PHA and EPS production from industrial wastewater by conventional activated sludge, membrane bioreactor and aerobic granular sludge technologies: a comprehensive comparison

Francesco Traina[1], Marco Capodici[1], Michele Torregrossa[1], Gaspare Viviani[1], Santo Fabio Corsino[1*]

Affiliation 1: Department of Engineering, University of Palermo, Viale delle Scienze, Ed. 8, 90128, Palermo, Italy

*Corresponding author: tel: +39091238xxx; fax: +39 09123861929

E-mail address: santofabio.corsino@unipa.it (Santo Fabio Corsino)

Abstract

The present study has focused on the mainstream integration of polyhydroxyalkanoate (PHA) production with industrial wastewater treatment by exploiting three different technologies all operating in sequencing batch reactors (SBR): conventional activated sludge (AS-SBR), membrane bioreactor (AS-MBR) and aerobic granular sludge (AGS). A full aerobic feast/famine strategy was adopted to obtain enrichment of biomass with PHA-storing bacteria. All the systems were operated at different organic loading (OLR) rate equal to 1-2-3 kgCOD/m^3·d in three respective experimental periods. The AS-MBR showed the better and stable carbon removal performance, whereas the effluent quality of the AS-SBR and AGS deteriorated at high OLR. Biomass enrichment with PHA-storing bacteria was successfully obtained in all the systems. The AS-MBR improved the PHA productivity with increasing OLR (max 35% w/w), whereas the AS-SBR reduced the PHA content (max 20% w/w) above an OLR threshold of 2 kgCOD/m^3·d. In contrast, in the 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 AS-SBR (max yield 51%) and MBR (max yield 61%), whereas additional stressors in 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's structure. The results of the present study 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 AS-MBR resulted 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: activated sludge; aerobic granular sludge; industrial wastewater; membrane bioreactor; mixed microbial culture; polyhydroxyalkanoate.

1. Introduction

In recent years, the current paradigm according to which wastewater treatment plants (WWTP) must solely comply the discharge limits imposed by environmental regulations is transposing to the biorefinery concept. Indeed, organic and inorganic matters present in the wastewater can be recovered and value-added products could be produced, thereby ensuring an innovative approach that guarantees the environmental sustainability of water purification processes (Shah, 2023). 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-linear 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 alginates-like polymers, or in the form of intracellular granules such as polyhydroxyalkanoates (PHA) (Burniol-Figols et al., 2020). These polymers play a crucial role in bacterial metabolism as they serve as a carbon and energy source for bacteria during periods of substrate scarcity. The microbial synthesis of these products occurs under stressful conditions induced by a strong surplus of carbon and a nitrogen deficiency (Rojas-Zamora et al., 2023). The most used system for PHA production from wastewater is mixed microbial culture (MMC) because it does not require sterile conditions as in the case of pure microbial cultures. It consists of three phases: 1) acidogenic fermentation, in which complex substrate 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 main challenge of this PHA production system is the selection of an MMC with high PHA accumulation potential. The strategy typically employed for this purpose 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 its direct utilization for synthesis processes (Valentino et al., 2017).

In recent years, researchers made significant efforts to develop new strategies to optimize the MMC enrichment in order to increase PHA production rates. Among these strategies, nitrogen limitation during the feast phase (double growth limitation) was proven to prevent the growth of non-PHA-accumulating bacteria during the famine phase, allowing for more efficient MMC selection (Argiz et al., 2022). Similarly, the introduction of a settling phase following the feast phase promoted the washout of non-accumulating bacteria (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 loads), which are required for a successful enrichment of the MMC (Corsino et al., 2022). Furthermore, the low biomass retention capacity of this system reduced the achievable PHA productivity (Burniol-Figols et al., 2020).

Another issue in MMC selection is that 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. 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 on the system's footprint and complexity.

Some recent studies in the literature explored the use of other biotechnologies to produce PHA. Among these, aerobic granular sludge (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). Additionally, some preliminary experiences reported promising results using membrane bioreactors (MBR) technology (Burniol-Figols et al., 2020; Corsino et al., 2021). 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, especially when dealing with industrial wastewater. Within this context, wastewater generated from citrus processing is an eligible organic feedstock in the production of PHA because of the high carbon/nitrogen ratio. Moreover, because of the high availability of soluble organic matter, the preliminary fermentation step commonly adopted in the PHA production process could be avoided, leading to a significant simplification of the plant layout (Morgan-Sagastume et al., 2015). For this reason, this study aimed to compare the performance of three biological systems: conventional activated sludge, activated sludge with ultrafiltration membrane (AS-MBR), and aerobic granular sludge, for PHA production. Specifically, their integration into the mainstream of an industrial wastewater treatment plant (WWTP) was assessed to simulate the wastewater treatment process and the MMC enrichment phase, simultaneously. An in-depth analysis was conducted on concurrent PHA-accumulation processes related to metabolic pathways of EPS production.

