Universidade de Aveiro Departamento de Química
2011 – 2012

Ana Francisca Osório de Almeida Coelho e Silva

Bioprodução de PHA a partir de águas residuais de lagares de azeite

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, realizada sob a orientação científica do Doutor Mauro Majone, Professor Associado do Departamento de Química da Universidade de Roma La Sapienza, e da Doutora Luísa Serafim, Professora Auxiliar Convidada do Departamento de Química da Universidade de Aveiro.

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palavras-chave

Polihidroxialcanoatos, Culturas Microbianas Mistas, Acumulação, Reator Descontínuo Sequencial, Regime de "Fome e Fartura", Águas residuais de lagares de azeite, Resíduos agro-industriais

resumo

O objetivo deste trabalho incidiu no desenvolvimento de um processo *multi-stage* económico para a produção de polímeros biodegradáveis, polihidroxialcanoatos (PHA), por culturas microbianas mistas, usando como matéria-prima águas residuais de lagares de azeite.

Neste processo foram utilizadas águas residuais de lagares de azeite pré-fermentadas, nas quais 60% do conteúdo de CQO (Carência Química de Oxigénio) solúvel foi convertido em ácidos gordos voláteis (AGV) que são substratos mais directos para a produção de PHA. Esta corrente rica em AGV foi utilizada como alimento na fase sucessiva do processo, efetuada num reator descontínuo sequencial (SBR) à escala laboratorial. O objetivo desta etapa foi a selecção e enriquecimento da cultura mista microbiana em microrganismos com capacidade de acumulação de PHA elevada e estável, recorrendo ao regime de "fome e fartura". Foram testadas duas cargas orgânicas diferentes no SBR – 2.37 e 4.74 g L^{-1} d^{-1} (referentes a CQO solúvel) – diluindo as águas residuais pré-fermentadas com meio mineral. A resposta de acumulação da cultura selecionada com a carga orgânica mais elevada foi estudada na etapa seguinte, tendo como objetivo o aumento do conteúdo intracelular de polímero na biomassa, através de uma estratégia de alimentação por pulsos sucessivos das águas residuais (as quais possuem baixo conteúdo de azoto) não diluídas. O polímero produzido consistia no poli β-hidroxibutirato-hidroxivalerato [P(HB-HV)] contendo entre 10 e 15% (w/w) de HV. Por fim foi desenvolvido o processo de extração do PHA obtido através de um processo de separação sólido-líquido seguido de digestão química com hipoclorito de sódio.

Este estudo permitiu determinar o potencial do processo proposto para transformar resíduos orgânicos, como águas residuais de lagares de azeite, em produtos de valor acrescentado.

keywords **Polyhydroxyalkanoates, Mixed Microbial Culture, Storage, Sequencing Batch Reactor, "Feast and famine" regime, Olive oil mill effluents, Agro-industrial wastes**

abstract

The aim of this research was the development of a low-cost multi-stage process for the production of biodegradable polymers, polyhydroxyalkanoates (PHAs), by using mixed microbial cultures (MMCs) and olive oil mill effluents (OMEs) as feedstock.

Prefermented OMEs were used, in which about 60% of the soluble chemical oxygen demand (COD) had been transformed into volatile fatty acids (VFAs) that are more direct substrates for PHA storage. This VFA-rich stream was fed to a successive stage performed in a lab scale sequencing batch reactor (SBR), 1 L working volume. The aim of this stage was to select and enrich MMCs in microorganisms with high and stable storage ability through the establishment of "feast and famine" regime. Two different organic loading rates (OLRs) – 2.34 and 4.74 g L^{-1} d^{-1} (referred to the soluble COD) – were assayed in the SBR by diluting the fermented OMEs (soluble COD = 23.7 ± 1.1 g L^{-1}) with mineral medium. The storage response of the microbial culture selected at the higher investigated OLR was analyzed in a following stage, aimed at maximizing the intracellular PHA content in the biomass by successive pulse feeding of no diluted OME (whose nitrogen content is very low). The produced polymer was a poly β-hydroxybutyrate-hydroxyvalerate [P(HB-HV)] with an HV content between 10 and 15 % (w/w). Finally, the attention has been paid at developing the further PHA recovery stage which consisted in a solid-liquid separation followed by chemical digestion using sodium hypochlorite.

In conclusion, this study pinpoints the great potential of the proposed process to convert organic wastes, such as OMEs, into valuable products.

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3H2MB	3-hydroxy-2-methylbutyrate
3H2MV	3-hydroxy-2-methylvalerate
3HHx	3-hydroxyhexanoate
ADF	Aerobic dynamic feeding
AN/AE	Anaerobic aerobic
COD	Chemical oxygen demand
CSTR	Continuous stirred tank reactor
DO	Dissolved oxygen
EBPR	Enhanced biological phosphorous removal
FF	"Feast and Famine"
GAOs	Glycogen accumulating organisms
HB	Hydroxybutyrate
HRT	Hydraulic retention time
HV	Hydroxyvalerate
K_La	Oxygen transference coefficient
MMC	Mixed microbial culture
N	Nitrogen
OLR	Organic loading rate
OME	Olive oil mill effluents
OUR	Oxygen uptake rate
PAOs	Polyphosphate accumulating organisms
PBBR	Packed bed biofilm reactor
PHA	Polyhydroxyalkanoates
P(HB-HV)	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
rbCOD	Readily biodegradable chemical oxygen demand
r_{PHA}	Polyhydroxyalkanoate storage rate
r_S	Substrate consumption rate
SBR	Sequencing batch reactor
SRT	Sludge retention time
SS	Suspended solids
TKN	Total Kjeldhal nitrogen
TOC	Total organic carbon
TSS	Total suspended solids
VFA	Volatile fatty acids
VSS	Volatile suspended solids
WWTP	Wastewater-treatment plant
Y_{GROWTH}	Growth yield
Y_{STO}	Storage yield

Chapter 1 | State of the art

1.1. Background

The disposal of agro-industrial wastes consists in a serious economic and environmental issue (Mahro and Timm 2007). In Europe, food industry produces huge volumes of by-products as well as solid and liquid wastes, in the order of 2.5×10^8 ton $year^{-1}$, being the major fraction managed as wastes affecting adversely the overall sustainability of the food processing industry (AWARENET 2004). In order to prevent the environmental problems caused by the disposal of these types of wastes, the possibility to bioconvert their organic matter gained status, since it is a great opportunity to combine the waste treatment with the production of value added chemicals and energy (Laufenberg *et al.* 2003; Federici *et al.* 2009).

According to the IEA Bioenergy Task 42 "Biorefinery is the sustainable processing of biomass into a spectrum of marketable products (food, feed, materials, and chemicals) and energy (fuels, power, and heat)". In other words, biorefineries use biomass obtained from agriculture, forestry, industry and aquaculture to produce a great variety of bio-based products using chemical, physical and, also, biotechnological methods. When compared with conventional refineries, biorefineries can produce a wider variety of products using a broader range of raw materials. However, they require a larger array of technologies, being most of them in a pre-commercial stage. Another disadvantage is the fact that biomass requests a depolymerization and deoxygenation step for the production of chemicals and biofuels. Lastly, like petroleum, its composition is not homogeneous and it is affected by seasonal changes (Cherubini 2010; Fitzpatrick *et al.* 2010).

In fact, the world has a massive dependence on fossil resources (petroleum, natural gas, coal) since they are considered the main feedstock for the industrial production of chemicals, energy and fuels. These non-renewable resources present alarming rates of depletion in a long-term period, an increasing cost trend over time and associated environmental issues due to emissions of greenhouse gases from fossil fuels and production processes of petrochemicals. As a consequence, fossil resources are not regarded as sustainable and biowastes can be exploited as renewable resources that have a great potential as feedstocks for a high range of industrial processes (Wyk 2001; Kamm *et al.* 2006; Cherubini 2010).

1.2. Bioplastics: a sustainable solution

During the past decades, petroleum-based plastics have grown at a faster rate than any other group of materials. Their versatility and durability enable a wide field of applications in industry, medicine and day-to-day. However, these plastic materials are dependent on limited fossil resources and represent a huge problem in disposal of solid waste because they are recalcitrant towards microbial degradation, being extremely persistent in environment. Regarding the problematic and damaging effects of the plastic wastes on the environment, biopolymers offer an ecological promising solution to synthetic plastics (Ojumu *et al.* 2004; Chanprateep 2010; Reis *et al.* 2011).

According to the European Bioplastics Association, bioplastics are a group of materials that are biobased, biodegradable or both, which means that are based on renewable resources and/or can be degraded under specific conditions, respectively. Among all the proposed and tested polymers for this purpose, polyssacharides, polylactides (PLAs), polyhydroxyalkanoates (PHAs), copolymers and blends of those may be included (Zinn *et al.* 2001; Reddy *et al.* 2003; Chanprateep 2010). Being both biobased and biodegradable and presenting similar properties to those of synthetic plastics, PHAs are presented as the most promising alternative since they can respond to the most concerning disadvantages of conventional plastics (Reis *et al.* 2011).

1.2.1. Polyhydroxyalkanoates (PHAs)

1.2.1.1. Properties, characteristics and fields of application

PHAs are biologically synthesized polyesters of R-hydroxyalkanoic acid (HA) monomers with the chemical structure shown in Figure 1 (Lee 1996). They can be produced from renewable resources and are natural, recyclable, biocompatible and completely biodegradable into water, carbon dioxide and other inorganic non-toxic compounds (Lee 1996; Jendrossek and Handrick 2002; Kuruppalil 2011). Only the bacterial production of PHAs allows a complete stereospecificity (R-configuration) which is necessary for the typical biodegradability and biocompatibility of these polymers (Zinn and Hany 2005).

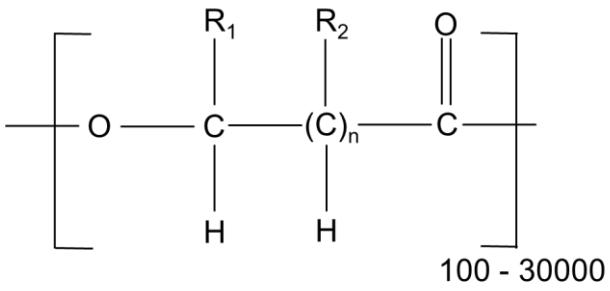


Figure 1: PHA chemical structure, where n is the number of carbon atoms in the linear polyester structure, and R is the hydrocarbon side chain (Valentino *et al.* 2012)

So far, more than 150 diverse types of PHA monomers have been described and there is a huge possible variation of the side chains in what regards length and composition (Steinbüchel and Lütke-Eversloh 2003; Verlinden *et al.* 2007). The most usual monomers and corresponding side chains (R_1 and R_2) are represented in Table 1. PHA monomers can be classified by chain length according to three different classes: short-chain-length PHAs (scl-PHAs) which range from C_3 to C_5, medium-chain-length PHAs (mcl-PHAs) varying between C_6 and C_{14} and long-chain-length PHAs (lcl-PHAs) that go above C_{14} (Zinn and Hany 2005). Depending on the type of incorporated monomers, PHAs can achieve a wide range of thermoplastic and elastomeric properties that are very similar to those of conventional plastics (Lee 1996).

Table 1: The most common HA monomers (adapted from (Loo and Sudesh 2007).

Size of the alkyl group (n)	Hydrocarbon side chain (R_1)	Hydrocarbon side chain (R_2)	Type of monomer
1	Methyl	Hydrogen	3-hydroxybutyrate (3HB)
	Ethyl	Hydrogen	3-hydroxyvalerate (3HV)
	Propyl	Hydrogen	3-hydroxyhexanoate (3HHx)

Poly(3-hydroxybutyrate), P(3HB), the most extensively studied PHA, is an example of scl-PHA. It presents high crystallinity (between 55 and 80%) and it is a rigid, but brittle material. Its extension to break is about 2 – 10% which is considered poor when compared to 400% of that for poly(propylene) (PP). On the other hand, its tensile strength of 40 MPa and Young's modulus of 3.5 GPa is really close to that of PP (38 MPa and 1.3 GPa, respectively) (Lee 1996; Sudesh *et al.* 2000). The melting temperature of this polymer is around 180 ºC and the glass transition temperature is around 4 ºC. However, it starts to be

thermally degraded at 170 ºC which means that during the processing of P(3HB) in melt, its mechanical properties may be deteriorated due to losses in molecular weight that usually ranges from 1×10^4 to 3×10^6 Da (Kunioka and Doi 1990; Reis *et al.* 2011). In fact, the incorporation into the polymer chain of different HA monomers, as 3HV or 3HHx, improves the material properties and decreases polymer crystallinity. When compared to P(3HB) homopolymer, the copolymers have higher flexibility and lower melting and glass temperatures, which means that they can be processed at lower temperatures than P(3HB), avoiding molecular weight losses (Carrasco *et al.* 2005).

One particular example of PHA copolymer is poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (P(HB-HV)). Its mechanical properties are reliant on the molar ratio of 3HV: increasing 3HV content from 0 to 25% mol, the Young's modulus drops (from 3.5 to 0.7 GPa) which means that the copolymer's flexibility rises; on the other hand, these copolymer is more resistant in the sense that the tensile strength decreases (from 40 to 30 GPa) with an increment of the 3HV molar ratio (Loo and Sudesh 2007). The Table 2 compares some properties between PHA polymers, copolymers and common plastics.

Table 2: Comparison between PHA polymer, copolymer and poly(propylene) (adapted from (Loo and Sudesh 2007).

Polymer	Glass transition temperature, T_g (ºC)	Melting temperature, T_m (ºC)	Tensile strength (MPa)	Young's Modulus (GPa)	Elongation to break (%)
P(3HB)	4	180	43	3.5	5
P(3HB-co-3HV) (20% HV, mol)	-1	145	20	1.2	50
PP	-10	176	38	1.3	400

As already mentioned, biodegradability is a unique characteristic of PHAs and it depends on their physical and chemical properties (Zinn *et al.* 2001; Tokiwa and Calabia 2004). Molecular weight (M_W) is one of the main factors affecting the polymer biodegradability, corresponding to a low M_W value a better condition of biodegradability. Another important influencing aspect in enzymatic degradability is the melting temperature as the biodegradability decreases with higher melting temperature values (Tokiwa and Calabia 2004). Furthermore, a wide variety of PHA degrading microorganisms has been isolated from different types of environments, such as soil, activated and anaerobic sludge as well as sea and lake waters being their ability to degrade influenced by environmental

conditions, such as microbial population, pH and temperature (Zinn *et al.* 2001). Microbial PHA polymerases and depolymerases are responsible for this process (Jendrossek and Handrick 2002).

The great possible variation in the length and composition of the pendant group allows the tailor-made of these polymers, making the PHA family suitable for a wide range of applications, including industry (e.g., packaging and compost bags), agriculture (e.g., mulch films) and medical activity (e.g., tissue engineering and heart valves) (Philip *et al.* 2007; Valentino *et al.* 2012).

1.2.1.2. Synthesis of PHAs by microbial cells

1.2.1.2.1. PHA granules

PHAs were first discovered, in 1926, by the french microbiologist Lemoigne who reported the occurrence of inclusion bodies of poly(3-hydroxybutyrate), P(3HB), in *Bacillus megaterium* (Lemoigne 1926). Nowadays, it is known that they are synthesized by a great variety of microorganisms, over 300 species, under both aerobic and anaerobic conditions (e.g. *Bacillus* sp., *Pseudomonas* sp. and *Methylobacterium* sp.) (Keshavarz and Roy 2010; Valentino *et al.* 2012). In nature, microorganisms can pass through unexpected changes that promote metabolic stress, like limitation of an essential nutrient (nitrogen, phosphorous, sulphur or oxygen) and restrictions in anabolic enzyme levels and activity (Loo and Sudesh 2007; Valentino *et al.* 2012). They are able to respond to those changes storing important compounds, like PHAs, for subsistence during deprivation periods (Zinn and Hany 2005). Also, there are bacteria that have the ability to store this polymer during growth. These two different inducing storage conditions must be considered when developing a fermentation strategy for PHA production (Khanna and Srivastava 2005).

PHAs function as carbon and energy storage polymers and they occur when the carbon sources are assimilated in the cell cytoplasm as insoluble granules (8 – 13 per cell) presenting diameters from 0.2 to 0.7 μm (Anderson and Dawes 1990; Braunegg *et al.* 1998). These granules can be surrounded by a phospholipid monolayer that integrates related proteins, like PHA synthase and polymerases, structural and regulatory proteins (Sudesh *et al.* 2000; Zinn *et al.* 2001).

1.2.1.2.2. Current technology for PHA production: Bottlenecks and Solutions

Up to date, the industrial production of PHAs is mainly based on bacterial pure culture (wild-type or recombinant strains) fermentations, operated in fed-batch mode (Grothea *et al.* 1999; Lee *et al.* 1999). This process includes a first stage of growth media supply until a high cellular density is achieved. At that point, growth limiting conditions are established in order to induce the storage of polymer. The obtained content of stored polymer is up to 80 – 90% of the cell dry weight and the volumetric productivities are considered high (up to 5 gPHA L^{-1} h^{-1}) (Choi and Lee 1997). However, the production costs remain too high affecting the overall economy of the process (Koller *et al.* 2011). The major factors that derail its competitiveness are the high costs related to the fermentation with pure cultures, which include the price of refined sugar feedstocks used as substrate, and to the downstream processing (Lee *et al.* 1999; Chanprateep 2010). Even though that a huge variety of microorganisms are recognized as PHA producers, just a few are suitable for the large-scale production of the polymer: *Cupriavidus necator*, *Alcaligenes latus* and recombinant *Escherichia coli*, among others (Lee 1996). Consequently, many efforts have been made to turn PHA production into a feasible alternative for the production of conventional plastics.

During the last decade, the development of alternative low-cost processes has been the main research scope (Salehizadeh and Van Loosdrecht 2004). These efforts include the use of genetic engineering strategies to increase process productivities and improve PHAs extraction that requires several separation steps as they are intracellular polymers. The application of low-cost substrates based on agro-industrial wastes and by-products is of an extreme interest too. Other alternative is the replacement of pure cultures by mixed microbial cultures (MMCs) that do not require sterile conditions or excessive operation control and have the ability to consume a wider variety of undefined substrates and produce PHA with a broader range of compositions (Serafim *et al.* 2008; Rehm 2010; Reis *et al.* 2011). In this context, it is possible to combine the use of both MMCs (Beccari *et al.* 1998; Dionisi *et al.* 2004; Serafim *et al.* 2004) and economic feedstocks (Kim 2000; Dionisi *et al.* 2005; Albuquerque *et al.* 2010) as a promising alternative since it has the potential to decrease operation costs once it uses economic substrate and saves energy from the sterilization step. Many recent research works are based on processes employing

MMCs, but the industrial implementation is not on course yet (Serafim *et al.* 2008; Reis *et al.* 2011).

1.2.1.2.3. Metabolisms for PHA synthesis

Currently, the PHA synthesis metabolism is well-known for pure microbial cultures, such as *C. necator* and *A. latus* that use the Etner-Doudoroff pathway for carbohydrate catabolic degradation (Khanna and Srivastava 2005; Reis *et al.* 2011). From this pathway results the production of pyruvate, energy as adenosine triphosphate (ATP) and reducing agents (reduced nicotinamide adenine dinucleotide – NADH). Under non-limiting growth conditions, pyruvate is converted into acetyl-CoA which is oxidized into carbon dioxide during the tricarboxylic acids (TCA) cycle, with complementary generation of anabolic precursors, supplementary energy and reducing agents. When growth limiting conditions are imposed, acetyl-CoA is not converted into carbon dioxide but P(3HB), as demonstrated by Figure 2a. Assuming that exist a lack of an external nitrogen source, the protein synthesis becomes slower; NADH and reduced nicotinamide adenine dinucleotide phosphate (NADPH) would accumulate in the cell, inhibiting the enzymes involved in TCA cycle (Reis *et al.* 2011). This phenomenon directs acetyl-CoA for P(3HB) synthesis (Dawes and Senior 1973).

There are three main stereospecific enzymes involved in P(3HB) synthesis from acetyl-CoA that guarantee the R configuration of HA monomers (Anderson and Dawes 1990): 3-ketothiolase (PhaA) that is responsible for the condensation of two molecules of acetyl-CoA into acetoacyl-CoA which pass through a reduction reaction catalyzed by acetoacetyl-CoA reductase (PhaB) producing (R)-3-hydroxybutyryl-CoA; this molecule is finally incorporated into the polymer linear chain as 3(HB) by PHA synthase (PhaC).

Besides pyruvate, also short-chain organic acids – volatile fatty acids (VFAs) – can be activated to the corresponding acyl-CoA molecules that act as precursors for diverse HA monomers, as represented in Figure 2c. Although just few studies considering the metabolic pathways for PHA production by MMCs using VFAs are reported (Lemos *et al.* 2003; Lemos *et al.* 2007), it is possible to assume that they are similar to those used by pure cultures. When using acetate as the carbon source, two acetyl-CoA molecules form P(3HB) as already described. Propionate can be activated into both propionyl-CoA and acetyl-CoA: two molecules of propionyl-CoA can form 3-hydroxy-2-methylvalerate

(3H2MV) or one unit of propionyl-CoA can condensate with a unit of acetyl-CoA forming 3HV or 3-hydroxy-2-methylbutyrate (3H2MB). Furthermore, butyrate and valerate can both be directly converted into 3-hydroxybutyril-CoA and 3-hydroxyvaleryl-CoA which originate, respectively, 3HB and 3HV (Reis *et al.* 2011). These four mentioned HA monomers are the most common scl-PHAs produced by MMCs.

