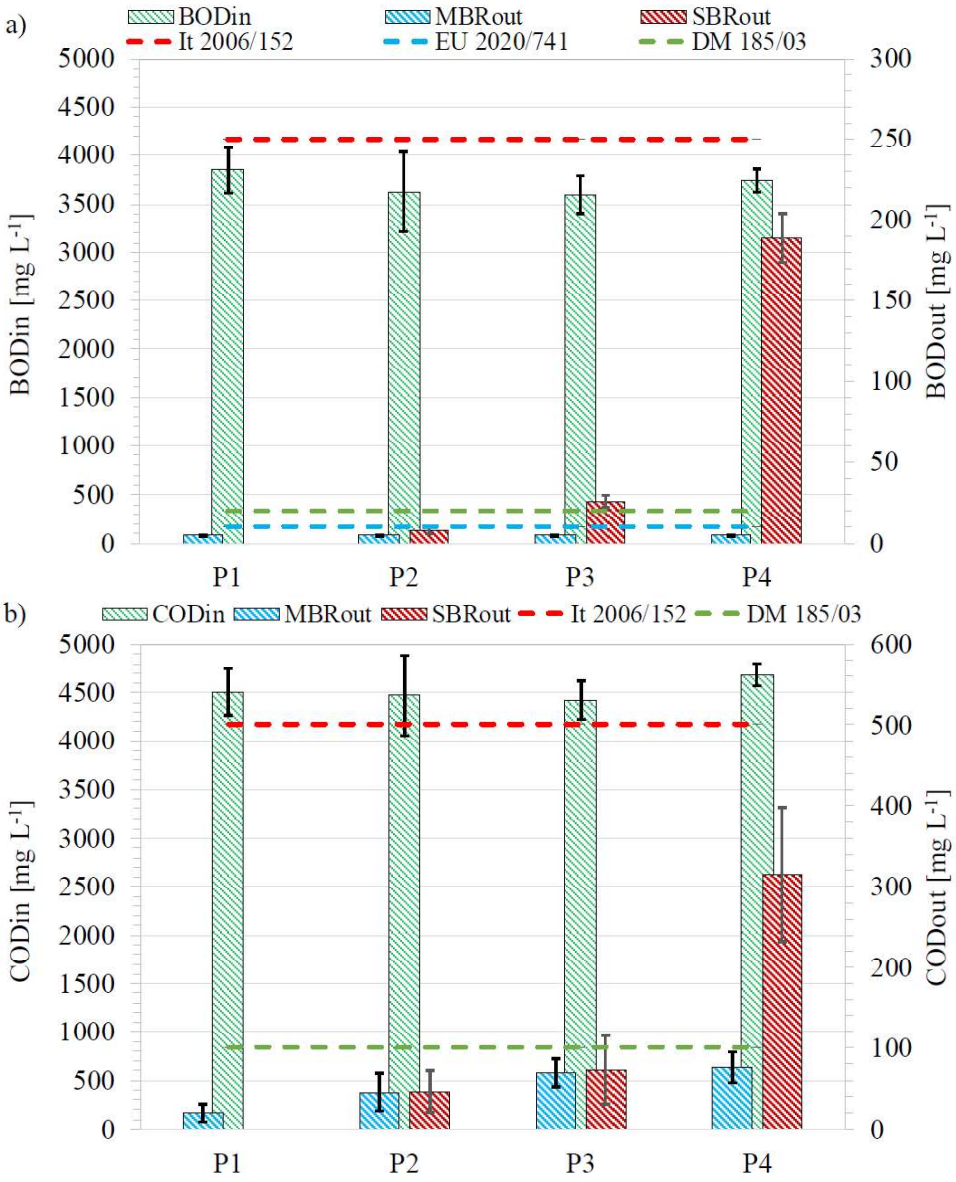

Figure 4.2: Average values of the total COD in the raw CPWW and the effluents of the SBMBR and SBR. The blue ( ), green ( ) and red ( ) dotted lines represent the limit imposed by the EU 2020/741, by the Italian Ministerial Decree (DM) 185/03 and by the Italian Legislative Decree 2006/152 implementing the CEE 91/271, respectively.

The enrichment MBR enabled to obtain very high BOD and COD removal in all the experimental phases, close to 99%, on average. Specifically, the BOD was always below the detection limit of the method (5 mg L^{-1}), whereas the average COD value in the permeate resulted lower than 100 mg L^{-1}, while showing a slightly increasing trend with the F/M applied. Indeed, the lowest COD values in the permeate were obtained during Period 1 (<

20±11 mgCOD L^{-1}), whereas the highest (76±19 mgCOD L^{-1}) was observed in Period 4 when the F/M was equal to 0.65 kgCOD $kgTSS^{-1}d^{-1}$. Therefore, as far as concerns BOD, COD, the enrichment MBR allowed obtaining an effluent permeate in compliance with the main reuse requirement established by all the in-force regulations in the field of wastewater reclamation. In contrast, BOD and COD removal performances in the SBR collapsed at F/M higher than 0.50 kgCOD $kgTSS^{-1}d^{-1}$, although the effluent quality complied with the discharge limits imposed by CEE 91/271. Overall, the enrichment MBR exhibited a greater process stability for a wider range of F/M referring to BOD and COD removal, whereas the enrichment SBR was more instable when increasing the F/M. Indeed, when increasing the OLR at the beginning of each period, the enrichment MBR achieved steady state in less than 3-4 days, corresponding to about twice the HRT. In contrast, the enrichment SBR required a slight longer adaptation period, close to 4-5 HRT. At the maximum F/M applied, the performance of the enrichment SBR deteriorated within 2 weeks, whereas as previously mentioned the enrichment MBR did not show any significant variation.

As reported in the previous chapter, high F/M ratio caused the modification of the activated sludge composition and the predominance of filamentous bacteria that contributed to worsen the sludge settleability. Consequently, the increase of the effluent turbidity and the wash-out of active biomass caused the deterioration in BOD and COD removal performances. The enrichment MBR allowed sustaining better BOD and COD removal at higher F/M than the enrichment SBR, likely because the biomass retention capacity provided by the ultrafiltration membrane (Di Bella et al., 2010). Therefore, MBR systems resulted better suited to operate at higher F/M than conventional activated sludge systems based on gravity solid-liquid separation process, especially with a view of being integrated in a mainstream process for simultaneous PHA production and water reuse. Indeed, regarding PHA production high F/M (> 0.6 kgCOD $kgTSS^{-1}d^{-1}$) are required to obtain an efficient selection of PHA accumulation organisms in the MMC (Cruz et al., 2022; Frison et al., 2021). In this respect, SBR was proven to be ineffective to provide high-quality effluent when operating at high F/M, whereas MBR enabled a permeate that in terms of residual organic content met the minimum standards for reuse. Moreover, it should

be stressed that applying high F/M allows to operate with reactor having smaller volume, thus contributing to reduce the plant footprint.

4.5.2 Compliance of enrichment MBR permeate with reuse requirements

Performance of enrichment MBR towards quality parameters for wastewater reuse are reported in the Table 4.2.
In more details, two Regulations, both in force, were considered in this chapter. The first regulates level of wastewater reclamation for irrigation, civil and industrial purposes in Italy (DM 185/03), whereas the second (EU 2020/741) refers to irrigation purposes in Europe. The European regulation divided reclaimed water into four quality classes. Specifically, the water quality decreases from the highest (class A), without any restriction of using in irrigation, to the worst (class D), which is restricted to be applied only on commercially processes crops that has no contact with humans or livestock. The Regulation sets out quality requirements for several parameters, namely E. Coli, BOD_5, TSS, turbidity. Furthermore, the Regulation considers additional requirements that may be imposed following a risk assessment for specific supplies, including heavy metals, pesticides, and others micropollutants. In contrast, the regulation in force in Italy (DM 185/03) sets restricted limits even for micropollutants independently from the reuse purpose, whereas for those mandatories provided for the EU Regulation (E.coli, TSS, BOD_5), quality parameters are less restrictive.

Table 4.2: Effluent quality of the enrichment MBR with reference to Italian and European regulations for wastewater reclamation

Parameter		DM 185/03	EU 2020/741	Raw CPW	SBMBR effluent			
					P1	P2	P3	P4
pH		6-9.5	n.r	4.8	8.3	8.2	8.4	8.3
E-coli	number 100 mL^{-1}	< 100	< 10	n.m.	0	0	0	0
Total suspended solids	mg L^{-1}	10	<10	625±131	<5	<5	<5	<5
Turbidity	NTU	n.r.	<5	n.m.	0.28	0.36	0.35	0.33
BOD_5	mg O_2 L^{-1}	20	<10	3026±114	<5	<5	<5	<5
COD	mg O_2 L^{-1}	100	n.r	4512±217	<10	18	68	79
Total phosphorous	mg TP L^{-1}	2	n.r	3.2±0.9	0.8	1.2	0.2	0.2
Total nitrogen	mg TN L^{-1}	15	n.r	15.3±2.8	1.3	1.6	1.4	1.2
Ammonium nitrogen	mg NH_4 L^{-1}	2	n.r	0.2±0.01	0.5	0.4	0.2	0.3
Electic condictivity	µS/cm	3000	n.r	1307±59	1.286	1305	1496	1833
Aluminium	mg/L	1	n.r	n.m.	n.m.	0.132	0.065	0.051
Arsenic	mg/L	0.02	n.r	n.m.	n.m.	0.0004	n.d	n.d.
Barium	mg/L	10	n.r	n.m.	n.m.	0.047	0.041	0.032
Boron	mg/L	1	n.r	n.m.	n.m.	0.021	0.038	0.078
Cadmium	mg/L	0.005	n.r	n.m.	n.m.	n.d	n.d	n.d
Chromium	mg/L	0.005	n.r	n.m.	n.m.	n.d	n.d	n.d
Iron	mg/L	2	n.r	n.m.	n.m.	0.113	0.027	0.03
Manganese	mg/L	0.2	n.r	n.m.	n.m.	0.002	0.002	0.045
Mercury	mg/L	0.001	n.r	n.m.	n.m.	n.d	n.d	n.d
Nichel	mg/L	0.2	n.r	n.m.	n.m.	0.004	0.001	0.003
Lead	mg/L	0.1	n.r	n.m.	n.m.	0.001	0.001	0.001
Copper	mg/L	1	n.r	n.m.	n.m.	0.014	0.007	0.045
Selenium	mg/L	0.01	n.r	n.m.	n.m.	n.d	n.d	n.d
Tin	mg/L	3	n.r	n.m.	n.m.	0.002	n.d	0.002
Zinc	mg/L	0.5	n.r	n.m.	n.m.	0.055	0.076	0.114
Grease, vegetable and animal oils	mg/L	10	n.r	51±16	n.m.	8.0	5	8.0
Total surfactants	mg/L	0.5	n.r	6.2±0.4	n.m.	0.22	0.25	0.36
Total pesticides	mg/L	0.05	n.r	1.0±0.08	n.m.	0.042	0.031	0.032
Total phenols (others)	mg/L	0.1	n.r	0.92±0.04	n.m.	0.053	0.083	0.096

Legend: n.m.: not measured; n.r.: not required; n.d.: not detected; others: includes parameters never detected in all the analysis.

As presented in Table 4.2, all the quality parameters of the enrichment MBR were much below the regulated levels of both Italian and European's regulations. More precisely, the high removal of the organic matter (as COD and BOD_5), surfactants, oils and phenols suggested that MBR should be the suitable approach for treating the industrial wastewater for reuse purposes. Numerous studies have suggested that different wastewaters have the potential to serve as a viable source of reusable water following treatment with an MBR system (Nguyen et al., 2023). Furthermore, microbial parameters (E.coli) indicated that the enrichment MBR effectively removed the pathogen in the wastewater, thus meeting the requirement of reuse purposes.

However, it should be stressed that the effluent quality slightly decreased with the F/M. This suggested that higher F/M values would have involved the exceedance of some quality limits, in particular COD, phenols, and surfactants, likely because limitations of the biological degradation capacity.

Therefore, further F/M increases were not considered in this experimentation. Overall, this experimentation confirmed that membrane ultrafiltration is one of the most suited technologies to produce reclaimed water from industrial wastewater, because the high TSS, organic matter, and pathogen removal capacity.

4.5.3 Selection of the PHA-storing mixed microbial culture in the enrichment MBR

At the beginning of the experiment, the enrichment MBR was operated under the uncoupling feeding strategy that consisted in limiting the availability on nutrients (N, P) during the feast phase, while supplying them at the end of the feast to ensure to the minimum needed for microbial growth. Although this strategy is considered more suited to favor the PHA accumulation pathway (Argiz et al., 2022), in this experimentation it was observed a severe biological foaming and associated membrane fouling (data not reported). This was due to overproduction of extracellular biopolymers because of the unbalanced nutrient condition that caused formation of dense and stable foam on the reactor surface and the membrane

fiber (Di Bella and Torregrossa, 2013), which hindered a sustainable management of the plant. For this reason, during the 210 days of the experiment, after the initial phase under "uncoupling" mode, the enrichment MBR was operated under the coupled feeding strategy that consisted in the simultaneous C, N and P addition at the beginning of the cycle. The enrichment of the MMC in the enrichment MBR was assessed by monitoring the length of the feast phase, and in particular the F/F ratio. Additionally, the PHA and EPS accumulated at the end of the feast phase were determined once for each experimental period. Results are summarized in Table 4.3.

Table 4.3: Performances of the enrichment SBR and MBR during the experimental periods

	P1		P2		P3		P4	
	SBR	**MBR**	**SBR**	**MBR**	**SBR**	**MBR**	**SBR**	**MBR**
F/F [-]	-	0.04	0.13	0.11	0.19	0.13	0.39	0.19
Max PHA* [%VSS]	-	2.41	1.86	7.81	3.34	9.0	2.19	7.23
Max EPS* [%VSS]	-	23.3	36.4	20.5	30.1	16.5	21.5	20.5

*: at the end of feast phase

The typical feast/famine profile was observed from the beginning of the experiment since biomass was already acclimated to the organic substrate of the CPWW. The length of the feast phase increased according to the F/M in both the enrichment reactors. The feast/famine ratio was lower than 0.20 in all the experimental periods in MBR, thereby evidencing the selection of PHA-accumulating bacteria in the MMC as suggested by previous studies (Morgan-Sagastume et al., 2014).

Similarly, the duration of the feast phase in the enrichment SBR increased with the F/M and the feast/famine ratio was between 0.08-0.39, thus indicating that successful enrichment of MMC with high PHA-accumulating capacity occurred only until Period 3. The analysis of the PHA and EPS during the cycle highlighted that the maximum values were obtained at the end of the feast phase in both the enrichment reactors, when most of 90% of the soluble COD in the supernatant of the mixed liquors was depleted. The PHA content in the enrichment MBR (2.41-9.0%) was higher than the enrichment SBR (1.86-3.34%) in each period, thus indicating a

better enrichment of PHA-accumulating organisms in the enrichment MBR. Specifically, the PHA content slightly increased with the F/M in both the reactors reaching a maximum value in correspondence to a F/M of 0.50 kgCOD $kgTSS^{-1}d^{-1}$ in Period 3. At higher F/M, a decrease of the PHA-storing capacity was observed in both the enrichment reactors, although this was greater in the enrichment SBR (-34%) than the enrichment MBR (-20%). A similar result was reported in a previous study, in which a noticeable decrease of PHA production was observed when a OLR higher than 7.5 gCOD $L^{-1}d^{-1}$ (meaning F/M > 2 kgCOD $kgTSS^{-1}d^{-1}$ approximately) was applied to the enrichment reactor (Simona et al., 2022). This indicated the existence of a threshold above which the F/M imposed represented a detrimental condition for the system in terms of microbial selection and PHA production. Nevertheless, in this experimentation the observed loss of PHA-storing capacity was limited by the presence of the ultrafiltration membrane. In contrast to what observed regarding PHA, the maximum EPS content obtained at the end of the feast phase (referred to VSS) showed a decreasing trend with the F/M in both the systems. This result was in line with previous literature, confirming that high substrate availability reduced the EPS production (Ekstrand et al., 2020). In general, the EPS content was higher in the SBR, apart from Period 4 in which a significant decrease in the EPS content in the SBR sludge was noted. The above result suggested that in presence of a hydraulic selection pressure imposed by the gravity-driven solid-liquid separation, bacteria are more stimulated to drive the organic matter into the EPS production pathway. Indeed, previous studies found that metabolic stressors (e.g., hydraulic selection pressure) activated quorum-sensing signals among microorganisms, resulting in a positive correlation between the signal activity and the EPS production (Tan et al., 2014). Based on the above results, the MMC enrichment process carried out with MBR system had a significant impact on the selection of bacteria with PHA-storing capacity. Moreover, it was reasonable to speculate that the absence of the settling phase promoted metabolic pathways that preferentially channeled the organic matter toward PHA rather than EPS.

4.5.4 Accumulation of PHA in the SBR

To assess the maximum PHA-accumulation capacity of the MMC, accumulation assays were carried out at the end of each experimental period. The results were compared with previous assays carried out with biomass from the enrichment SBR. The maximum PHA contents obtained with biomass from both the enrichment MBR and enrichment SBR are shown in Figure 4.3a in relationship with the F/M applied in the relative enrichment reactors.

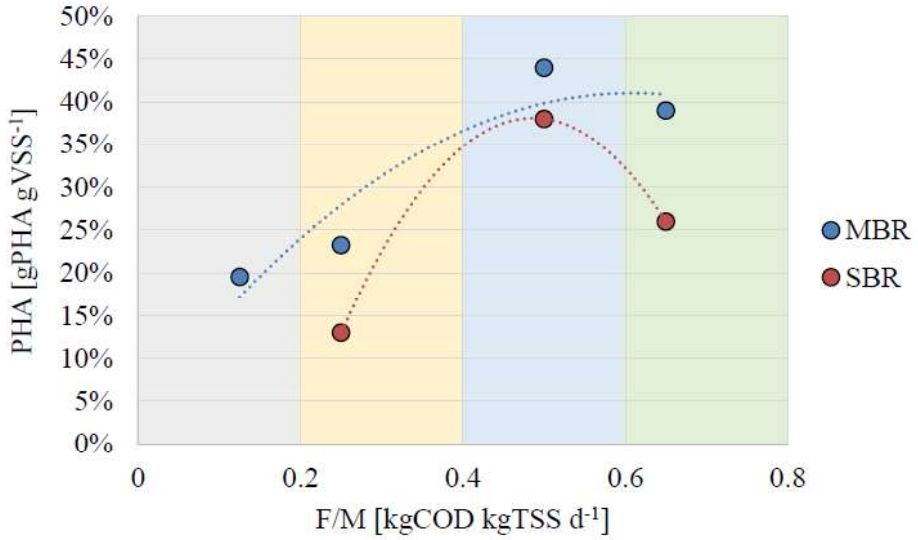

Figure 4.3: Maximum PHA content obtained with the enriched biomass from the enrichment MBR and enrichment SBR in the accumulation assays performed at steady state in each experimental period (Period 1; Period2; Period 3; Period 4)

The accumulation assays confirmed that PHA-storing microorganisms were successfully selected in the enrichment MBR. Biopolymers obtained in the accumulation assays of both the enrichment reactors were composed by HB monomers, whereas HV and other comonomers were absent. Previous studies reported that the composition of PHA depends on the VFA composition. More precisely, the fraction of HB increased if sugar and carbohydrates-rich wastewater were used as feedstock (Carvalho et al., 2014; Morgan-Sagastume et al., 2015). Therefore, the prevalence of HB monomers in this experiment was attributed to the wastewater composition. The PHA content was higher in the enrichment MBR than the enrichment SBR biomass in all the experimental periods, whereas both showed a similar

trend in relationship with the F/M applied to the enrichment reactors. Specifically, the maximum PHA content (%VSS) was reached when the F/M applied to the enrichment reactors was 0.50 kgCOD $kgVSS^{-1}d^{-1}$, whereas lower PHA contents were obtained for lower and higher F/M. In more detail, when the MMCs were selected under lower F/M (0.25 kgCOD $kgVSS^{-1}d^{-1}$) the maximum PHA contents achieved at the end of the accumulation assays resulted close to 24% and 13% with the biomass of the enrichment MBR and enrichment SBR, respectively. Similarly, MMC selected under the highest F/M (0.65 kgCOD $kgVSS^{-1}d^{-1}$) showed a better capacity to store PHA in the enrichment MBR (39% vs 26%). Consequently, the biomass enriched in the enrichment MBR allowed achieving higher PHA content, even at high F/M. The above results were in line with what previous observed regarding the PHA accumulation capacity in the enrichment MBR enrichment reactor, where a decrease of the PHA-storing capacity was observed as the F/M was increased. Previous studies demonstrated a correlation between the maximum PHA yield and the F/M applied to the enrichment reactors. Specifically, literature studies reported that limiting carbon conditions (low F/M) led to lower PHA yields, whereas higher yields were obtainable once not limiting conditions were reached (Palmeiro-Sánchez et al., 2019). Nevertheless, the same authors suggested the existence of a F/M threshold above which the PHA-storing capacity noticeably decreased. Indeed, Dionisi et al. (Dionisi et al., 2006) suggested that biomass competition occurring at high F/M determined the loss of PHA-accumulating capacity of microbial cultures, as the fast-storing microorganisms were favoured and became predominant in the MMC. Moreover, it should be stressed at high F/M the readily biodegradable substrate is not entirely removed during the feast phase, thus resulting in residual available substrate for non-storing microorganisms during the famine. Therefore, as residual substrate accumulated within the enrichment reactor, non-storing microorganisms competed with PHA-storing population for the available substrate, resulting in a decrease of PHA-accumulation capacity of the MMC. In a recent study, it was demonstrated that the selective pressure induced by the applied OLRs strongly influenced the microbiome composition revealing a high content of bacteria with low PHA-storing capacity in the MMC (Simona et al., 2022). The results obtained with the SBR enriched biomass were in line with the above

discussion (chapter 3), thereby confirming the role of the F/M in MMC selection. However, it was interesting to note that the decrease of the PHA accumulation capacity with the F/M was significantly lower in the enrichment MBR, suggesting that the implementation of a complete cell-retention system in the enrichment reactor promoted an increase in the PHA accumulation capacity of the enriched MMC compared to a conventional enrichment system.