2. Materials and methods

2.1 Experimental setup

Three laboratory-scale SBR plants were operated in parallel to evaluate the PHA accumulation in AS, AS-MBR and AGS systems treating wastewater from a citrus processing industry.
A schematic layout of the plant is shown in Figure 1.

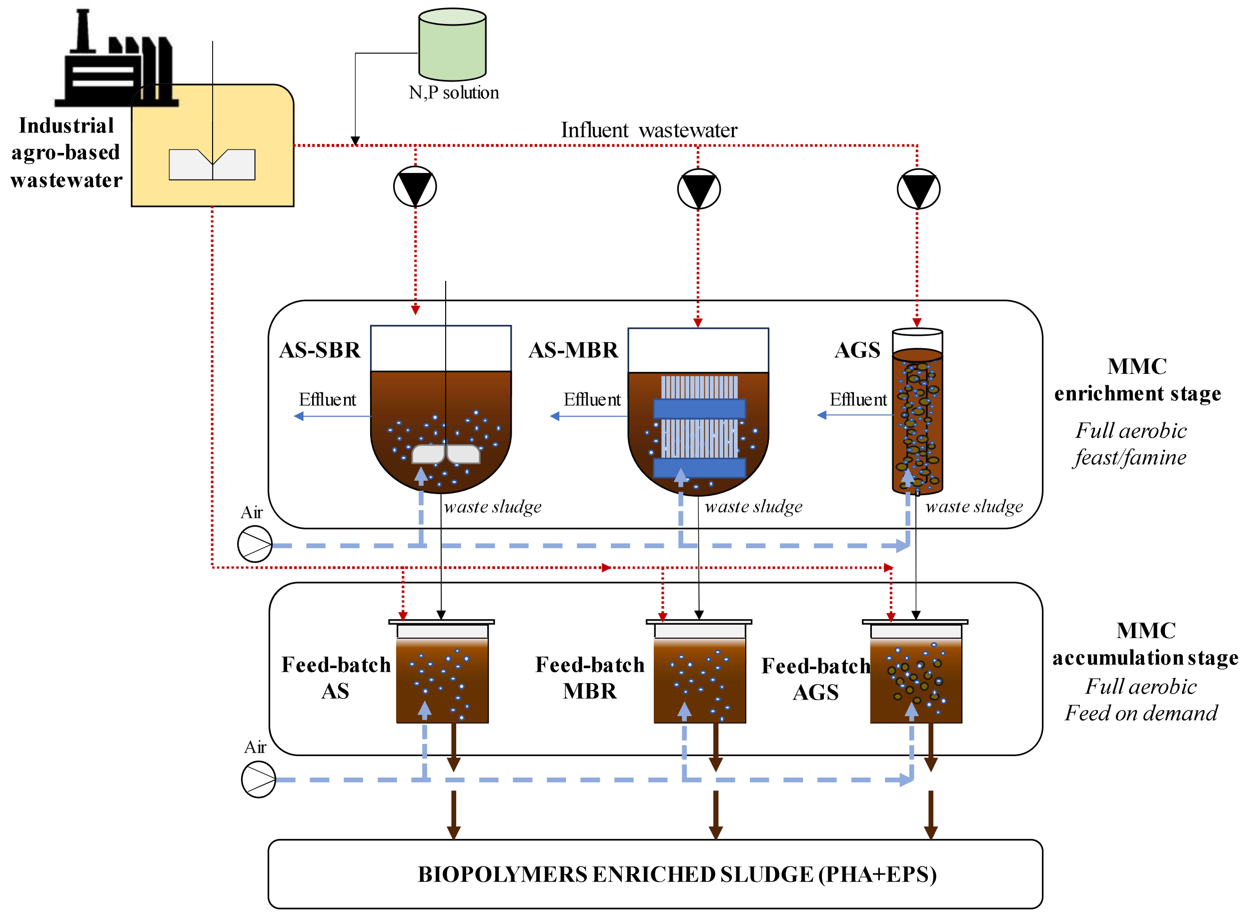

Figure 1: Layout of the plant for the comparison of the AS-SBR, AS-MBR and AGS enrichment systems

Each of the systems was configured according to a "two stage" process that consisted of an enrichment reactor and a dedicated accumulation reactor, both operating in batch mode. The fermentation step was not included in the process as high concentrations of readily biodegradable organic carbon was already present in the raw wastewater (Morgan-Sagastume et al., 2015).

The AS-SBR was constituted by an acrylic tank with a working volume of 20 L. The AS-MBR consisted of a fiberglass tank (working volume 40 L) in which an ultrafiltration membrane (0.03 µm of porosity, 1.4 m^2 of surface, PURON® triple bundle Demo) was installed in submerged configuration. The AGS was a column-type acrylic reactor having an operating volume of 4 L.