It is possible to obtain precursors for PHA production through other three metabolic pathways: *de novo* fatty acid synthesis originate precursors for PHA production, as suggested by Figure 2b; medium- and long- chain fatty acids, in particular butyrate and valerate, can be metabolized by β-oxidation forming shorter chain fatty acids – Figure 2d; alkanes can be oxidized, as represented in Figure 2e, to the equivalent alkanoate, which will be activated to the corresponding acyl-CoA unit to be used to synthesize PHA (Reis *et al.* 2011).

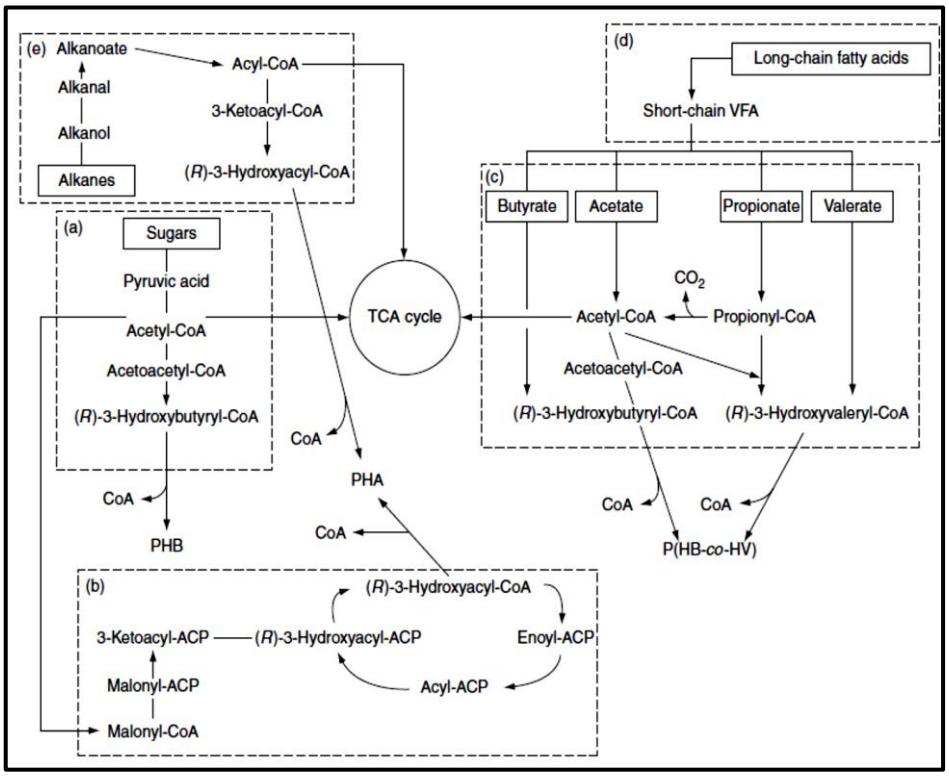


Figure 2: Metabolic pathways for PHA synthesis from sugars, fatty acids and alkanes. a) glycolysis; b) *de novo* fatty acids biosynthesis; c) directly from fatty acids; d) fatty acids β-oxidation; e) alkane oxidation (Reis *et al.* 2011).

1.2.1.2.4. Mechanisms that induce PHA storage in MMCs

Mixed cultures are microbial populations that operate in open biological systems. Their composition depends on the substrate and operation conditions imposed on the

system. These fundamentals have been employed in biological wastewater-treatment plants (WWTP) (Reis *et al.* 2011).

PHA storage by mixed cultures was first detected in enhanced biological phosphorous removal (EBPR) systems that operate under alternated anaerobic and aerobic (AN/AE) cycles (Wallen and Rohwedder 1974). The main groups of selected bacteria are polyphosphate accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs) (Levantesi *et al.* 2002). For both groups, PHA storage has an important function on metabolism (Serafim *et al.* 2008): under anaerobic conditions, PAOs and GAOs take up the carbon source and store it as PHA, while glycogen is consumed; microbial growth, maintenance and glycogen pool replenishment occurs during the aerobic cycle. The main difference between these two microbial groups is the cycling of phosphate by PAOs. Under anaerobic conditions, this group release phosphate, gaining useful energy for PHA storage and, under aerobic conditions, takes up phosphate in excess for polyphosphate pool replenishment (Pereira *et al.* 1996). On the other hand, GAOs only acquire energy through the catabolism of glycogen, under oxygen limiting conditions.

Also, in aerobic WWTP where selectors for bulking control were introduced was observed a considerable ability of sludge to store PHA. This process configuration creates alternated conditions of external carbon source availability that enhances the selection of floc-formers microorganisms (Majone *et al.* 1996). At lab-scale, were operated reactors simulating periods of substrate excess ("feast") and lack of it ("famine") to better understand the mechanisms that induce this phenomenon (Serafim *et al.* 2004). So, after a period under growth limiting conditions, the amount of intracellular components required for cell growth (RNA and enzymes) decreases. Hence, when the conditions are changed to excess of substrate, the amount of available enzymes does not ensure that a maximum growth rate is reached and the storage response becomes dominant (Serafim *et al.* 2008; Reis *et al.* 2011). The physiological adaptation needed after each starvation period is considered the main mechanism inducing PHA storage by microorganisms under aerobic "feast and famine" (FF) conditions (Anderson and Dawes 1990).

Fully aerobic activated sludge cultures show higher capacity to channel carbon toward storage rather than growth and maintenance, after a long period of starvation (Beun *et al.* 2002). During the "feast" phase, the culture uptakes ammonia and carbon sources with simultaneous PHA synthesis. After depletion of the external carbon substrate, the

intracellular PHA granules serve as carbon and energy sources used for cell growth and maintenance. As result, the faster microorganisms to store the substrate and to reuse it for growth have a greater competitive advantage. This process approach using transient conditions of external substrate availability is known as aerobic dynamic feeding (ADF) or FF regime and it allows the selection of cultures with high ability of PHA production during the "feast" phase and the capacity to use PHA to grow during the "famine" phase (Salehizadeh and Van Loosdrecht 2004; Serafim *et al.* 2008; Reis *et al.* 2011).

There are significant differences between the mechanisms for PHA storage under AN/AE conditions by PAOs and GAOs and in the aerobic FF process in terms of presence of final electron acceptor: in the latter, PHA storage happens due to internal growth limitation, since both electron donor and acceptor are present during the feast phase, while in the AN/AE process the storage response is a result of electron acceptor (oxygen, nitrate) absence. Thus, fully aerobic conditions (Figure 3b) create a strong selective pressure for PHA storage, whereas in AN/AE process (Figure 3a), when oxygen is provided, the culture has already depleted the external substrate. This suggests that the single imposition of AN/AE conditions does not select for PHA storing cultures, falling under the definition of an FF regime (Dias *et al.* 2006; Serafim *et al.* 2008; Reis *et al.* 2011).

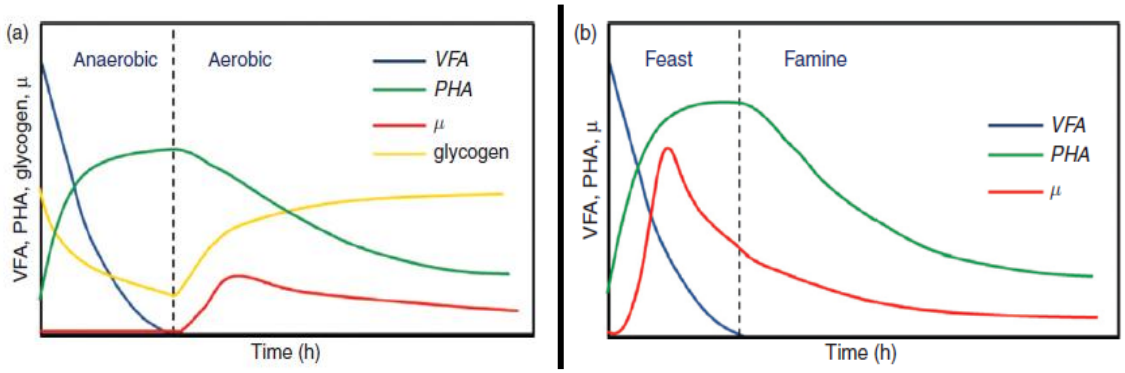


Figure 3: Mechanisms for storage of PHA by MMCs under different conditions: a) AN/AE and b) fully aerobic FF (Reis *et al.* 2011).

Moreover, under ADF conditions is observed a higher cell growth rate, once cellular growth by consumption of the external substrate and PHA storage are concomitant (Reis *et al.* 2011). The polymer composition also varies with the imposed operation conditions: under AN/AE conditions, glycogen is present and its degradation produces precursors for PHA synthesis (Bengtsson *et al.* 2010a). To conclude, ADF systems can

select for cultures with higher productivities, but with a poorer range of polymer compositions when compared with AN/AE.

1.2.1.3. MMC processes to convert organic by-products into PHAs

As already explained, many are the efforts in finding cost-effective alternative processes for PHA production. In this context, the application of MMC processes to transform organic by-products into PHAs seems to be a promising solution, once cheap substrates reduce the overall cost process in about 40% (Choi and Lee 1997). Those processes can be operated in either two or three stages, depending on the type of substrate employed as feedstock (Dias *et al.* 2006).

The two-step process includes a first step of microbial culture selection which is followed by a second step of maximization of PHA accumulation by the selected culture (Serafim *et al.* 2008). The two steps are carried out separately, enabling the process optimization, since each step requires different operational conditions (Albuquerque *et al.* 2007). The PHA obtained at the end of accumulation step is then extracted and purified. This kind of process is predominantly suitable for PHA production when organic acids such as acetate, propionate and butyrate, are used as feedstock (Beccari *et al.* 1998; Dionisi *et al.* 2004; Serafim *et al.* 2004). On the contrary, when agro-industrial wastes are employed as feedstock, a preliminary treatment may be required to convert their organic matter into suitable substrates for PHA storage, like VFAs (Reis *et al.* 2011). The reason for this prerequisite is that some agro-industrial wastes are sugar-rich materials, which are not used by MMCs, unlike pure cultures, to store PHAs under FF conditions. So, an additional acidogenic fermentation step is added, resulting in a three-stage process (Dionisi *et al.* 2005), which is represented in Figure 4.

Another important parameter influencing the process is the nitrogen (N)-content of the feedstock. In this context, if the waste presents a low N content it is necessary to supply the SBR with nitrogen, allowing the microorganisms to growth. In the further accumulation step, the N limiting conditions are favorable for the polymer storage. On the contrary, if the feedstock consists of a N-rich waste, it is not necessary the additional N supply step, but at the end of the accumulation step the PHA content in the biomass will be slightly low (Valentino *et al.* 2012).

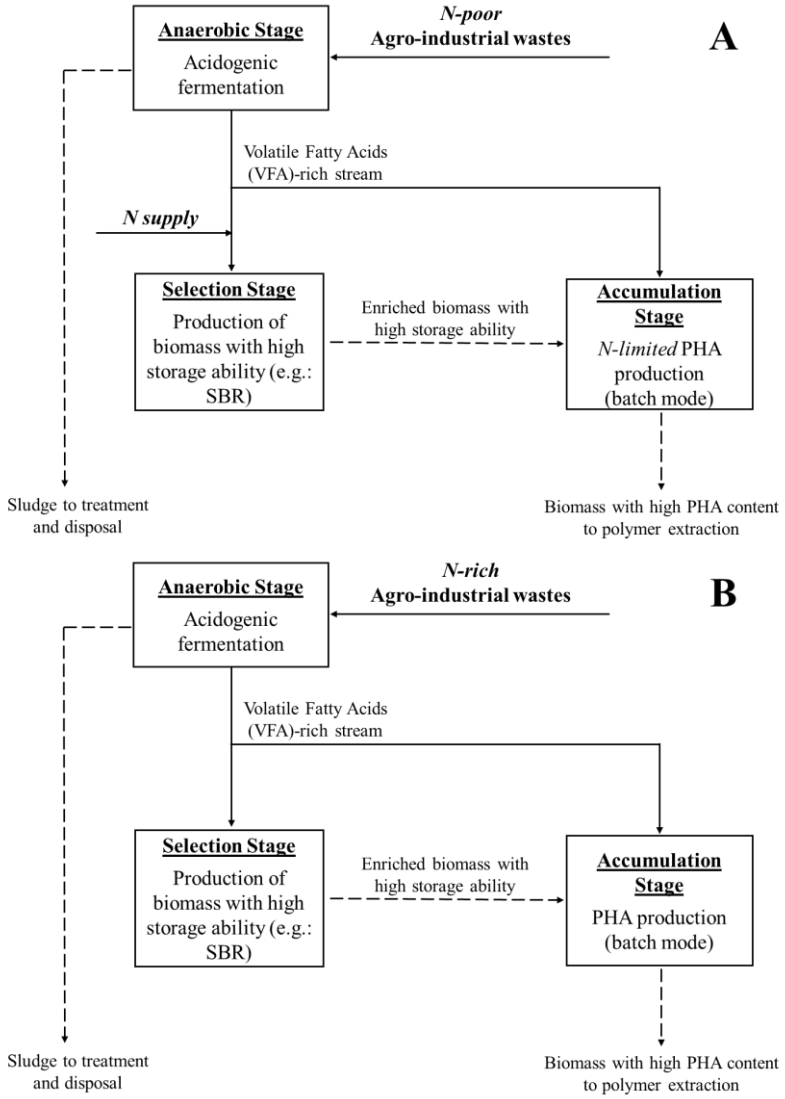


Figure 4: Schematic representation of a three-step PHA production process using N-poor agro-industrial wastes (A) or N-rich agro-industrial wastes (B) as feedstock (Valentino *et al.* 2012).

1.2.1.3.1. The first stage: Acidogenic fermentation for VFA production

The acidogenic fermentation corresponds to the initial step of the anaerobic digestion of organic matter to methane and carbon dioxide (Figure 5). Under anaerobic conditions, soluble organic matter is converted in VFAs (such as acetic, propionic, butyric and valeric acids), lactic acid (Bouallagui *et al.* 2005; Serafim *et al.* 2008) and other fermentation products. On the contrary, insoluble organic matter needs to be first hydrolyzed, being the hydrolysis rate limiting for the further acidogenic fermentation step (Reis *et al.* 2011; Valentino *et al.* 2012). In order to obtain a VFA-rich stream, it should be guaranteed that intermediate products are not converted in biogas. So, it is possible to

prevent methanogenic activity by controlling the reactor operating conditions at low hydraulic retention time (HRT), low temperature (Beccari *et al.* 2009; Bertin *et al.* 2010) and low pH (Albuquerque *et al.* 2007) or combining different approaches (Bengtsson *et al.* 2008a).

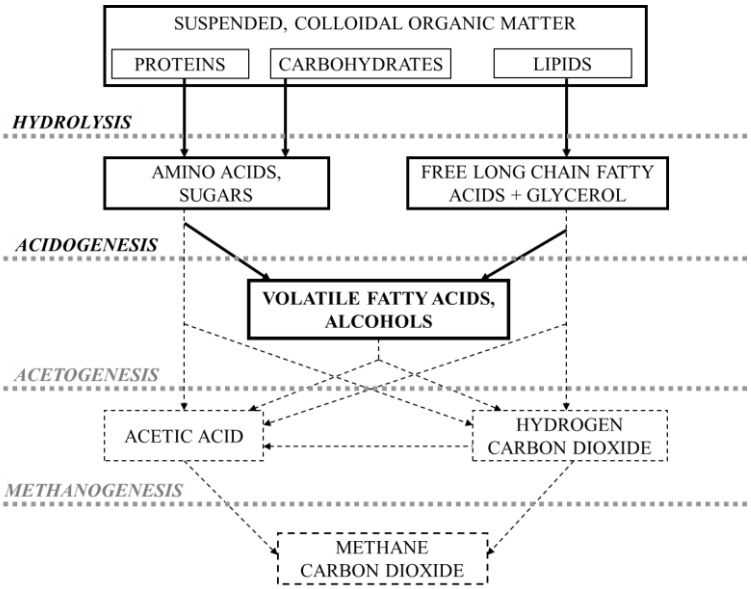


Figure 5: Scheme of the anaerobic digestion of organic wastes (adapted from (Villano *et al.* 2012).

Moreover, aiming for the optimization of VFA yield and the exploitation of the produced VFA's blend, it is necessary a careful adjustment of the operative parameters. This aspect plays a pivotal role in what regards the overall process, since the polymer composition and characteristics are highly dependent on the used substrate. So, further research is needed to correlate process conditions of acidogenic fermentation of wastes with the range of obtained VFAs (Valentino *et al.* 2012). Many are the factors that influence the final products obtained through acidogenic fermentation, namely the type of employed feedstock, the structure of microbial culture as well as the reactor configuration mode and operational conditions (HRT, sludge retention time (SRT), pH, temperature, organic loading rate (OLR)) (Reis *et al.* 2011). In this context, several agro-industrial streams have been investigated to be used as feedstock in MMC processes for PHA production and they include sugar cane molasses (Albuquerque *et al.* 2011), cheese whey and pulp mill effluents (Bengtsson *et al.* 2008a) and wastewaters from olive oil (Dionisi *et al.* 2005) and paper industries (Bengtsson *et al.* 2008b). The most common reactor operation mode for acidogenic fermentation is the continuous, since it allows for the complete adaptation of anaerobic mixed culture to the feedstock.

Operating both chemostat and batch operation mode for pretreatment through acidogenisis of cheese whey and paper mill effluents, Bengtsson and co-workers obtained higher VFAs yield for the first mode (0.75 to 0.87 gCOD $gCOD^{-1}$ compared to 0.59 to 0.60 gCOD $gCOD^{-1}$) (Bengtsson *et al.* 2008a), what suggest that continuous mode allows for a more efficient conversion by the anaerobic mixed culture. Increasing pH from 5 to 6 and HRT from 8 to 95 hours, the propionate content in the VFAs mixture increased (Bengtsson *et al.* 2008a). Also, Albuquerque *et al.* evaluated the pH effect on the acids composition of fermented sugar cane molasses. They reported that varying the pH value from 5 to 7 the acids profile changes, since at lower pH values butyric and valeric acids prevail, while at higher pH the main acids were acetic and propionic (Albuquerque *et al.* 2007). From these studies it is possible to notice that operative parameters can be controlled to exploit the VFAs profile obtained through acidogenic fermentation and, consequently, control the composition of the polymer produced in the successive steps.

Bertin *et al.* studied the performance of anaerobic packed bed biofilm reactors (PBBR) on acidogenic digestion of olive oil mill effluents (OME) to obtain VFAs, varying biofilm support material (ceramic filters and granular activated carbon), temperature and OLR (Bertin *et al.* 2010). The maximum VFA produced yield (85%) was obtained with the ceramic filters at 25 ºC, operating at an OLR of 13 g L^{-1} d^{-1}. Besides that, the authors reported that the main produced VFA were acetic, propionic and butyric acids and their relative concentrations were affected by the OLR parameter. Interestingly, the employed biofilm support material had a great effect in the system performance as it affects the biofilm community structure (Bertin *et al.* 2010). In addition, with this reactor configuration, it is possible to avoid shock loading and wash-out problems associated to suspended growth reactors and perform highly efficient acidogenic fermentation with just a small fraction of substrate (around 8%) being converted into biogas or used for cellular growth (Beccari *et al.* 2009).

1.2.1.3.2. The second stage: MMC selection

The MMC selection stage main purpose is to enrich the activated sludge in stable PHA-accumulating organisms, determining the productivity of the subsequent stage of the process (Reis *et al.* 2011). This enables working the third step at high cell densities, which improves final PHA productivity (Valentino *et al.* 2012). Its effectiveness is influenced by

operating conditions that should impose high selective pressure for PHA-storing organisms because those with low storage ability may affect the downstream process (Serafim *et al.* 2008). The best strategy to achieve it is the FF regime which can be performed in sequencing batch reactors (SBR), plug flow reactors or in two continuous stirred tank reactors (CSTR) in series with recirculation of biomass (Valentino *et al.* 2012). Up to date, more than 80% of PHA-storing organisms in the biomass were obtained after MMC enrichment (Johnson *et al.* 2009). However, when compared with pure cultures, MMC PHA volumetric productivities is still short, since the cellular density achieved in the SBR and in the further stage is low.

The most widely used is the SBR, since it enables to operate a succession of repeated cycles, each composed by different phases, in a single vessel. More in detail, these phases usually consist of feed, reaction, settling and treated effluent discharge, giving to the reactor the necessary versatility to operate in FF regime: during the feed and beginning of the reaction phases microorganisms are able to deplete the substrate ("feast" phase), while during the remainder phases microorganisms are submitted to stress conditions due to the exhaustion of substrate ("famine" phase) (Valentino *et al.* 2012). Operating a SBR under FF conditions, Majone and co-worker obtained an activated sludge with 20-fold higher storage ability after the selection stage (Majone *et al.* 2006).