Table 4.4 shows the comparison between the main results found in the literature using MMC and real feedstocks with the present study. The results achieved in the enrichment MBR were comparable to those reported in the literature using agro-based wastewater as feedstocks in terms of maximum PHA content achieved (Bengtsson et al., 2010; Campanari et al., 2017a; Guventurk et al., 2020). Higher values were obtained when high F/M were applied to the enrichment reactors (> 1 kgCOD $kgTSS^{-1}d^{-1}$) (Albuquerque et al., 2010; Argiz et al., 2020; Morgan-Sagastume et al., 2020). Such values of F/M were not applicable in the present experimentation since they would have determined a significant worsening of the effluent enrichment MBR quality. Another noticeable improvement observed in the enrichment MBR was the increase in PHA productivity. Indeed, the maximum volumetric PHA production (0.63 gPHA $L^{-1}h^{-1}$) observed with the enrichment MBR enriched biomass was significantly higher than the majority of that reported in previous literature using MMC and industrial agro-based wastewaters as feedstock (Albuquerque et al., 2011; Valentino et al., 2017). In general, organic feedstocks deriving from fruit waste enabled higher PHA productivity in comparison with other carbon sources (Silva et al., 2022). It has been previously reported that the complexity of real wastewaters could negatively affect the production of biopolymers, in terms of maximum PHA accumulation and productivity (Campanari et al., 2017b; Palmeiro-Sánchez et al., 2019). Indeed, if compared to oil-mill, fish-canning or cheese-way wastewaters, in CPWW the protein/carbohydrate ratio is much lower because of the greater abundance of carbohydrates (Corsino et al., 2021). Proteins and lipids are more difficult to be channelled into the PHA biosynthesis route compared to sugar and carbohydrates, since they required additional time to assure carbon source hydrolysis and its accessibility to the culture (Argiz et al., 2022; Tepari et al., 2020). In this sense, the use of citrus processing wastewater could have been beneficial to obtain high process

yields. Overall, dealing the same CPWW, PHA productivity was higher when using a membrane bioreactor in the enrichment stage. Indeed, even comparing the specific PHA productivity (referred to the VSS) obtained with the enriched biomass from the MBR and the SBR, in the former it was 0.125 gPHA $gVSS^{-1}h^{-1}$, whereas in the SBR it resulted equal to 0.08 gPHA $gVSS^{-1}h^{-1}$. Therefore, the above findings suggested that the MMC enrichment strategy carried out by MBR allowed improving the selection of PHA-accumulating bacteria, thus enabling higher productivity than conventional enrichment systems. The reason for these results could be likely linked to a higher diversity and richness in the MMC of the enrichment MBR than the enrichment SBR with PHA-accumulating organisms, which included dispersed bacteria or not able to form settleable flocs that would be discharged in biological system in which a gravity settling stage is imposed.

Table 4.4: Results of accumulation assays carried out with MMC under different feeding/enrichment strategies and F/M

Carbon source	Feeding/enrichment strategy	F/M [kgCOD kgTSS^{-1} d^{-1}]	Max PHA content [gPHA gVSS^{-1}]	PHA productivity [gPHA L^{-1}h^{-1}]	References
Paper Mill Wastewater	Continuous flow aerobic reactors Selector + main reactor	0.40-0.50	0.43-0.48	0.09-0.13	(Bengtsson et al., 2008)
Sugar Cane Molasses	Sequencing batch reactor Aerobic dynamic feeding	0.51-1.10	0.33-0.61	0.13-0.46	(Albuquerque et al., 2010b)
Olive Oil Mill Wastewater	Sequencing batch reactor Fed-batch multi-spike	0.75	0.30	0.06	(Campanari et al., 2014)
Cheese Whey	Fed-batch multi-spike	n.a.	0.35	0.20	(Asunis et al., 2022)
Potato starch	Sequencing batch reactor Aerobic dynamic feeding	0.40-0.48	0.40-0.77	n.a.	(Morgan-Sagastume et al., 2020)
Cooked mussel processing wastewater	Aerobic dynamic feeding + settling after feast phase	1.21-2.27	0.59	0.19	(Argiz et al., 2020)
Fruit waste	Sequencing batch reactor Aerobic dynamic feeding	n.a.	0.71	1.48	(Silva et al., 2022)
Winery waste	Sequencing batch reactor Aerobic dynamic feeding	n.a.	0.63	0.12	(Kovalcik et al., 2020)
Citrus processing wastewater	Sequencing batch reactor Aerobic dynamic feeding	0.25-0.65	0.38	0.47	Chapter 3
Citrus processing wastewater	Sequencing batch reactor + ultrafiltration membrane	0.125-0.65	0.44	0.63	This experiment

To confirm this, microscopic analysis was carried out for both the enriched biomasses. Specifically, Figure 4.4 shows the activated sludge pictures

(fresh samples and after Sudan Black staining) during Period 3, in which the maximum PHA accumulation capacity was achieved in both the systems.

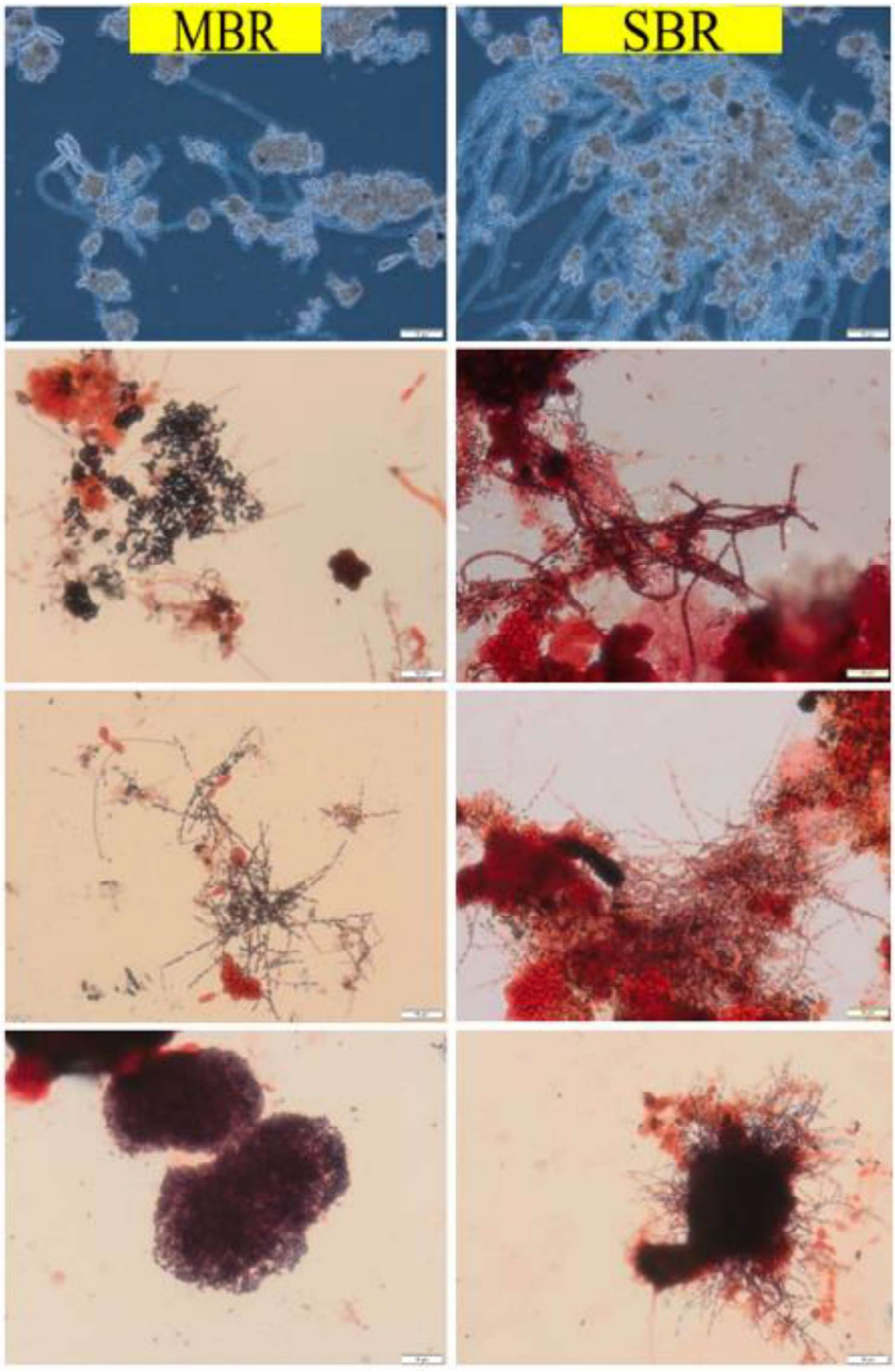

Figure 4.4: Microscopic pictures of the enrichment MBR (left column) and the enrichment SBR (right column) during

The enrichment MBR sludge was characterized by small flocs (15-35 μm), having an open and weak structure with a large abundance of filamentous bacteria. In contrast, sludge from the enrichment SBR was characterized by larger flocs (90-120 μm) having also large abundance of filamentous bacteria forming inter-bridging connections between the flocs that caused bulking phenomena. Sudan Black stain enabled to highlight a great abundance of dispersed and filamentous bacteria (e.g., nocardioform actinomycetes, Eikelboom morphotypes Nostocoida Limicola II and Type 021N) in the enrichment MBR with PHA-storing capacity, whereas in the enrichment SBR such bacteria were only found within the floc structure. Previous literature have reported the capacity of some filamentous bacteria (e.g, nocardioform actinomycetes) and other dispersed microorganisms (e.g., zoogleas) (Jenkins et al., 2003) to store PHA granules intracellularly. Such microorganisms, because of their scarce capacity to form dense and settleable flocs are generally washed-out from those reactors in with a gravity-based process is used to perform solid-liquid phase separation. Additionally, in conventional activated sludge systems, filamentous bacteria are responsible of filamentous bulking which causes severe biomass washout and the worsening of the effluent quality (Wanner, 2017). Such microorganisms showed a good capacity to accumulate PHA, thus their retention within the enrichment MBR could have been beneficial to achieve a higher PHA productivity compared to the enrichment SBR.

4.5.5 Membrane fouling behaviour

Long-term operation of membrane could be affected by fouling phenomena. To address how membrane fouling evolved simultaneously with the biomass enrichment with PHA-storing bacteria, residual membrane permeability was calculated throughout the experiment. At the end of each period, fouling characterization was carried out by applying the RIS model. The results are reported in Figure 4.5.

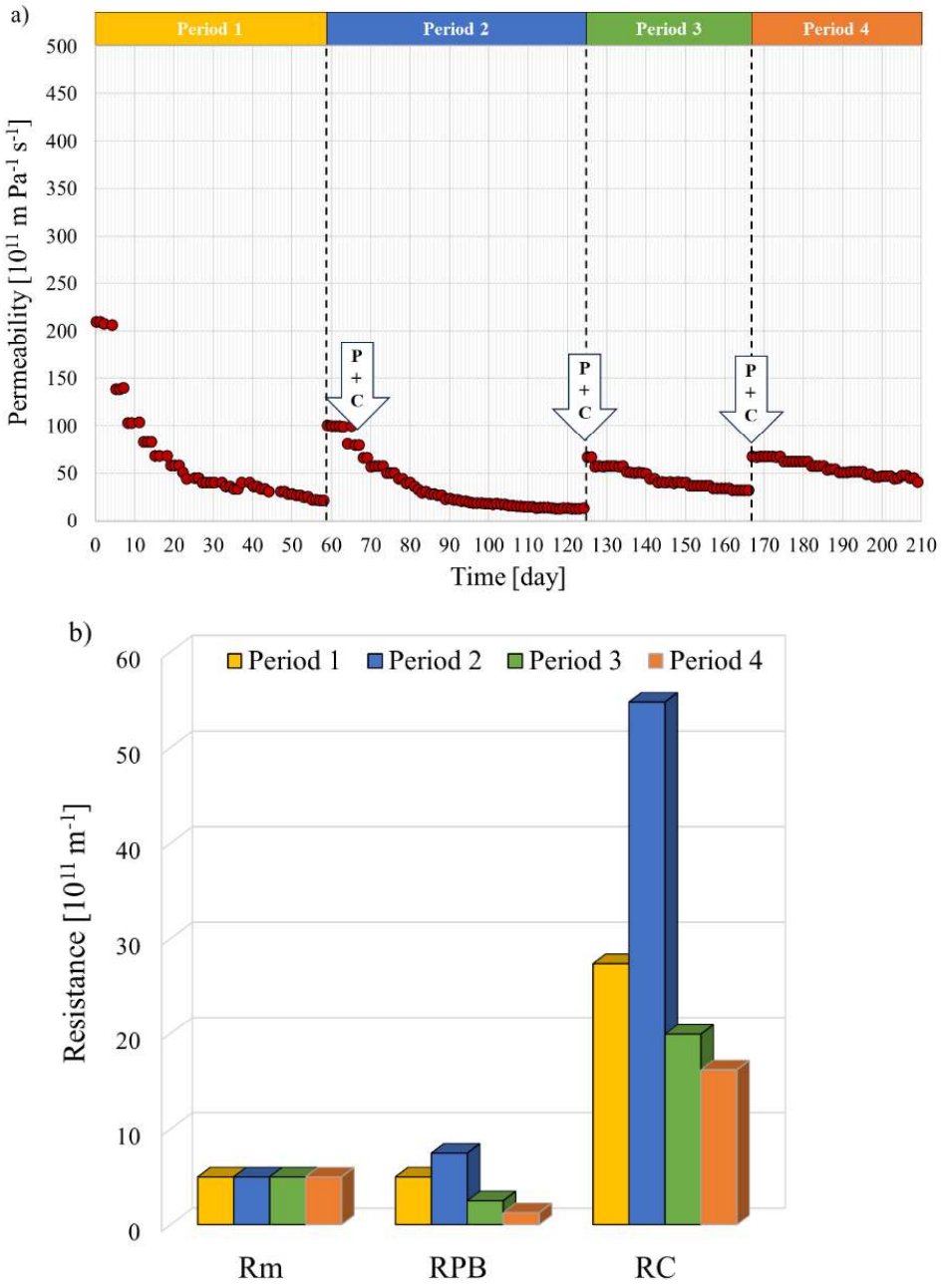

Figure legend: R_m: resistance to filtration due to the membrane; R_{PB}: resistance due to pore blocking, including thefouling removable with chemical cleanings only; R_C: resistance due to cake deposition, including the fouling removable with ordinary backwashings and manual cleaning operations.

Figure 4.5: Trends of membrane permeability during the experiment (a) and membrane fouling mechanisms in each period. The arrows in Figure 6a indicated the physical and chemical cleaning operations (P+C)

The trend of membrane permeability was significantly different during each experimental period (Fig. 4.5a). A noticeable permeability loss was observed at the beginning of Period 1, whereas it decreased more gradually in the following days, attesting to about 35 10^{11} m $Pa^{-1}s^{-1}$ at the end of the period. In the following periods, the loss in permeability with time noticeably decreased, suggesting a lower tendency of membrane fouling formation. The results of the RIS model (Fig. 4.5b) indicated that the effects of fouling mechanisms varied during each period. In more detail, the resistance due to irreversible fouling (R_{PB}), indicating the formation of the pore blocking fouling mechanism, decreased from Period 1 to Period 4, thus once the biomass was enriched in PHA storing microorganisms. Similarly, even the fouling due to the cake deposition mechanism led to a lower membrane fouling when the PHA accumulation capacity of the biomass was increased. As was previously discussed, selection of PHA-accumulating organisms in the biomass of the enrichment MBR favoured the channelling of organic substrate towards intracellular rather extracellular storage pathways, thus limiting the formation of EPS that are widely recognized as the main foulant agents in MBR systems. These results indicated that biomass enrichment with PHA-storing bacteria was favourable to reduce the membrane fouling tendency.

4.6 Conclusions

This experimentation demonstrated feasibility in the production of PHA and water eligible for reuse purposes from a citrus processing wastewater using a MBR system in which wastewater treatment and PHA-enrichment stage were coupled in a single unit.

The effluent of the MBR system was complied with the requirements provided by the main national (Italy) and European regulations (DM 185/03 and EU 2020/741), thus indicating the suitability of its use for different reuse purposes (e.g., irrigation, civil and industrial uses, etc.). Additionally, the enrichment MBR appeared a more technically feasible alternative to conventional SBR for the enrichment stage of PHA production process. Maximum intracellular storage of 45% and a production yield of 0.63 gPHA $L^{-1}h^{-1}$ were reached when the enrichment MBR was operated under a F/M close to 0.50 kgCOD $kgTSS^{-1}d^{-1}$, which resulted higher of approximately

35% than those achieved in the enrichment SBR. Besides, the F/M was observed to play an important role in the enrichment stage. Indeed, at high values of F/M (> 0.50 kgCOD $kgTSS^{-1}d^{-1}$) a noticeable loss of PHA-accumulating capacity of the MMC was noted, albeit this was minimized in the enrichment MBR due to complete cell-retention capacity that avoided the washout of dispersed and filamentous bacteria able to store PHA. Finally, if on the one hand maximization of PHA productivity in conventional enrichment SBR caused the occurrence of process dysfunctions (e.g., filamentous bulking), on the other hand in the enrichment MBR it promoted their mitigation through reduction of membrane fouling behaviour. Therefore, given the potential of the enrichment MBR process, it should be advisable to move on full-scale application to validate its performance and assess its economic feasibility.