All the enrichment reactors operated under the feast/famine regime in full aerobic conditions. The operating cycle was set to 12 h consisting of 30 minutes of feeding under static not-aerated conditions, common to all the reactors, followed by a period of aeration, solid/liquid separation and effluent discharge that was different based on the specificity of the system. In more detail, the aeration in the AS-SBR lasted 540 minutes and it was followed by 60 minutes of settling and 30 minutes of effluent discharge. In the AS-MBR, the aerobic reaction was set to 640 minutes and the last 90 minutes of the cycle were for permeate extraction and membrane relaxation. Finally, the AGS operated with 690 minutes of aeration, 5 minutes of settling and 15 minutes for effluent discharge and idle. The effluent discharge from the AS-SBR and the AGS reactors was performed with dedicated peristaltic pumps, whereas the permeate extraction from the AS-MBR was carried out by two peristaltic pumps that alternated 5 minutes of permeate extraction and 1 minute of backwashing to prevent membrane fouling. Aeration was supplied by three different air blowers through porous stone diffusers located at the bottom of the reactors. A programmable logic controller handled the phases' alternation. The temperature was not controlled, and reactors operated at room temperature. All the reactors were equipped with dissolved oxygen (DO) and pH sensors for online monitoring.

The waste sludge from each of the enrichment reactor was accumulated within a dedicated reactor operating under a fed-batch mode to perform PHA accumulation assays. This consisted in a 1.5 L jacketed glass reactor, maintained at temperature of 20 °C by means of a thermostatic bath. The aeration of these reactors was provided by a blower.

2.2 Operational conditions

All the reactors were inoculated with the activated sludge collected from the citrus processing industry and fed with the same wastewater.

The wastewater was collected from a citrus processing industry that processed mandarins, oranges and lemons. The main operations included juices and essential oils extraction as well as peels processing. In this process, the most wastewater volume is generated from fruit and machineries washing, and cooling operations. Such water is characterized by a low carbon content. In contrast, juice and essential oils extraction operations, produce low wastewater volume with significantly higher organic carbon content (Lucia et al., 2022).

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 the same organic loading rate (OLR). In more detail, three different OLRs were tested in respective experimental periods: 1 kgCOD/m^3·d in Period 1, 2 kgCOD/m^3·d in Period 2 and 3 kgCOD/m^3·d 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 AS-SBR treated 5, 10, 15 L/d, the AS-MBR 10, 20, 30 L/d and the AGS 1,2, 3 L/d in Period 1, Period 2 and Period 3, respectively. Accordingly, volumetric exchange ratio ranged between 0.125-0.375 during the experiment. Process conditions were changed once steady performances were observed in each system, but not before a time higher to three times the sludge retention time (SRT) lasted. Overall, the AS-SBR, the AS-MBR and the 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.

Specifically, the achievement of steady state was assumed once the duration of the feast/famine cycle was below 0.20 and this was maintained at least three times the sludge retention time (Conca et al., 2020). The enrichment reactors operated with different biomass concentration, according to the specificity of the technology. In table 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. Specifically, the SRT was determined by mass balance between the amount of biomass (as TSS) into the reactor and those discharged daily (as waste sludge, effluent TSS and samples for analysis).

A detailed summary of the above information is reported in Table 1.

Table 1: Summary of the main operating conditions for the AS-SBR, AS-MBR and AGS systems

		Duration [d]	Flow rate [L/d]	Biomass [gTSS/L]	SRT [d]
		Period 1:			
OLR 1 [kgCOD /m^3]	AS-SBR	78	5	4.56 ± 0.12	14 ± 3
COD 4.5 ± 0.1 [g/L]	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]	AS-SBR	32	10	4.42 ± 0.09	7 ± 2
COD 4.46 ± 0.09 [g/L]	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]	AS-SBR	62	15	4.46 ± 0.09	5 ± 1
COD 4.38 ± 0.16 [g/L]	MBR	43	30	6.3 ± 0.2	9 ± 1
VER 0.375	AGS	41	3	5.06 ± 0.11	9 ± 2

The dissolved oxygen concentration, the chemical oxygen demand, the PHA and EPS content during the enrichment cycle were periodically monitored. Specifically, samples at regular intervals were withdrawn from the enrichment reactors and subjected to EPS and PHA extraction procedures.

The achievement of steady state was assumed once the duration of the feast/famine ratio was below 0.2, which indicated that MMC was successfully enriched in PHA-accumulating organisms (Conca et al., 2020).

At this stage, accumulation assays were performed in each experimental period. These assays were carried out in an accumulation reactor, named fed-batch reactor, one dedicated for each line, in which the excess sludge produced in the respective enrichment reactor (1.5 L) was used to produce PHA. The accumulation fed-batch reactor was aerated continuously, maintaining the DO concentration above 4 mg/L. The raw wastewater was used as feedstock without the addition of nutrients to prevent bacterial growth and promote PHA accumulation. The wastewater was supplied adopting the “feed on demand” strategy according to the literature (Morgan-Sagastume et al., 2014). Each accumulation assay lasted at least 8 hours. At the end of the accumulation assay, the sludge was separated from the supernatant though centrifugation and PHA was extracted from the sludge pellet.