In literature are reported some works focusing in the selection step of PHA production process mainly ADF (Dionisi *et al.* 2005), but also AN/AE (Dai *et al.* 2007), employing both synthetic substrates (Dionisi *et al.* 2004; Serafim *et al.* 2004) and real substrates (Bengtsson *et al.* 2008b; Beccari *et al.* 2009). Dai and co-workers, using AN/AE production process employing acetate as substrate, selected a microbial culture composed mainly by GAOs that are considered more robust microorganisms than PAOs. This selected culture was able to produce P(HB-HV) (Dai *et al.* 2007). Dionisi *et al.* operated a SBR under fully aerobic conditions using a mixture of acetic, propionic and lactic acids for activated sludge selection. The maximum PHA content in the biomass was 50% and the obtained polymer was composed by 69% of 3HB and 31% of 3HV (Dionisi *et al.* 2004). In what regards real substrates, Albuquerque and co-workers compared the selection stage using both acetic acid and fermented molasses in SBR. Despite of the selected culture with fermented molasses showed a lower capacity of accumulation in the subsequent stage of the process than that with acetate, the overall process cost worth as the substrate price can

balance the lower obtained productivity (Albuquerque *et al.* 2007). In other work, Bengtsson *et al.* operate a two CSTR in series system under FF conditions using fermented paper mill wastewater. The enriched culture produced an average PHA content of 11% of cell dry weight (Bengtsson *et al.* 2008b).

As reviewed by Reis and co-workers (Reis *et al.* 2011), besides reactor operation mode and type of feedstock, there are some factors that govern the culture behavior during the feast phase, when both polymer storage and cellular growth occur, influencing the MMC selection stage: feast to famine length ratio (F/F ratio), limitation or inhibition by the used carbon substrate and the operation under carbon or nitrogen-limiting conditions. In order to study such influences, some different reactor conditions were investigated by changing SRT, HRT, pH, temperature (T), cycle length, OLR, influent substrate and nutrient concentrations (Table 3).

Table 3: The most common operating parameters studied in PHA-storing culture selection in MMC processes (Reis *et al.* 2011).

Operating Parameters		**Range**
Related to reactor operating parameter	SRT	1 – 20 d
	HRT	1 – 3 d
	SBR cycle length	2 – 12 h
	pH	7 – 9.5
	T	20 – 30 ºC
Related to feedstock	OLR	1.8 – 31.25 gCOD L^{-1} d^{-1}
	Substrate concentration	0.9 – 31.25 gCOD L^{-1}
	C/N ratio	9 – 120 gC gN^{-1}
Related to both reactor parameters and feeding	F/F ratio	0.1 – 1.15

Particularly about F/F ratio, it plays a crucial role for the success of the selection stage, since it affects the selective pressure for PHA-storing organisms (Valentino *et al.* 2012). Therefore, should be assured that the famine phase is long enough to achieve the internal growth limitation inducing high PHA storage. F/F ratio can be exploited by varying OLR and/or influent substrate concentration (Reis *et al.* 2011). High OLR, influent substrate concentration or shorter SBR cycles result in a higher F/F ratio which may be responsible for physiological adaptation losses that will focus the substrate uptake towards microbial growth. Thus, the selection stage is usually operated at low F/F ratios to obtain PHA-storing enriched cultures (Serafim *et al.* 2004; Johnson *et al.* 2009). Dionisi and colleagues studied the effect of the OLR on the operation of a SBR with a SRT of 1 day

using as feedstock a mixture of organic acids (Dionisi *et al.* 2004). As the OLR varied between 8.5 and 31.25 gCOD L^{-1} d^{-1}, also the F/F ratio ranged from 0.10 to 1.15. As predictable, biomass concentration raise, while PHA production rate declined when OLR increased. According to the OLR effects, an intermediate value (20 gCOD L^{-1} d^{-1}) caused the highest storage ability, even if the process was unstable. In what regards F/F ratio, for ratios of up to 0.26 and higher than 0.90 the culture showed a storage and growth response, respectively. By changing the influent substrate concentration (30 – 60 Cmmol VFA L^{-1}), Albuquerque and co-workers operated an SBR with an HRT of 10 days and obtained the predominance of the storage response at the lower substrate concentrations (corresponding to low F/F ratio – 0.21) (Albuquerque *et al.* 2010). Another parameter that has to be considered during the selection stage performer is the HRT, which is the reciprocal of the effective specific growth rate. It is assumed that lower HRT values result in a higher availability of substrate for growth, instead of polymer storage (Valentino *et al.* 2012). This reactor parameter (varying between 1 and 0.21 d) and also OLR (ranging from 8.5 to 40.8 gCOD L^{-1} d^{-1}) effects in culture selection phase were studied by Villano and colleagues (Villano *et al.* 2010b). The FF conditions establishment which is needed for the culture enrichment was influenced by both studied parameters. The maximum PHA production rates and yields were obtained at OLR of 8.5 gCOD L^{-1} d^{-1} and HRT of 1 day.

Even know the culture selection stage operated in SBR under FF conditions by imposition of internal growth limitation without the need of external limiting conditions (Valentino *et al.* 2012), it was also studied the influence of nutrient limiting conditions by varying the C/N ratio. In this context, Serafim and colleagues selected an activated sludge with high PHB storage ability, operating an SBR with acetate as substrate (Serafim *et al.* 2004). They studied the effect of carbon and ammonia concentration in PHB storage and concluded that for the highest concentration of acetic acid (180 Cmmol L^{-1} released in three pulses of 60 Cmmol L^{-1}) was obtained the maximum of PHB content (78.5%) and that the lower the ammonia concentration, the higher the PHB content.

In addition, some studies reported the effect of either pH or temperature in the selection stage (Chua *et al.* 2003; Johnson *et al.* 2010c; Villano *et al.* 2010a), but the influence of these parameters is not clear yet (Valentino *et al.* 2012). As an example, Villano and co-workers using a SBR fed with a mixture of acetate and propionate inferred that the variation of pH between 7.5 and 9.5 made the storage rates and yields fall down

(Villano *et al.* 2010a). On the contrary, Chua *et al.*, by using activated sludge to treat a municipal wastewater, reported no substantial variations in the culture storage ability, when the selection phase was performed by changing the pH from 7 to 8 (Chua *et al.* 2003).

1.2.1.3.3. The third stage: PHA accumulation

PHA accumulation stage highly depends on the storage ability of the enriched culture and it is commonly carried out in batch mode. The PHA content obtained in the biomass has a great influence in the cost of the further extraction phase (Valentino *et al.* 2012). In Table 4 are reported some studies that used enriched cultures selected under ADF conditions for the subsequent polymer accumulation stage by using synthetic and/or real substrates.

Table 4: Different enriched MMC performance in the end of the accumulation stage using synthetic or real substrates (Valentino *et al.* 2012).

MMC selection stage (ADF)			**PHA accumulation stage (Batch)**				**Ref**
Substrate	System	SRT (d)	Substrate	Nutrient conditions	% PHA content	Polymer composition (mol:mol)	
Acetate	SBR	10	Acetate	N-limitation	65 gPHA $gVSS^{-1}$	P(3HB)	(Serafim *et al.* 2004)
Acetate	SBR	1	Acetate	N-starvation	89 gPHA $gVSS^{-1}$	P(3HB)	(Johnson *et al.* 2010b)
Acetic, propionic and lactic acids	SBR	1	Acetate, propionate and lactate	Nutrient excess	50 gCOD $gCOD^{-1}$	P(HB-HV) (69:31)	(Dionisi *et al.* 2004)
Fermented molasses	SBR	10	Fermented molasses	N-limitation	74.6 gPHA $gVSS^{-1}$	P(HB- HV) (74:26)	(Albuquerque *et al.* 2010)
Fermented paper mill effluents	2-CSTR in series	7	Fermented paper mill effluents	N-limitation	40 gCOD $gCOD^{-1}$	P(HB -HV) (41:59)	(Bengtsson *et al.* 2008b)
Acetic, propionic and lactic acids	SBR	1	Centrifuged and fermented OME	Nutrient excess	54 gPHA $gVSS^{-1}$	P(HB-HV) (89:11)	(Dionisi *et al.* 2005)

The nutrient availability is determinant for the final obtained PHA content, being the nutrient limiting conditions, using both synthetic and real substrates, more favorable for

polymer accumulation. The performance of the accumulation stage with high availability of nutrients will direct the biomass response to growth and the maximum PHA content will decrease. Instead, if nutrient limiting conditions are imposed, the biomass response will be driven towards polymer storage and the final PHA content will be higher (Reis *et al.* 2011). Up to date, the maximum biomass polymer content, 89%, was obtained by Johnson and colleagues using acetate as substrate of a MMC under nitrogen starvation conditions. Surprisingly, this value fits in the range of those already obtained for pure cultures (Johnson *et al.* 2010b). In what regards real substrates, the highest biomass polymer content was achieved using fermented molasses and operating under nitrogen limiting conditions (74.6%) (Albuquerque *et al.* 2010). So far, Dionisi and co-workers obtained the highest PHA content under nutrient excess conditions by using a MMC selected with a mixture of organic acids and by using fermented OME as substrate in the accumulation step (Dionisi *et al.* 2005). Despite being lower the achieved biomass PHA content values using nutrient excess conditions, this alternative seems to be really promising, in the sense that there is no need of eventual nutrient supply during the selection stage. At the same time, it still allows for the effectiveness of the PHA accumulation stage (Valentino *et al.* 2012).

The PHA production using raw materials as feedstock presents a great advantage at the level of the final polymer composition (Rehm 2010). As already mentioned, the variety of physical properties and characteristics of PHA that leads to a wider range of fields of application is highly influenced by its composition (Philip *et al.* 2007). So, other monomers than 3HB were obtained in the end of the accumulation stage by using fermented sugar molasses (Albuquerque *et al.* 2010), OME (Dionisi *et al.* 2005) and paper mill effluents (Bengtsson *et al.* 2008b). The 3HV content in the produced copolymer fluctuate a lot (ranging from 11 to 59%) depending on the feedstock used for the polymer. Concluding, the use of complex substrates in PHA production processes allows the adjustment of polymer properties by exploiting the important parameters of each stage (Valentino *et al.* 2012). However, those feedstocks are significantly affected by seasonal and process variations, being of a great importance the development of operational strategies, which may involve the all process stages in order to solve these problems (Albuquerque *et al.* 2007; Serafim *et al.* 2008).

1.2.1.4. Polymer extraction step

The polymer obtained in the end of the accumulation stage needs to be extracted and purified (Reis *et al.* 2011). Thus, several technologies have been reported aiming the sustainability of the selected method as well as the maximum purity of the recovered polymer (Jacquel *et al.* 2008).

Solvent extraction, a simple and fast technique, is the most used method to recover the polymer from the biomass, being chlorinated hydrocarbons (i.e. chloroform) and cyclic carbonates (i.e. ethylene carbonate) the most common used solvents (Kunasundari and Sudesh 2011). It involves two main steps, namely PHA release and solubilization by modifications in the permeability of the cellular membrane followed by non-solvent precipitation (Jacquel *et al.* 2008). This method has a great effectiveness in terms of the obtained polymer purity and Mw, but has some environmental issues as well as high associated costs both related to the used solvents (Jacquel *et al.* 2008). As a consequence, digestion methods (chemical and enzymatic) are considered a promising alternative (Kunasundari and Sudesh 2011).

The mainly used chemicals in chemical digestion are sodium hypochlorite and surfactants to solubilize non-PHA cellular mass (Jacquel *et al.* 2008). The first presents strong oxidizing properties and non-selectivity that can be manipulated in order to improve PHA recuperation from the biomass (> 97% of purity) (Yu and Chen 2006). Chemical digestion technique presents low operating costs and good levels of polymer purity that are not yet balanced by the relatively high price of the involved chemical agents (Jacquel *et al.* 2008). In the other hand, enzymatic digestion is an approach based on the use of hydrolytic enzymes (i.e. lysozyme) and effective under mild conditions (Kunasundari and Sudesh 2011). Although this technique involves a more complicated recovery process and high costs related to the enzymes it presents great eco-efficiency and levels of achieved purity (Kapritchkoff *et al.* 2006). As reviewed by Jacquel and colleagues, some promising approaches involving the combination of these techniques were developed like the recovery by using dispersions of chloroform and sodium hypochlorite as well as the combined use of enzymes and sodium hypochlorite (Jacquel *et al.* 2008).

The efficiency and cost of the extraction step strongly depend on the PHA content in the biomass (Serafim *et al.* 2008). When high contents of PHA in the biomass are achieved, less digesting agent is needed as well as less carbon source is wasted, decreasing

the associated cost (Reis *et al.* 2011). So, this step is highly influenced by the previous stages involved in PHA production. In addition, as MMC produce lower PHA concentrations than pure cultures, the extraction step should be particularly studied and optimized to be employed in MMC processes that convert complex feedstocks into PHAs (Reis *et al.* 2011).

1.3. OME as a suitable feedstock for PHA production

1.3.1. Olive oil production

Mediterranean countries govern the major part of the worldwide olive oil production, being Spain the biggest olive oil-producing country followed by Italy, Greece, Turkey and Portugal (Roig *et al.* 2006). The extraction of olive oil from olives may involve two different technologies, either traditional discontinuous pressing or continuous centrifugation. Traditional pressing is an obsolete technique but is still in use by some producers. Currently, olive oil industrial production is based on the second, which can be classified in three-phase and two-phase systems that are represented in Figure 6 (Kapellakis *et al.* 2006; Morillo *et al.* 2009).

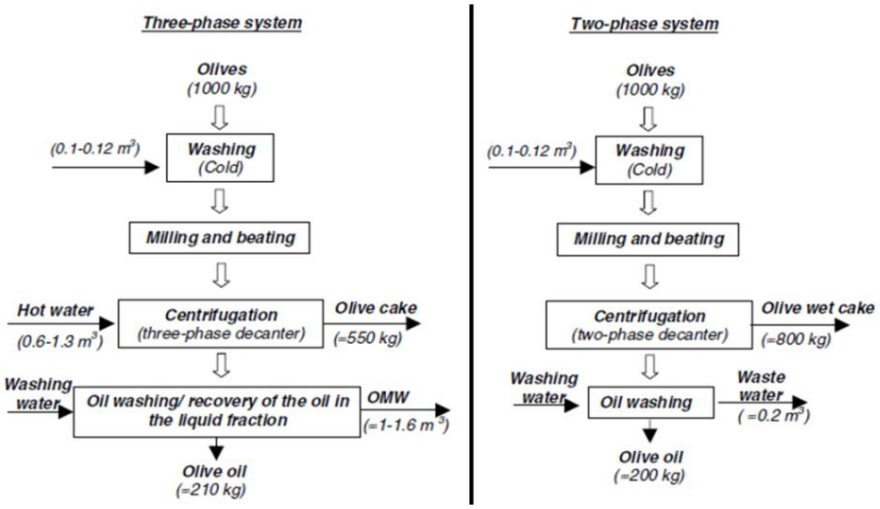


Figure 6: Three- and two-phase continuous systems for olive oil production (Roig *et al.* 2006).

In Spain, the two-phase system is the most common used technology, while in Italy almost all olive oil production is based on three-phase system (Alburquerque *et al.* 2004; Roig *et al.* 2006). On the other hand, in Portugal the traditional pressing technology is being replaced by the more recent ones. In this context, in 2010 it accounted 44% for traditional pressing, 33% for two-phase system and 23% for three-phase system (INE

2010). These facts are relevant because the two-phase system is considered an ecological approach for the reason that reduces the amount of generated wastewater and produces a solid by-product called olive wet cake that can be used to produce additional yields of oil through chemical extraction and energy generation (Alburquerque *et al.* 2004). On the contrary, both traditional and three-phase systems produce large amounts of highly pollutant liquid stream – called olive oil wastewaters (OMW) or olive oil mill effluents (OME) – that have been illegally discharged to the nearby soil or water streams, consisting in a great environmental concern along the Mediterranean basin (Roig *et al.* 2006).

1.3.2. Properties, characteristics and environmental issues of OME

The high environmental impact of OME is due to its high organic load and chemical characteristics (Table 5) (Roig *et al.* 2006). The OME composition and characteristics are not consistent, varying with cultivation, harvesting, ripening, seasonality and climatic aspects. Also, its propensity to be fermented facilitates some variations of composition during storage (Valentino *et al.* 2012). Direct discharge of these effluents is not allowed since they are very recalcitrant in the environment, cause deterioration of soil and water bodies, present high toxicity to plants and cause odor nuisance when disposed in the environment (Morillo *et al.* 2009; Valentino *et al.* 2012).

Table 5: Main characteristics of OME produced through three-phase system. Ranges were determined based on 8 studies reviewed by (Valentino *et al.* 2012).

Parameter	Range	Parameter	Range
VSS (g L^{-1})	30 – 38	Total phenols (g L^{-1})	0.1 – 17.5
TSS (g L^{-1})	35 – 45	Ammonia (g L^{-1})	0.1 – 0.9
COD (g L^{-1})	40 – 196	Phosphorous (g L^{-1})	0.2 – 0.4
pH	4.6 – 5.7	COD:N ratio	100:0.1 – 100:0.9

OME composition is approximately based on water (83 – 84%), organic matter (14 – 15%) and mineral salts (1 – 2%), being composed by both dissolved and suspended substances. The organic fraction contains lipids, proteins, sugars, alcohols, pectin and phenolic compounds, while the mineral content is mainly composed by carbonates, phosphate, sodium and potassium (Valentino *et al.* 2012).

The components of the phenolic fraction can be divided into two main groups: simple phenolic compounds (i.e. tannins and flavonoids) and dark-colored polymers resulting from the polymerization and autoxidation of the first (Morillo *et al.* 2009). The

presence of this recalcitrant organic fraction is not regular and is the main responsible for the problems involved in detoxification and purification of OME as well as some biological concerns (i.e. phytotoxicity). Moreover, the proportion between the two phenolic groups determines the intensity of dark-colored appearance (Valentino *et al.* 2012). Alongside with phenolic compounds, also the acidic pH, high chemical oxygen demand (COD) content and low nutrient concentration contributes for this environmental concerning problem (Roig *et al.* 2006).

1.3.3. Biological treatment and valorization approaches for OME

During the last years, many efforts have been made aiming at the reduction of organic load and environmental negative effects of OME as well as their conversion into value added products (Valentino *et al.* 2012).

Due to the low biodegradability and high toxicity, the common techniques used for urban wastewater treatment are not suitable to be used in OME management (Mantzavinos and Kalogerakis 2005). The use of conventional activated sludge systems for wastewater treatment in OME case is conditioned by the high organic content, the presence of inhibiting compounds and low nutrient concentration. On the contrary, those characteristics allow for the choice of anaerobic processes with biogas production for biological treatment of OME, since under anaerobic conditions the growth yields are lower and the nutrient content is not decisive (Paraskeva and Diamadopoulos 2006). However, in both types of treatment, the presence of oily and phenolic compounds, as they are inhibitory to the cells, represents a great challenge to the application of biological wastewater treatment (Valentino *et al.* 2012).

Nowadays, the development of integrated approaches is considered of great interest in the context of OME and the concerning disposal in the environment (Scoma *et al.* 2011). So, many alternatives have been proposed regarding OME employment as a renewable feedstock in biorefinery processes (Federici *et al.* 2009). The removal of polyphenols from OME through physicochemical or biological approaches represents a potential alternative to valorize such agro-industrial stream (Scoma *et al.* 2011). Accordingly, the extraction of polyphenols can be performed aiming two main situations: the posterior application in food industry (Bertin *et al.* 2011) or to improve the performance of the subsequent biological processes (i.e. acidogenic fermentation) (Ena *et al.* 2009). However, this approach can lead

to losses of other constituents of the organic fraction, minimizing the possibility to exploit OME as feedstock for a broad variety of bioprocesses (Ena *et al.* 2009). OME have been studied as promising suitable growth-media for lipase-producing microorganisms (D'Annibale *et al.* 2006). The energetic valorization of raw OME through anaerobic fermentation for biogas production is also of great interest (Sampaio *et al.* 2011). Another promising innovative approach to valorize these agro-industrial effluents is by producing biodegradable polymers, such as PHA (Dionisi *et al.* 2005; Beccari *et al.* 2009).

1.3.3.1. PHA production from fermented OME

The production of biodegradable polymers from fermented OME consists in a promising alternative to use an economic feedstock to obtain high-value products (Dionisi *et al.* 2005). As already explained, the organic content into OME can be transformed through acidogenic fermentation in VFAs, the most direct substrates for PHA production (Reis *et al.* 2011). Furthermore, their lipid and phenolic contents do not inhibit the acidogenic fermentation step. OME are liquid streams which can easily be pumped in slurry-phase reactors (Valentino *et al.* 2012). In Table 6 are summarized two research works that investigated the three-stage process for PHA production (Figure 4A) by using OME as feedstock.

According to Dionisi and colleagues, the OME pretreatment through centrifugation before acidogenic fermentation leaded to the production of a more preferable substrate, inducing higher PHA storage yields. Also, this pretreatment induced the production of propionic and valeric acids, besides acetic and butyric acids which is advantageous for the production of copolymer P(HB-HV). Furthermore, in the end of the accumulation stage, the obtained storage yield was high (≈1.0 $mgCOD_{PHA}$ $mgCOD_{VFA}^{-1}$), even if compared to those obtained using synthetic substrates (Dionisi *et al.* 2005). In what regards Beccari and co-workers research, the value obtained for PHA storage yield during the selection stage (0.36 COD COD^{-1}) was very similar compared to the value obtained with synthetic mixture of VFAs (Beccari *et al.* 2009). In the end of the accumulation stage, the PHA storage yield (≈ 0.35 COD COD^{-1}) was within the range for those determined using real substrates such as fermented molasses (Albuquerque *et al.* 2007) and paper mill effluents (Bengtsson *et al.* 2008b).