4.7 References

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Chapter 5: Utilization of AGS for the integration of PHA production in a mainstream WWTP: comparison with SBR and MBR technologies

Summary

The present experimentation has focused on integrating PHA production into the mainstream of industrial wastewater treatment using an AGS reactor, aiming to compare it with the SBR and MBR enrichment reactors from previous chapters. The enrichment MBR showed better and more stable carbon removal performance, while the effluent quality of the enrichment SBR and enrichment AGS deteriorated at high OLR. Biomass enrichment with PHA-storing bacteria was successfully achieved in all systems. The enrichment MBR improved the PHA productivity with increasing OLR (max 35% w/w), whereas the enrichment SBR reduced the PHA content (max 20% w/w) above an OLR threshold of 2 kgCOD m^{-3} d^{-1}. In contrast, in the enrichment AGS, increasing OLR resulted in a significant decrease in PHA productivity (max 14% w/w) and a concomitant increase of extracellular polymers (EPS) production (max 75% w/w). Results demonstrated that organic carbon was mainly driven towards the intracellular storage pathway in the enrichment SBR (max yield 51%) and enrichment MBR (max yield 61%), whereas additional stressors in enrichment AGS (e.g., hydraulic selection pressure, shear forces) induced bacteria to channel the COD into extracellular storage compounds (max yield 50%) necessary for maintaining the granule structure. The results suggested that full-aerobic feast/famine strategy was more suitable for flocculent sludge-based technologies, although biofilm-like systems could open new scenarios for other biopolymers recovery (e.g., EPS). Moreover, the enrichment MBR resulted the most suitable technology for the integration of PHA production in a mainstream industrial wastewater treatment plant, considering its greater process stability and the potential for reclaimed treated wastewater.

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5.1 Introduction

The granular sludge system is another advanced biological process with the potential to enhance biomass retention capacity, increase overall PHA productivity, and achieve higher purification performance compared to a conventional activated sludge system, especially at higher OLRs. These systems exhibit significant resistance to organic load shocks, as they can withstand sudden changes in organic load without compromising the stability of the PHA production process. Additionally, AGS, similarly to MBR systems, are well-suited for operating under more challenging process conditions than conventional activated sludge systems, overcoming issues related to sludge settling and tolerating higher organic loads. The aim of this experimentation was to integrate AGS technology into the mainstream of a citrus industrial wastewater treatment plant to simulate the wastewater treatment process and the MMC enrichment phase for PHA production simultaneously, comparing it with SBR and MBR technologies. However, a comparative analysis of these three technologies for PHA production is currently lacking in the literature, especially when dealing with industrial wastewater. Furthermore, given that the production of EPSs is a mechanism underlying the granulation process of AGSs, focus was also placed on the EPSs production as they represent additional potentially recoverable products.

5.2 Experimental setup

The enrichment AGS consisted of a 4L poly-methyl-methacrylate column-type reactor (700 mm of height, 60 of inner diameter) equipped with an internal riser according to a sequencing batch-airlift-reactor (SBAR) configuration (Beun et al., 2002). This reactor operated aerobically under the typical feast/famine (F/f) regime, as enrichment SBR and MBR. Continuous aeration (3.5 LPM, corresponding to 2.4 cm sec^{-1} of flux velocity) was provided by an air stone diffuser placed at the bottom of the reactor and coaxial with the riser, connected to an air blower.
The effluent was discharged at different heights to modify the volumetric exchange ratio (VER) according to operational needs. All electrical devices were connected to a programmable logic controller (PLC) that handled cycle

operations. The AGS was operated in cycles of 12 h, consisting of 10 min of influent feeding, 690 min of aeration, 5 min of settling, 10 min of effluent discharge and lastly, 5 min of idle. The volume of CW2* and discharged in each cycle changed according to the operating period (equal to those of the two previous experimentations), thus resulting in different hydraulic retention times (HRT). Information on characteristics and operating conditions of enrichment SBR and enrichment MBR can be found in chapter 3 and chapter 4, respectively. The storage capacity of the enriched MMC was assessed by carrying out several accumulation assays in the same reactor SBR of the previous experimentations (described in Chapter 2). A schematic layout of the plant is shown in Figure 5.1.

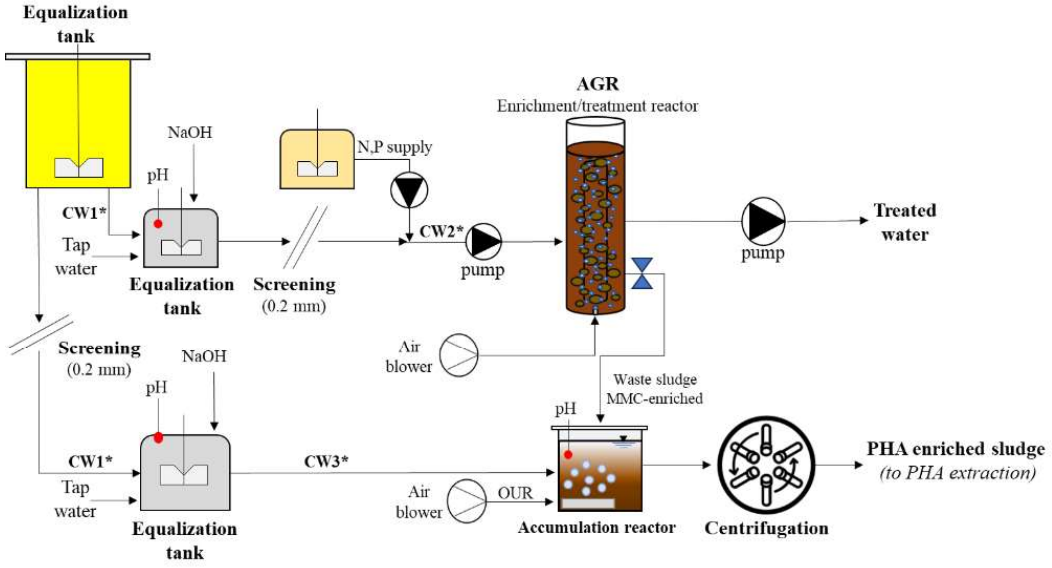

Figure 5.1: Layout of the AGS enrichment system

5.3 Operational conditions

The AGS reactor operated under the same operating conditions as the other two enrichment reactors (Chapter 3 and 4) in order to compare the three different technologies used for PHA production at the concomitant wastewater treatment.

Three different OLRs were tested in respective experimental periods: 1 kgCOD $m^{-3}d^{-1}$ in Period 1, 2 kgCOD $m^{-3}d^{-1}$ in Period 2 and 3 kgCOD $m^{-3}d^{-1}$ in Period 3.

Considering that the volume of the enrichment reactors was not equal in each of the systems, the volume of wastewater treated in a day was different. Specifically, the enrichment SBR treated 5, 10, 15 L d^{-1}, the enrichment 10, 20, 30 L d^{-1} and the enrichment AGS 1, 2, 3 L d^{-1} in Period 1, Period 2 and Period 3, respectively. Accordingly, volumetric exchange ratio ranged between 0.125-0.375 during the experiment.

Overall, SBR, MBR and AGS enrichment reactors were operated for 172, 150 and 204 days, respectively. The different duration of the observation period was due to the different times each system took to reach steady-state conditions. The enrichment reactors operated with different biomass concentration, according to the specificity of the technology. In Table 5.1 the average TSS concentration in the enrichment reactors during the experiment are summarized. A regular sludge withdrawn was performed to maintain a constant TSS concentration in the enrichment reactors according to the biomass growth yield. Consequently, the SRT was not controlled but calculated by means of a mass balance on TSS. A detailed summary of the above information is reported in Table 5.1.

Table 5.1 Summary of the main operating conditions for the SBR, MBR and AGS systems

		Duration [d]	Flow rate [L d^{-1}]	Biomass [gTSS L^{-1}]	SRT [d]
		Period 1:			
OLR 1 [kgCOD m^{-3}]	SBR	78	5	4.56 ± 0.12	14 ± 3
COD 4.5 ± 0.1 [g L^{-1}]	MBR	65	10	8.1 ± 0.2	14 ± 2
VER 0.125	AGS	126	1	4.89 ± 0.21	23 ± 1
		Period 2:			
OLR 2 [kgCOD m^{-3}]	SBR	32	10	4.42 ± 0.09	9 ± 2
COD 4.46 ± 0.09 [g L^{-1}]	MBR	42	20	6.3 ± 0.1	11 ± 2
VER 0. 25	AGS	37	2	5.11 ± 0.09	10 ± 3
		Period 3:			
OLR 3 [kgCOD m^{-3}]	SBR	62	15	4.46 ± 0.09	5 ± 1
COD 4.38 ± 0.16 [g L^{-1}]	MBR	43	30	6.3 ± 0.2	9 ± 1
VER 0.375	AGS	41	3	5.06 ± 0.11	9 ± 2

Table legend: OLR: Organic loading rate; COD: Chemical Oxygen Demand; VER: Volumetric Exchange Ratio.

5.4 Analytical methods

All the physical-chemical analyses for the assessment such as TSS, VSS, COD, BOD, NH_3-N, TN, TP, pH, DO, electrical conductivity, concentration of acetate, biokinetic parameters were carried out according to Chapter 2.

5.5 Results and discussion

5.5.1 Organic carbon removal

Enrichment of the MMC requires high OLR in order to channel the organic carbon towards PHA production rather than growth (Oliveira et al., 2017). However, such condition is not always suitable for the achievement of high purification efficiency of wastewater. To this purpose, COD removal performances were monitored in the three enrichment reactors. Figure 5.2 shows the average COD concentration in the effluents and removal efficiencies obtained at steady state in each period, and its COD fractions resulting from the fractionation assays.

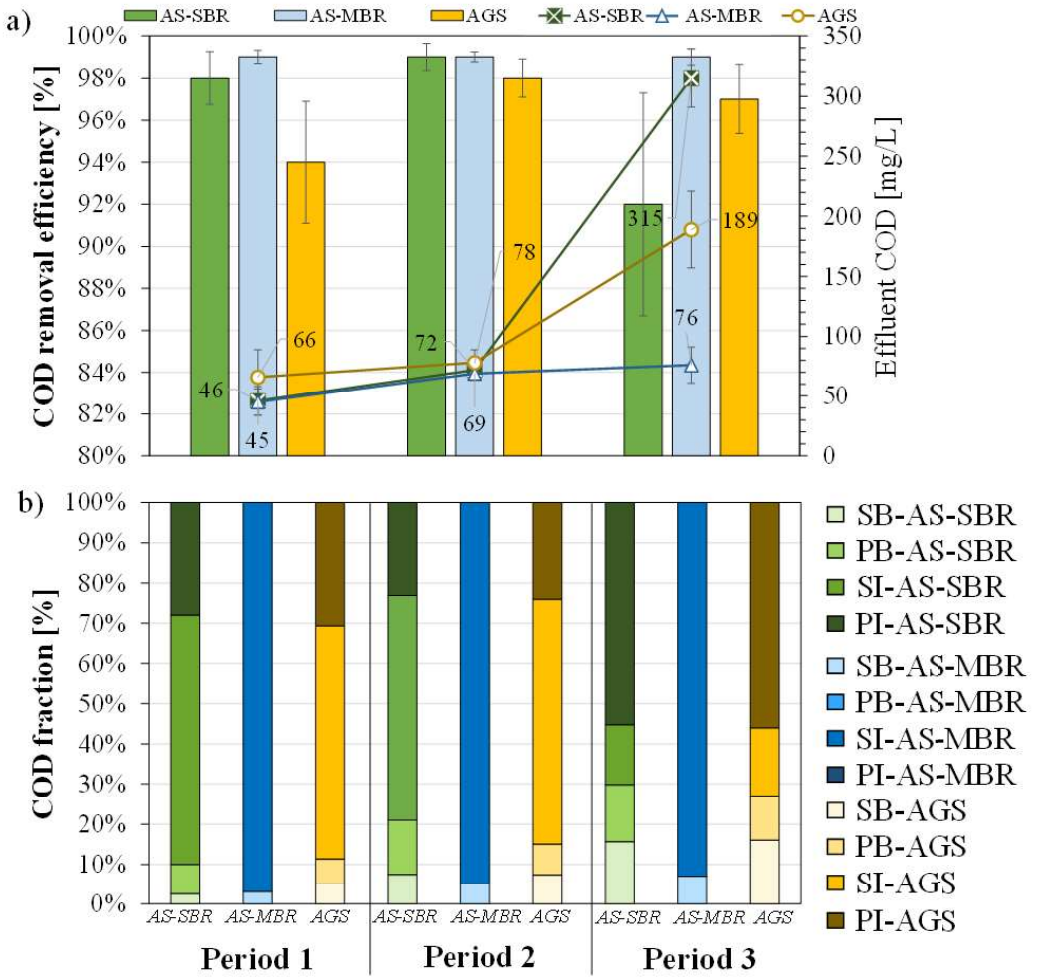

Figure legend: SB: Soluble Biodegradable carbon; PB: Particulate Biodegradable carbon; SI: Soluble Inert carbon; PI; Particulate Inert carbon.

Figure 5.2: Average COD concentrations in the effluent (lines) and removal efficiencies (histograms) at steady state in the enrichment reactors (a); COD fractions in the effluent of the enrichment reactors (b). Bars in figure a) indicate the standard deviations.

COD removal efficiency was higher than 90% in all the reactors in Period 1 (1 kgCOD $m^{-1}d^{-1}$), resulting in an effluent concentration between 50-75 mg L^{-1} (Fig. 5.2a). The SBR and the MBR enrichment reactors exhibited better performances and a greater stability in this period than the AGS, whose COD removal efficiency showed a slightly higher variability as indicated by the standard error. Granulation process occurred in Period 1 in the AGS system and granules stability was obtained by reducing the settling time to 5 minutes to prevent the accumulation of flocculent sludge. Therefore, it was assumed that washout of small and poor settling flocs caused a slightly

COD increase in the effluent of the AGS system in this period. When increasing the OLR in Period 2 (2 kgCOD $m^{-1}d^{-1}$), all the reactors showed high COD removal capacity, which resulted close to 99%. The effluent COD concentration was below 75 mg L^{-1} in all the reactors. A noticeable decrease in COD removal was noted referring to the AS-SBR in Period 3 when operating at 3 kgCOD $m^{-1}d^{-1}$. The average COD removal efficiency was 90% approximately, although the effluent concentration rose to 300 mg L^{-1}. Similarly, the AGS showed a slight worsening of COD removal compared with the previous periods, although the effluent concentration was below 200 mg L^{-1}. In contrast, the enrichment MBR demonstrated a greater stability for COD removal within the OLR range investigated. Indeed, the effluent COD concentrations in the permeate of the MBR were always below 100 mg L^{-1}, thereby enabling removal efficiencies of 99% during the entire experiment. As reported in Fig. 5.2b, when increasing the OLR, the fraction of the soluble COD in the effluent increased in the enrichment SBR and AGS, more significantly than the enrichment MBR, suggesting a not efficient utilization of the readily biodegradable organic matter by PHA-accumulating organisms. The lower concentration of soluble COD in the enrichment MBR was also confirmed in the supernatant beside on the permeate. Similarly, the incidence of the particulate fractions (e.g., inert and biodegradable) were greater in the enrichment SBR and AGS systems at high OLR, indicating a gradual loss of the sludge flocculation capacity. Contextually, occurrence of flocs and granules deflocculation was noted in those reactors especially during Period 3, mainly due to the overgrowth of filamentous bacteria. This resulted in a noticeable worsening of sludge settling properties and a consequently deterioration of the effluent quality (Table 5.2).

Table 5.2: Average data of the effluent TSS concentration, sludge volume index (SVI) and flocs/granules size for the SBR and AGS

	AS-SBR			AGS		
	Period 1	Period 2	Period 3	Period 1	Period 2	Period 3
Effluent TSS [mg L^{-1}]	10±7	12±9	135±27	28±12	51±17	56±44
SVI* [mL $gTSS^{-1}$]	51±9	48±18	250±76	25±8	32±6	56±31
Size [mm]	0.139±0.015	0.207±0.021	0.069±0.032	1.8±0.2	1.7±0.1	2.2±0.4

* SVI was calculated at 30 minutes for enrichment SBR and 5 minutes for enrichment AGS. Note that data referred to enrichment MBR are not reported as effluent TSS was always 0 mg L^{-1} and SVI was not measured.

In the enrichment MBR the particulate fraction of the COD was entirely trapped within the reactor as the porosity of the ultrafiltration membrane was lower than 0.45 μm. It should be considered that at the same OLR, the MBR system operated under a lower F/M ratio than the other enrichment reactors due to the higher biomass concentration. As an excess of organic load could overcome the metabolic need of bacteria, operating under lower carbon availability allowed for a better substrate utilization that resulted in lower COD effluent concentrations. Overall, the enrichment SBR and the enrichment AGS showed a higher process instability when the OLR was increased over 2 kgCOD $m^{-3}d^{-1}$, indicating that higher values would result in a probable collapse of process performances. On the other hand, the higher biomass retention capacity of the MBR system enabled higher and more stable COD removal while operating at higher OLR. In previous literature, it was emphasized that high OLR is desirable as a means to enhance PHA productivity (Oliveira et al., 2017). Different optimum values of OLR for an efficient MMC enrichment were reported in the literature. Oliveira et al. (2017) found an optimal OLR of 2 kgCOD $m^{-3}d^{-1}$ using fermented cheese-way. Lorini et al. (2020) and Crognale et al. (2022) achieved a stable selection of MMC at 8 kgCOD $m^{-3}d^{-1}$ although operating with a synthetic mixture. More recently, Isern-Cazorla et al. (2023) found that an increase in the OLR applied in the enrichment reactor up to 1.8 kgCOD $m^{-3}d^{-1}$ improved the MMC selection while reducing the purification performances. The wide range of OLR reported probably indicated that the ideal OLR likely depends on the characteristics of the wastewater used as feedstock. Nevertheless, the literature findings suggested that optimum OLR should be greater than 2-3 kgCOD $m^{-3}d^{-1}$ when dealing with industrial

wastewaters (Valentino et al., 2017). Hence, SBR and AGS systems could be susceptible to process instability when operating at OLR above this threshold. If on the one hand high PHA productivity could be achieved when operating at OLR higher than 2 kgCOD $m^{-3}d^{-1}$, on the other the poor purification performances would limit the implementation of MMC-enrichment in the mainstream of a WWTP. In this context, MBR system could have a greater potential for this purpose, as it was proven to be effective to provide high purification efficiency at OLR higher than the suggested threshold.

5.5.2 Dynamics of PHA and EPS storage in the enrichment reactors

The enrichment reactors were seeded with the same activated sludge and operated under the feast/famine strategy to enrich the biomass in microorganisms with storage capacity. The coupled feeding strategy, involving the simultaneous supply of nutrients with carbon, was adopted to prevent the occurrence of process dysfunctions (e.g., viscous bulking and foaming). Typical F/F profiles were observed in all the enrichment reactors after few operational cycles. The operation of the reactors became stable after the 50th day and 37th day in the SBR and MBR, respectively, whereas a longer time was observed in the AGS (98 days) as the granulation process was completed later. The effects of OLR on the profiles of DO concentration, PHA and EPS contents during a representative enrichment cycle at steady state are depicted in Figure 5.3.