2.3 Characteristics of wastewater

The wastewater collected from the citrus processing industry was characterized by a COD close to 4.5 g/L, on average. Respirometric techniques were used to address the amount of soluble readily and slowly biodegradable organic content in the citrus wastewater (Capodici et al., 2016). The organic content of the citrus wastewater 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. The pH of the raw wastewater was close to 5.1 and it was adjusted to neutral by adding NaOH (0.1 M) before it was fed to the enrichment and accumulation reactors. Total nitrogen and total phosphorous concentrations in raw wastewater were on average 43 mg/L and 13 mg/L, respectively. Hence, the C/N ratio in the raw wastewater was about 100. For this reason, additional nitrogen and phosphorous sources were provided to the enrichment reactors only.

2.4 Analytical procedures

Mixed liquor total and volatile suspended solid (TSS and VSS, respectively), total COD, TN and TP were measured by the standard methods (APHA, 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). All the samples for determination of soluble substrates were filtered through a 0.45 μm filter paper. The DO concentration was monitored online with a probe (model FDO® 915, WTW), equipped with a digital thermometer, whereas the pH was measured by a pH-meter (model SenTix 940-3, WTW). Sudan black stain was performed to assess the presence of PHA within the bacterial cells (Jenkins et al., 2003). Microscopic observations were carried out by a phase contrast optical microscope (Olympus).

2.5 PHA and EPS extraction and characterization

PHA was extracted from wet biomass samples according to the literature 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 VSS (% w/w).

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 VSS (% w/w).

Organic carbon mass balance (as total COD) was carried out in the accumulation reactor to assess the amount of COD converted into intracellular (PHA) and extracellular (EPS) biopolymers. To this aim, the following stoichiometric coefficients were assumed for referring PHA mass as COD: 1.67 gCOD per gram of polyhydroxybutyrate (PHB) and 1.92 gCOD per gram of polyhydroxyvalerate (PHV) (Conca et al., 2020). Referring to EPS, the stoichiometric coefficients for proteins and carbohydrates were 1.40 gCOD/gPN and 1.36 gCOD/gPS, respectively (Corsino et al., 2022).

3. Results and discussion

3.1 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 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.