Table 6: Main data of two research works about PHA production from OME.

First Stage				Second Stage	Third Stage			Ref.
OME characterization		VFA (gCOD L^{-1})	VFA yield (%)	Substrate	Substrate	PHA content	Storage yield	
pH	5.2	7 – 16	22 – 44	Mixture of acetic, propionic and lactic acids (40, 20 and 40%)	Centrifuged OME	0.54 gPHA $gVSS^{-1}$	≈ 1.0 $mgCOD_{PHA}$ $mgCOD_{VFA}^{-1}$	(Dionisi *et al.* 2005)
Total COD	113.8 g L^{-1}							
Soluble COD	34.5 g L^{-1}							
Soluble polyphenols	2.2 g L^{-1}							
Soluble carbohydrates	3.1 g L^{-1}							
Kjeldhal nitrogen	2 g L^{-1}							
Lipids	11.1g L^{-1}							
OME were both pretreated (centrifugation, bentonite addition or bentonite addition + centrifugation) and non-pretreated								
pH	5.5	10.7	32	Fermented OME (OLR = 8.5 gCOD L^{-1} d^{-1})	Fermented OME	20% gCOD $gCOD^{-1}$	≈ 0.35 COD COD^{-1}	(Beccari *et al.* 2009)
COD	37 g L^{-1}							
VSS	16.6 g L^{-1}							
TSS	22.6 g L^{-1}							
Polyphenols	1.5 g L^{-1}							
Kjeldhal nitrogen	4.97 g L^{-1}							
OME were fermented in a PBBR								

A recent proposed approach aims the integrated biohydrogen and biopolymers production from OME, prospecting the simultaneous energetic and economical valorization of such effluents (Ntaikou *et al.* 2009).

Chapter 2 | Scopes and objectives of the research

The main objectives of this research were the development and optimization of an innovative low-cost multi-stage process for the production of polyhydroxyalkanoates (PHAs) by using mixed microbial cultures and olive mill effluents (OME) as feedstock. Prior to being used for PHA production, OME were dephenolized and anaerobically fermented into volatile fatty acids (VFAs). In particular, the OME pretreatment was carried out at the University of Bologna.

In order to accomplish the target objectives, the research plan has been organized as follows:

- Initially, the pretreated OME were characterized in terms of total and soluble COD content, VFA composition and amount, nutrient composition (e.g. N and P). This characterization was performed by means of a standardized analytical procedures and techniques.
- After characterization, batch tests were carried out with an activated sludge in order to study the possible presence of inhibitory effect on the microbial degradation of OMEs.
- Prior to exploiting the characterized OME as process feedstock, a lab-scale reactors' configuration, simulating the PHA production steps, has been completely assembled and set-up. This operation consisted in the development of a sequencing batch reactor (SBR) and a PHA accumulation reactor, both monitored through a software which allowed to control each operational phase (e.g., reactors' feeding, sludge withdrawal, aeration, among others).
- Different reactors' operating conditions have been investigated, in order to identify the best operational conditions of the process. In this context, a key parameter affecting the SBR performance is the organic loading rate (OLR) which has been investigated at two different values (2.37 and 4.74 gCOD L^{-1} d^{-1}) to evaluate the effect of substrate influent concentration on the culture selection, in terms of produced PHA, VFA and nitrogen consumption as well as kinetic parameters. As for the accumulation stage, it was operated under nitrogen limiting conditions (i.e., with no further addition of N to that contained in the OME) and pulse feeding strategy with undiluted OME. Here, the effectiveness of the studied feeding strategy as well as the suitability of the fermented OME for the PHA accumulation was evaluated.

- After the optimization of the polymer accumulation step, main attention has been paid at developing a PHA recovery procedure. For this purpose, the PHA-rich biomass (from the accumulation reactor) was recovered through a solid-liquid separation method with a subsequent chemical digestion with sodium hypochlorite, aiming at disrupting the cell wall and releasing the intracellularly accumulated PHA. This stage was optimized through its operation set-up to improve the quality of the final recovered polymer.
- Finally, in order to obtain a dry powder of PHA granules, a lyophilization operation was set up and lyophilized samples were analyzed by GC in order to determine the PHA content and composition.

Chapter 3 | Materials and methods

3.1. Olive oil mill effluents pretreatment and characterization

Olive mill effluents (OME) were provided by the University of Bologna, where they were submitted to a pretreatment consisting of a first dephenolization step followed by an acidogenic fermentation step. The fermented OME was characterized in terms of COD, VFA concentration as well as nitrogen and phosphorous content.

3.2. Assays of microbial degradation of OME

The microbial degradation of OME was tested by means of respirometric assays, performed by alternating sludge aeration. An activated sludge from the "Roma Nord" full-scale wastewater treatment plant was used for these tests. The sludge was diluted in mineral media (the composition is described in section 3.3.1.), more in detail 200 mL of sludge were added to 200 mL of mineral medium, resulting in a final biomass concentration of 2357.5 mgVSS L^{-1}. Initially, the sludge was aerated for 1 hour. The aeration was then stopped and after 1 min the dissolved oxygen (DO) concentration was measured each 10 sec in order to determine the endogenous oxygen uptake rate ($OUR_{endogenous}$). Subsequently, 20 mL of fermented OME (corresponding to a final concentration of 1.13 gCOD L^{-1}) were added to the sludge under aeration, after 10 min the aeration was turned off again and the DO values were measured again each 10 sec to determine the OUR. This procedure was constantly repeated during about 6 hours. The same sludge was submitted to these assays for 3 days. The pH was controlled at 7.5 by adding sodium hydroxide (1 M) or sulfuric acid (0.8 M) according to the system requirements.

The OURs at each 10 min were determined by applying a linear function of dissolved oxygen concentration plotted over time.

3.3. Experimental set-up

3.3.1. Microbial Mixed Culture selection in Sequencing Batch Reactor

The culture enrichment stage was studied in a lab-scale sequencing batch reactor (SBR, 1L) operated under aerobic conditions at controlled temperature (25 ºC). A

schematic representation of the SBR is shown in Figure 7. The length of each cycle was 6 hours and its structure was initially composed by an initial feed phase of 10 min (0.25 L $cycle^{-1}$ resulting in 1 L day^{-1}), a reaction phase of 338 min, a withdrawal phase of the mixed liquor (0.25L $cycle^{-1}$ resulting in 1 L d^{-1}) of 2 min and a regeneration phase of 10 min. By the 46^{th} day of operation, the withdrawal was anticipated, resulting in a cycle composed by an initial feed phase of 10 min, a reaction phase of 50 min, a withdrawal phase of the mixed liquor of 2 min and a regeneration phase of 298 min. Unlike usual SBR operations, the settling phase was not performed, being all excess biomass discharged with the mixed liquor. As result, the sludge retention time (SRT) was the same as the hydraulic retention time (HRT) and consisted of 1 day. The pH of the reactor was controlled at approximately 7.6 by carbon dioxide bubbling through a compressed gas cylinder. During each cycle, the reactor was aerated by means of air pumps through ceramic diffusors and stirred by a mechanical impeller to obtain an oxygen transference coefficient ($K_La \approx 0.3$ min^{-1}) which should allow at guarantee a non-oxygen-limiting concentration (> 2 mg L^{-1}). During each SBR cycle the value of DO concentration was continuously monitored. The change in its slope from negative to positive along time was indicative of the substrate depletion, thus defining the feast phase duration and the beginning of the famine phase, allowing for the determination of F/F (feast and famine) ratio. All the peristaltic pumps used to feed and to draw the reactor, as well as the aeration pumps, were controlled by a software program which was also allowed to continuously record the values of oxygen and pH concentration in the reactor.

Initially, the reactor was inoculated with 1 L of activated sludge from the "Roma Nord" full-scale treatment plant (780,000 p.e.; 354,000 m^3 d^{-1}) which treats almost entirely domestic wastewater. During the initial 21 days, the reactor was fed with fermented OME diluted with mineral medium to obtain a final concentration of 2.37 gCOD L^{-1}, resulting in an organic load rate (OLR) of 2.37 gCOD L^{-1} d^{-1}. Subsequently, the feed was changed to a more concentrated one that was 4.74 gCOD L^{-1} of influent concentration and 4.74 gCOD L^{-1} d^{-1} of OLR. The OLR values were determined based on the soluble COD. In order to obtain those concentrations, fermented OME were diluted about 10 and 5 times, respectively, with the mineral medium. Its composition was $(NH_4)_2SO_4$ (1500 mg L^{-1}), $FeCl_3 \cdot 6H_2O$ (2 mg L^{-1}), EDTA (3 mg L^{-1}), thiourea (20 mg L^{-1}), K_2HPO_4 (335 mg L^{-1}), KH_2PO_4 (259 mg L^{-1}), $MgSO_4 \cdot 7H_2O$ (100 mg L^{-1}), $CaCl_2 \cdot 2H_2O$ (50 mg L^{-1}), $ZnSO_4 \cdot 7H_2O$

(0.1 mg L^{-1}), $MnCl_2 \cdot 4H_2O$ (0.03 mg L^{-1}), H_3BO_3 (0.3 mg L^{-1}), $CoCl_2 \cdot 6H_2O$ (0.2 mg L^{-1}), $CuCl_2 \cdot 2H_2O$ (0.01 mg L^{-1}), $NiCl_2 \cdot 6H_2O$ (0.02 mg L^{-1}) and $Na_2MoO_4 \cdot 2H_2O$ (0.03 mg L^{-1}). Importantly, starting from the 63rd operation day, the $(NH_4)_2SO_4$ concentration in the mineral medium was reduced to 750 mg L^{-1}, in order to avoid that residual ammonia concentration from the SBR could allow for biomass growth in the following accumulation stage (described below), affecting negatively the concentration of stored polyhydroxyalkanoates (PHAs). The feed was kept in a refrigerated vessel at 4 ºC.

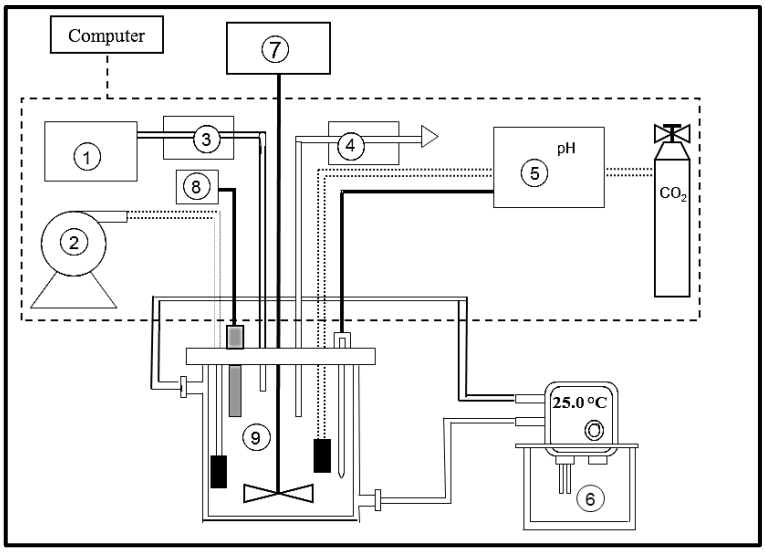


Figure 7: SBR layout. (1 – feed tank; 2 – aerator; 3 – pump for substrate feeding; 4 – withdrawal pump; 5 – pH meter and carbon dioxide pump for pH control; 6 – thermostat bath for temperature control; 7 – mechanical stirring; 8 – dissolved oxygen meter; 9 – reactor vessel).

Each SBR run, at the two different investigated OLR (2.37 gCOD L^{-1} d^{-1} and 4.74 gCOD L^{-1} d^{-1}), was characterized by determination of total and volatile suspended solids (TSS and VSS, respectively) as well as PHA and substrates concentrations at the moment of the substrate depletion (end of the feast phase) and at the moment of the mixed liquor withdrawal (end of the cycle). During the last period of the run at the higher OLR, samples for suspended solids (SS), PHA and substrates determination were also taken after one hour from the beginning of the feed phase.

To obtain more detailed profiles about the trend of PHA and substrates during an overall cycle, two kinetic tests for each SBR run were performed. During kinetic tests, the reactor was sampled before and after the feed phase, at the moment of substrate depletion and at regular intervals of 30 or 60 minutes along the remaining cycle.

3.3.2. PHA accumulation stage

The PHA accumulation stage of the process was simulated in a 0.35 L working volume reactor (Figure 8) operated at 25 ºC. The operation of the accumulation reactor started from the 30th day of the SBR operation (SBR run performed at 4.74 gCOD L^{-1} d^{-1} of OLR). The enriched sludge withdrawn from the SBR (0.25 L) was directly sent to the accumulation reactor, which was fed with 25 mL of fermented OME during 20 min each 90 min for 6 hours (4 feeding pulses), corresponding to a final concentration of 6.78 gCOD L^{-1}. After 33 days of accumulation stage operation, in order to maximize PHA production and avoid polymer internal consumption by the cells, the feeding strategy was changed from 4 to 5 feed pulses, consisting of 25 mL of fermented OME during 20 min each 72 min (corresponding to a final concentration of 7.91 gCOD L^{-1}). As a result, the reactor performance structure was based on alternating various pulses of feed with accumulation periods. The accumulation reactor was operated twice per day (i.e. by using the biomass deriving from two SBR cycles) under continuous aeration and mechanical stirring (the oxygen concentration was always higher than 2 mg L^{-1}). At the end of each accumulation assay (6 hours), the biomass was sent to further polymer extraction and purification. The feeding and withdrawal pumps as well as aerator were automatically controlled by a software program.

The description of the accumulation reactor performance was made by means of sampling for SS, PHA and substrates determination both at the beginning (in correspondence to the SBR mixed liquor withdrawn) and at the end of the accumulation assay. Differently from the SBR, pH was not controlled in the accumulation reactor and it generally raised to around 9.

The kinetic characterization of the accumulation reactor performance was made during the period of 4 feed pulses strategy. Two kinetic tests were performed by sampling the reactor each 30 min during the 6 h of each assay for PHA, VFA and ammonium determination.

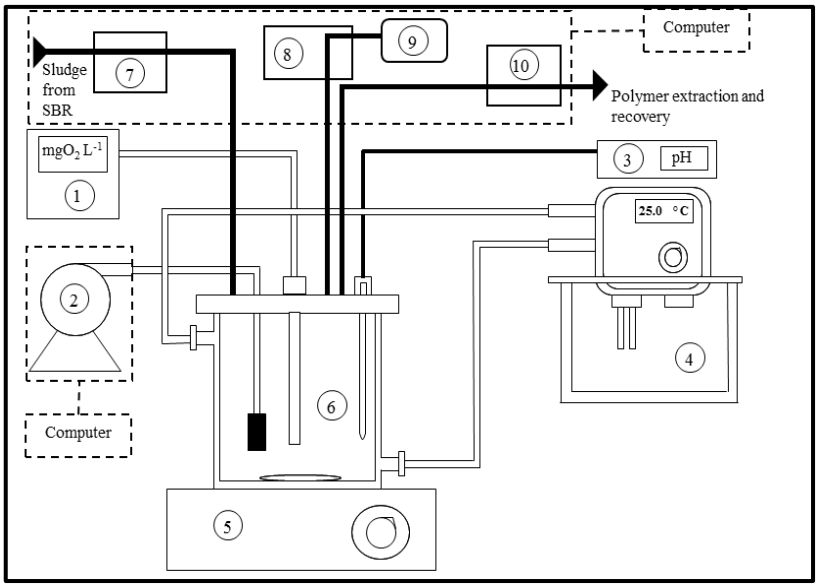


Figure 8: Accumulation reactor layout (1 – dissolved oxygen meter; 2 – aerator; 3 – pH meter; 4 – thermostatic bath; 5 – magnetic stirring; 6 – reactor vessel; 7 – SBR withdrawal pump; 8 – feeding pump; 9 – feeding tank; 10 – withdrawal pump).

3.3.3. Polymer recovery

At the end of each accumulation assay, the mixed liquor was withdrawn and the cells were separated from the supernatant by centrifugation (8000 rpm, 20 min). To the pellet was added a medium volume of 60 mL per accumulation assay of a solution of sodium hypochlorite (NaClO) (with 5% Cl_2) and kept under continuous mixing for several hours. The NaClO solution allowed the disruption of cell wall, the release of intracellular PHA, and the solubilization of non-PHA cellular mass, while preventing polymer degradation. The mixture was then centrifuged (8000 rpm, 15 min) in order to separate the non-polymer cellular mass from the polymer, which was frozen at -4 ºC. After a few days of operation of the accumulation stage, before to be frozen, samples started to be washed with water and centrifuged (6000 rpm, 25 min). Finally, the frozen pellet samples were unfrozen at room temperature and samples deriving from several accumulation assays were collected to be dried by means of a lyophilization process, performed at 5 mmHg and at -40 ºC for 96 h.

3.4. Analytical procedures

3.4.1. Suspended solids determination

The dry weight of the overall biomass was measured as TSS and VSS according to standard methods (APHA 1995), modified due to the little amount of available sludge. Thus, 10 mL of SBR biomass and 5 mL of biomass from the accumulation stage were taken from the respective reactor at the specific moments of the cycle, as already described. The samples were filtered under vacuum through GF/C filters (47 mm of diameter and 10 μm of porosity). Those filters were pretreated (at 100 ºC in incubator during 24 h) and stored in exsicator. Prior of measurements, filters were weighted (P_1) and, after sample filtration, were incubated at 100 ºC at least for 4 h, then treated for 30 min in exsicator and weighted (P_2). Finally, filters were treated in muffle at 550 ºC, kept in exsicator for 30 min and weighted (P_3). The corresponding TSS, inert suspended solids (ISS) and VSS were calculated as follows:

$$\text{TSS}\ (\text{mg L}^{-1}) = \frac{(P_2 - P_1)}{V} \qquad \text{ISS}\ (\text{mg L}^{-1}) = \frac{(P_3 - P_1)}{V} \qquad \text{VSS}\ (\text{mg L}^{-1}) = \text{TSS} - \text{ISS}$$

The obtained non-polymer VSS (which corresponds to the difference between VSS and PHA concentrations) was converted in COD according to the oxidation stoichiometry (1.42 mgCOD mgbiomass^{-1}).

3.4.2. Polyhydroxyalkanoate (PHA) determination

For determination of PHA concentration, the sampled sludge (5 mL from the SBR and 2.5 mL from the accumulation stage) was immediately treated with NaClO (7% of active Cl_2) (1 mL and 0.5 mL for the SBR and accumulation samples, respectively) and stored at -4 ºC. PHA was extracted, hydrolyzed and esterified to 3-hydroxyacyl methyl esters to be assayed by gas-chromatography according to the method proposed by Braunegg modified (Braunegg *et al.* 1978). So, the unfrozen samples were centrifuged (10000 rpm, 30 min) to separate the polymer from the remaining supernatant. A solution (2 mL) containing methanol, sulfuric acid (3% v/v) and 0.05 mg mL^{-1} benzoic acid, functioning as internal standard was added to the pellets recovered by centrifugation. Finally, 1 mL of chloroform was also added and the prepared samples were thermostated at

100 ºC for 4 h. After cooling at ambient temperature, 1 mL of water was added to induce two phase (aqueous and organic) separation by mixing during 10 min.

The analysis were performed through injection of 1 µL of the organic phase in the gas-chromatograph (Perkin Elmer 8410 Gas Chromatograph) equipped with a flame ionization detector (FID) set to 200 ºC, a 6 ft·1/4 inch·2 mm glass column with 2% Reoplex 400 on 60/80 Chromosorb GAW. The temperature program used was 100 ºC at the initial point, rising at 8 ºC min^{-1} until 148 ºC. The carrier gas was N_2 at 120 KPa. H_2 and air pressures were 120 and 150 KPa, respectively. The injector temperature was 200 ºC. The peaks present in the chromatograms were automatically integrated by means of laboratory computing integrator (Perkin Elmer LCI-100). The retention times were about 4.5 for HB, 5.6 for HV and 6.5 for benzoic acid.

The concentration of stored polymer as well as the relative amounts of HB and HV monomers were determined by using a polymer of P(HB-HV) containing 5% on weight basis of HV content (Sigma) as standard from 0 to 3000 mg L^{-1}. Since benzoic acid was used as internal standard, the determined calibration curves reported the concentration of each HA monomer as a function of the ratio of peak area of the corresponding HA monomer and that of internal standard. The obtained concentrations (in mg L^{-1}) were converted into COD in accordance with oxidation stoichiometry: 1.67 mgCOD mgHB $monomer^{-1}$ and 1.92 mgCOD mgHV $monomer^{-1}$. PHA concentration corresponded to the addition of HB and HV monomers concentrations.

3.4.3. Organic acids determination

The organic acids contained in OME were acetic, propionic, butyric, isobutyric and valeric acids. Their concentration was measured after samples filtration through syringe filters (0.45 µm porosity). Filtered samples were stored at -4 ºC prior to being analyzed. Samples of OME as well as samples were taken from both the SBR and the accumulation reactor at the specific moments were analyzed. To 1 mL of unfrozen sample were added 100 µL of 0.33 M concentrated solution of oxalic acid and 100 µL of a 2 g L^{-1} concentrated solution of acrylic acid (functioning as internal standard) to further analysis. If necessary, samples were diluted.