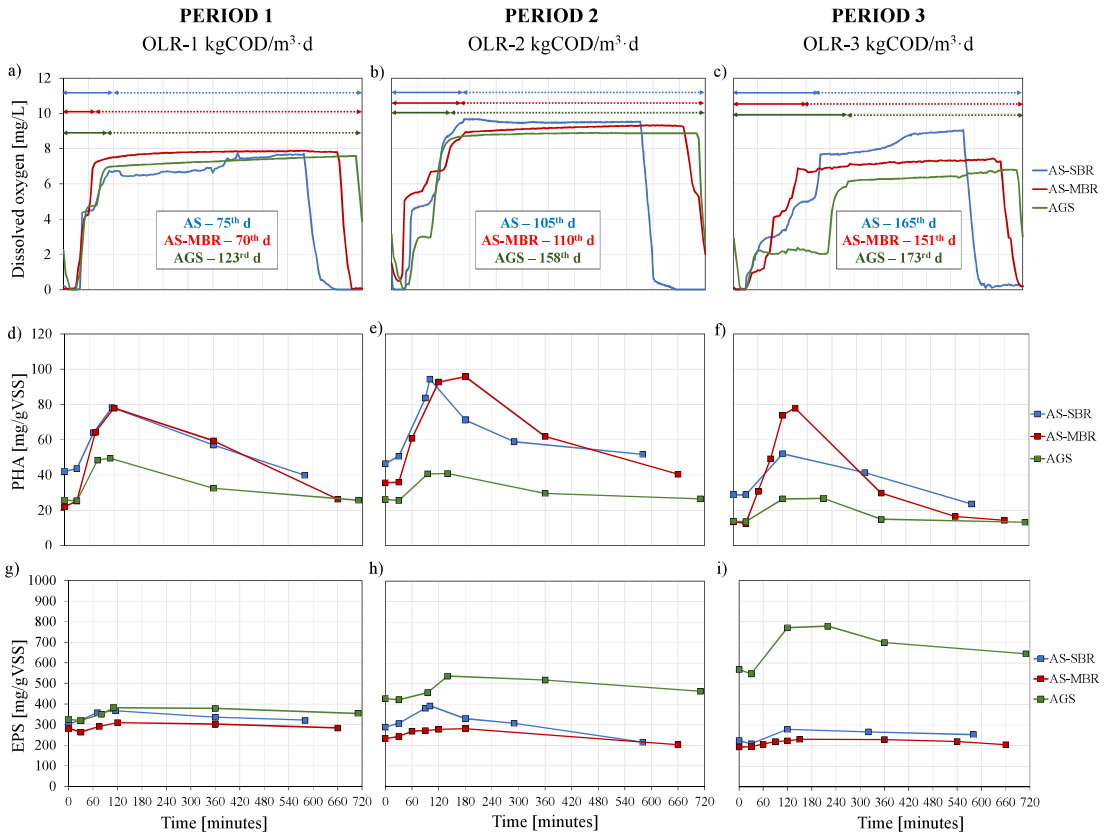

Figure 5.3: Characterization of a typical enrichment cycle in the AS-SBR, AS-MBR and AGS, at steady state during Period 1 (a,d,g), Period 2 (b,e,h) and Period 3 (c,f,i), showing the profile of DO, feast (⟷) and famine (⇠⇢) (a-c), PHA (d-f) and EPS (g-i). Data within the caption box indicate the day to which the DO, PHA and EPS profiles are referred (Period 1: AS-SBR 75th day, AS-MBR 70th day, AGS 123rd day; Period 2: AS-SBR 105th day, AS-MBR 110th day, AGS 158th day; Period 3: AS-SBR 165th day, AS-MBR 151st day, AGS 173rd day).

In Period 1 the length of the feast phase presented a low variability among the three systems. The ratio between the feast and famine lengths was on average 0.13, although the enrichment MBR showed a slightly lower value (0.11). This result indicated that microorganisms developed a high storage capacity and MMC was successfully enriched in PHA storing bacteria (Conca et al., 2020). Indeed, during the feast phase, PHA (Fig. 5.3d) and EPS (Fig. 5.3g) increased in all the enrichment reactors, indicating that bacteria converted the external carbon source into extracellular and intracellular storage compounds. During the famine phase, the content of both PHA and EPS decreased, suggesting the occurrence of biomass growth

on storage compounds. Overall, the enriched biomass of enrichment SBR and enrichment MBR exhibited a similar PHA production capacity that was higher of that in AGS, which instead showed a greater EPS productivity. In Period 2, the length of the feast phase slightly increased in all the reactors, although maintaining the F/F ratio below 0.20. A noticeable improvement of PHA production was observed in the enrichment SBR and enrichment MBR, in which the maximum PHA content at the end of the feast phase was 94 mg $gVSS^{-1}$ and 95 mg $gVSS^{-1}$, respectively, whereas a slight decrease of PHA in the AGS was observed. Contrarily, a significant increase of EPS content was noted in the biomass of the AGS, which suggested alterations in the cellular metabolism as the OLR was increased. Any significant variation of the EPS content in the enrichment SBR and enrichment MBR biomasses was noted. The further increase of OLR in Period 3 caused an extension of the feast phase duration in all the enrichment reactors. The F/F ratio was still lower than 0.20 in the enrichment MBR only, whereas in the SBR and AGS systems it resulted higher than 0.30. As previously reported, residual organic carbon in soluble form was measured in the effluent of the enrichment SBR and enrichment AGS in Period 3. This suggested that soluble COD was not entirely depleted during the feast phase in those reactors, and it remained available during the famine phase. Therefore, this reduced the selective pressure on not-accumulating organisms that could be outcompeted for substrate with PHA-storing bacteria. Consequently, this reduced the use of the organic carbon for accumulation purposes, favouring also metabolic pathways that required longer time (e.g., cellular synthesis) (Huang et al., 2018). If on a side the PHA content in the enrichment MBR slightly decreased compared with the previous period, on the other in the SBR and the AGS systems it noticeably reduced. A different behaviour was noted referring to EPS. Indeed, the EPS content decreased in the SBR and MBR systems, consistently with the decrease of the storage capacity above highlighted referring to PHA. In contrast, the EPS content in the aerobic granules at the end of the feast phase further increased to over 780 mg $gVSS^{-1}$, showing an increment of approximately 50% respect to Period 2. It should be observed that the EPS production in the AGS occurred in the early of the feast phase, indicating a high activity of EPS storing bacteria. Comparing the overall production of PHA and EPS in the enrichment cycle of the three

systems, a greater production of EPS was observed. This result was in good agreement with previous literature in which is reported that organic carbon was channelled towards extracellular polymeric substances when operating with low C/N ratio (20) (Zhao et al., 2021). The above results indicated also that the OLR increase caused a reduction of PHA accumulation capacity in all the enrichment reactors, although of different magnitude, especially when the OLR threshold of 2 kgCOD $m^{-3}d^{-1}$ was exceeded. Nevertheless, the enrichment MBR maintained a good PHA accumulation capacity during the entire experiment. Biomass washout and the availability of soluble organic substrate during the famine phase were assumed as the main reasons for the decrease in PHA accumulation in the SBR and AGS systems at high OLR. This result confirmed what stated in previous literature referring to the existence of an OLR threshold in the enrichment stage, above which the culture selection was more difficult to achieve (Crognale et al., 2022; Valentino et al., 2017). In contrast, the higher biomass concentration, and the membrane retention ability towards poor settling flocs in the enrichment MBR, allowed to completely deplete the organic substrate during the feast phase and avoided the washout of sludge enriched in PHA storing bacteria.

5.5.3 Assessment of maximum PHA accumulation

Accumulation assays were carried out on the enriched cultures once steady performances in the respective enrichment reactors were achieved. Figure 5.4 shows the overall biopolymers content, as sum of PHA and EPS, accumulated by the enriched cultures during each experimental period.

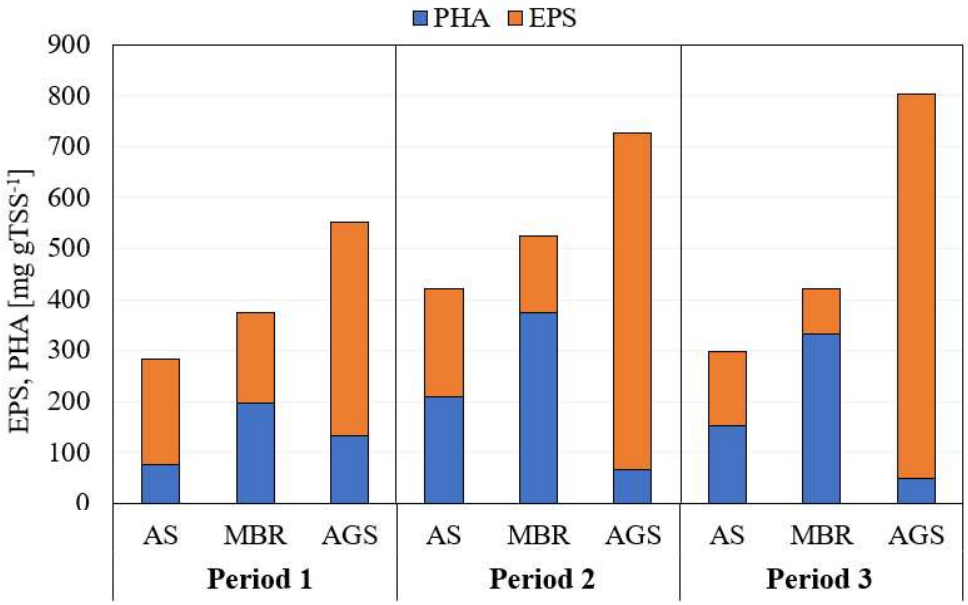

Figure 5.4: Maximum polymers storage and composition in the three MMC cultures enriched at different OLRs.

In Period 1, the maximum biopolymers accumulation capacity was observed in the enriched biomass from the AGS system (61.2% w/w), followed by the enrichment MBR (45% w/w) and the enrichment SBR (35%). The same tendency was observed in the other periods. In more detail, while in Period 2 the overall biopolymer content in all the enriched cultures increased (49% in the enrichment SBR, 63% in the enrichment MBR, and 82% in the AGS), in Period 3 the maximum accumulation capacity decreased in the enrichment SBR (34.9% w/w) and enrichment MBR (48.8% w/w) and still increased in the AGS (90.3% w/w). Based on the above, the biopolymer accumulation capacity resulted the highest in the AGS system, while showing a positive correlation with the OLR applied in the enrichment stage. Biofilm systems are recognised to have higher biopolymer content than conventional flocculent activated sludge, especially as EPS, since the secretion of such compounds is the basic principle of biofilm formation (Wang et al., 2020). A significant difference was noted in terms of biopolymer composition. While the EPS was prevalent in the AGS system, PHA prevailed in the flocculent-based systems, especially in the enrichment MBR. More precisely, PHA content in AGS decreased with the OLR, whereas that of EPS showed an opposite trend. The maximum PHA content

was observed in Period 1, while resulting lower than 15% (w/w), whereas the minimum was obtained in Period 3 (5.5% w/w). This suggested that when increasing the OLR, organic carbon was mainly channelled towards the extracellular accumulation pathway. An opposite trend was noted in the SBR and MBR systems. Indeed, the PHA content showed an overall increasing trend with the OLR, reaching a maximum of 45% w/w in the enrichment MBR and 24.5% in the enrichment SBR. These values were significantly higher than those obtained in the enrichment reactors, since in the accumulation fed-batch reactors the C/N was higher than 80, thus promoting formation of internal storage compounds (Silva et al., 2017). The EPS content decreased with the OLR revealing an opposite trend to that observed in the AGS. Decrement in EPS production was reported in previous studies, and it was attributed to substrate inhibition at high concentrations of organic carbon (Zhao et al., 2021) and to overgrowth of filamentous bacteria (Li et al., 2020) as occurred in Period 3 in the present study. Based on the obtained results, the SBR and MBR systems exhibited a better capacity of PHA accumulation than the enrichment AGS, in which EPS were the main biopolymers produced by the enriched culture. In a previous study, activated sludge floc size was found inversely correlated with PHA accumulation capacity (Li et al., 2019). This finding was confirmed by the present experiment, while extending the results also to biofilm-like systems (AGS). The growth in sludge floc size resulted in a reduction in specific surface area and mass transfer rate, causing adverse impacts on microbial life activities. In this sense, the smaller flocs size that characterize activated sludge in MBR systems could have been beneficial in promoting culture selection with high PHA accumulation capacity. Nevertheless, in larger bio-aggregates as aerobic granules, EPS play a key role in their formation and stability in the long term (Corsino et al., 2016). According to the literature, as external stressors increase (e.g., OLR), the EPS secretion is stimulated, especially if a full aerobic cultivation strategy is applied. Indeed, as reported in previous studies, anaerobic feeding is favourable to the enrichment of microorganisms that could take up organic matter and convert it to internal carbon source during the anaerobic phase (Carrera et al., 2019; Corsino et al., 2017; Sun et al., 2024). In contrast, aerobic feeding led to the development of fast-growing microorganisms and

promoted more production of more EPS (Li et al., 2021). Moreover, increase in OLR was often associated with larger size granules (Di Bella and Torregrossa, 2013), which could further hamper the PHA accumulation capacity. Overall, the PHA productivity from the SBR and MBR systems aligned with previous researches (Argiz et al., 2022; Isern-Cazorla et al., 2023; Morgan-Sagastume et al., 2020; Valentino et al., 2017). However, the results from the AGS system indicated a significant gap from the average results reported in the literature, although at low OLR results were comparable with other studies (Amorim de Carvalho et al., 2021; Rojas-Zamora et al., 2023). Therefore, it is necessary to optimize the enrichment stage in the AGS system to enhance culture selection and maximize the content of PHA.

5.6 Assessment of carbon channelling towards PHA and EPS

As previously discussed, two organic carbon accumulation pathways mainly outcompeted in the accumulation reactors, involving formation of intracellular storage compounds (PHA) or extracellular (EPS). To evaluate the percentage of COD directed towards PHA and EPS production, mass balances in the fed-batch reactors were performed. Figure 5.5 shows the results obtained in the accumulation assays performed at the end of each experimental period at steady state.

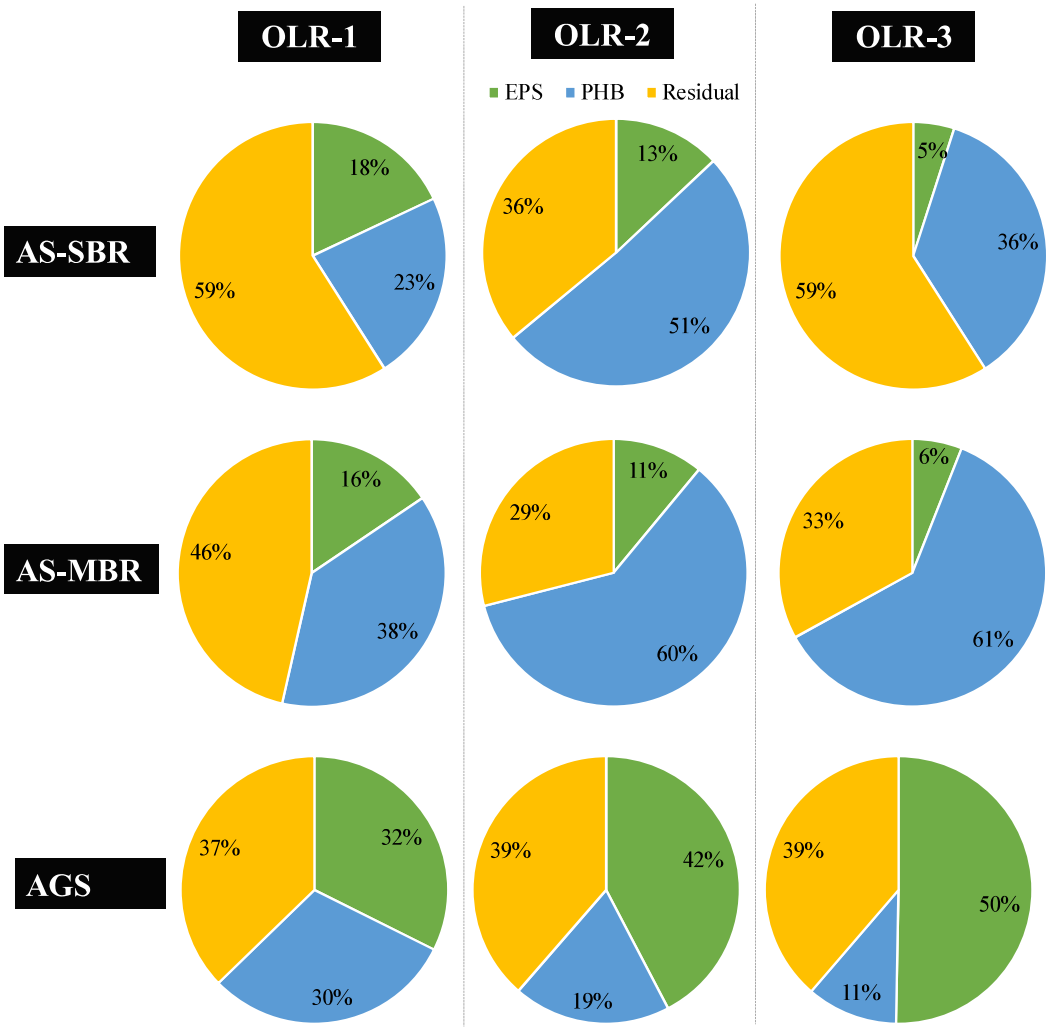

Figure 5.5: Percentage of COD converted into PHA and EPS during the accumulation assays

In Period 1, the highest fraction of the COD converted into intracellular storage compounds was found in the enrichment MBR (38% w/w), while lower values were obtained in the enrichment AGS (30% w/w) and the enrichment SBR (23% w/w). In contrast, the AGS showed the highest percentage of COD conversion towards EPS (32% w/w), whereas the enrichment MBR (16% w/w) the lowest. When increasing the OLR in the enrichment reactors at OLR of 2 kgCOD $m^{-3}d^{-1}$, the COD channelled towards PHA significantly rose in the enrichment SBR (51% w/w) and the enrichment MBR (60% w/w), consistently with the results previously discussed. Indeed, as the PHA was mainly constituted by PHB monomers,

the COD conversion yield to PHA reflected the yields (as gPHA $gVSS^{-1}$) reported in the previous sections. It is worth noticing that at high OLR in the SBR and MBR systems, the organic carbon was mainly directed towards PHA storage pathways at the expense of the EPS. Indeed, the COD converted into EPS significantly decreased at high OLR, standing at about 5% (w/w), indicating that MMC in the SBR and MBR systems were mainly enriched in PHA-accumulating organisms. Therefore, increasing the OLR in the enrichment reactors of the flocculent sludge-based systems was found a suitable solution to tackle biopolymers accumulation via the extracellular pathway. These results clearly reflected the findings previously presented about the biopolymers production observed in the three systems. This suggested that the enrichment MMC strategies applied did not produce the same results in terms of MMC speciation when dealing with flocculent sludge-based systems and AGS. Theoretically, in AGS the higher bacterial diversity could sustain both the production of EPS and PHA. In this respect, a previous study reported that simultaneous PHA and EPS production was obtained in AGS system, although EPS was the main component (3.92 g L^{-1} vs 2.62 g L^{-1}) (Kopperi et al., 2021). The same authors found that EPS production increased when operating at high OLR, at the expense of PHA. Moreover, it was hypothesized that EPS hydrolysis products were used by other bacteria to support PHA production, thus indicating that EPS production was a priority process in AGS systems. Substrate competition for EPS and PHA accumulation was reported in previous studies (Koller and Rodríguez-Contreras, 2015; Oliveira et al., 2021; Rojas-Zamora et al., 2023). Specifically, the authors reported that competition for organic matter in AGS was intensified with the increase of environmental stressors that induced microorganisms to produce more EPS to maintain the granule structure. Previous literature studies reported that overstressed OLR induced more EPS production in full aerobic granular sludge reactors (Liu and Tay, 2015). Moreover, compared with the other systems, the higher hydraulic selection pressure induced by the low settling time, is known to be a key stressor that induced bacteria to produce more EPS to sustain the granule structure (Adav et al., 2009, 2008; McSwain et al., 2004). In this sense, previous literature demonstrated that the hydraulic selection pressure (or the minimum settling velocity) resulted in granular sludge having a higher EPS

content (McSwain et al., 2004). In the present experiment, a full-aerobic strategy was implemented to achieve aerobic granulation instead of the up-flow anaerobic. The former is used when a fast granulation is required and involve the growth of fast-forming microorganisms able to secrete larges EPS amount. Contrarily, up-flow anaerobic feeding is helpful to selection of slow-growing microorganisms able to convert easily biodegradable carbon into storage polymers (Iorhemen and Liu, 2021). Therefore, based on the findings of the present experiment, feast/famine selection strategy performed under full aerobic conditions resulted more suitable to enrich the MMC with PHA-accumulating organisms in the activate-sludge based systems rather than biofilm-like. In this respect, previous literature studies suggested that such conditions in AGS system are favourable for the growth of fast-growing microorganisms and promoted production of more EPS (Li et al., 2021). Full aerobic conditions and high OLR are beneficial to formation of large granules (Di Bella and Torregrossa, 2013), which are reported to present lower PHA yields since the scarcity of substrate inside the granules would prevent the bacteria located in that region to produce PHA (Amorim de Carvalho et al., 2021). Nevertheless, further studies are required to clarify this aspect. Overall, tackling extracellular pathways for carbon storage was easier to achieve in the MBR system. Furthermore, minimize the EPS content in the sludge represents a significant advantage for MBR systems as membrane fouling is mitigated. On the other hand, minimize the EPS content in biological system in which the solid-liquid separation is gravity-driven could be detrimental to process performance, especially if a mainstream PHA-enrichment stage is implemented. Indeed, if on the one hand EPS are essential for AGS formation and stability in the long term, they are even fundamental to form activated sludge flocs with high settling properties (Tian et al., 2006). Nevertheless, it should be noted that EPS extraction from AGS has been recently recognized as a new frontier on biopolymers recovery from AGS (Campo et al., 2022). Indeed, EPS showed promising applications as flame retardants, bio-flocculants and other environmental applications. In this sense, given the great potential of AGS to produce EPS, carbon channelling towards extracellular storage pathways should not be seen as a competitive reaction to PHA production

rather an alternative to diversify the biopolymers recovery from waste sludge.