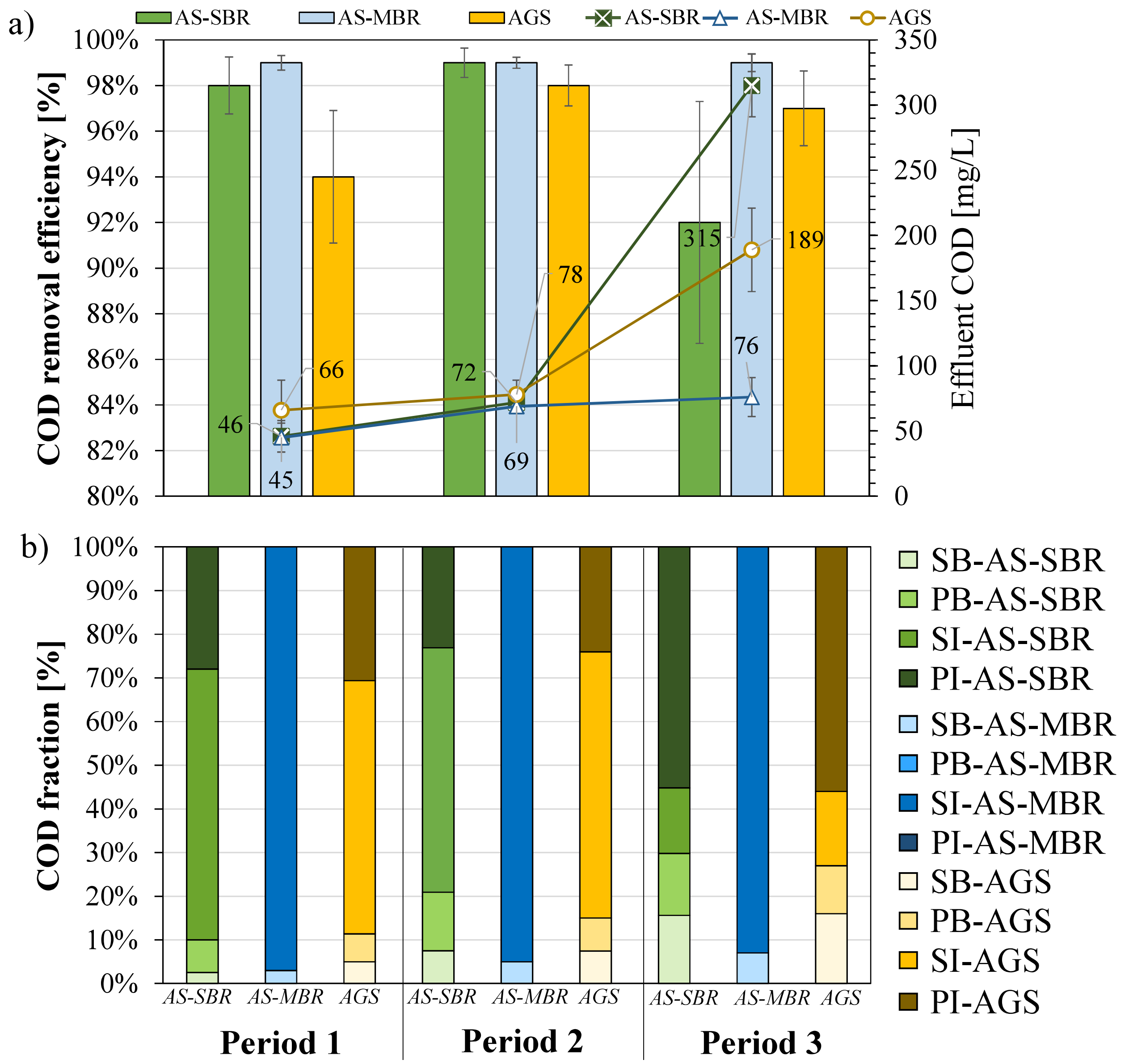

Figure legend: SB: Soluble Biodegradable carbon; PB: Particulate Biodegradable carbon; SI: Soluble Inert carbon; PI; Particulate Inert carbon.

Figure 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, resulting in an effluent concentration between 50-75 mg/L (Fig. 2a). The AS-SBR and the AS-MBR 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, all the reactors showed high COD removal capacity, which resulted close to 99%. The effluent COD concentration was below 75 mg/L in all the reactors. A noticeable decrease in COD removal was noted referring to the AS-SBR in Period 3 when operating at 3 $kgCOD/m^3 \cdot d$. The average COD removal efficiency was 90% approximately, although the effluent concentration rose to 300 mg/L. Similarly, the AGS showed a slight worsening of COD removal compared with the previous periods, although the effluent concentration was below 200 mg/L. In contrast, the AS-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, thereby enabling removal efficiencies of 99% during the entire experiment. As reported in Fig. 2b, when increasing the OLR, the fraction of the soluble COD in the effluent increased in the AS-SBR and AGS, more significantly than the AS-MBR, suggesting a not efficient utilization of the readily biodegradable organic matter by PHA-accumulating organisms. The lower concentration of soluble COD in the AS-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 AS-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 (supplementary data).

Table S1: Average data of the effluent TSS concentration, sludge volume index (SVI) and flocs/granules size for the AS-SBR and AGS

	AS-SBR			AGS		
	Period 1	Period 2	Period 3	Period 1	Period 2	Period 3
Effluent TSS [mg/L]	10±7	12±9	135±27	28±12	51±17	236±44
SVI* [mL/gTSS]	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 AS-SBR and 5 minutes for AGS

Note that data referred to AS-MBR are not reported as effluent TSS was always 0 mg/L and SVI was not measured.

In the AS-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 AS-MBR operated under a lower food to microorganisms' ratio (F/M) 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 AS-SBR and the AGS systems showed a higher process instability when the OLR was increased over 2 kgCOD/m^3·d, 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 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. (Oliveira et al., 2017) found an optimal OLR of 2 kgCOD/m^3·d using fermented cheeseway. Lorini et al. (Lorini et al., 2020) and Crognale et al. (Crognale et al., 2022) achieved a stable selection of MMC at 8 kgCOD/m^3·d although operating with a synthetic mixture. More recently, Isern-Cazorla et al. (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 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 when dealing with industrial wastewaters (Valentino et al., 2017). Hence, AS-SBR and AGS 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, on the other the poor purification performances would limit the implementation of MMC-enrichment in the mainstream of a WWTP. In this context, membrane bioreactors 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.

3.2 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 feast/famine 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 AS-SBR and AS-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 3.