The analyses were performed by injection of 1 µL of the prepared sample in the gas chromatograph (DANI Master GC) equipped with a FID set to 200 ºC and a 200 cm glass

column with 2 mm in diameter with packed stationary phase Carbopack at 175 ºC. The carrier gas was He at a flow rate of 25 mL min^{-1}. The obtained chromatograms were integrated by using the computer software Clarity. The retention times (in min) were about 3 for acetic acid, 7 for propionic acid, 9 for acrylic acid, 12 for isobutyric acid, 16 for butyric acid and 38 for valeric acid.

The concentration of volatile fatty acids (VFA) in the samples were determined by preparing multi-standards containing well defined amounts of each acid (acetic acid: from 0 to 210 mg L^{-1}; propionic acid: from 0 to 100 mg L^{-1}; isobutyric acid: from 0 to 95 mg L^{-1}; butyric acid: from 0 to 193 mg L^{-1}; and valeric acid: from 0 to 94 mg L^{-1}) to determine the corresponding calibration curves. As acrylic acid was used as the internal standard, the determined calibration curves reported the concentration of each VFA as a function of the ratio of peak area of the corresponding VFA and that of acrylic acid. The obtained concentrations (in mg L^{-1}) were converted into COD in accordance with oxidation stoichiometry: 1.067 mgCOD mg $acetic^{-1}$, 1.51 mgCOD mg $propionic^{-1}$, 1.81 mgCOD mg $butyric^{-1}$, 1.81 mgCOD mg $isobutyric^{-1}$ and 2.04 mgCOD mg $valeric^{-1}$. Total VFAs concentration was calculated by the sum of the concentration of all identified acids.

3.4.4. Ammonium ion determination

The ammonium ion (NH_4^+) concentration was assayed spectrophotometrically through the direct Nessler method – available in the range from 0.4 to 4 mgN L^{-1} – (APHA 1995). The samples taken from the SBR and the accumulation reactor that were used for ammonium analysis were the same as those used for VFA analysis, but prepared according to a different procedure: to 100 µL of sample were added 5 mL of water, 100 µL of Nessler reagent and 2 drops of Seignette salt. After at least 15 min, the samples were analyzed in spectrophotometer (SHIMADZU Spectrophotometer UV-1800) at 410 nm. The Nessler reagent consists of a solution of potassium iodomercurate and reacts with the ammonia content of the sample, originating a colored complex (NH_3HgI_2), accordingly to the following reactions:

$$2(HgI_2 + KI) + 2\ NH_3 \rightarrow 2(NH_3HgI_2) + 2KI$$

$$2(NH_3HgI_2) \rightarrow NH_2Hg_2I_3 + NH_4I$$

Following the same procedure, ammonium sulfate standards with concentrations from 0 to 200 mgN L^{-1} were prepared and analyzed to determine a calibration curve which allowed for calculation of nitrogen content in each sample.

3.4.5. Total Kjeldhal Nitrogen determination

Total Kjeldhal nitrogen (TKN) consists of organic nitrogen, ammonia and ammonium ion present in the sample. Its determination involved a first degradation step in order to liberate the reduced nitrogen as ammonia sulfate by heating 1 g of the sample with 25 mL of a concentrated solution of sulfuric acid and 0.5 mL of selenic mixture at 230 ºC for 2 hours. To the cooled sample were added 40 mL of distillated water. In the subsequent step, 50 mL of a solution of boric acid (0.5%) were added to capture the ammonia by means of a distillation process. The obtained solution was diluted 20 times with water.

The TKN concentration was determined based on a calibration curve determined by preparing sodium glutamate standards (within the 3 to 16 mgN L^{-1} range) by the Nesslerization method (APHA 1995), already described (paragraph 3.4.4). Here, to 2.5 mL of previously diluted sample were added 0.5 mL of Nessler reagent and 1 drop of Seignette salt.

3.4.6. Total phosphorous determination

Total phosphorous occurs in wastewaters mainly as phosphates, which can be divided into orthophosphates, condensed phosphates and organically bound phosphates. Its determination involves a first step of conversion of the total phosphorous to dissolved orthophosphate that can be determined through colorimetric method in the subsequent step. So, to liberate the organic matter as dissolved orthophosphate, 5 mL of sample were boiled with 1 mL of sulfuric acid solution and 5 mL of nitric acid solution, until its volume was reduced to 1 mL. To the cooled sample, 20 mL of water and one drop of phenolphthalein indicator solution were added and further neutralized with sodium hydroxide to a faint pink color.

The total phosphorous determination was based on the ascorbic acid method. 2.5 mL of ammonium molybdate react in acid medium (2.5 mL of sulfuric acid) with the orthophosphate in solution (50 mL), forming the phosphomolybdic acid which is reduced to a blue colored compound by ascorbic acid (2.5 mL). To be spectrophotometrically

assayed at 650 nm, the sample was suitably diluted and dipotassium phosphate based standards were prepared (within the 1.7 and 17.8 mgP L^{-1} range).

3.4.7. Phosphate anion determination

The concentration of phosphate anion (PO_4^{3-}) was assayed by means of ionic chromatography (Dionex ICS-1000 IC equipped with AS15 column and AG14 pre-column, conductibility revelator, MMS suppressor, working at 1.2 mL min^{-1} and 1800-2000 psi and an auto-sampler *Dionex AS40* associated). The eluent composition was 4.8 mM Na_2CO_3/ 0.6 mM $NaHCO_3$. The samples were suitably diluted using deionized water. The determination was made by means of preparation of dipotassium phosphate standards within the 10 – 50 mg L^{-1} range.

3.4.8. Chemical oxygen demand determination

The chemical oxygen demand (COD) determination was carried out with COD cell tests (Merck) of two different measuring ranges, 300 – 3500 mgCOD L^{-1} (Cat. No. 114691) and 500 – 10000 mgCOD L^{-1} (Cat. No. 114555) depending on the analyzed samples. This method is based on the oxidation of the sample by a hot sulfuric acid solution of potassium dichromate, with silver sulfate as the catalyst. The originated green Cr^{3+} ions concentration is then assayed spectrophotometrically (SHIMADZU Spectrophotometer UV-1800) at 605 nm.

The determination of correspondent calibration curves, to calculate COD concentration in mg L^{-1}, was done based on glucose standards with concentrations ranging from 500 – 2000 mgCOD L^{-1} and 2000 – 8000 mgCOD L^{-1} depending on the used kit. The reactors sampling procedure was the same as that described for VFA determination.

3.4.9. Total organic carbon content determination

The total organic carbon (TOC) content in OME was analyzed using the total organic carbon analyzer (TOC-V CSN SHIMADZU). The carrier gas was oxygen, which also functioned as oxidizing agent, at a flow rate of 140 mL min^{-1} and 200 KPa of pressure. The acidifying agent was HCl (1 M). The instrument determines the TOC concentration by determining the difference between the total carbon (TC) and inorganic carbon (IC). In this context, a potassium ftalic acid solution (1000 mgC L^{-1}) was used to prepare standards for

TC calculation; whereas a solution containing Na_2CO_3 and $NaHCO_3$ (total of 1000 mgC L^{-1}) was used to determine IC concentration.

3.5. Calculation of kinetic parameters

The performance of both SBR and accumulation reactors was characterized by calculation of relevant kinetic parameters.

The PHA content in the biomass on weight basis was given by the ratio between the PHA concentration (as mg L^{-1}) and total VSS concentration (as mg L^{-1}) and expressed as percentage.

In the SBR, the storage rate (r_{PHA}) was determined as the ratio of the stored PHA (ΔX_{PHA}, as mgCOD L^{-1}) and the length of the feast phase (t, h), per unit of non-polymer biomass ($VSS_{non\text{-}polymer}$ as gCOD L^{-1}); whereas, during the accumulation stage as the ratio of the stored PHA and the duration of each accumulation assay, per unit of non-polymer biomass. In some cases, it was not possible to determine the concentration of non-polymer biomass, so the average value was taken.

$$r_{PHA} = \frac{\Delta X_{PHA}}{VSS_{non-polymer} \times t}$$

The substrate consumption rate ($-r_s$), referred to VFAs, was determined only for the kinetic tests by linear regression of acids concentrations ($-\Delta S$, mgCOD L^{-1}) vs time (h) data and was expressed as specific rates with respect to the initial biomass concentration (for the SBR) or to the achieved biomass concentration at the end of the accumulation stage. The initial substrate concentration was assumed to be the theoretical concentration value by adding the residual concentration present at the beginning of the SBR cycle to that provided by the feed. In what regards the accumulation it was considered to be the substrate provided by each pulse feed and the remaining substrate from the previous pulse.

$$-r_s = \frac{(-\Delta S)}{VSS_{non-polymer} \times t}$$

The consumption rate of nitrogen was only calculated for kinetic tests, when relevant, and it was determined by the ratio between the consumed nitrogen during either "feast" or "famine" phase and the duration of the corresponding phase (h). The consumed nitrogen was given by the difference between the concentration present at the end of the phase and that existing at the beginning. The initial nitrogen content was determined by

adding the nitrogen content that was present in the reactor before feeding to that given by feeding (OME and mineral media).

The percentage of removed COD was only determined for the SBR and it was calculated as follows:

$$\%\mathrm{COD}_{\mathrm{removed}} = \frac{\mathrm{COD}_{\mathrm{in}} - \mathrm{COD}_{\mathrm{out}}}{\mathrm{COD}_{\mathrm{in}}}$$

in which COD_{in} and COD_{out} (gCOD d^{-1}) are the daily amount in the influent and effluent streams.

The storage yield was calculated only for the kinetic tests during the feast phase, for the SBR (Y_{STO}^{SBR}), and after each feed pulse or the overall kinetic test, for the accumulation reactor (Y_{STO}^{acc}). It was given by the ratio of stored PHA and the removed substrate (-ΔS, as either soluble COD or total VFAs) at the respective moments.

$$\mathrm{Y}_{\mathrm{STO}} = \frac{\Delta \mathrm{X}_{\mathrm{PHA}}}{(-\Delta S)}$$

The growth yield (Y_{GROWTH}) was calculated by the ratio between the formed biomass (as COD) and consumed substrate (as COD_{VFA}). The consumed nitrogen was converted into formed biomass based on the general chemical composition of the biomass ($C_5H_7O_2N$) and its content in nitrogen (≈12.4 %).

$$\mathrm{Y}_{\mathrm{GROWTH}} = \frac{\Delta \mathrm{X}}{(-\Delta S)}$$

Chapter 4 | Results and discussion

4.1. Characterization of the pretreated olive oil mill effluent

In this research the production of PHAs with mixed cultures has been investigated by using OME, which are a high volume by-product of olive oil industry, as feedstock of the process. Prior to be converted into PHAs, OME were dephenolized and their polyphenolic fraction, which can be toxic to microorganisms, was recovered by means of a solid-phase extraction procedure. Moreover, after the dephenolization stage, OME were fermented to convert their organic content in VFA, which are more direct substrates for PHA production. Particularly, the OME pre-treatments were performed at University of Bologna. The provided dephenolized and fermented OME were characterized in terms of COD, VFA, nitrogen and phosphorous content (Table 7).

Table 7: Characterized parameters of the pretreated OME.

Parameter	Value	Parameter	Value
Total COD	28.03 ± 0.78 gCOD L^{-1}	Total Kjeldhal Nitrogen	0.15 g L^{-1}
Soluble COD	23.73 ± 1.07 gCOD L^{-1}	Ammonium ion	0.018 gN L^{-1}
Total VFA	14.16 ± 0.81 gCOD L^{-1}	Total phosphorous	0.3 ± 0.05 g L^{-1}
Acetic acid	5.60 ± 0.12 gCOD L^{-1}	Phosphate anion	0.2 gP L^{-1}
Propionic acid	1.92 ± 0.04 gCOD L^{-1}	COD:N:P ratio	100:0.54:1.07
Isobutyric acid	1.11 ± 0.03 gCOD L^{-1}	Total phenols	0.2 – 0.3 g L^{-1}
Butyric acid	4.84 ± 0.10 gCOD L^{-1}	TOC	11.7 g L^{-1}
Valeric acid	0.99 ± 0.14 gCOD L^{-1}	pH	6.4

The main parameters are within the range of those reported in literature, as shown in in Table 5. As exception, COD content is lower and pH is higher. This fact is not unexpected, since OME characteristics are not consistent varying with some aspects related to seasonality and cultivation. The optimal COD:N:P ratio for biomass growth during the selection stage is 100:5:1. The COD:N ratio was low (about 100:0.54) as usual for OME, being required an extra nitrogen supply through the mineral media. The COD:P ratio is 100:1.07 matching the optimal ratio. Furthermore, 59.67% of soluble COD refers to VFA

that are the main substrates for PHA synthesis indicating the potential of OME as a suitable feedstock for biopolymer production.

4.2. Assays of microbial degradation of OME

Before using the fermented OME as feedstock of the following stages of the PHA production process, respirometric tests were performed in order to evaluate the ability of microorganisms to degrade this substrate. To accomplish this objective, 200 mL of activated sludge diluted in 200 mL of mineral media (corresponding to 2357.5 mgVSS L^{-1}) were aerated in presence of OME. More in detail, initially 20 mL of OME (corresponding to 1.13 gCOD L^{-1}) were added; and subsequently, after one and two days, further additions of 20 mL, respectively were supplied. The microbial activity was evaluated by means of OUR (oxygen uptake rate) measurements. The OUR variation during each day of the test are represented in Figure 9.

The values corresponding to the negative time moments are those obtained without any external substrate supplement and represent the oxygen consumption due to the endogenous respiration of the sludge. As observed, after adding the substrate (at time 0 h) the OUR increased significantly, tending to stabilize during the remaining period of each assay. It was observed that the maximum achieved OUR increased over time, reaching a value of around 30, 130 and 170 mgO_2 L^{-1} h^{-1} during the three different days of the test, respectively. These results suggest that during the first assay (Figure 9A) the OME had an inhibiting effect on the activity of the microbial culture or that an acclimation period was required to let microorganisms degrade the substrate. During the second and third assays (on the second and third days), an abrupt upturn in OUR value was observed, after addition of the substrate (Figure 9B and C), likely due to the consumption of readily biodegradable COD (rbCOD) contained in the OME. The successive observed lower OUR peaks were probably due to the slower consumption of the residual COD present in the OME. At the end, it was possible to conclude that the sludge acclimation was effective, since the cells were assimilating the OME to metabolic activity, without significant inhibition effects to the culture.

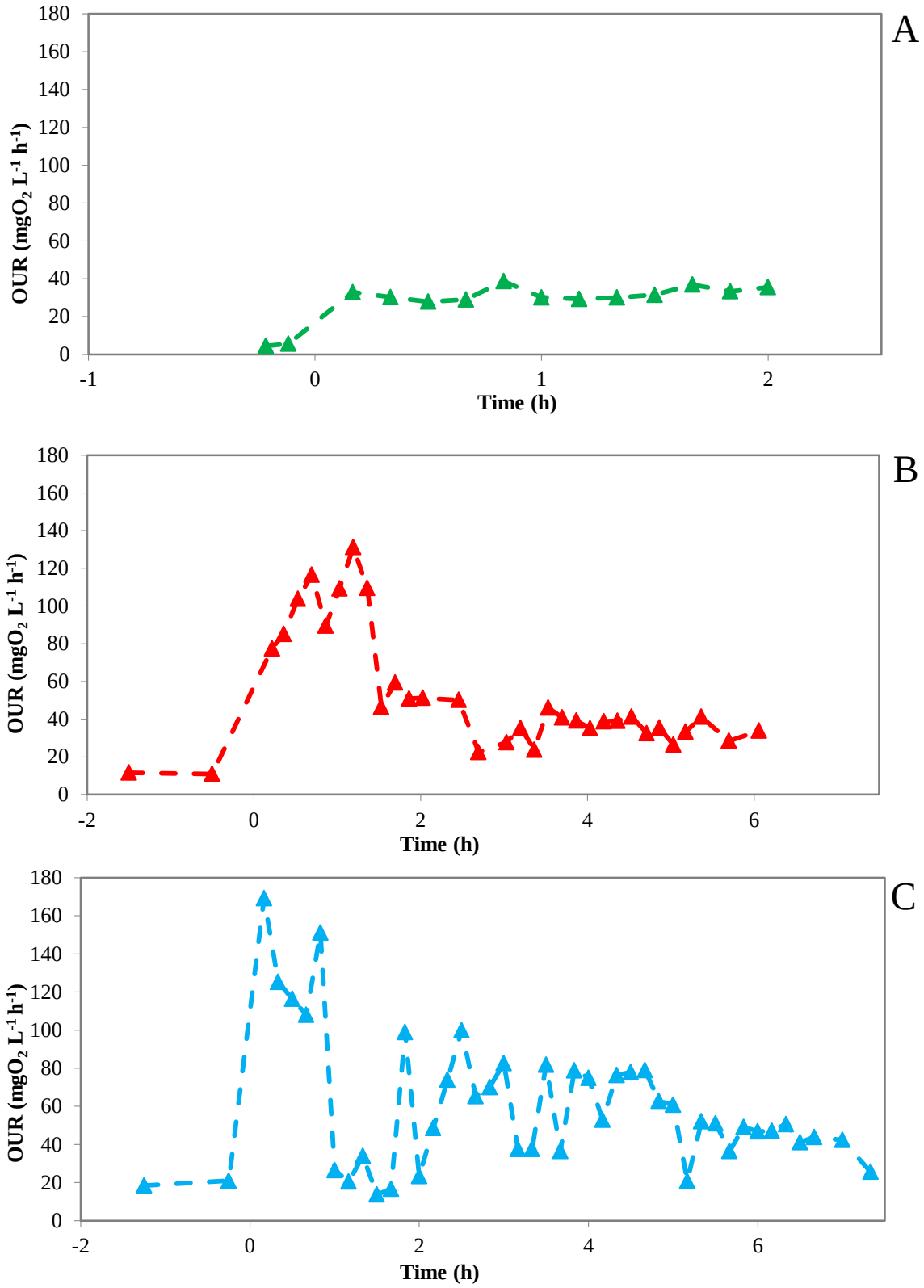


Figure 9: OUR variation during respirometric tests with activated sludge and fermented OME namely 1st day (A), 2nd day (B) and 3rd day (C).

4.3. The selection phase in Sequencing Batch Reactor

4.3.1. SBR performance

The fermented OME was fed to a SBR aimed at establishing the FF conditions required to select MMC with high storage ability. The SBR was operated at two organic OLRs, of 2.37 and 4.74 gCOD L^{-1} d^{-1} (referred to the soluble COD), changed by increasing the OME concentration, the cycle length (6 hours) being the same. At both OLRs no microbial inhibition by OME was observed.

The pH and dissolved oxygen (DO) profiles during a typical SBR cycle at the different studied OLRs are shown in Figure 10. The DO profile is indicative of substrates consumption. Indeed, as shown in the Figure 10, a sudden decrease in DO concentration was observed in correspondence to the start of the SBR feed phase (0 min), due to the increase of the metabolic activity for the presence of the external substrate. As for the lower investigated OLR (Figure 10A), a DO drop under 2 mg L^{-1} was observed, that may be considered a DO-limiting condition for aerobic processes. The reason for this fact was the insufficient oxygen supply by the used diffusers. To optimize this aspect, a second aerator was added as well as oxygen diffusers were replaced. During the run at the higher OLR (Figure 10B), the DO concentration was maintained at higher values. Once most of substrates were depleted, the DO concentration started to increase and this corresponded to the end of the "feast" phase and the beginning of the famine phase. However, it can be noticed that the DO rise was not abrupt and it partially divided in two moments, likely due to the complexity of the feedstock. More in detail, the initial rapid increase in the DO concentration was likely due to the consumption of the readily biodegradable COD (rbCOD), mainly consisting in VFAs; whereas the subsequent increase could be due to the remaining COD. For the sake of clarity, the duration of the feast phase was determined in correspondence to the depletion of the rbCOD and it accounted for about 20 minutes at both OLR runs. The change in the DO concentration slope confirms the operation under FF regime that is required for PHA-accumulating microorganisms' selection.

Besides that was observed a percentage of removed COD of around 81% during the run at the lower investigated OLR and around 83% for the higher OLR. Indeed, the reactor was treating the OME by removing its COD content at the same time that microorganisms were storing the polymer. As an example, in a previous work in which a SBR was operated

by using OME as substrate at an OLR of 8.5 gCOD L^{-1} d^{-1} the percentage of removed COD was 85 ± 1% (Beccari *et al.* 2009), indicating the potential of the proposed process, which integrate the wastewater treatment with PHA production by using OME.

The pH was always maintained at the optimum value, between 7.5 and 7.6. As already described in materials and methods (chapter 3), at the beginning of the SBR operation, the withdrawal of the mixed liquor was imposed in proximity at the end of the SBR cycle (at 350 min – withdrawal 1). This option was supported by the complexity of the substrate and unexpected culture response to it. After feast phase stabilization (at the 46th operation day), the withdrawal was anticipated and imposed at 60 min (withdrawal 2) which is after the depletion of the rbCOD (≈ 20 min in both runs) in order to maximize PHA accumulation in the accumulation stage.