5.7 Conclusions

The present experiment has found that flocculent sludge-based systems (AS, MBR) had higher PHA productivity, whereas in the AGS, the organic carbon was mainly driven towards EPS pathways accumulations. Integration of PHA production in the mainstream of an industrial WWTP resulted limited when using the SBR and AGS systems, since at certain OLR a simultaneous drop of purification performances and PHA accumulation potential was observed. On the other hand, the results demonstrated the full potential of membrane bioreactor technology that was proven to exhibit a greater robustness in terms of PHA productivity and process performances.

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Chapter 6: Preliminary study on changes in the characteristics of PHAs as a function of OLR

Summary

A laboratory-scale enrichment SBR-type reactor was used to produce polyhydroxyalkanoates with the aim of studying changes in the polymer characteristics such as monomer composition and M_w under different operating conditions applied in the enrichment reactor. M_w and monomer composition are crucial characteristics of PHA as they determine its applicability in industrial sectors and consequently its commercialization purposes (Koller, 2020). Specifically, these parameters affect melting point, thermal properties, and mechanical properties like tensile strength, elastic modulus, and crystallinity (Singh et al., 2015). On the other hand, M_w and monomeric composition depend on many factors during the PHA production process: substrate, feeding strategies, PHA synthase specificity to a substrate, type of bacterial strain, and physiological conditions (Tsuge, 2016; Albuquerque et al. 2011; Bengtsson et al., 2010a; Dai et al., 2008). In particular, the metabolic pathway followed depends on the type of the substrate and the type of bacteria consuming it. The molecular weight of PHA can vary from about 50 kDa to well over 1.000 Da (Bugnicourt et al, 2014; Reddy et al, 2003). In this experiment, the molecular weight fluctuated as the OLR applied in the enrichment reactor for biomass selection increased (2, 4 and 6 gCOD L^{-1} d^{-1}). Specifically, the results of the accumulation assays performed in both fed-batch and continuous strategies at the end of each observation period showed a similar trend where M_w tended to reach a minimum for an intermediate OLR value (4 gCOD L^{-1} d^{-1}) and then increase again for an OLR value of 6 gCOD L-1 d-1. Specifically, the mean values obtained were 462.34, 269.91, 396.94 KDa for period 1, 2 and 3, respectively. In contrast, the mean M_w values of the continuous accumulation assays were 484.7.9, 289.1, 408.9 KDa for the same observation periods. The mean productivity, on the other hand, was 0.86, 0.45, and 0.84 gPHA $L^{-1}h^{-1}$ for fed-batch assays and 0.59, 0.46, 0.73 gPHA $L^{-1}h^{-1}$ for continuous assays. Additional fed-batch accumulation assays were performed at the end of period 3 when the characteristics of the fed substrate were changed. Specifically, the average M_w values obtained were 377.08 KDa using the same synthetic substrate but with N&P, while they were 307.1 KDa in the case of real fermented agro-industrial wastewater with a different VFA composition. The use of different substrates affected the molecular weight, composition of the polymer and volumetric productivity. The latter tended to decrease, probably due to the presence of N&P directing some of the carbon used to bacterial synthesis. In the case of fermented wastewater, metabolic stress conditions occurred because the selected MMCs were not accustomed to consuming certain VFAs. The productivity values obtained were 0.75 gPHA $L^{-1}h^{-1}$ and 0.58 gPHA $L^{-1}h^{-1}$ for the synthetic substrate with N&P and for the real fermented wastewater, respectively.

6.1 Materials and methods

6.1.1 Culture selection stage

An SBR with working volumes of 2 L was operated under the feast and famine regime with the uncoupled ammonia feeding strategy for 107 days. The reactor was inoculated with activated sludge from the municipal wastewater treatment plant of Almada, Portugal. After inoculation, the sludge was aerated for about 5 h. The initial concentrations of total solids and volatile solids were 2.74 ± 0.11 g L^{-1} and 2.33 ± 0.15 g L^{-1}, respectively. The SBR cycle was divided as follows: 11 hours of reaction phase, 50 minutes of settling phase, 5 minutes of withdrawal phase and 5 minutes of idle phase. In the first 5 minutes of the reaction phase, the carbon source was fed together with a mineral solution with activated stirring and without aeration. A nitrogen and phosphorus solution was added after a time ranging between 120 minutes and 240 minutes from the beginning of the reaction phase. However, these macronutrients were always fed after the feast phase. In the final minute of the reaction phase, a volume of 250 ml of mixed liquor was purged to maintain a SRT of 4 days. During the withdrawal phase, half the volume of the reactor was discharged and replaced with feeding and mineral solutions at the beginning of the next cycle, resulting in a hydraulic retention time (HRT) of 1 d. The OLR was increased in three steps: 2, 4 and 6 gCOD L^{-1} d^{-1} (49, 98 and 147 Cmmol L^{-1}) corresponding to period 1, period 2 and period 3, respectively. The carbon source was a mixture of volatile fatty acids consisting of 60 % butyric acid, 20 % valeric acid and 20 % hexanoic acid. The concentration of the organic carbon solution was on average 25 gCOD L^{-1} and the pH was adjusted to 6.5 with NaOH. The mineral solution fed into the reactor was composed of ATU, EDTA, $MgSO_4.7H_2O$, $CaCl_22H_2O$, and trace elements. These solutions were dosed in such a way as to obtain concentrations that remained constant within the enrichment reactor during all experimental periods. Specifically, concentrations were fixed at 0.01 g L^{-1}, 0.1 g L^{-1}, 0.6 g L^{-1} and 0.07 g L^{-1} for ATU, EDTA, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, respectively. The trace element solution was composed of $FeCl_3.6H2O$, H_3BO_3, $CoCl_2.6H_2O$, $MnCl_2.4H_2O$, $ZnSo_4.7H_2O$, $Na_2MoO_4.2H_2O$, $CuSO_4.5H_2O$ and KI. The latter was dosed such that the reactor had the concentrations of 1.5 g L^{-1}, 0.15 g L^{-1}, 0.15 g L^{-}

^{1}, 0.12 g L^{-1}, 0.12 g L^{-1}, 0.06 g L^{-1}, 0.03 g L^{-1} e 0.03 g L^{-1} of $FeCl_3$.6H2O, H_3BO_3, $CoCl_2.6H_2O$, $MnCl_2.4H_2O$, $ZnSo_4.7H_2O$, $Na_2MoO_4.2H_2O$, $CuSO_4.5H_2O$ and KI, respectively. The pH of the mineral solution was adjusted to 7.2–7.3 using NaOH whenever it was prepared. The fed volume, comprising mineral solution and carbon solution, varied based on the applied OLR in the three experimental periods. Specifically, the volume of carbon solution was 80 ml, 160 ml and 240 ml while mineral solution was always the complement to 1000 ml in Period 1, Period 2 and Period 3, respectively. A N&P solution composed of NH_4Cl and KH_2PO_4 was prepared to correspond to a C:N:P ratio of 100:7:1 and added after the end of the feast phase. The experimentation was carried out at room temperature (20 ± 1 ◦C), and the pH was controlled so as not to exceed 8.5 by automatic addition of 1 M HCl as needed. Air was supplied by an air compressor through a silicone tube spiral disperser and the DO level was maintained above 2 mg L^{-1} by adjusting the air flow rate. DO concentration and pH were measured online using analog sensors (Mettler Toledo, LLC, Columbus, OH, USA). The length of the feast phase was identified based on the trend of oxygen level. Specifically, low levels of DO indicate the presence of substrate due to biological oxidation reactions. When the substrate was consumed (end of feast phase) there was a rapid increase in oxygen concentration, marking the beginning of the famine phase. Stirring was performed with a stainless-steel shaft with two impellers at 250 rpm. Weekly monitoring of the cycle was performed throughout the reaction phase by collecting samples to analyze TSS, VSS, VFA, N, P and PHA. The frequency of monitoring increased when the accumulation assays were carried out to ensure that no problems occurred in the SBR enrichment reactor.

6.1.2 PHA accumulation assays

Fed-batch assays were performed for each experimental period to evaluate the maximum storage capacity of the selected MMC and to determine any changes in the composition and molecular weight of the polymers. The reactor used for this purpose was a BioFlo®/CelliGen® 115 system (Eppendorf AG, Germany) with a 1 L working volume glass vessel and

controlled aeration and agitation, using 500 mL of surplus biomass from the enrichment reactor at the end of the famine phase. For each accumulation assays, aeration was adjusted to maintain a DO level above 2 mg L^{-1} and mixing was set to 250 rpm. The pH value was maintained between 7.5 and 8.5 by automatic addition of 1 M HCl and 0.5 M NaOH as needed. The temperature of the accumulation reactor was controlled and maintained at 21 ± 1 ◦C. Duplicate accumulation assays were performed using both pulse-wise and continuous strategies for each experimental period. The same OLR value of the enrichment reactor was used for each accumulation test. The carbon solution used had the same composition as that fed into the enrichment reactor (60 % butyric acid, 20 % valeric acid, and 20 % hexanoic acid on a Cmol basis). All accumulation assays with the pulse-wise strategy were performed using 6 pulses, after which the reaction was considered to have slowed significantly. The total duration of the accumulation assays ranged from 3.32 to 4.68 hours. The continuous accumulation assays were tested based on the average volume value of each pulse and the resulting average reaction time of the previous two accumulation assays. Thus, it was possible to calculate the six flow rates to be applied during the continuous accumulation assays. However, to ensure that no polymer accumulated by the bacteria was consumed due to lack of substrate, each calculated flow rate was multiplied by a security factor. The latter was 25 % for the first flow rate, 20 % for the second and third pulses, 15 % for the fourth and fifth, and 10 % for the sixth flow rate. In addition, during the third period (OLR equal to 6 gCOD $L^{-1}d^{-1}$), duplicate accumulation assays were performed with the same synthetic substrate but with the presence of N&P. A C:N:P ratio of 100:3.5:0.5 was used for the first two pulses to avoid bacterial inhibition phenomena due to too high concentrations of these macronutrients, while a C:N:P ratio of 100:7:1 was used for the other four pulses as in the enrichment reactor. Finally, two other fed-batch accumulation assays with real fermented agro-industrial wastewater were performed during the same period. The organic load of the real fermented wastewater was on average 24.99 gCOD L^{-1} (620.417 Cmmol L^{-1}) and consisted of acetate, propinate, isobutyrate, butyrate, isovalerate, valerate, and caproate, in proportions of 11.42% (2.854 gCOD L^{-1}), 3.89% (0.974 gCOD L^{-1}), 7.06% (1.765 gCOD L^{-1}), 15.87% (3.966 gCOD L^{-1}), 0.07% (0.018 gCOD L^{-1}), 1,66% (0.416 gCOD L^{-1}), 60% (14.995 gCOD L^{-1}), respectively. The corresponding

values in Cmmol L^{-1} are: 89.187 Cmmol L^{-1}, 26.082, 44.133 Cmmol L^{-1}, 99.147 Cmmol L^{-1},0.429 Cmmol L^{-1}, 9.99 Cmmol L^{-1}, 351.446 Cmmol L^{-1}, respectively. The concentration of N and P was 0.547 g L^{-1} and 0.323 g L^{-1}, thus having a C:P:N ratio of 100 : 2.19 : 1.29. Samples were collected over the full duration of the accumulation assays to follow the VFA consumption, the PHA content, the characteristics of the polymer obtained (e.g. molecular weight), and nitrogen and phosphorus trends in the case of synthetic substrate with N&P and in the case of real wastewater. The accumulation reactor worked only at the end of each experimental period when bacterial selection was considered completed i.e., when the F/F ratio was lower than 0.20 (Morgan-Sagastume et al., 2014) and in any case not earlier than 3 times the SRT.

6.1.3 Analytical methods

TSS and VSS were determined according to Standard Methods (APHA, 1998). Samples collected during enrichment phase monitoring and during accumulation assays were immediately centrifuged at 11000 rpm for 3 minutes. The pellets were lyophilized while the supernatant was filtered through a 0.45 micron membrane filter (Whatman®, Cytiva, Marlborough, MA, USA). VFA concentration of filtered samples was determined by high-performance liquid chromatography (HPLC) using a VWR Hitachi Chromaster chromatographer as described by Oliveira et al. (2017). Ammonium and phosphate concentrations were quantified using a colorimetric method implemented in a segmented flow analyser (Skalar San++ automated system, Skalar Analytical B.V, Breda, The Netherlands). Lyophilised biomass pellets were processed to quantify 3-hydroxybutyrate (3-HB), 3-hydroxyvalerate (3-HV) and 3-hydroxyhexanoate (HHx) using a Bruker 430-GC gas chromatographer following the method described by Lanham et al. (2013). Size exclusion chromatography (SEC) (Waters Millennium system) was used to determine molecular mass distribution and polydispersity indices (PDI) of biopolymers (Pereira et al. 2019).

6.1.4 Calculations

The F/F ratio was calculated by dividing the length of the feast phase by that of the famine phase. The PHA content in the biomass (%, w/w) was determined in terms of percentage of VSS by dividing the PHA content by the VSS determined at the same sampling time. Active biomass concentrations (X_A, g L^{-1}) were calculated by subtracting the PHA concentration (g L^{-1}) from VSS concentration (g L^{-1}). VFA concentration (Cmmol L^{-1}) is given by the sum of the concentrations of butyric, valeric and hexanoic acids in the medium at a given time. PHA concentration (Cmmol L^{-1}), on the other hand, is equal to the sum of 3-HB, 3-HV and HHx. Specific substrate uptake rate (-qHorg, Cmol-VFA.Cmol-X_A^{-1} h^{-1}) was calculated from the slope of the linear regression of total VFA concentrations over time. Specific PHA accumulation rate (q_{PHA}, CmolPHA Cmol X_A^{-1} h-^{1}) and specific substrate consumption rate (-qPHAfamine, CmolPHA CmolX_A^{-1}.h^{-1}) were determined by dividing the slope of the linear regression of total PHA concentrations over time by the average X_A concentration during the feast phase, respectively. Storage yields ($Y_{PHA/VFA,}$ CmolPHA CmolVFA^{-1}) were determinate by dividing the q_{PHA} (total amount of PHA produced) by the -qHorg (total amount of PHA consumed). The volumetric PHA production rate (gPHA $L^{-1}h^{-1}$) was calculated by dividing the concentration of PHA produced by the time elapsed. Growth yields on PHA during the famine phase ($Y_{X/PHA}$, CmolX_A CmolPHA^{-1}) were calculated by dividing the total amount of X_A produced by the total amount of PHA consumed during the famine phase.

All concentrations (3-HB, 3-HV, HHx, VFA and X_A) were converted into COD in accordance with the following factors: 1.67 gCOD g3-HB^{-1}, 1.92 gCOD g3-HV^{-1}, 2.08 gCOD gHHx^{-1}, 1.07 gCOD g-$HLac^{-1}$, 1.07 g-COD g$HAce^{-1}$, 1.51 gCOD g$HPro^{-1}$, 2.08 gCOD g-$EtOH^{-1}$, 1.82 gCOD g$HBut^{-1}$, 2.04 gCOD g$HVaç^{-1}$, 2,20 gCOD g$HCap^{-1}$ and 1.41 gCOD gXa^{-1} (Xa formula of $C_5H_7NO_2$ (Gujer and Henze, 1991)). Alternatively, all concentrations (3-HB, 3-HV, HHx, VFA and Xa) were converted into Cmol in accordance with the following factors: 0.046 Cmol.g3-HB^{-1}, 0.050 Cmol.g3-HV^{-1}, 0.048 Cmol gHHx^{-1}, 0.033 Cmol g$HLac^{-1}$, 0.033 Cmol g-$HAce^{-1}$, 0.040 Cmol g-$HPro^{-1}$, 0.043 Cmol g$EtOH^{-1}$, 0.049 Cmol g$HBut^{-1}$,

0.049 Cmol g HVaç$^{-1}$, 0.052 Cmol gHCap^{-1} and 0.044 Cmol gX_A^{-1} (Gujer and Henze, 1991).

6.2 Result

6.2.1 PHA production

During steady-state conditions, kinetic parameters were calculated by referring to data acquired during at least two complete monitoring cycles of enrichment reactor cycles during that given period. The value of the F/F ratio in the start-up phase of the system was 0.37 and then decreased below the value of 0.2 after the first day of experimentation (Fig 6.1).

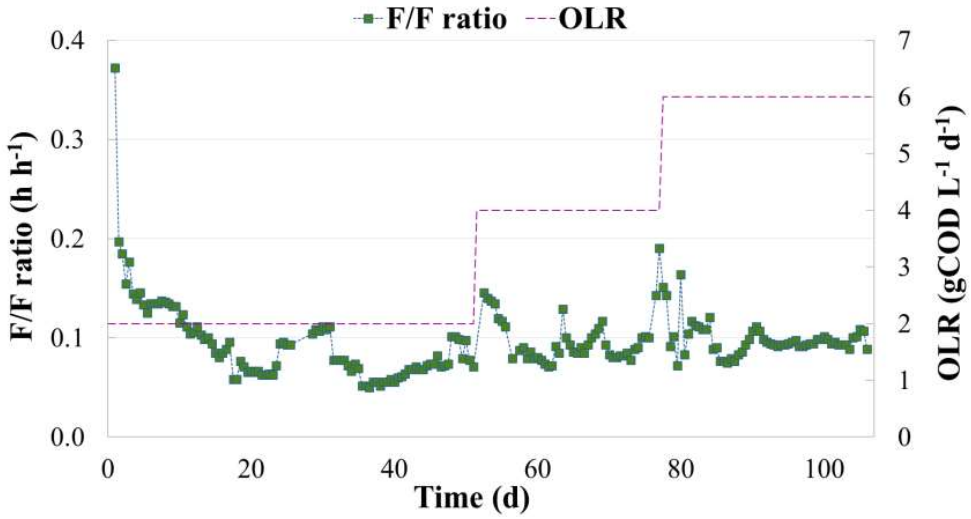


Figure 6.1: Feast to famine ratio and OLR change over time in SBR

This value was almost always less than 0.1 throughout the first period. The value of the F/F ratio tended to increase as the OLR was increased. The increase in organic load stimulated the growth of biomass, whose concentration tended to increase until it reached steady-state conditions again. In general, after each increase in OLR, the F/F ratio remained below 0.2 and tended over time to settle at values close to 0.1. Overall, therefore, the F/F ratio did not increase significantly despite the increase in the OLR parameter. The performance obtained by the bacterial culture regarding the achievement of steady-state conditions for each experimental period is shown in Figure 6.2 and Table 6.1.