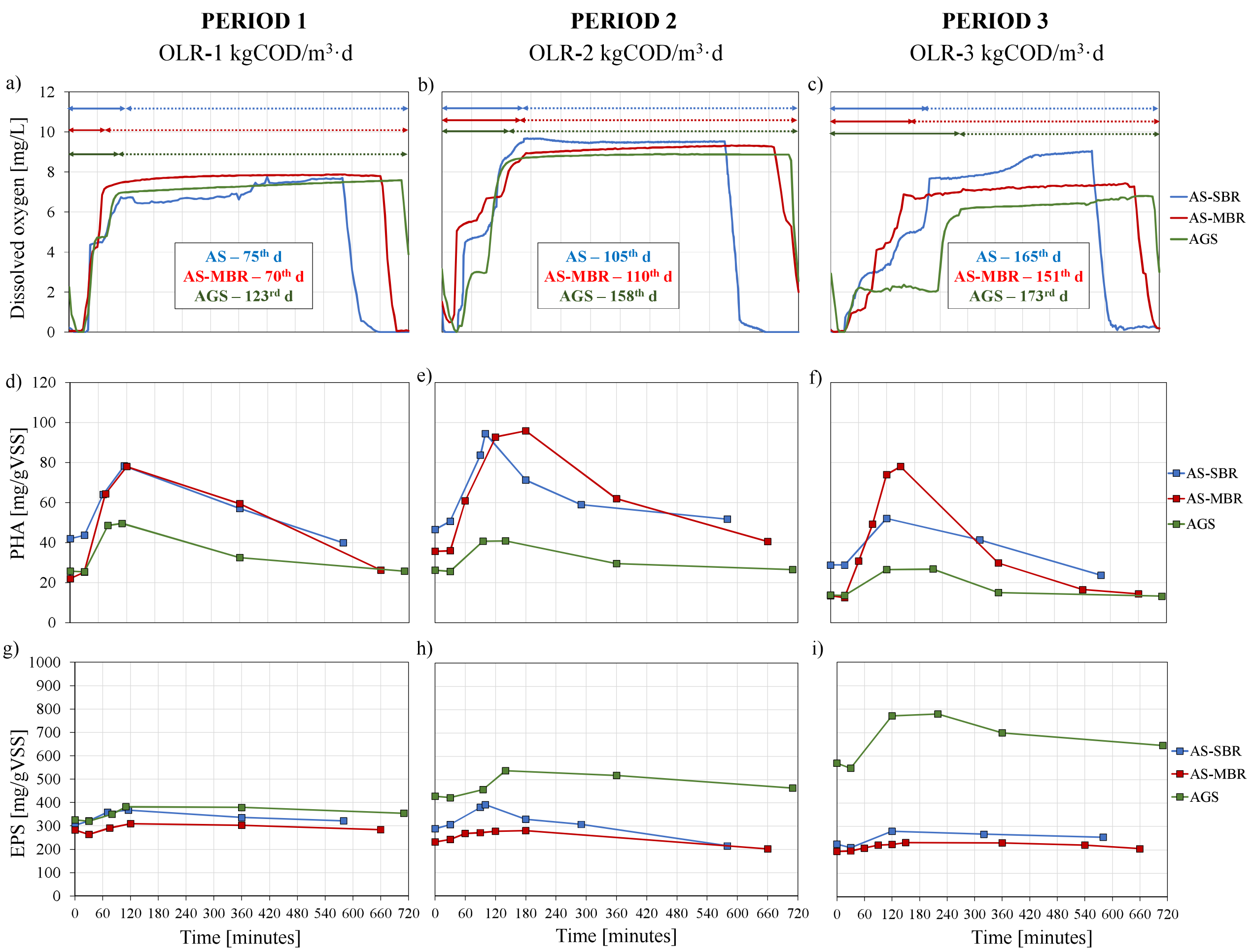

Figure 3: Characterization of a typical enrichment cycle in the AS-SBR, AS-MBR and AGS, at steady state during Period 1 (a,d,g), Period 2 (b,e,h) and Period 3 (c,f,i), showing the profile of DO, feast (⟷) and famine (⇠⇢) (a-c), PHA (d-f) and EPS (g-i). Data within the caption box indicate the day to 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 AS-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. 3d) and EPS (Fig. 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 AS-SBR and AS-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 feast/famine ratio below 0.20. A noticeable improvement of PHA production was observed in the AS-SBR and AS-MBR, in which the maximum PHA content at the end of the feast phase was 94 mg/gVSS and 95 mg/gVSS, 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 AS-SBR and AS-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 feast/famine ratio was still lower than 0.20 in the AS-MBR only, whereas in the AS-SBR and AGS it resulted higher than 0.30. As previously reported, residual organic carbon in soluble form was measured in the effluent of the AS-SBR and 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 AS-MBR slightly decreased compared with the previous period, on the other in the AS-SBR and the AGS it noticeably reduced. A different behaviour was noted referring to EPS. Indeed, the EPS content decreased in the AS-SBR and AS-MBR, 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, 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 was exceeded. Nevertheless, the AS-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 AS-SBR and AGS 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 AS-MBR, allowed to completely deplete the organic substrate during the feast phase and avoided the washout of sludge enriched in PHA storing bacteria.

3.3 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 4 shows the overall biopolymers content, as sum of PHA and EPS, accumulated by the enriched cultures during each experimental period.