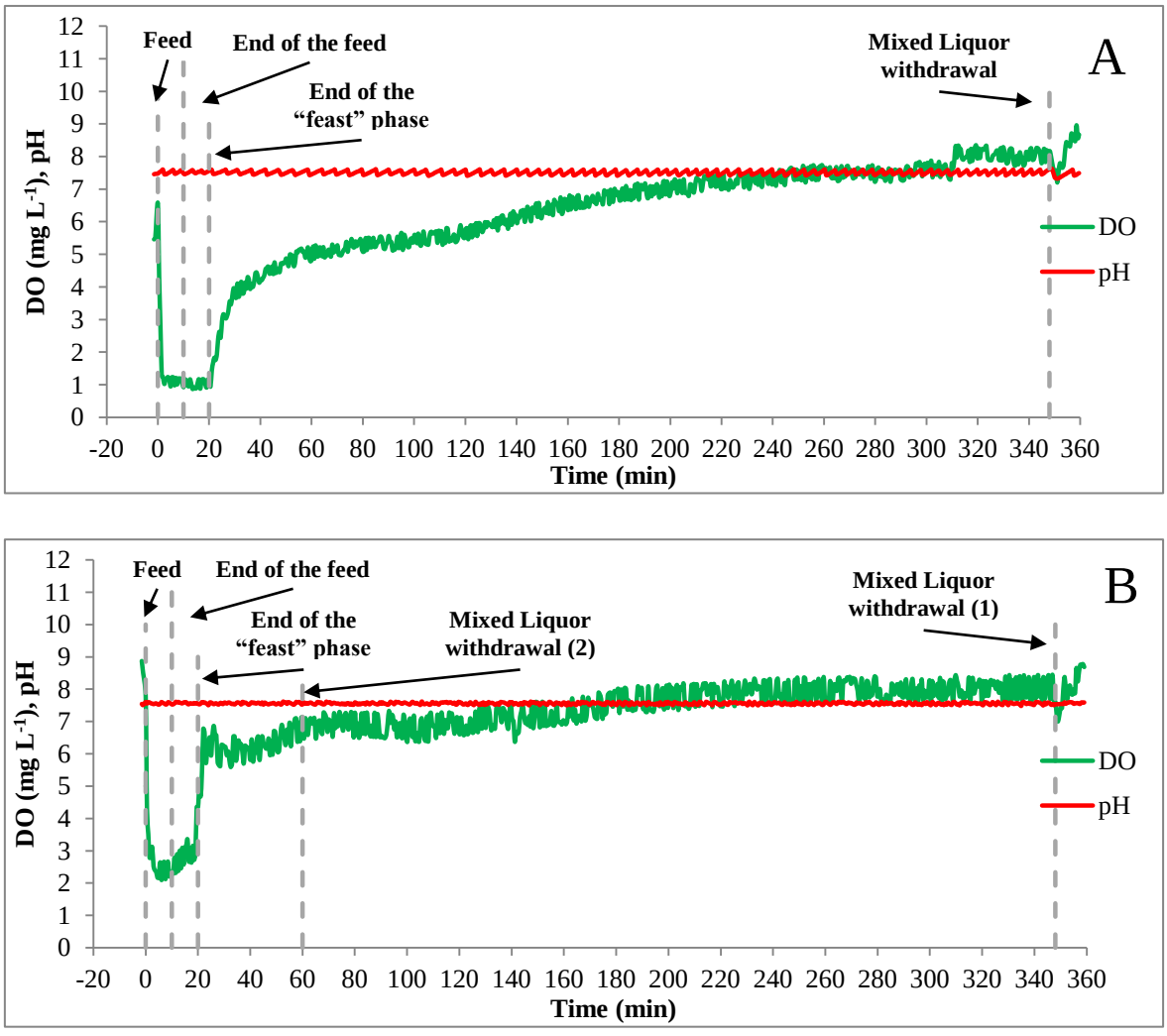


Figure 10: Profiles of DO and pH during a typical SBR cycle at an OLR of 2.37 gCOD L^{-1} d^{-1} (A) and 4.74 gCOD L^{-1} d^{-1} (B), respectively.

The length of the feast phase, based on daily average values, during the entire SBR performance period is reported in Figure 11. During the first operation days, at 2.37 gCOD L^{-1} d^{-1}, its value was fluctuant (between 50 and 140 min) likely due to the acclimation of the microbial culture to the operating conditions. In correspondence to the 9^{th} operation day, the feast phase duration started to stabilize at an average value of 26.2 ± 1.6 min, which corresponded to an average F/F ratio of 0.08 ± 0.01. When the OLR was doubled, after 22 days of operation, new operating conditions were imposed to the microbial culture and thus a new acclimation period occurred. Indeed, the feast phase length destabilized again reaching values up to 230 min. However, after 7 days of operation at 4.74 gCOD L^{-1} d^{-1}, the value stabilized again at around 22.1 ± 1.9 min, corresponding to an average F/F ratio of 0.07 ± 0.01. Some fluctuations were observed over the process operation, due to possible fluctuations of biological processes. Even though in literature is reported that higher OLRs induce higher F/F ratios (Reis *et al.* 2011), in this study the F/F ratio did not significantly change by varying the OLR and this could be due to the fact that the length of the feast phase was referred to the rbCOD. As an example, Dionisi and coauthors (Dionisi *et al.* 2004), with a mixture of synthetic organic acids obtained F/F ratios in the range 0.1 and 1.15, for OLRs between 8.5 and 31.25 gCOD L^{-1} d^{-1}. The authors concluded that for lower OLR (F/F ratio up to 0.26) the storage response was high. In this work the tested OLRs were lower as well as the calculated F/F ratios (which were close to 0.1), suggesting that the OLRs used in the present study were suitable for culture selection.

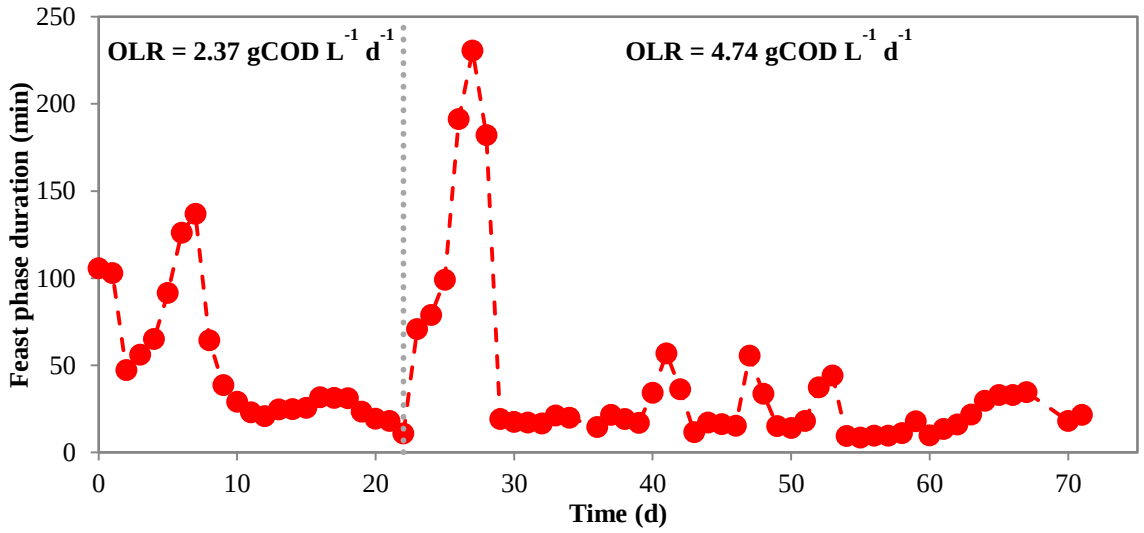


Figure 11: Average daily value of the feast phase duration over the SBR operation period.

Figure 12A shows the trend of both the total and volatile suspended solids (TSS and VSS, respectively) at the end the cycle during the first 46 days of SBR operation. In correspondence to the inoculum of the reactor, the TSS and VSS concentrations in the activated sludge were 5350 mg L^{-1} and 3690 mg L^{-1}, respectively. During the 1st operation day, those concentrations declined abruptly (to 2340 mg L^{-1} and 1910 mg L^{-1}). After this initial drop, the TSS and VSS concentrations remained approximately constant until the end of the first SBR run, which was performed at the lower OLR, with an average value of 1255 ± 47 mgTSS L^{-1} and 1197 ± 48 mgVSS L^{-1}. As expected, when OLR was doubled, also the suspended solids concentration increased about 2 times reaching an average value of 2607 ± 96 mgTSS L^{-1} and 2430 ± 77 mgVSS L^{-1}. This finding is in agreement with the very similar values of the duration of the feast phase at the different OLRs (Figure 11), since at the higher substrates concentration corresponds a higher biomass concentration (in terms of VSS).

In Figure 12B is reported the concentration of the non-polymer VSS (which refers to the active biomass) during the same operation period. A similar trend to that obtained for TSS and VSS was observed. At the end of the cycle the medium active biomass concentration was 1155 ± 47 mg L^{-1} (corresponding to 1640 ± 86 mgCOD L^{-1}) at the lower OLR, while 2401 ± 83 mg L^{-1} (corresponding to 3409 ± 133 mgCOD L^{-1}) was achieved for the higher OLR.

Another important parameter for the SBR performance characterization is the VSS/TSS ratio as it indicates the biomass content in the mixed liquor (Figure 12C). In this context, higher the ratio higher the biomass concentration within the mixed liquor what is favorable to the process. As shown in figure 14C, during the first 7 days of operation, the VSS/TSS ratio value increased substantially until 0.98, then stabilizing at an average value of around 0.94. When OLR was changed (day 22), the biomass passed through a new period of acclimatization that was shown by the VSS/TSS ratio reduction (until around 0.87). From the 28th day it stabilized again at around 0.93 demonstrating that almost the totality of SS in the reactor was VSS.

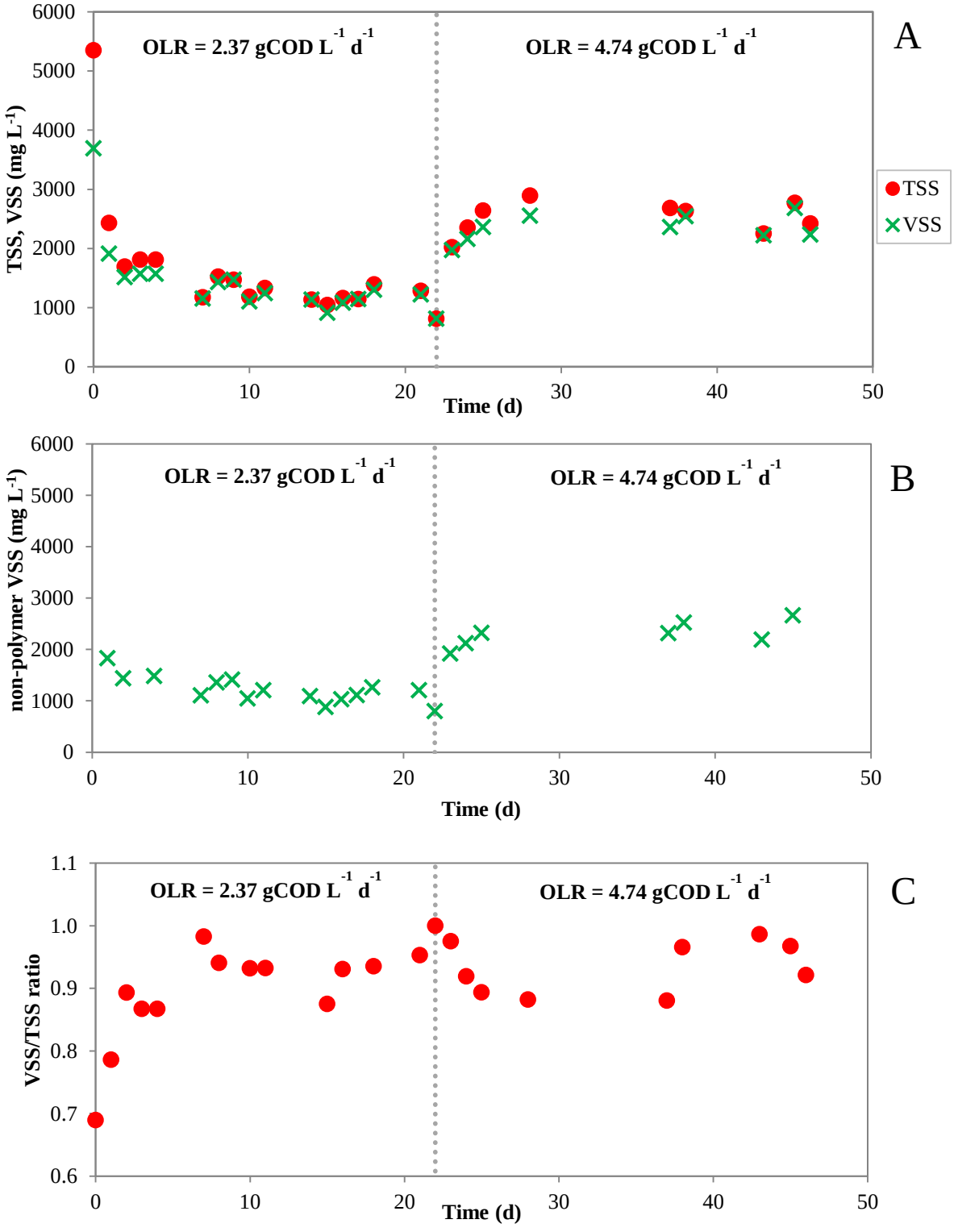


Figure 12: TSS and VSS concentration (A), non-polymer VSS concentration (B), and VSS/TSS ratio (C) during the first 45 days of SBR operation period. All parameters refer to the end of the SBR cycle.

At the higher investigated OLR, on 46th day of operation, the withdrawal of the mixed liquor was shifted from the end of the cycle to one hour after the beginning of the

SBR feeding (which also corresponded to the beginning of the cycle); in order to study the differences of the operating parameters at that moment and the end of feast phase (22.1 ± 1.9 min). The results obtained in terms of SS concentration (between 46th and 73rd operation days) are reported in Table 8.

At the end of the feast phase the average values for TSS and VSS (2380 ± 123 mgTSS L^{-1} and 2218 ± 119 mgVSS L^{-1}) were similar to those obtained after 1 hour from the beginning of the SBR feeding (2186 ± 161 mgTSS L^{-1} and 2108 ± 167 mgVSS L^{-1}). The obtained medium value for non-polymer VSS concentration was 2136 ± 101 mg L^{-1} at the end of the feast phase and was higher when compared with 1835 ± 133 mg L^{-1} obtained after 1 hour of SBR feed. This behavior reflects that after the 1st hour from the feed, PHA content is still high whereas most of the microbial growth occurs during the famine phase. In what regards VSS/TSS they presented a regular trend achieving values within the range between 0.93 – 0.96.

Table 8: Average values of suspended solids concentrations at two different times of the SBR cycle, operated at OLR = 4.74 gCOD L^{-1} d^{-1}.

OLR = 4.74 gCOD L^{-1} d^{-1} Operation period between 46th and 73rd day				
	TSS (mg L^{-1})	**VSS (mg L^{-1})**	**Non-polymer VSS (mg L^{-1})**	**VSS/TSS ratio**
End of the feast phase	2380 ± 123	2218 ± 119	2136 ± 101	0.93
1 hour after SBR feed	2186 ± 161	2108 ± 167	1835 ± 133	0.96

In Figure 13A is reported the trend of PHA concentration (in terms of COD) during the entire SBR operation period. It is worth noting that PHAs were always higher at the end of the feast phase than at the end of the SBR cycle, clearly indicating the ability of the FF conditions to select MMCs able to store PHAs. On the other hand, as expected, very similar values of PHA concentration were obtained at the end of the feast phase (732 ± 176 mgCOD L^{-1}) and after 1 h from the beginning of the SBR cycle (712 ± 152 mgCOD L^{-1}), since there was not time enough to observe a considerable consumption of the produced polymer between these two moments of the cycle.

The variation of the content of HV monomer in the stored polymer throughout the operation period is represented in Figure 13B. As already explained this is an important parameter since it significantly affects polymer properties. During the first run (at OLR of 2.37 gCOD L^{-1} d^{-1}), no significant differences were observed in terms of HV content in the

polymer at both end of the cycle (10%) and end of the feast phase (14%). Throughout the second run, it could be observed a similar trend of HV content, but revealing more considerable fluctuations along the operation. At the end of the cycle, the HV content presented a medium value of 13% and at 1 hour after feeding 15%, while at substrate depletion moment, the HV medium content was 20%, which was slightly higher.

The PHA content in the biomass along the SBR operation is reported in Figure 13C. During the first run, the PHA content in the biomass at the end of the cycle was constant presenting an average value of 4%. During the second run, from the 22nd until 46th day of work, the PHA content in the biomass at the end of the cycle was 1.3%, while at the end of the feast phase that value was higher, on average 14%. However, during the last period of the SBR operation, the obtained values for PHA content in the biomass obtained at the end of the feast phase were significantly higher, up to around 40% (w/w).

From the 46th operation day, it was measured the intracellular polymer content also in correspondence to 1 hour from the beginning of the SBR feeding (18%). The differences observed between these different moments of the cycle (end of the feast phase and 1 hour after feeding), suggests that the strategy to anticipate the withdrawal of the mixed culture from SBR allows recovering high amounts of polymer in the following accumulation stage.

As already discussed, for the obtained non-polymer VSS values at the end of the cycle and at the end of the feast phase no significant differences were observed. So, the PHA storage rate was calculated depending on the sampled moments for non-polymer VSS and assumed that it does not influence the determined rate. The overall operation results were used to determine the average value. The average obtained storage rate at the lower investigated OLR was 192 ± 42 mgCOD gCOD h^{-1}, while its average value at the higher studied OLR was 155 ± 39 mgCOD $gCOD^{-1}$ h^{-1}. As an example, Beccari and coworkers compared the production of PHA by a culture enriched at 8.5 gCOD L^{-1} d^{-1} using synthetic mixture of organic acids and fermented OME (Beccari *et al.* 2009). They obtained a storage rate of 146 ± 12 mgCOD $gCOD^{-1}$ h^{-1} that was really close to that achieved with the synthetic mixture. These results indicate that the OME as well as OLR used in this study were suitable for the selection of a culture with high storage ability.

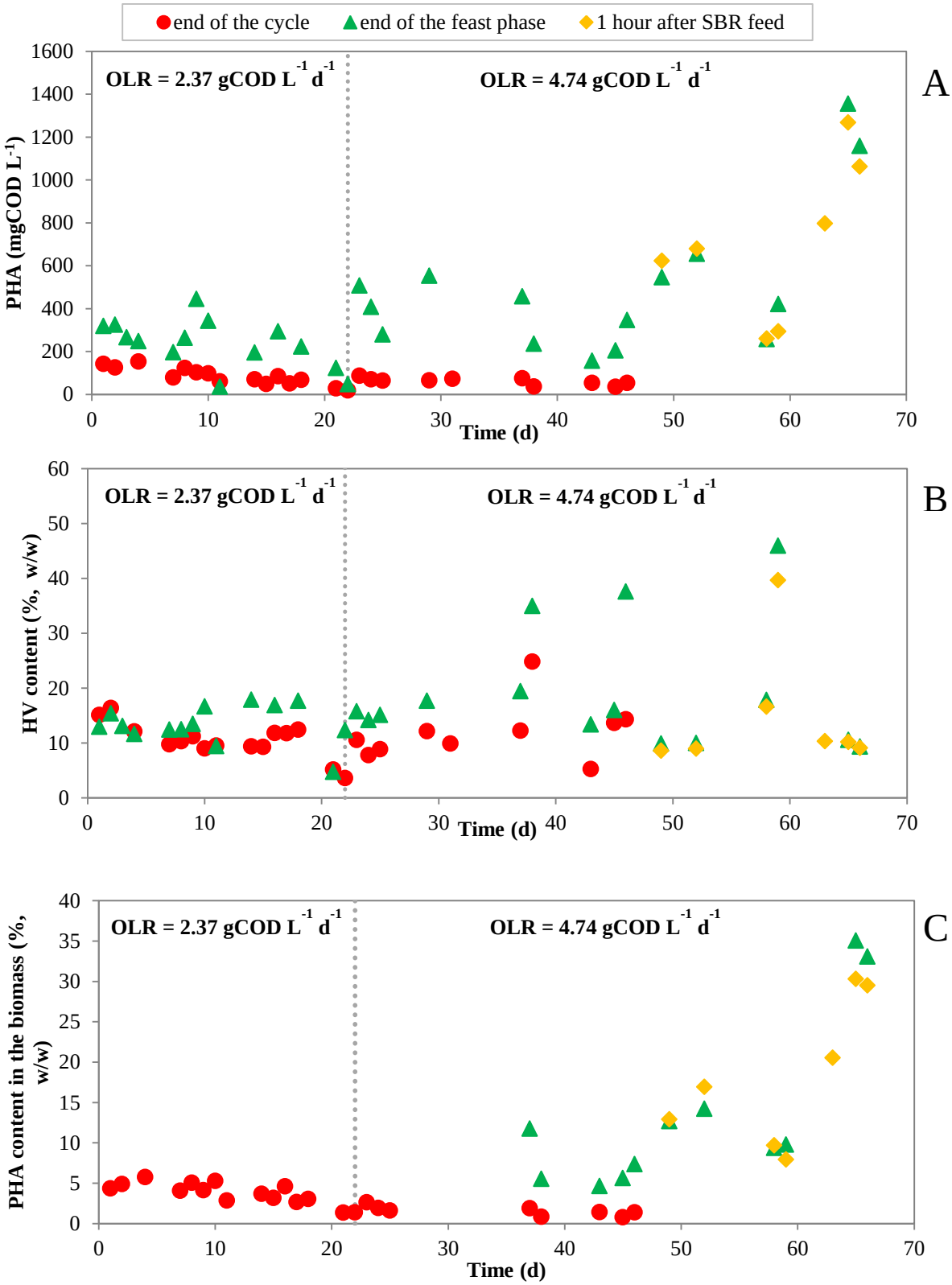


Figure 13: PHA concentration (A), HV content in the polymer (B) and PHA content in the biomass (C) at different moments of the SBR cycle, during the entire period of operation.