Figure 6.2a illustrates an example of MMCs' behaviour in the period 3 cycle once steady-state conditions were reached. Specifically, the profiles of TSS, VSS, X_A, H.Orgs, PHA, N and P over the 12-hour period are depicted.

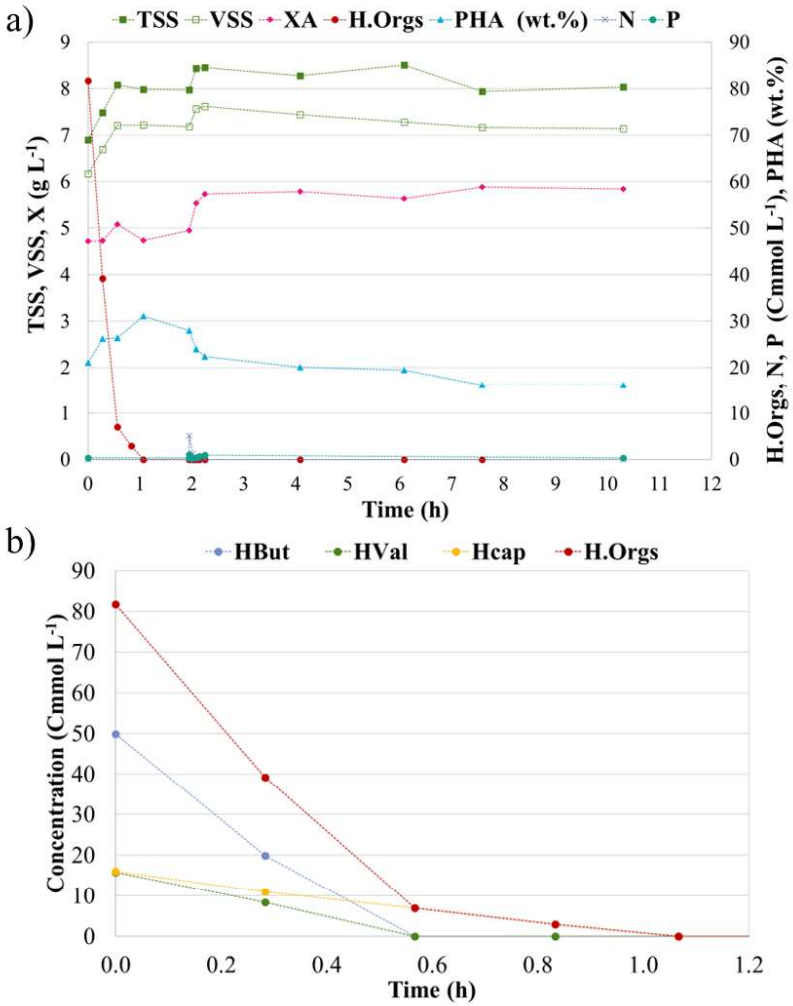


Figure 6.2: Concentration profiles of TSS, VSS, X_A, H. Orgs, PHA, N and P (a) and VFAs consumption during a typical cycle at pseudo-steady state in Period 3 (b)

A VFA solution was fed at the beginning of the feast phase in the absence of a nitrogen source. As a result, carbon was consumed to support the production and storage of PHA, while no significant cell growth was observed. After 2 hours from the start of the cycle, N&P dosing occurred, which was consumed simultaneously with the stored PHA leading to active biomass growth. The nitrogen supplied was consumed entirely, unlike P. Increasing the OLR parameter while maintaining the same value of SRT (4

days) resulted in an increase in the concentration of active biomass. The latter rose from a value of 1.809 $gX_A\ L^{-1}$ to 5.200 $gX_A\ L^{-1}$, passing through 3.347 $gX_A\ L^{-1}$ (Period 2).
The selected MMCs were specialized in butyrate and valerate consumption while caproic acid was consumed last (Figure 6.1b). Table 6.1 shows the mean values and standard deviation of the kinetic and stoichiometric parameters of the feast phase and famine phase calculated for all three experimental periods under steady-state conditions.

Table 6.1: Main paramters of SBR operation

	Period 1	Period 2	Period 3
OLR [gCOD $L^{-1}\ d^{-1}$]	2	4	6
OLR [Cmmol Horgs $L^{-1}\ d^{-1}$]	49	98	147
F/F [h h^{-1}]	0.055 ± 0.003	0.089 ± 0.003	0.085 ± 0.012
F/M [kgCOD $kgSST^{-1}\ d^{-1}$]	0,696 ± 0,053	0,829 ± 0,008	0,866 ± 0,004
TSS [g L^{-1}]	3.054 ± 0.228	4.833 ± 0.341	7.881 ± 0.337
VSS [g L^{-1}]	2.745 ± 0.225	4.364 ± 0.322	7.088 ± 0.279
X_A [Cmmol]. Average	159.929 ± 20.320	295.912 ± 36.999	459.679 ± 33.246
X_A [g]. average	3.618 ± 0.460	6.694 ± 0.837	20.799 ± 0.877
PHA [wt.%]	39.139 ± 6.119	31.688 ± 2.414	32.038 ± 0.905
ΔPHA feast [%. w/w]	18.057 ± 4.529	19.676 ± 1.841	13.848 ± 3.743
$-q_S$ [CmolS $CmolX_A^{-1}\ h^{-1}$]	0,747 ± 0,003	0,440 ± 0,057	0,561 ± 0,038
qPHA [CmolPHA $CmolX_A^{-1}\ h^{-1}$]	0,560 ± 0,021	0,449 ± 0,092	0,492 ± 0,080
$Y_{PHA/S}$ [CmolPHA $CmolS^{-1}$]	0,749 ± 0,029	1,020 ± 0,248	0,877 ± 0,155
Famine phase			
-qPHAfamine [CmolPHA $CmolX_A^{-1}\ h^{-1}$]	0,197 ± 0,022	0,237 ± 0,061	0,149 ± 0,018
qX|famine [Cmol $CmolX_A^{-1}\ h^{-1}$]	0,119 ± 0,017	0,073 ± 0,007	0,129 ± 0,015
$Y_{X/PHA}$ [$CmolX_A$ $CmolPHA^{-1}$]	0,601 ± 0,111	0,306 ± 0,083	0,866 ± 0,144

The specific substrate uptake (-qS) and storage (qPHA) rates had a maximum value of 0,747 ± 0,003 CmolS $CmolX_A^{-1}\ h^{-1}$ and 0,560 ± 0,021 CmolPHA $CmolX_A^{-1}\ h^{-1}$, respectively, during period 1. Then the values tended to decrease to 0,440 ± 0,057 CmolS $CmolX_A^{-1}\ h^{-1}$ and 0,449 ± 0,092 CmolPHA $CmolX_A^{-1}\ h^{-1}$ in period 2 before increasing again in period 3. However, the values were lower than in the first experimental period: q_S equal to 0,561 ± 0,038 CmolS $CmolX_A^{-1}\ h^{-1}$ and q_{PHA} equal to 0,492 ± 0,080 CmolPHA $CmolX_A^{-1}\ h^{-1}$.

The storage yield $Y_{PHA/S}$ showed an opposite trend, whereby the highest conversion of supplied carbon to PHA was observed during period 2, resulting in 1.020 ± 0.248 CmolPHA $CmolS^{-1}$.
An intermediate value of 0.877 ± 0.155 CmolPHA Cmol-S^{-1} was reported in period 3, while the lowest conversion was observed during period 1 (0.601 ± 0.111 CmolPHA $CmolS^{-1}$).
The maximum PHA content reached during the feast phase was highest in period 1 (39.139 ± 6.119 wt.%), while it maintained lower values in the remaining periods (31.688 ± 2.414 and 32.038 ± 0.905 wt.% for period 2 and 3, respectively).
Regarding the famine phase, maximum specific PHA consumption (-qPHA famine), maximum growth rates (qX_{famine}) and growth yield on PHA ($Y_{X/PHA}$) showed variations as the OLR changed (Table 6.1). The first parameter tended to reach a minimum value (0.237 ± 0.061 CmolPHA $CmolX_A^{-1}$ h^{-1}, while the second reached a maximum value (0.073 ± 0.007 Cmol Cmol X_A^{-1} h^{-1}) in period 2. The result of these trends returned a value of $Y_{X/PHA}$ equal to 0.601 ± 0.111 CmolPHA $CmolX_A^{-1}$ h^{-1}, 0.306 ± 0.083 Cmol-PHA $CmolX_A^{-1}$ h^{-1}, and 0.866 ± 0.144 CmolPHA $CmolX_A^{-1}$ h^{-1} in period 1, period 2 and period 3, respectively.

6.2.2 Accumulation assays with different OLRs

Accumulation assays with the selected MMCs were performed both in fed-batch and continuous modes in duplicate to evaluate the maximum PHA accumulation capacity in the accumulation reactor at all experimental periods and to assess any changes in the composition and characteristics of the PHAs produced. The OLR value used for the accumulation tests was the same as that imposed in the enrichment reactor. All accumulation assays were conducted without the addition of nitrogen and phosphorus to prevent bacterial growth.
In all accumulation assays, the biomass was fed and aerated until a stable PHA content was attained, corresponding to their maximum PHA accumulation capacity.
As an example, Fig. 6.3a illustrates trend of PHA content trend (wt%), TSS and VSS concentration, and H.Orgs over time, while Fig.6.3b shows the

variation in VFA concentration during a Fed-Batch accumulation assay of period 3.

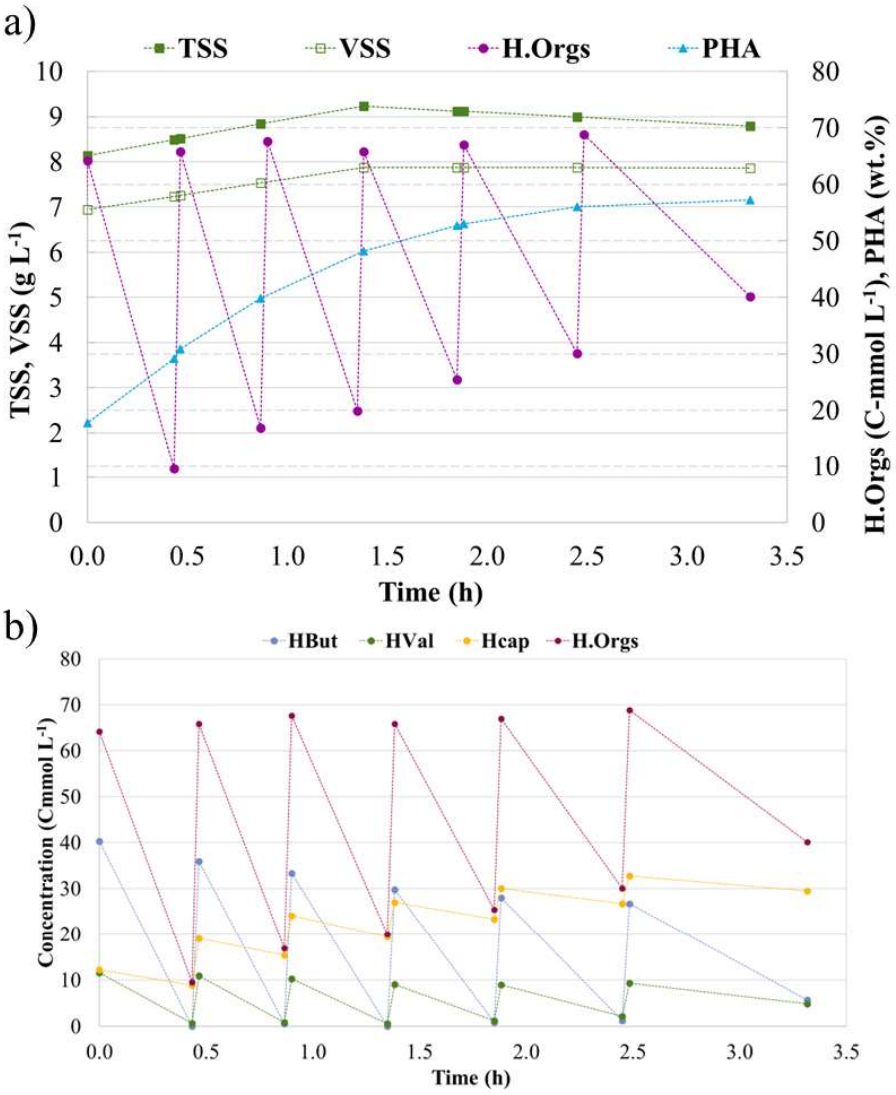


Figure 6.3: Example of the PHA, TSS, VSS, H.Org trends (a) and amount of VFA over time (b) during Fed-Batch accumulation assays performed using biomass from enrichment reactor. The maximum values of VFA and H.Org. correspond to the addition of feed pulses.

PHA content tended to increase over time until it reached a plateau, indicating that maximum PHA content had been reached. TSS and VSS remained almost constant as bacterial growth was limited by the lack of N&P. Similarly, in the enrichment reactor, the preferred substrate for MMCs was butyric acid, while caproic acid tended to accumulate during the accumulation assay (Figure 6.3b).

Figure 6.4a and 6.4b show similar trends related to continuous accumulation assays during period 3.

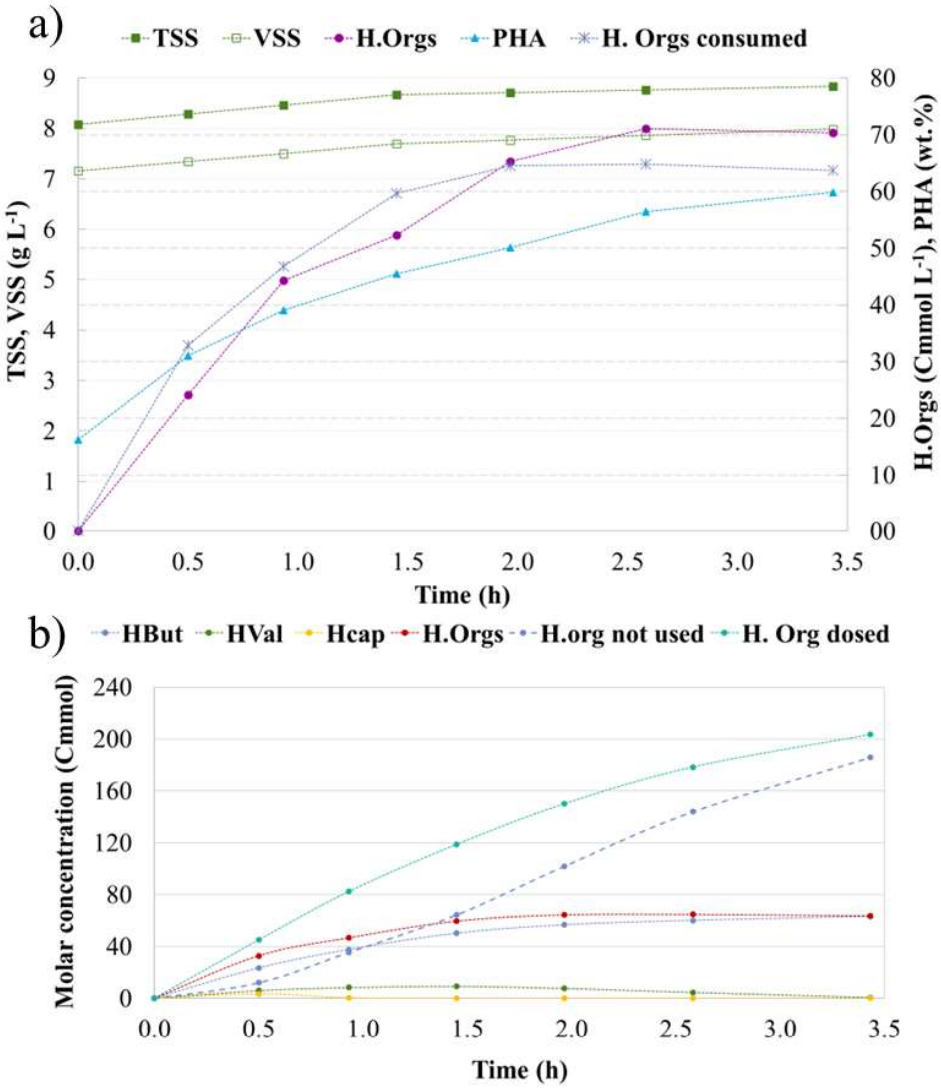


Figure 6.4: Example of the a) PHA, TSS, VSS, H.Org trends and b) amount of VFA over time during Fed-Batch accumulation assays performed using biomass from enrichment reactor. H.Org. and H.Org. not used are cumulative curves while the remaining values refer to the sampling moment.

In the continuous assay, it is even more evident how the biomass prefers butyric acid. Caprotate consumption was very limited and equal to zero after one hour from the start of the test while valerate consumption was zero after 3.5 hours. The advantage of continuous accumulation assays is related to the fact that carbon supply occurs throughout the assay run so there is no consumption of the accumulated polymer by the biomass. Again, the

preferred VFA of biomass was butyric acid, followed by valeric acid and butyric acid.

Table 6.2 summarizes the mean values of the parameters obtained during the different accumulation assays as storage yields ($Y_{PHA/S}$), PHA productivity, elapsed time, PHA content on biomass (PHAmax). The $Y_{PHA/S}$ values of the fed-batch assays are the maximum values reported during the accumulation assays that coincided with the first pulse. In fact, as the accumulation assay progressed, MMC became enriched in PHA, and consequently the specific PHA production rate and the capacity of biomass to produce PHA both decreased. This behaviour has also already been observed by other authors (Cruz et al., 2022; Lorini et al., 2021; Tamis et al., 2014).

The highest value of $Y_{PHA/S}$ was observed in the first period, being equal to 1.112 ± 0.125 CmolPHA $CmolS^{-1}$, suggesting that the supplied substrate was converted entirely to PHA. This value tended to decrease as the OLR applied in the enrichment reactor increased, being equal to 0.944 ± 0.018 and 0.843 ± 0.050 CmolPHA $CmolS^{-1}$ in the second and third periods, respectively.

The volumetric productivity of PHA of both types of accumulation assays decreased to a minimum value in period 2 and then increased again in period 3, but still that for batch assays was always higher than for continuous assays except in period 2 when they were comparable (0.45 ± 0.008 and 0.46 ± 0.016 g-PHA L^{-1} h^{-1}). In the case of batch assays, the productivity was comparable in period 1 and 2, being 0.86 ± 0.037 and 0.84 ± 0.014 g-PHA L^{-1} h^{-1}. In contrast to the batch assays, productivity was higher in period 3 (0.73 ± *0.054* g-PHA L^{-1} h^{-1}) than in period 1 equal to 0.59 ± *0.011* g-PHA L^{-1} h^{-1}.

PHA_{max} of the batch assays, on the other hand, presented the same trend and comparable values to the PHAmax of the continuous assays, except for the first experimental period in which $PHA_{max\text{-}Fed\text{-}Batch}$ was greater than $PHA_{max\text{-}Continuous,}$ amounting to 65.57 ± 4.356 and 59.63 ± 0.0003 wt.%, respectively.

The trend of PHAmax was similar to that of volumetric productivity; in fact, for both accumulation assays, there was a maximum value at period 1, a minimum value at period 2 (equal to 52.12±1.446 and 53.56 ± 1.379 wt.%, respectively) and an intermediate value at period 3 equal to 58.08 ± 0.826 and 59.33 ± 0.474 wt.%, respectively.