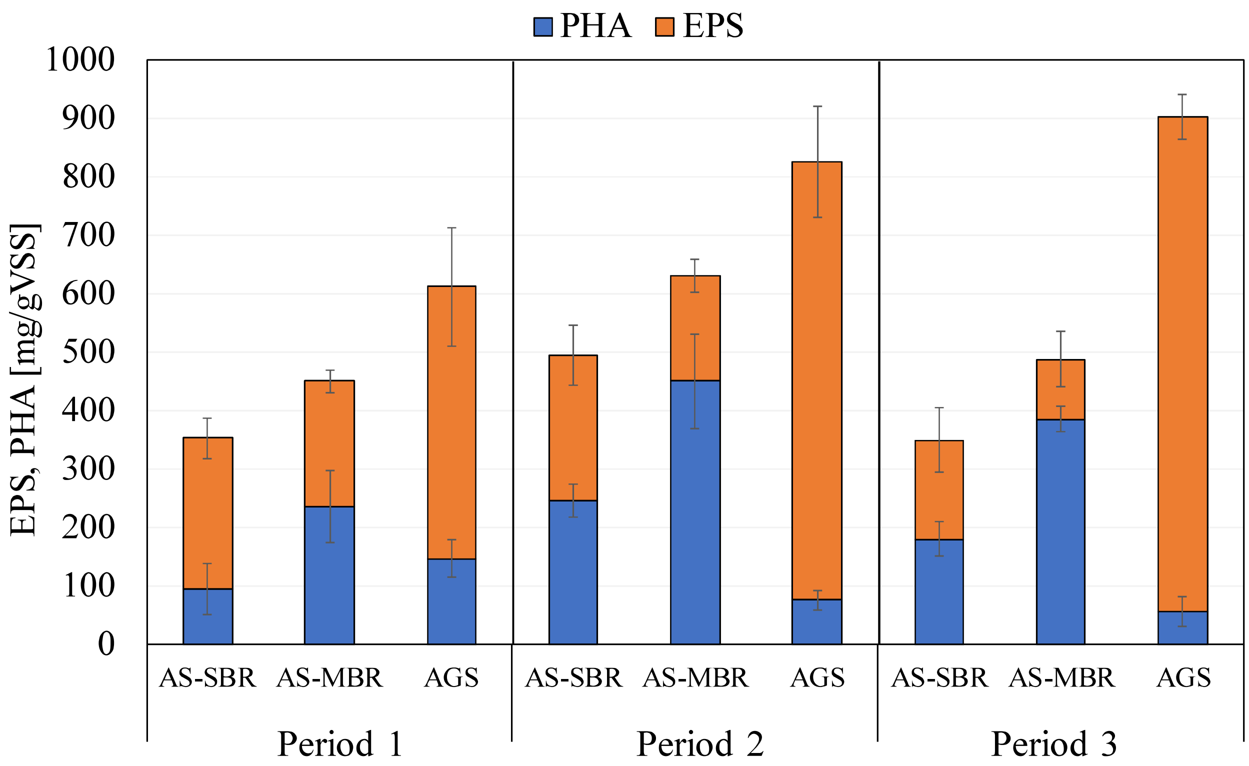

Figure 4: Maximum polymers storage and composition in the three MMC cultures enriched at different OLRs.

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 AS-MBR (45% w/w) and the AS-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 AS-SBR, 63% in the AS-MBR, and 82% in the AGS), in Period 3 the maximum accumulation capacity decreased in the AS-SBR (34.9% w/w) and AS-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 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 AS-SBR and AS-MBR. Indeed, the PHA content showed an overall increasing trend with the OLR, reaching a maximum of 45% w/w in the AS-MBR and 24.5% in the AS-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 AS-SBR and AS-MBR systems exhibited a better capacity of PHA accumulation than the 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 study, 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 AS-SBR and AS-MBR 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.

3.4 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 shows the results obtained in the accumulation assays performed at the end of each experimental period at steady state.