In Figure 14 is reported the trend of ammonium concentration during SBR performance. Generally, at the end of the feast phase the ammonium concentration was slightly higher than that at the end of the corresponding cycle, because during the feast phase, microorganisms' storage response is dominant, while in the famine phase they might growth consuming the available nutrients present in the medium. When OLR was doubled (22nd operation day), the concentration of ammonium in the reactor decreased. This was due to the higher request of nutrients for microbial growth, in agreement with the higher values of VSS observed when the SBR was operated at 4.74 gCOD L^{-1} d^{-1} (as previously discussed) (Figure 12A). On the 63rd operation day, the decrease of nitrogen content in the reactor was due to a change of the nitrogen concentration in the mineral medium supplied to dilute OMEs. Unfortunately, N data at the end of the cycle are lacking. However, it is not possible to exclude that N was fully depleted at the end of the cycle. As an hypothesis, a N lack could be the cause of progressive accumulation of PHA in the SBR (Figure 13A).

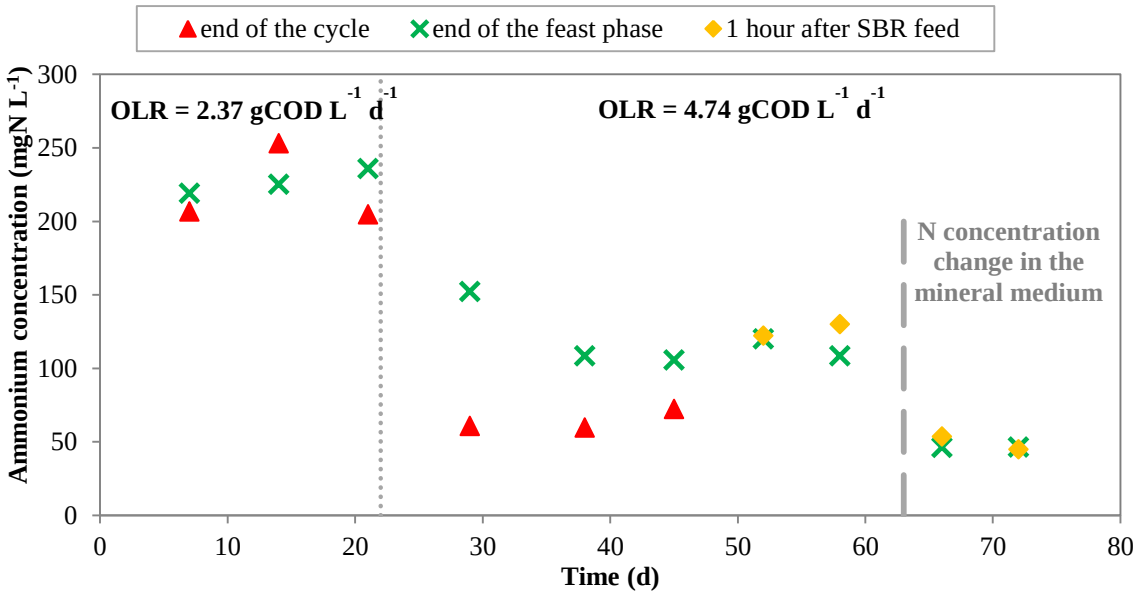


Figure 14: Trend of the ammonium concentration throughout the SBR performance.

4.3.2. Kinetic tests

4.3.2.1. SBR run at 2.37 gCOD L^{-1} d^{-1}

In order to deeply understand the performance of the SBR during a typical cycle, two kinetic tests were performed at each investigated OLR. The results of one kinetic carried out at 2.37 gCOD L^{-1} d^{-1} are discussed in this paragraph.

Figure 15 shows the profiles of DO, PHA and VFA concentration during the entire SBR cycle. From the DO profile, it can be observed that the length of the feast phase (in this specific cycle) was 27 minutes, and this most likely corresponded to the microbial consumption of the readily biodegradable COD (e.g. VFAs) contained in OMEs. Indeed, the slight increase of DO concentration during the remained of the cycle suggests a further microbial consumption of residual COD. As for polymer production, the PHA concentration rapidly started to increase during the reactor feeding, achieving its maximum (450 mgCOD L^{-1}) in correspondence to the end of the feast phase. This was in agreement with the complete consumption of total VFAs, which were converted into PHA by microorganisms during that phase. Therefore, during the famine phase no VFAs were present in the medium whereas the PHA concentration decreased to around 90 mgCOD L^{-1} at the end of the cycle. This fact was a consequence of carbon substrate exhaustion in the medium, so microorganisms started to consume the polymer as it functions as internal carbon and energy reserve during starvation periods.

During the feast phase (duration of 35 min), the determined storage rate was 301.27 mgCOD gCOD $^{-1}$ h^{-1}. This result indicates the ability of the selected culture to store the polymer. The storage yields were 0.71 (COD COD^{-1}) and 0.89 (COD COD_{VFA}^{-1}), indicating the OME as a suitable feedstock for PHA production by MMC. Indeed, 89% of COD content due to VFA concentration in the OME was converted into PHA, while a lower fraction of the overall soluble COD was consumed to produce the polymer. From these results, it was possible to confirm the preferable status of the VFAs as substrates for PHA production and, even if VFAs were only a fraction (59.67%) of the soluble COD of the fermented OME, the maintenance of a selective pressure in the SBR towards the enrichment of MMCs able to store PHAs.

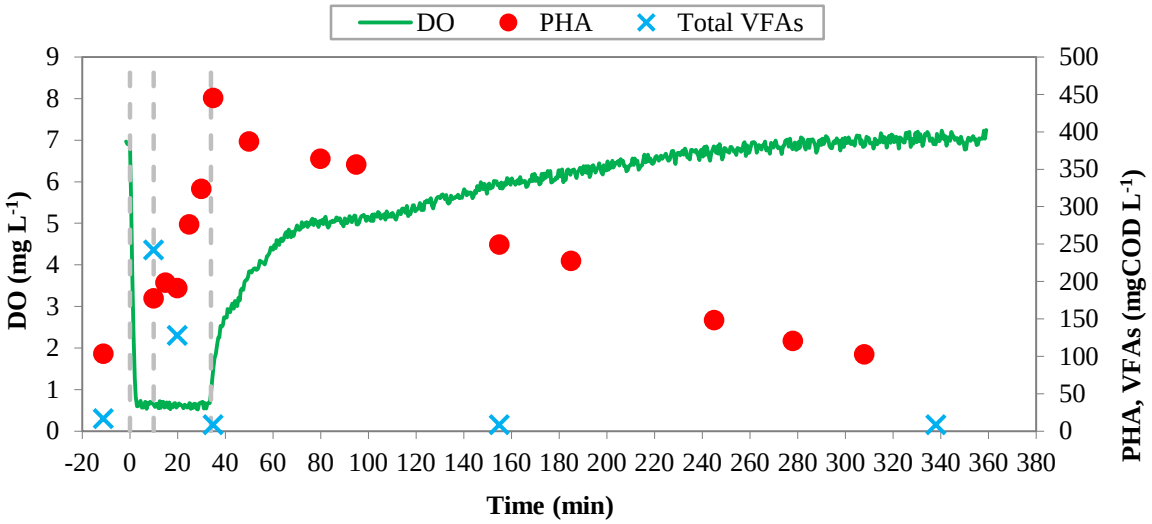


Figure 15: Profiles of DO, PHA and total VFAs concentration during a SBR kinetic carried out when the reactor was operated at 2.37 gCOD L^{-1} d^{-1}.

The variation of HV content in the polymer, which is represented in Figure 16, rapidly increased during the feast phase, from around 11% (w/w) to about 14% (w/w) after 25 min of the reactor feeding. During the famine phase, its value slightly decreased to around 12% (w/w), likely due to its consumption by the microbial culture.

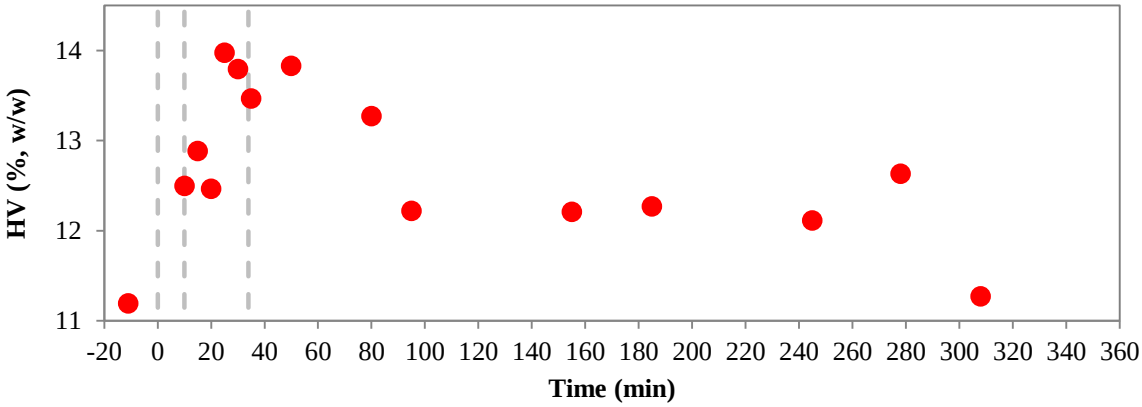


Figure 16: Profile of HV content in the polymer throughout a SBR kinetic carried out at 2.37 gCOD L^{-1} d^{-1}.

More in detail, the variation of each VFA concentration is presented in Figure 17A. The trend presented by each acid was the same as that described for total VFAs, being completely exhausted until the end of the feast phase. Acetic and butyric acids were the more abundant acids in the substrate, while valeric acid, the less abundant, was the first to exhaust.

These results are in accordance with the calculated substrate consumption rates during the feast phase (Figure 17B), assuming a theoretical initial VFA concentration

(obtained by adding to determined VFA concentration present in the feeding solution and that present before feeding the reactor). It was possible to notice that acetic and butyric acids were consumed at the higher rates (122 and 104 mgCOD $gCOD^{-1}$ h^{-1}) followed by valeric, propionic and isobutyric acids (72, 37 and 24 mgCOD $gCOD^{-1}$ h^{-1}). Indeed, is reported that acetic and butyric acids are channeled in a different metabolic pathway than that of propionic and valeric acids (Dionisi *et al.* 2004). Furthermore, HV content started to decrease in correspondence with the exhaustion of both valeric and propionic acids. As observed in Figure 17B, isobutyric acid was the last VFA started to being consumed by the microorganisms, likely due to the greater abundance in the OME and/or more suitableness as substrates for HB production of acetic and butyric acids.

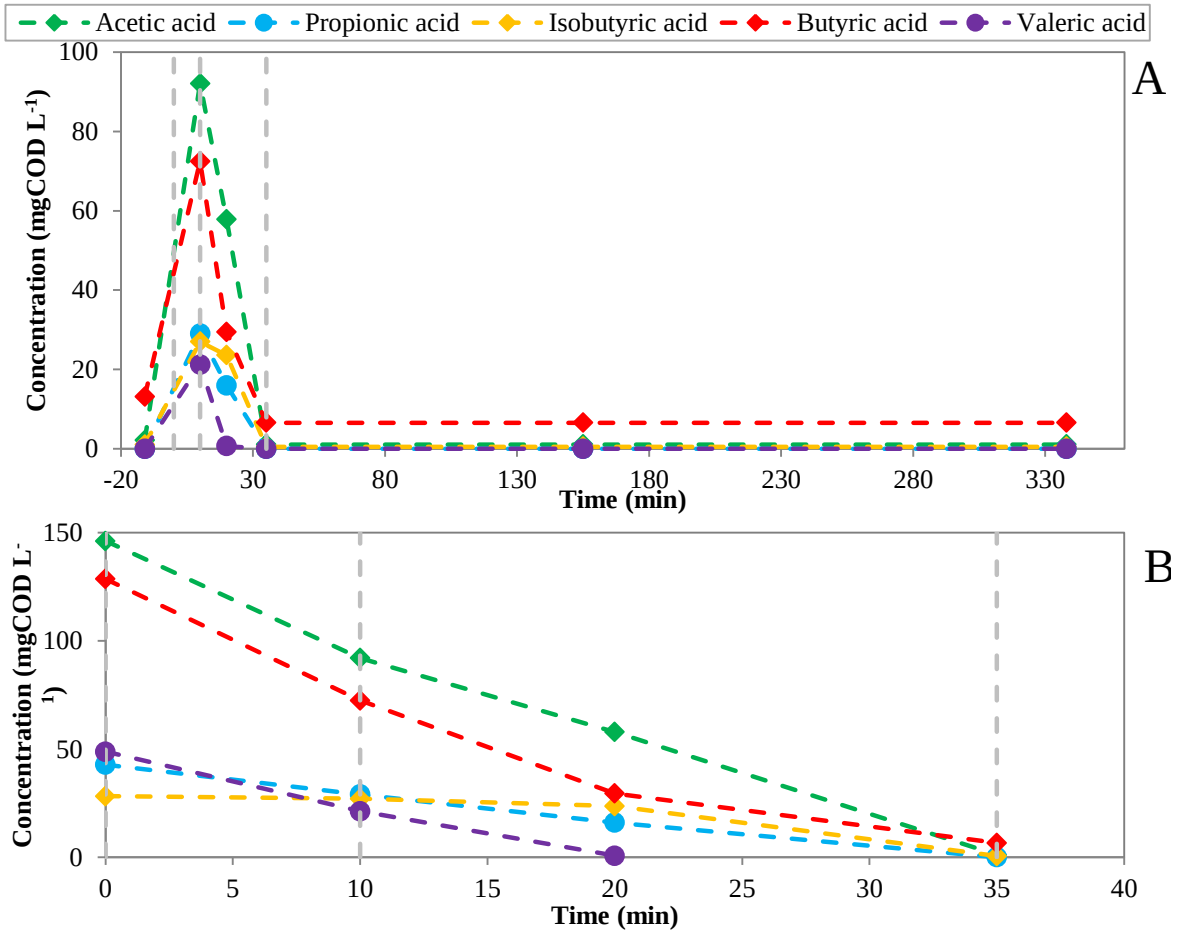


Figure 17: Trend of each acid concentration (A) and of VFA consumption during the feast phase (B) in a SBR kinetic carried out at 2.37 gCOD L^{-1} d^{-1}.

Finally, Figure 18 shows the variation of nitrogen content during the SBR kinetic. This represents an important parameter to the SBR performance since nitrogen is an

essential nutrient for microbial growth. As shown in the graph, the ammonium concentration was higher during the feast phase, achieving a maximum concentration of 245 mgN L^{-1} at 15 min, and it slightly decreased (down to about 180 mgN L^{-1}) along the remaining cycle, giving a further indication of the occurrence of microbial growth during the famine phase by using the stored PHAs as internal carbon source.

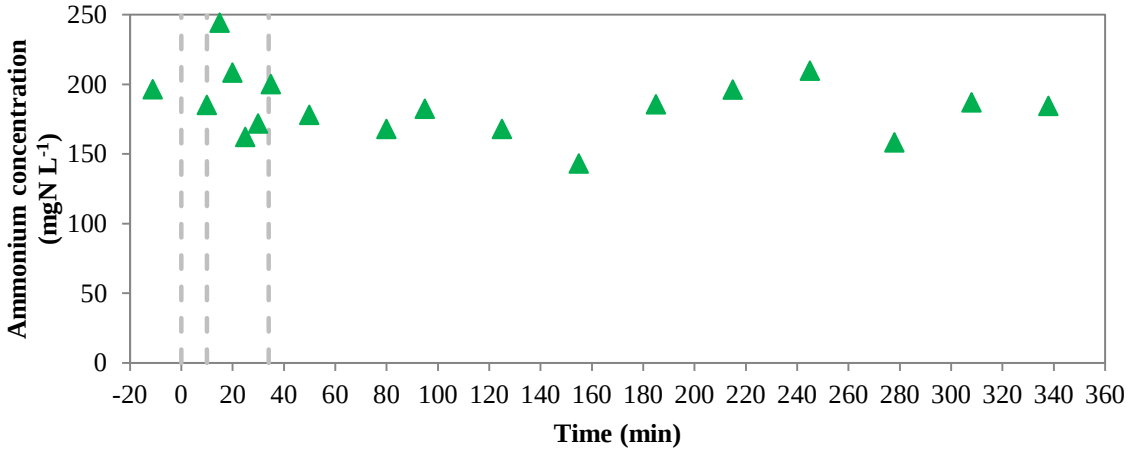


Figure 18: Trend of ammonium concentration during a SBR kinetic carried out at 2.37 gCOD L^{-1} d^{-1}.

4.3.2.2. SBR run at 4.74 gCOD L^{-1} d^{-1}

As previously explained, two kinetic assays were also performed during the SBR run at the higher investigated OLR. The results of one kinetic carried out at 4.74 gCOD L^{-1} d^{-1} are hereafter discussed.

The trends of DO, VFA, and PHA concentration during the SBR kinetic are reported in Figure 19 and were similar to those observed for the first SBR run. As already described, at this day, the culture was already acclimated since the daily average of feast phase duration started to stabilize (Figure 11). Particularly, in the present cycle it was 18 minutes, probably due to the consumption of the readily biodegradable COD content in the OME by the microbial culture. During the remaining cycle, it was observed a slight increase of DO concentration, suggesting that the culture was consuming the residual COD present in the reactor. In what regards PHA production, polymer concentration started to increase as soon as the reactor was feeding, achieving its maximum (550 mgCOD L^{-1}) at the end of the feast phase, while VFAs were being consumed. During the famine phase, VFAs were present in the medium in lower concentrations, being completely depleted at around 150 min after reactor feeding. As a consequence of this lack of carbon substrate in

the medium, PHA concentration decreased achieving 90 mgCOD L^{-1} due to its consumption as internal source of carbon and energy by microbial cells.

During the present SBR cycle, the polymer storage rate was calculated based on an average value of non-polymer VSS, since no sample was taken for VSS analysis. In this way, the obtained value was 466 mgCOD $gCOD^{-1}$ h^{-1} that was much higher than the average value obtained for the overall SBR run (155 ± 39 mgCOD $gCOD^{-1}$ h^{-1}) that can be explained by the typical observable fluctuations when operating with MMCs and real substrates. The storage yields were 0.52 (COD COD^{-1}) and 0.69 (COD COD_{VFA}^{-1}) that indicate a more direct conversion of VFA towards PHA production. Curiously, the increase in the OLR had a negative influence on the determined storage yields (0.71 (COD COD^{-1}) and 0.89 (COD COD_{VFA}^{-1}) obtained for the lower investigated OLR). This might be due to the higher content of removed COD when OLR increases, since produced PHA concentration didn't vary significantly. In agreement, a previous work (Beccari *et al.* 2009), revealed that the obtained storage yield was 0.36 (COD COD^{-1}) at 8.5 gCOD L^{-1} d^{-1} by using fermented OMEs.

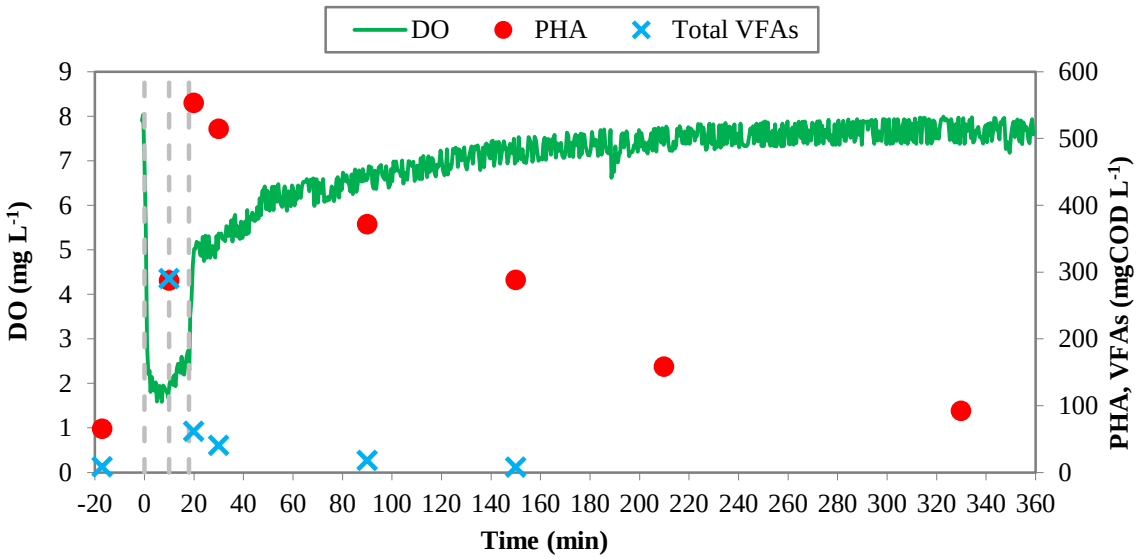


Figure 19: Profiles of DO, PHA and total VFAs concentration during a SBR kinetic carried out at 4.74 gCOD L^{-1} d^{-1}.