Despite the different OLRs applied, the results obtained indicate that the MMCs selected in the three experimental periods had similar specific capacities per cell to accumulate PHA, although higher volumetric PHA production rate was obtained in period 1.

Table 6.2: Main parameters determined in the accumulation reactor

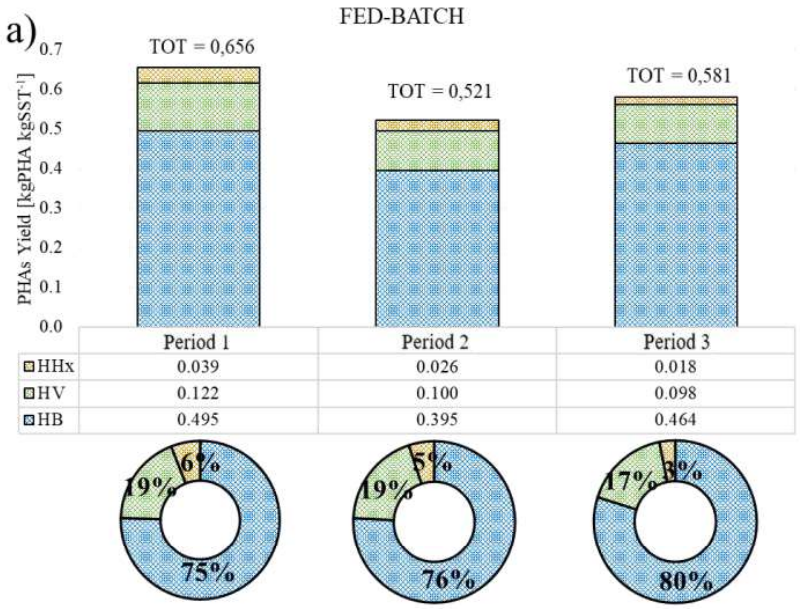
	Period 1		Period 2		Period 3	
	Fed-Batch	Cont. Fed	Fed-Batch	Cont. Fed	Fed-Batch	Cont. Fed
X_A (Cmmol). average	33.13	39.03	81.26	55.27	91.81	88.94
dev. st. p.	*2.33*	*2.70*	*0.68*	*6.81*	*1.24*	*3.60*
Maximum biomass activity						
$Y_{PHA/S}$ [CmolPHA CmolS-1]	1.112	N/A	0.944	0.655	0.843	N/A
dev. st. p.	*0.125*	-	*0.018*	*0.024*	*0.050*	-
PHA productivity [gPHA L^{-1} h^{-1}]	0.86	0.59	0.45	0.46	0.84	0.73
dev. st. p.	*0.037*	*0.011*	*0.008*	*0.016*	*0.014*	*0.054*
Overall accumulation performance						
Elapsed time (h)	3.74	3.86	4.55	4.68	3.33	3.44
dev. st. p.	*0.058*	*0.075*	*0.033*	*0.008*	*0.008*	*0.008*
PHAmax (wt.%)	65.57	59.73	52.12	53.56	58.08	59.33
dev. st. p.	*4.356*	*0.100*	*1.446*	*1.379*	*0.826*	*0.474*

Figures 6.5a,b show the PHA yield and polymer composition obtained for the different accumulation assays. PHA content related to total solids reached the maximum value in the first experimental period for both batch assays (0.66 KgPHA $KgTSS^{-1}$) and continuous assays (0.58 KgPHA $KgTSS^{-1}$). Polymer composition remained very similar for all accumulation assays carried out during the experimentation in that the percentage composition of VFAs fed was always the same.

Indeed, it is well known that the composition of carbon source e types of microorganisms in MMCs affect the monomeric composition (Sabapathy et al., 2020). Carvalho et al., (2014) noted that the 3-HV content of the polymer was correlated with the composition of the microbial population. The monomeric composition of PHA (3HB:3HV) is a key factor for possible polymer applications (Cruz et al., 2022).

Indeed, copolymers are generally used in packaging production if the 3-HV content is about 20%, while they can be used as adhesives or elastomers if the 3-HV content is higher (Arcos-Hernàndez et al., 2013; Philip et al., 2007).As expected, a terpolymer with a composition of 3-HB/3-HV/HHx was obtained; in fact, Silva et al., (2022), by promoting the production of caproate in the fruit waste fermentation process, demonstrated that caproate is the precursor for HHx production. Copolymers containing both SCL-PHA

and MCL-PHA, such as PHBHx poly(3-hydroxybutyrate-co-hydroxyoctanoate), are very interesting because they have elastomeric properties similar to rubber, which are not typical of PHB and PHBV poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (Pereira 2019; Volova et al., 2016). In general, an increase in 3-HV and HHx monomers improves the characteristics of pure PHB as it leads to a reduction in the degree of crystallinity, an increase in extension to break, a better impact resistance, lower melting temperature and tensile strength (Volova et al., 2016; Loo et al., 2007). 3-HB was always present in a higher percentage (75, 76, 80 % in period 1, 2 and 3, respectively) due to the high presence in the substrate of butyric acid (60 %), which is its precursor. The 3-HV and medium chain HHx tended to decrease slightly from 19 to 17 % and 6 to 3 %, respectively.



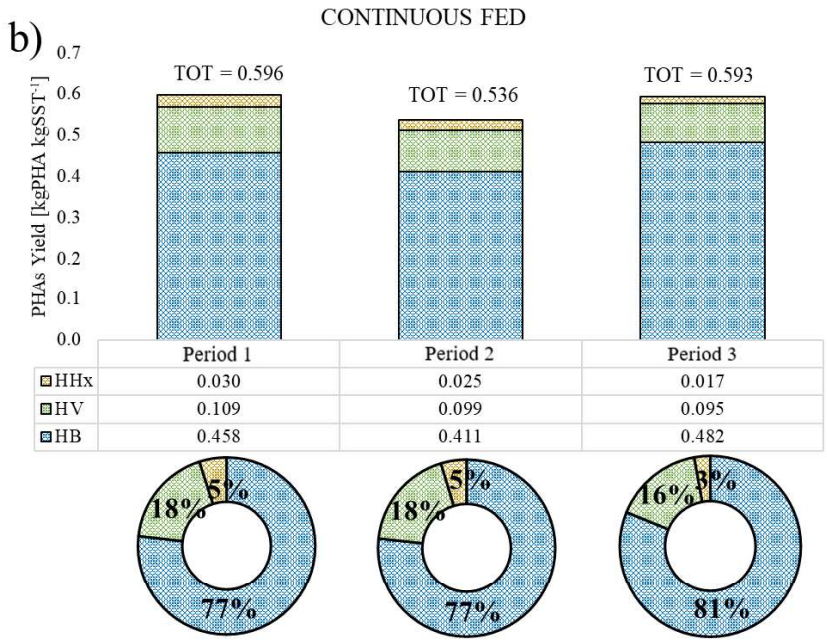


Figure 6.5: Polymer composition with different OLR (a) Fed-Batch accumulation assay (b) Continuous accumulation assays

The results obtained from comparing Fed-Batch and continuous accumulation assays were different from those of the Albuquerque et al., (2011) study, which employed fermented molasses for PHA production. In that study, it was found that the continuous strategy in the accumulation stage could result in increased volumetric productivity of PHA and increased hydroxyvalerate content. Specifically, the 3-HV content increased by about 9 percent compared with pulse-wise feeding and resulted in a change in the molecular weight and thermal properties of the P(3-HB-co-3-HV) copolymer. However, in the study by Albuquerque et al., (2011), the authors initiated the continuous accumulation assay by providing an initial pulse of substrate at the beginning of the test, which was not done in the present experiment.

6.2.3 Accumulation assays with different substrate

The parameters of the accumulation assays performed using different substrates are presented in Table 6.3.

Table 6.3: Main parameters determined in the accumulation assays in period 3

	Period 3		
	Fed-Batch	Fed-Batch w/N&P	Fed-Batch w/Real WW
X_A (Cmmol), average	91.81	101.38	106.88
dev. st. p.	*1.24*	*6.32*	*0.65*
Maximum biomass activity			
$Y_{PHA/S}$ [Cmol-PHA Cmol-S-1]	0.843	0.833	0.584
dev. st. p.	*0.050*	*0.152*	*0.080*
PHA productivity [g-PHA L-1 h-1]	0.84	0.75	0.58
dev. st. p.	*0.014*	*0.045*	*0.130*
Overall accumulation performance			
Elapsed time (h)	3.33	4.97	2.98
dev. st. p.	*0.008*	*0.033*	*0.100*
PHAmax(wt.%)	58.08	50.77	31.85
dev. st. p.	*0.826*	*1.013*	*0.100*

The maximum value of $Y_{PHA/S}$ was comparable in the case of synthetic substrate with or without N and P, amounting to 0.843 and 0.833 Cmol-PHA Cmol-S-1, respectively, while it had a lower value in the case of real fermented wastewater. In addition, significant differences were observed in both PHA productivity and PHAmax. As expected, the best performance was obtained in the case of synthetic substrate in the absence of macronutrients, where the productivity was 0.84 ± 0.014 gPHA L^{-1} h^{-1} and the maximum PHA content was around 58.08 wt%. These values decreased to 0.75 ± 0.045 gPHA L^{-1} h^{-1} and 50.77 wt% in presence of N and P and to 0.58 ± 0.130 gPHA L^{-1} h^{-1} and 31.85 ± 0.100 wt% in the case of real wastewater. The low values obtained in the case of the substrate with N and P and fermented waters could be explained because of two different phenomena. In the first case, the presence of N&P, allowed the biomass to direct some of the carbon supplied for bacterial synthesis, consequently limiting the accumulation of PHA, but keeping the composition of the polymer obtained almost unchanged because the carbon source was still the same. In the second case, the fermented effluent had a very different composition in terms of VFAs than the synthetic substrate; consequently, carbon utilization may have switched the pathway from PHA polymerization to EPS synthesis or to other competing reactions (Figure 6.5a), or the different composition of VFAs may have even inhibited bacterial activity (Figure 6.5b). However, it cannot be ruled out that the

presence of N and P, albeit limited, directed some of the supplied carbon toward the synthesis of new biomass. Substrate composition plays a key role in the bacterial composition of MMCs. For instance, Bengtsson et al. (2010b) treated fermented molasses with different VFA composition and produced a copolymer consisting of various monomers, such as: 3-HB, 3-HV, $3H_2MB$, $3H_2MV$ and HHx. In this way, a correlation between the composition of the PHA and VFA composition of substrate was found, resulting in 56-70 mol% 3HB, 13-43 mol% 3HV, 1-23 mol% 3HHx and 0-2 mol% 3H2MB and 3H2MV. In this case, the most produced monomer was always 3-HB, and the presence or absence of the other monomers depended on the amount of propionic acid and valeric acid in the effluent.

Notably, the bacterial strains that can most efficiently utilize the VFAs present in the substrate have a competitive advantage over the others and consequently may prevail in the total bacterial composition.

In general, PHA-accumulating bacteria in activated sludge are divided into aerobic bacteria, phosphate accumulating organisms, glycogen accumulating organisms (GAOs) and filamentous bacteria (Coats et al., 2016).

Different bacterial strains may use various metabolic pathways to produce specific PHA (Wei and Fang, 2022). For example, Aeromonas, Acinetobacter, Pseudomonas in PAOs, and Azotobacter, Alcaligenes, Pseudomonas, and Spirillum in GAOs have been employed to accumulate PHB (Ciesielski et al., 2011). At the same time Aeromonas and Alcaligenes strains have been employed in pure cultures to produce PHHx and SCL-PHA, respectively.

Xanthobacter and Neomegalonema may utilize distinct metabolic pathways for synthesizing 3-HV from acetate and propionate, or solely from propionate, resulting in variation in the percentage composition of 3-HB and 3-HV monomers (Cruz et al., 2022). Therefore, the most common choice is to select a particular microbial community by supplying specific volatile fatty acids to MMCs to regulate the population metabolism and the quality of PHAs synthesis (Khan et al., 2019; Luzi et al., 2019).

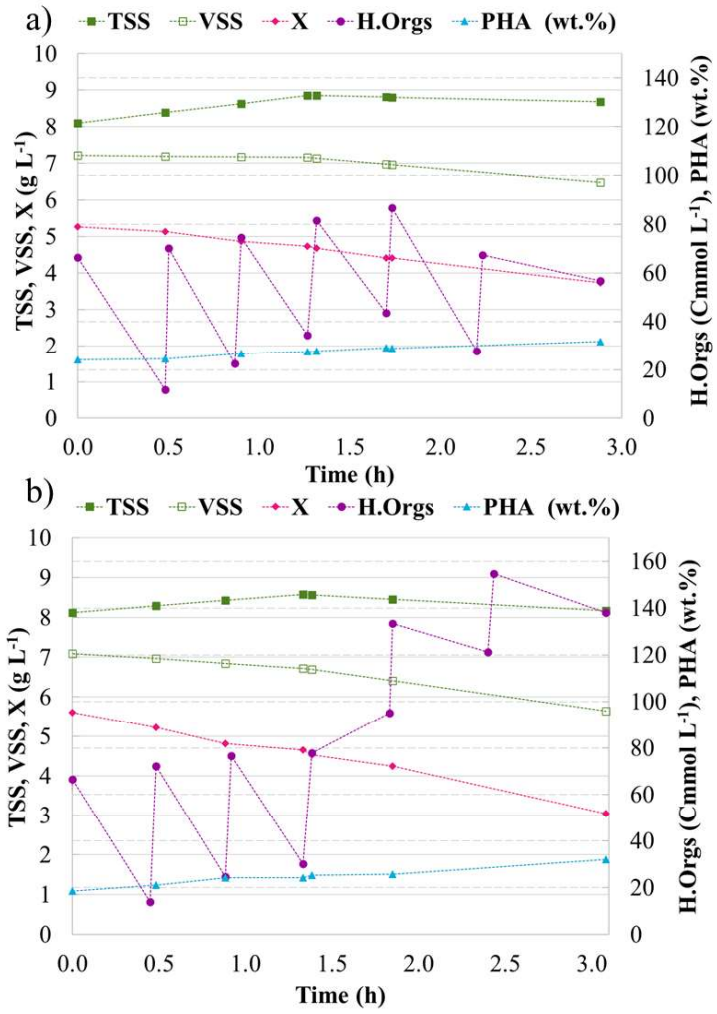


Figure 6.6: Trends of SST, SSV, XA, H.Org. and PHA in agro-industrial wastewater accumulation assays

As previously discussed, the fermented effluent had a very different composition than that of the synthetic substrate. Another relevant factor is that the acid least preferred by the selected MMCs was present in greater quantities. The dosage and eventual accumulation of these acids, therefore, generated stress situations such that PHA production was significantly lower than in the other accumulation assays.

The production of PHA in relation to total solids reached a maximum value of 0.581 KgPHA $KgTSS^{-1}$ in the accumulation assay without added nutrients, an intermediate value with N&P of 0.508 KgPHA $KgTSS^{-1}$, and finally, a minimum value of 0.318 Kg PHA $KgTSS^{-1}$ was observed in the

accumulation assay using real wastewater (Figure 6.7). The different composition of the fed effluent also resulted in variations in the composition of PHA. In fact, whereas in the assays with synthetic substrate, the percentage of 3-HV was about 16%, this was halved in the case of the agro-industrial wastewater (8%). At the same time, the percentage of HHx increased to 6% as its precursor was VFA available in larger quantities.

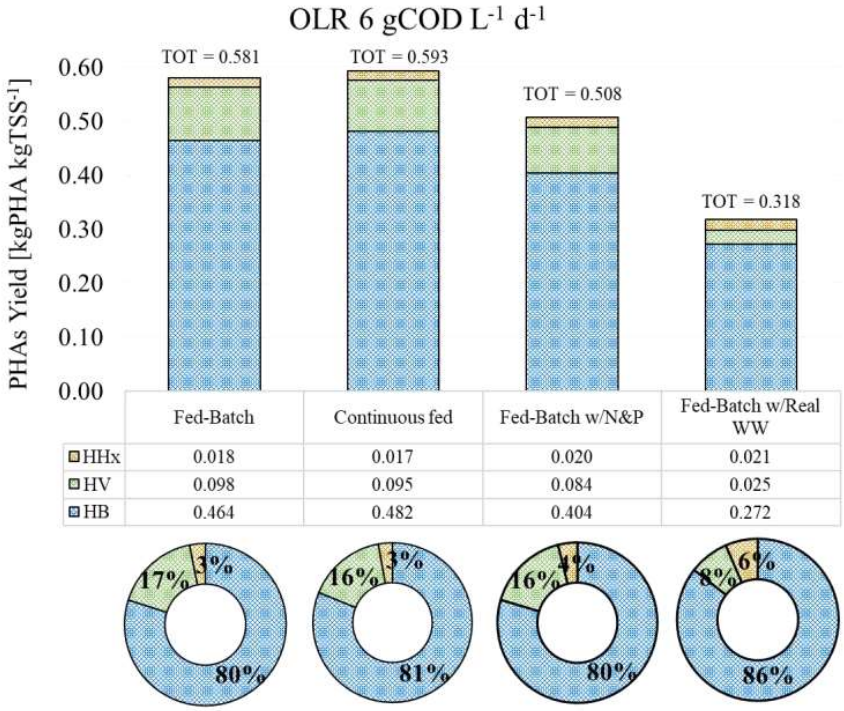


Figure 6.7: Polymer composition with different substrate

The polymer composition was very similar when using synthetic substrate, while it varied slightly with real wastewater. Specifically, the 3-HB fraction increased by about 6 percentage points, while the 3-HV fraction decreased by about 8 percentage points compared with the other two accumulation assays.

6.2.4 Impact of the operational conditions on PHA production

To investigate a potential correlation between M_w and the operating parameter OLR, unlike in Chapter 2, the SRT was always kept constant and consequently, the biomass concentration within the enrichment reactor

increased for each observed period until it reached a stable concentration under steady-state conditions. This prevented any influence from F/M ratio, which was maintained within a limited range to ensure it did not affect the M_w value. Specifically, this was 0.696 ± 0.053 kgCOD $kgSST^{-1}d^{-1}$, 0.829± 0.008 kgCOD $kgSST^{-1}d^{-1}$ and 0.866 ± 0.004 kgCOD $kgSST^{-1}d^{-1}$ in period 1, period 2 and period 3, respectively. The molecular weight value exhibited an unexpected trend in the accumulation assays using the synthetic substrate was used (Figure 6.8; Table 6.4)

The polymers obtained in the accumulation assays had different M_w and PDI due to the varying microbial composition, which changed based on the OLR of the enrichment reactor.

The M_w of the polymers obtained from the Fed-Batch accumulation assays was 462.34 ± 42.812 kDa, 295.91 ± 10.239 kDa, and 396.94 ± 1.244 kDa, respectively. In the case of continuous accumulation assays, it was 484.75 ± 42.812 kDa, 289.09 ± 0.931 kDa, and 408.92 ± 25.297 kDa.

Table 6.4: M_w and PDI of accumulation assays with synthetic substrate

	Period 1		Period 2		Period 3	
	Fed-Batch	Cont. Fed	Fed-Batch	Cont. Fed	Fed-Batch	Cont. Fed
Polymer M_w (kDa)	462,34	484,75	296,91	289,09	396,94	408,92
dev. st.	*42,812*	*18,460*	*10,238*	*0,931*	*1,243*	*25,927*
Polymer PDI	3,01	3,18	2,35	2,15	3,57	3,22
dev. st.	*0,052*	*0,180*	*0,229*	*0,019*	*0,004*	*0,191*

Specifically, it reached its maximum in period 1, decreased to a minimum value in period 2, and finally reached an intermediate value in period 3 (Figure 6.8) in both Fed-Batch and continuous feed strategies. Moreover, results suggest the continuous accumulation assays produced PHA with slightly higher molecular weight than Fed-Batch accumulation assays in period 1 and period 3, indicating that accumulation assay modality may influence this parameter.