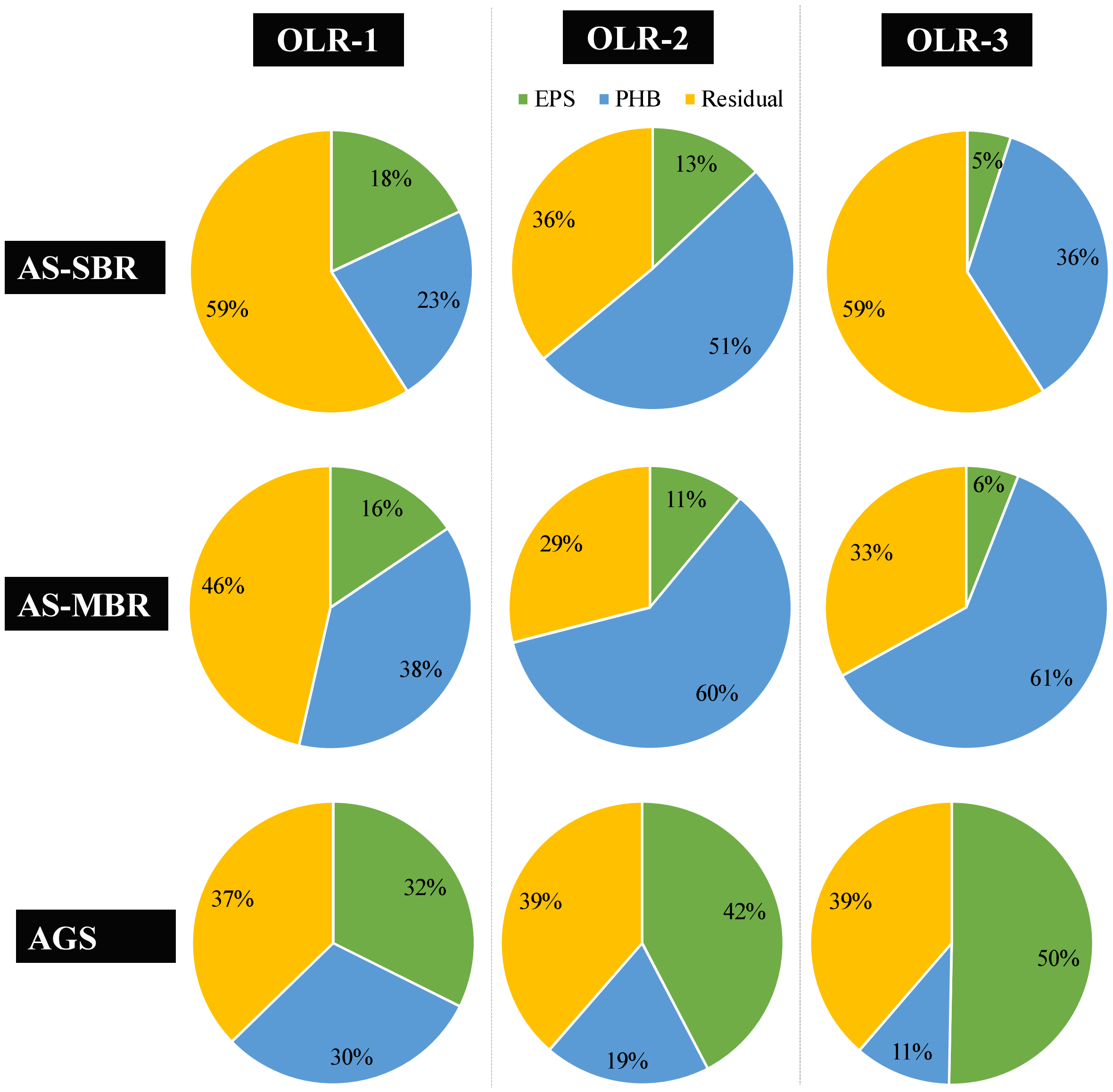

Figure 5: Percentage of COD converted into PHA and EPS during the accumulation assays.

In Period 1, the highest fraction of the COD converted into intracellular storage compounds was found in the AS-MBR (38% w/w), while lower values were obtained in the AGS (30% w/w) and the AS-SBR (23% w/w). In contrast, the AGS showed the highest percentage of COD conversion towards EPS (32% w/w), whereas the AS-MBR (16% w/w) the lowest. When increasing the OLR in the enrichment reactors at OLR of 2 kgCOD/m^3·d, the COD channelled towards PHA significantly rose in the AS-SBR (51% w/w) and the AS-MBR (60% w/w), consistently with the results previously discussed. Indeed, as the PHA was mainly constituted by hydroxybutyrate monomers (PHB), the COD conversion yield to PHA reflected the yields (as gPHA/gVSS) reported in the previous sections. It is worth noticing that at high OLR in the AS-SBR and AS-MBR, 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 AS-SBR and AS-MBR 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 vs 2.62 g/L) (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 study, 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 study, feast/famine selection strategy performed under full aerobic conditions resulted more suitable to enrich the MMC with PHA-accumulating organisms in the AS-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.

3.5 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 volatile fatty acids (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 (Corsino et al., 2022; 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 AS-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 (AS-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 AS-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 analyzed 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. In this context, while the technical feasibility of implementing a process to produce PHA-enriched sludge within a WWTP for the treatment of industrial wastewater is achievable with various technologies, the implementation of the extraction and purification processes is far from straightforward. The processes used for PHA extraction entail high management costs. On one hand, the application of high temperatures, and on the other hand, the use of chemicals, involves substantial expenses, not mentioning those required for the disposal of waste resulting from the extraction process. In light of these aspects, additional efforts are needed to make the production of PHA from wastewater technically and economically sustainable. The establishment of centralized facilities where PHA-enriched sludge from different WWTPs can be deposited, and within which biopolymers can be extracted, could certainly reduce specific costs and make the entire process economically viable.

Conclusions

The present investigation has found that the conventional full aerobic feast-famine feeding strategy for enrichment of MMC can be used to produce biomass with high biopolymers producing potential in AS, MBR and AGS systems, while meeting the water effluent quality standards. 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 pathways accumulations. Integration of PHA production in the mainstream of an industrial WWTP resulted limited when using the AS-SBR and AGS, 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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