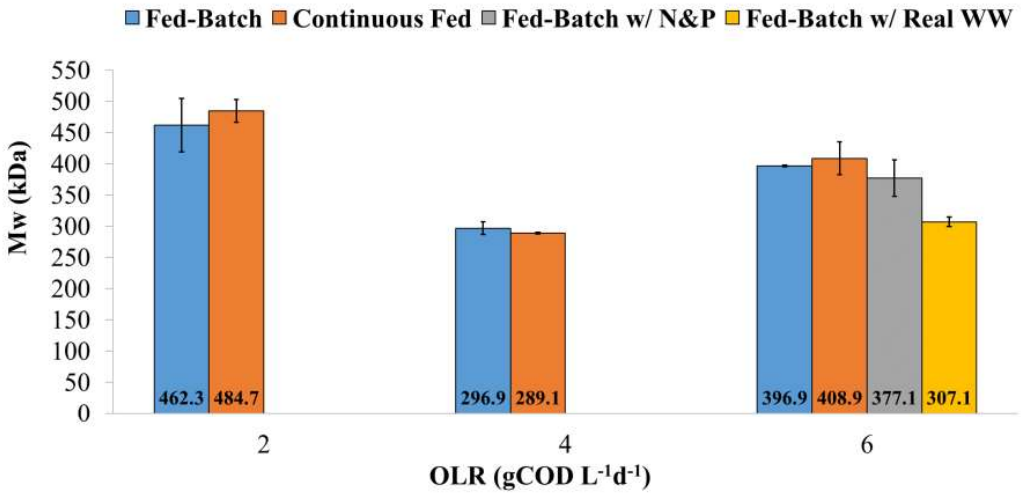


Figure 6.8: M_w of PHAs produced in accumulation assays

M_w was at its maximum in period 1, reached a minimum value in period 2, and finally attained an intermediate value in period 3 in both Fed-Batch and continuous accumulation assays. Furthermore, based on the results obtained, the continuous accumulation yielded PHA with slightly higher molecular weight than the Fed-Batch accumulation assays in period 1 and period 3.
Indeed, variations in the OLR of the enrichment reactor result in changes in the composition of the bacterial community, which in turn affects the characteristics of the polymer, including M_w, as already observed in the experimentation in Chapter 3. Therefore, this trend could be explained by knowledge of the prevailing microbial communities for each period observed.
M_w and PDI values of the accumulation assays with different substrates are shown in Table 6.5. In this case M_w was lower in the presence of nitrogen and phosphorus and with agro-industrial wastewater, probably for the same reasons discussed above.

Table 6.5: Mw and PDI of accumulation assays with different substrate

	Period 3		
	Fed-Batch VFA	Fed-Batch w/N&P	Fed-Batch w/Real WW
Polymer M_w (kDa)	396.94	377.07	307.10
dev. st.	1.243	29.189	7.737
Polymer PDI	3.57	2.85	2.26
dev. st.	0.004	0.005	0.063

The M_w obtained in all accumulation assays of this experimentation varied between about 290 to 485 kDa. These values fall within the same range as those reported in previous studies for commercial PHA produced from pure cultures and for PHA produced from MMCs using waste-based raw materials (200-660 kDa and 220-650 kDa, respectively) (Matos et al., 2021). Additionally, a potential correlation was observed between PHA productivity of accumulation assays and M_w (Figure 6.9).

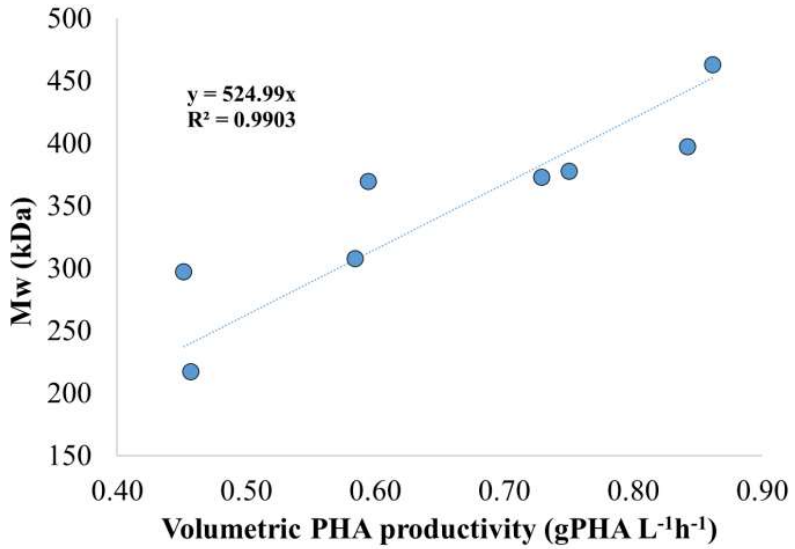


Figure 6.9: Correlation between volumetric productivity and molecular weight of polymers produced

In particular, it appears that molecular weight increases as volumetric productivity increases. To the best of the author's knowledge, no study in the literature has observed such a correlation; thus, further research is needed to explore a wider range of PHA productivity values. This aspect has not been well clarified and will be the subject of further studies.

6.3 Conclusions

A laboratory-scale SBR reactor was utilized for the enrichment step of MMCs with PHA-accumulating organisms. The maximum PHA content for both types of accumulation assays using synthetic substrate was reached in period 1. Specifically, it was 65.57 ± 4.356 wt% for Fed-Batch assays and 59.73 ± 0.1 wt% for continuous accumulation assays. Similar values were obtained in Period 3: 58.08 ± 0.826 for Fed-Batch assays and 59.33 ± 0.474

wt% for continuous accumulation assays, respectively. A similar observation was made regarding productivity, which was highest for Fed-Batch assays in period 1 (0.86 ± 0.037 g-PHA L-1 h-1), while continuous assays for showed the maximum value in period 3 (0.73 ± 0.054 g-PHA L-1 h-1). Maximum PHA content and volumetric productivity of PHA decreased when different substrates were used at the same OLR (6gCOD $L^{-1}d^{-1}$). For the synthetic substrate with nitrogen and phosphorus, this decrease occurred because some of the carbon supplied was used for bacterial growth instead of PHA storage. In the case of fermented wastewater, this limitation was probably due to the different composition of the wastewater, which contained different VFAs than those used for MMC selection. Consequently, the bacterial populations probably experienced metabolic stress condition, limiting PHA production. The polymers produced exhibited consistent characteristics on the composition throughout the experiment. Additionally, the presence of N and P likely limited the accumulation of PHA in this case. These results suggest that the different feeding mode, Fed-Batch or continuous, does not lead to significant variations in polymer composition and M_w when considering standard deviation.

However, the latter varied as a function of OLR, reaching a maximum value in the first period of about 462 KDa and 484 KDa for Fed-Batch and continuous accumulation assay, respectively. A variation on polymer composition and molecular weight was observed when fermented wastewater with a different composition was used, in agreement with other studies in the literature (Cruz et al., 2022; Sabapathy et al., 2020). While the addition of N and P did not seem to result in significant changes, the percentage of 3-HV was halved using fermented wastewater (about 8%) compared to the value observed with synthetic substrate (about 17%). Meanwhile, the % HHx increased from 3% with synthetic substrate increased to 6% with fermented wastewater. Nevertheless, the molecular weight reached medium to high values, enabling potential industrial reuse. Further investigations are required to evaluate other polymer characteristics, such as crystallinity and stiffness. In summary, it is desirable to conduct

further studies incorporating a wider range of variation in terms of OLR, along with analyses of microbial community composition.

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Chapter 7: Implementation of PHA production in the mainstream of an industrial WWTP: challenges and perspectives

Integration of PHA production within existing WWTP infrastructure was a concept already introduced in previous literature (Anterrieu et al., 2014). These authors exploited the treatment of a sugar factory wastewater to produce biomass enriched in PHA accumulating organisms while still meeting the effluent water quality goal. More recently, this concept was applied to a WWTP treating municipal wastewater (Morgan-Sagastume et al., 2015). The authors successfully obtained activated sludge with PHA accumulation potential, while obtaining average removals of 70% COD. An innovative process named SCEPPHAR (ShortCut Enhanced Phosphorous and PHA Recovery) was proposed within a EUH2020 project (Smart-Plant), implementing a novel plant in which simultaneous recovery of PHA and phosphorous was obtained (Conca et al., 2020). These schemes involve a noticeable modification of the original plant layout, mainly attributable to the production of VFA. When dealing with certain types of industrial wastewaters, the aerobic feast-famine strategy on the readily biodegradable COD is sufficient to select a PHA-storing biomass, thus avoiding a VFA production step (Morgan-Sagastume et al., 2015). If on the one hand this constituted a definite advantage, on the other, industrial wastewaters are characterized by seasonal, weekly or daily variations in their composition. Certainly, this could have a remarkable impact on effluent water quality and PHA productivity (Larriba et al., 2020). Therefore, technologies able to provide stable performances even under sudden variability of the wastewater quality is essential to ensure high reliability to the entire process. Moreover, it should be bearing in mind that industrial facilities often require treatment technologies with low footprint. In this scenario, MBR is a compact technology that was proven to be effective in obtaining high PHA-production potential, not entailing the carbon removal efficiency. Indeed, it enabled to meet effluent quality standards within a wide range of OLRs, allowing to overcome operational challenges highlighted by other systems.

Although both the SBR and AGS systems showed a good potential for implementation in a mainstream process for PHA production, their stability was limited within a small range of OLR. It is worth to be reminded that instability in such systems occurred in terms of worsening of settling properties due to the occurrence of filamentous bulking (SBR) and granules breakage (AGS). From a practical perspective, this could involve not only the overcome of the effluent discharge limits, but also the washout of the PHA-accumulating biomass. This would imply the occurrence of transitional periods during which the PHA potential would significantly decrease. Considering the noticeable load variations typical of industrial wastewaters, nor the conventional activated sludge neither the granular sludge systems ensure, so far, a sufficient robustness and resilience to be considered as suitable technologies for mainstream implementation within an industrial WWTP. Form this point of view, membrane retention in MBR systems would ensure a greater guarantee towards the washout of PHA-accumulating organisms' occurrence. Also, the higher biomass retention capacity, just because it does not affect the solid-liquid separation phase, allows better withstand the effect of high OLRs, thereby enabling a higher process resilience and shorter response time to load variations. In addition, it should be bearing in mind that MBR provides the potential possibility to recover the treated wastewater for reuse purposes (Di Trapani et al., 2019). This is of a matter importance, with a view to implement environmentally friendly technologies that allow maximizing resource recovery from wastewater treatment, thus coping with circular economy requirements. Therefore, MBR could be considered a real and practical opportunity to integrate PHA production in the mainstream of an industrial WWTP.

Nonetheless, further studies are still needed to clarify several aspects. First, the eventual variation of the extracted PHA quality should be analysed and put in relationship with the variability of the wastewater used as feedstock or other operating parameters. In addition, economic assessment including the potential recovery of other resources (e.g., wastewater for reuse, EPS, etc) should be encouraged.

7.1 References

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Chapter 8: Conclusions

Food waste industries produce many wastes during primary production and processing. The citrus industry also plays an important role in the agro-industrial sector, especially in Mediterranean countries such as Italy and Spain. The citrus industry produces large amounts of CPWW with high polluting potential, which poses severe environmental and economic constraints for the citrus processing industry. Sustainable residue management, which entails technological innovation based on environmental and economic issues, sustainability, and economic resilience, has the potential to significantly increase the competitiveness of the citrus business.

This thesis evaluated the feasibility of integrating biopolymer production into the mainstream of a citrus industrial wastewater treatment plant using three types of biological reactors: conventional activated sludge (SBR), activated sludge with ultrafiltration membrane (MBR), and aerobic granular sludge (AGS). Specifically, the performance of the three systems was compared from both water treatment and biopolymer production perspectives using the conventional full aerobic feast-famine feeding strategy for the enrichment of MMC. The experiments were carried out using citrus wastewaters from a citrus wastewater treatment plant (potential of 12,500 PE) located in Palermo, Italy, characterized by both qualitative and quantitative seasonal variations. Three different OLR values of 1, 2, and 3 kgCOD $m^{-3}d^{-1}$ were used to reproduce summer, autumn/spring, and winter conditions, respectively.

The results obtained with the SBR system demonstrated the potential feasibility of using citrus wastewater as a low-cost substrate for the synthesis of biopolymers, although the variability in the quality of this wastewater led to different production yields of the biopolymers with different mechanical characteristics. Maximum production yields of biopolymers were comparable with those obtained in many other studies, although productivity was strongly affected by the OLR. Specifically, it was observed that the optimal OLR in terms of both COD removal performance (98%) and biopolymer production (0.38 gPHA $gTSS^{-1}$) was equal to 2 kgCOD $m^{-3}d^{-1}$. At higher OLR, a gradual decrease was observed in terms of both

purification performance and biopolymers production, although their quality increased as indicated by the lower degree of crystallinity. Nevertheless, it was demonstrated that the microbial diversity of real sludge provides both enough degradation potential and PHA accumulating strains to support integrated wastewater treatment and biopolymers production. Monitoring the MMC by using high-throughput DNA sequencing allowed the identification of PHA accumulating taxa that were enriched during the treatment. In order to improve the selection of MMCs and PHA productivity, advanced bio-based technologies (membrane bioreactors, granular aerobic sludge) were tested, as these systems operate with higher TSS concentrations.

The use of an MBR reactor for water treatment and simultaneous biomass enrichment not only increased biomass productivity but also limited membrane fouling. Specifically, it was deduced that:

- the use of F/M values in the range of 0.40-0.50 kg COD kg TSS^{-1} d^{-1} promotes the conversion of exogenous carbon into intracellular compounds, maximizing PHA storage and minimizing EPS production;
- lower EPS production significantly reduced the tendency for membrane fouling. Moreover, this contributed to reducing irreversible cake deposition;
- lower EPS content led to increased fouling removal with ordinary backwashings and consequently extraordinary cleaning operations were less frequent.

The effluent of the MBR system complied with the requirements provided by the main national (Italy) and European regulations (DM 185/03 and EU 2020/741), thus indicating the suitability of its use for different reuse purposes (e.g., irrigation, civil and industrial uses, etc.). Additionally, the MBR appeared to be a more technically feasible alternative to the conventional SBR for the enrichment stage of the PHA production process. Maximum intracellular storage of 45% and a production yield of 0.63 gPHA $L^{-1}h^{-1}$ were reached when the MBR was operated under a F/M close to 0.50 kgCOD $kgTSS^{-1}d^{-1}$, which was approximately 35% higher than those achieved in the SBR. Besides, the F/M was observed to play an important role in the enrichment stage. Indeed, at high values of F/M (> 0.50 kgCOD

$kgTSS^{-1}d^{-1}$) a noticeable loss of PHA-accumulating capacity of the MMC was noted, although this was minimized in the MBR due to its complete cell-retention capacity that avoided the washout of dispersed and filamentous bacteria capable of storing PHA. Finally, if on the one hand the maximization of PHA productivity in the conventional SBR caused the occurrence of process dysfunctions (e.g., filamentous bulking), on the other hand, in the MBR this maximization promoted the mitigation of these dysfunctions through a reduction of membrane fouling behaviour. Therefore, given the potential of the MBR process, it should be advisable to move on to full-scale application to validate its performance and assess its economic feasibility.

The AGS System has not proved particularly suitable for PHA recovery only; however, this system could be convenient for simultaneous PHA and EPS recovery. In effect, the AGS system allowed for a higher accumulation capacity with respect to conventional activated sludge in flocculent form. The maximum biopolymers accumulation capacity was close to 0.60 $mgPHA\text{-}EPS\ gVSS^{-1}$ in the AGS operating at an OLR of 3 $kgCODm^{-3}d^{-1}$, whereas, in the SBR, it was about half (0.35 $mgPHA\text{-}EPS\ gVSS^{-1}$). Biopolymers extracted from the AGS were mainly composed by EPSs (>70%), with the percentage increasing up to 95% at the maximum OLR applied in the enrichment reactor. Thus, organic carbon was mainly channeled toward metabolic pathways for extracellular storage, resulting in a significant prevalence of EPS in the extracted biopolymers. In contrast, SBR enabled a higher PHA production (50% of the biopolymers). The presence of metabolic stressors (e.g., hydraulic selection pressure, shear forces) as key factors for promoting aerobic granulation was supposed to be the main force for organic carbon channeling toward EPSs rather than PHAs. In contrast, flocculent sludge was more favorable for the selection of the PHA-storing population, and the OLR applied in the enrichment reactor was found to be a key operating factor in driving the process toward PHA recovery.

In conclusion, this thesis demonstrated the feasibility of producing PHA and water eligible for reuse purposes using AS, MBR, and AGS systems, in which wastewater treatment and the PHA-enrichment stage were coupled in a single unit to comply with environmental regulations for effluent quality and apply circularity concepts to waste streams. Flocculent sludge-based systems (AS, MBR) were found to have higher PHA productivity, whereas

in the AGS, the organic carbon was mainly driven towards EPS pathway accumulations. Integration of PHA production in the mainstream of an industrial WWTP showed limited results when using the SBR and AGS, since at certain OLR there was a simultaneous drop in purification performance and PHA accumulation potential was observed. On the other hand, the results demonstrated the full potential of membrane bioreactor technology, which proved to exhibit greater robustness both in terms of PHA productivity and COD removal.

Future studies are necessary to investigate the effect of operational conditions on the composition of the mixed microbial culture and to optimize PHA productivity by AGS and MBR, focusing on the role of the main operating parameters affecting the polymer production process.

Published articles used in this thesis

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

Corsino, S.F.[a], Di Trapani, D.[a], Traina, F.[a], Cruciata, I.[b], Scirè Calabrisotto, L.[b], Lopresti, F.[a], La Carrubba, V.[a], Quatrini, P.[b], Torregrossa, M.[a], Viviani, G.[a]

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➢ **Membrane Fouling Mitigation in MBR via the Feast-Famine Strategy to Enhance PHA Production by Activated Sludge**

Corsino, S.F.[a], Di Bella, G.[b], Traina, F.[a], Argiz, L.[a], Val del Rio, A.[c], Corral, A. M.[c], Torregrossa, M.[a], Viviani, G.[a]

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Membranes 2022, 12(7), 703.
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Author Contributions: Conceptualization, methodology, software, formal analysis, data curation, writing and original draft preparation.

➢ **Biopolymer Recovery from Aerobic Granular Sludge and Conventional Flocculent Sludge in Treating Industrial Wastewater: Preliminary Analysis of Different Carbon Routes for Organic Carbon Utilization**

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Water 2023, 15, 47. https://doi.org/10.3390/w15010047
Author Contributions: Conceptualization, methodology, software, formal analysis, data curation, writing and original draft preparation.

➢ **Combined recovery of polyhydroxyalkanoates and reclaimed water in the mainstream of a WWTP for agro-food industrial wastewater valorisation by membrane bioreactor technology.**

Traina, F. [a]; Corsino, S.F. [a]; Capodici, M. [a], Licitra, E. [b], Di Bella, G. [b], Torregrossa, M. [a], Viviani, G [a]

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Journal of Environmental Management 351 (2024) 119836. https://doi.org/10.1016/j.jenvman.2023.119836
Author Contributions: Formal analysis, Methodology, Data Curation, Writing - Original Draft

➢ **Comparison PHA and EPS production from industrial wastewater by conventional activated sludge, membrane bioreactor and aerobic granular sludge technologies: A comprehensive comparison.**

Traina, F. [a]; Capodici, M. [a]; Torregrossa, M. [a]; Viviani, G. [a]; Corsino, S.F. [a]

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Chemosphere 355 (2024) 141768. https://doi.org/10.1016/j.chemosphere.2024.141768.
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