The variation of HV content is represented in Figure 20. During the feast phase, it increased, from 12% (w/w) until reach a maximum content of almost 18% (w/w). During the famine phase, HV content decreased down to 11% (w/w) at 210 min after the beginning of the SBR cycle. At the end of the cycle, the polymer had an HV content of

about 15% (w/w), suggesting that the microorganisms from this specific moment stopped to consume HV as internal carbon source.

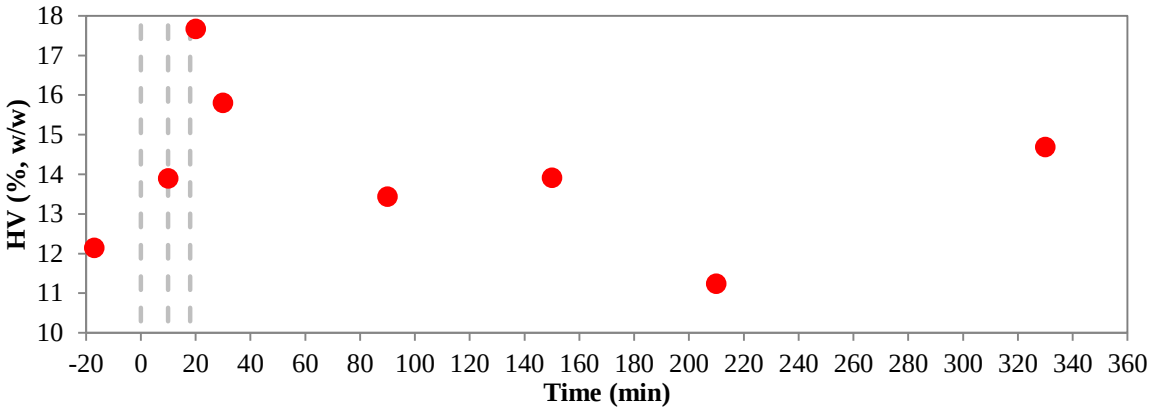


Figure 20: Profile of HV content in the polymer throughout a SBR kinetic carried out at 4.74 gCOD L^{-1} d^{-1}.

In Figure 21A are detailed the profiles of each VFA concentration until the end of the feast phase. Each acid presented a trend that was the same of that described for total VFAs. Butyric acid was not completely consumed during the feast phase remaining part of the residual COD content in the OME, suggesting that it may contribute for the two moments of oxygen increase observed in the DO profile (Figure 19).

As showed by Figure 21B, a similar trend of that obtained for the acids consumption rate at the lower studied OLR was observed during the feast phase of this cycle (Figure 17B). It is worth noting the effectiveness of microbial culture acclimation when operating at the higher investigated OLR, since the consumption rate of almost VFAs (excepting for valeric acid) increased with higher initial substrate concentration. Acetic and butyric acids were the fastest VFAs to be consumed (266 and 184 mgCOD $gCOD^{-1}$ h^{-1}, respectively), followed by propionic, valeric and isobutyric acids (79, 71 and 48 mgCOD $gCOD^{-1}$ h^{-1}). Furthermore, a great acclimation of the culture was observed when operating at the higher investigated OLR, since the consumption rate of almost VFAs (excepting for valeric acid) increased with higher initial substrate concentration. The obtained results are related with HV content in the produced polymer, since it increases during the feast phase until complete exhaustion of valeric and propionic acids. Once more, isobutyric acid was consumed at the lowest rate suggesting that its ramified structure gives a more preferable status to both acetic and butyric acids present in the OME to be converted into HB monomer by the microbial culture.

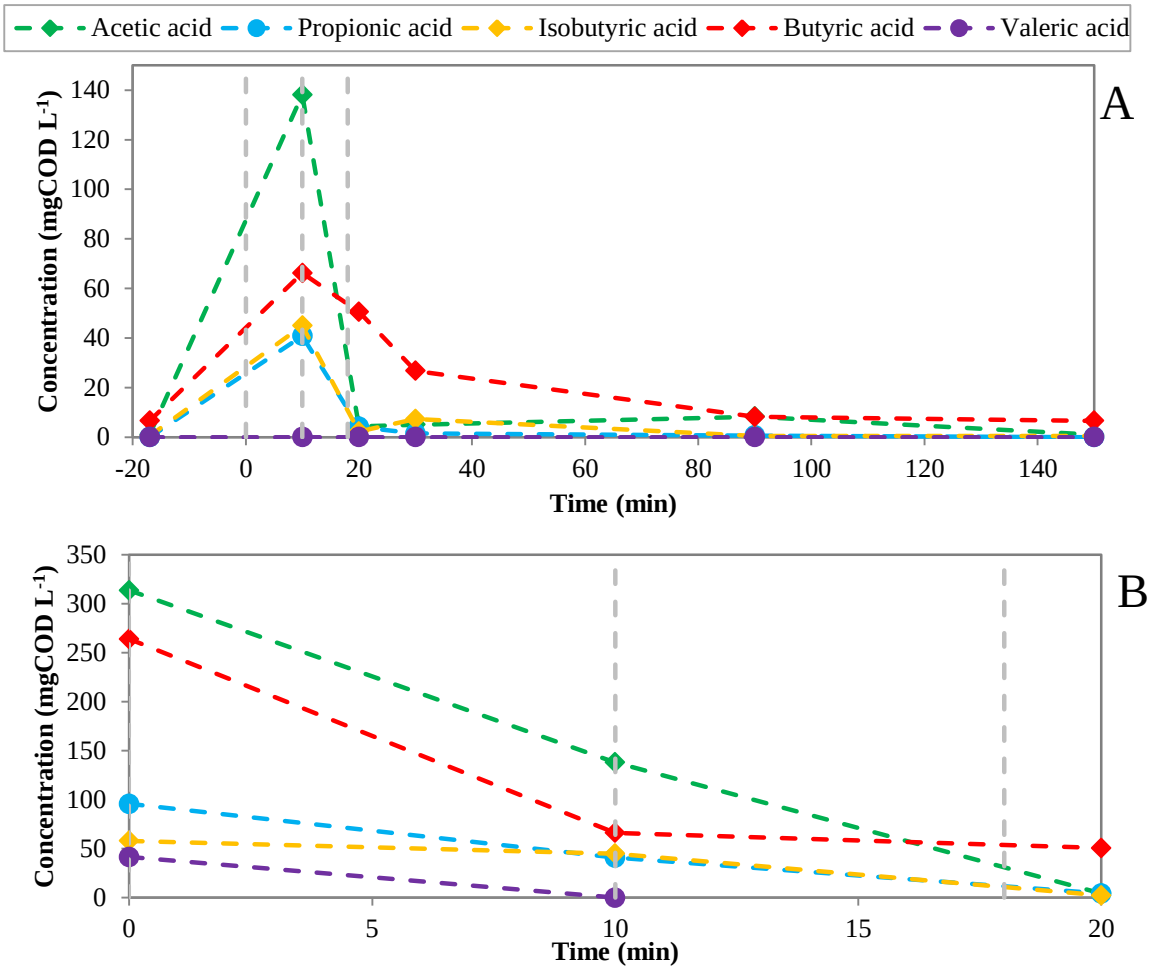


Figure 21: Trend of each acid (A) and of VFA consumption during the feast phase (B) throughout a SBR kinetic carried out at 4.74 gCOD L^{-1} d^{-1}.

Finally, Figure 22 represents the content of nitrogen in the reactor throughout the SBR kinetic assay, which is an important parameter for microbial growth during the selection stage. As shown in the graph, nitrogen concentration increased during the feast phase (from 55 to 99 mgN L^{-1}) achieving almost 140 mgN L^{-1} about 10 min after the end of the feast phase, suggesting that until that moment no or little nitrogen was consumed. In fact, the N fed to the reactor was consumed at a rate close to 0 mgN L^{-1} h^{-1} during this phase. Evidences of its consumption by microbial cells appeared during the remaining famine phase in which the nitrogen was consumed at a rate of 24 mgN L^{-1} (between 20th and 210th minutes of the kinetic test), achieving a constant value (around 60 mgN L^{-1}). These results indicate the occurrence of microbial growth during the famine phase. From the 210th min of the kinetic test, no N uptake was observed along with any PHA production (Figure 19), suggesting that at this time the SBR was not working effectively any more.

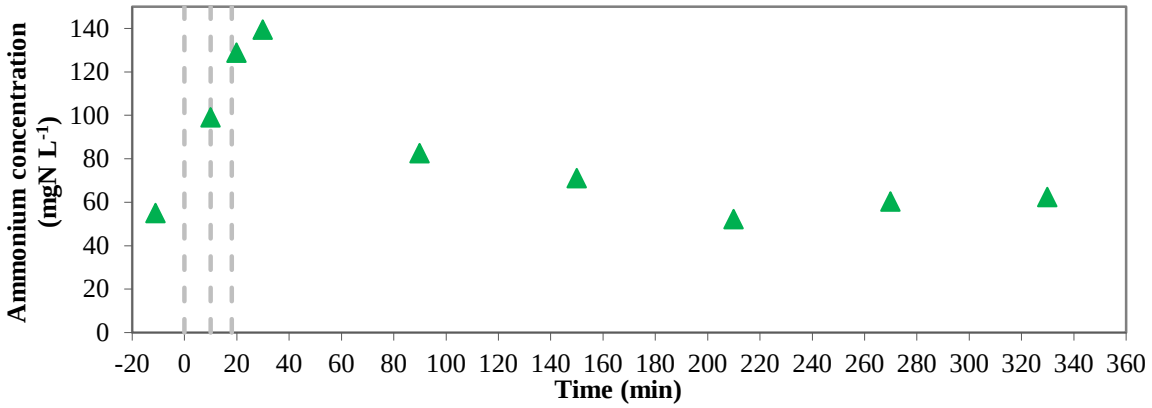


Figure 22: Trend of ammonium concentration trend during a SBR kinetic assay carried out at 4.74 gCOD L^{-1} d^{-1}.

4.4. PHA accumulation stage

4.4.1. Accumulation stage performance

The PHA accumulation stage was performed by automatically sending the culture selected in the SBR to a lab-scale reactor fed with successive pulses of undiluted OME – pulse feeding regime. More in detail, the accumulation lasted 6 hours and consisted of 250 mL of sludge from the SBR and four pulses of substrate, each one accounting for 25 mL of OMEs (corresponding to 6.78 gCOD L^{-1}). This strategy was adopted to overcome potential inhibition effects of undiluted OME on the selected culture during the accumulation stage. In correspondence to the 63rd operation day of the SBR, the periodicity of feeding was changed to 5 pulses, accounting for a final concentration of 7.91 gCOD L^{-1}. Due to practical limitations, the PHA accumulation stage was only performed once or twice during working days, whereas was omitted during night and weekend.

The operation of the PHA accumulation reactor started in correspondence to the 30th day of SBR operation, when the selection stage was already working at the higher investigated OLR. The performance of the accumulation stage was evaluated in terms of polymer and suspended solids production. As for PHA production, a significant increase in the concentration from the SBR to the accumulation reactor was observed from the beginning of the accumulation stage operation and the maximum reached value was 4350 mgCOD L^{-1} (Figure 23A). In spite of PHA concentration in the accumulation reactor was fluctuant, on average the PHA accounted for of 2365 ± 288 mgCOD L^{-1}, which is substantially higher than the average value reached in the SBR (732 ± 176 mgCOD L^{-1}).

These results indicate that the pulse feeding strategy adopted in the accumulation reactor was an effective tool to increase PHA concentration. In a previous study, Beccari and coworkers simulated the accumulation stage by feeding the SBR with higher amounts of substrate and obtained a PHA maximum concentration of about 1620 mgCOD L^{-1} by using fermented OMEs as substrate (Beccari *et al.* 2009).

During the last days of operation (from the 59th day referred to the SBR operation), the performance of the accumulation stage in terms of produced polymer started to decrease according to an excessive polymer accumulation in the SBR (as discussed in paragraph 4.3.1, Figure 13A). As a hypothesis, the reduction of N concentration (on the 63rd operation day, Figure 14) probably contributed for the decrease in the efficiency of the process. Accordingly, in a recent study, was reported the decrease of the storage capacity in SBR operating under N-limiting conditions, which indicates the importance of N availability during the selection stage and, consequently, for the success of the overall process (Johnson *et al.* 2010a).

The PHA content in the biomass during the operation of the accumulation reactor is reported in Figure 23B and it varied between 12% (w/w) and 76% (w/w) on the 58th operation day; however, on average, its value 31 ± 8% (w/w), was substantially higher that obtained in the SBR at the end of the feast phase (14 ± 3%, w/w). It was not possible to calculate PHA content in the biomass during the entire operation period, since VSS were just determined from the 45th operation day.

In terms of PHA composition, the HV content in the polymer (Figure 23C) during the accumulation stage performance was constant, varying in the range of 10 – 15% (w/w), when compared with the greater fluctuations that occurred during the SBR performance for this parameter.

The average storage rate during the accumulation stage was calculated based on the average value of non-polymer VSS. The obtained value was 102 ± 15 mgCOD $gCOD^{-1}$ h^{-1}, which was similar to that obtained in a previous study (106 mgCOD $gCOD^{-1}$ h^{-1}) (Beccari *et al.* 2009). Higher storage rates have been reported when using other wastes, e.g. 320 mgCOD $gCOD^{-1}$ h^{-1} for fermented sugar cane molasses (Albuquerque *et al.* 2007). Also, in a precedent study, by testing different OMEs using a mixed culture enriched with a synthetic mixture of organic acids were obtained higher storage rates (420 mgCOD $gCOD^{-1}$ h^{-1}) (Dionisi *et al.* 2005). Thus, when dealing with real wastes, which consist of complex

mixtures of several substrates, it is likely that the overall performance is also affected by the unidentified COD, which may not directly contribute for the storage response.

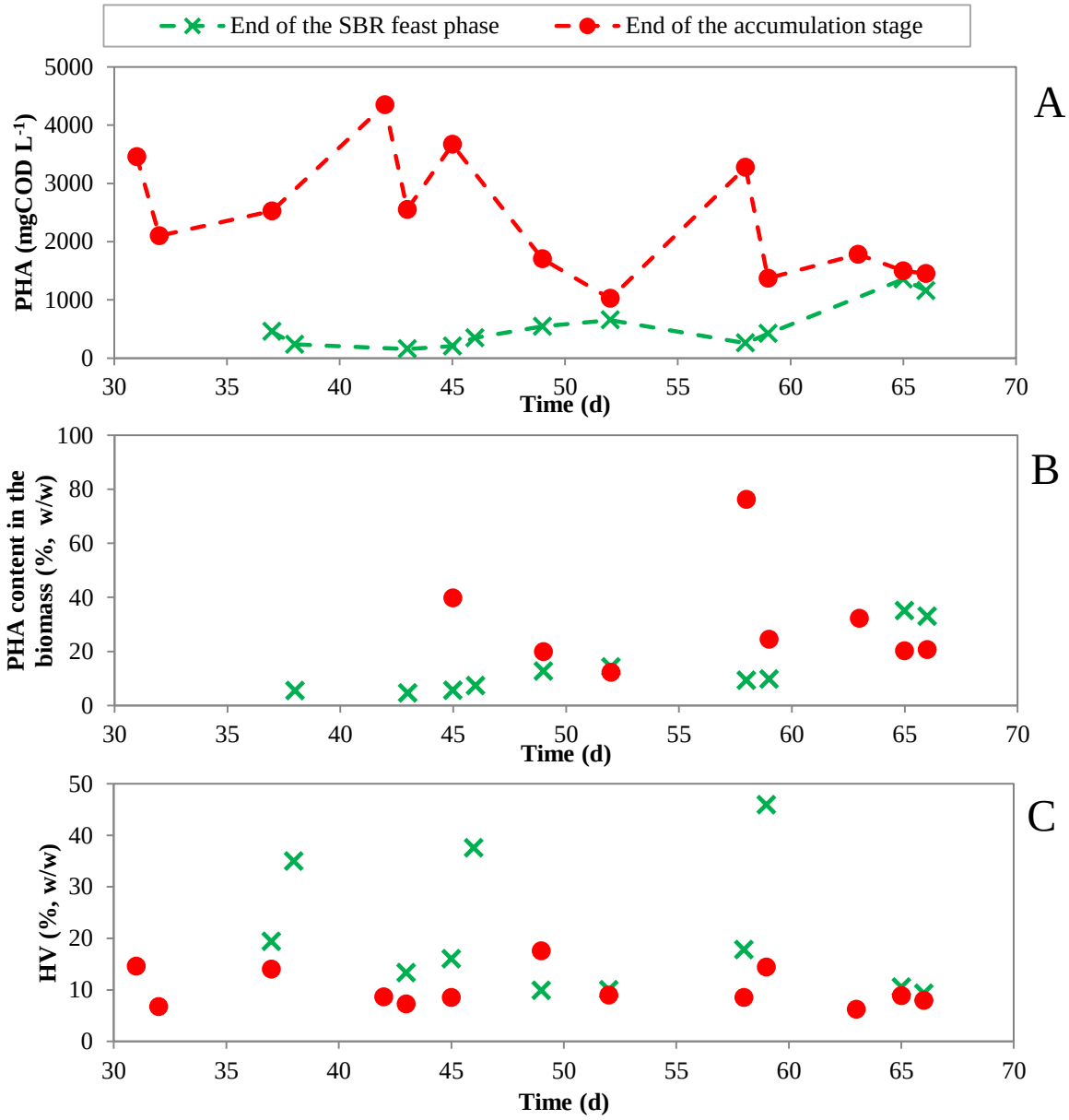


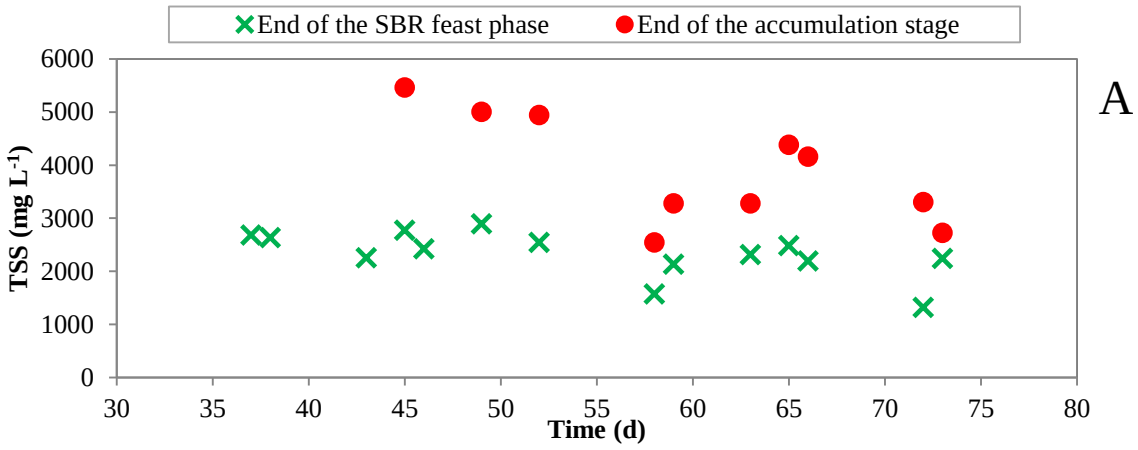
Figure 23: Performance of the accumulation stage in terms of produced PHA (A), PHA content in the biomass (B) and HV content in the polymer (C).

As shown in Figure 24A and B, the SS concentration in correspondence to the end of the accumulation stage (4428 ± 346 mgTSS L^{-1} and 3906 ± 323 mgVSS L^{-1}) is higher than that present at the end of the SBR feast phase (2380 ± 123 mgTSS L^{-1} and 2217 ± 119 mgVSS L^{-1}). The difference between those two values (more than 2000 mg L^{-1}) is mostly

due to the higher polymer concentration achieved during the accumulation stage (Figure 23A).

Indeed, the average non-polymer VSS concentration obtained at the end of the accumulation stage (2967 ± 395 mg L^{-1} or 4213 ± 561 mgCOD L^{-1}) is slightly higher than that observed at the end of the SBR feast phase (2136 ± 101 mg L^{-1} or 3034 ± 144 mgCOD L^{-1}) (Figure 24C). This fact indicated that microbial growth also occurred during the accumulation stage, in spite of N-limiting conditions were imposed due to the very low nitrogen content of the OMEs. However, the microbial growth could be related to the evidence that some residual amounts of nitrogen from the SBR were being withdrawn to the accumulation reactor. These results clearly indicate the importance to prevent microbial growth in the accumulation reactor, since it significantly affects the process performance in terms of PHA content in the biomass (Figure 23B), as recently reported in literature (Johnson *et al.* 2010b).

In what regards VSS/TSS ratio (Figure 24D) it is possible to notice a slight different trend during the performance of each stage: while, during the SBR performance the VSS/TSS was constant, during the operation of the accumulation stage it decreased (in a range from 0.99 and 0.81). However, its average value at the end of SBR feast phase (0.93 ± 0.02) was slightly higher than that obtained during the accumulation stage (0.88 ± 0.02).



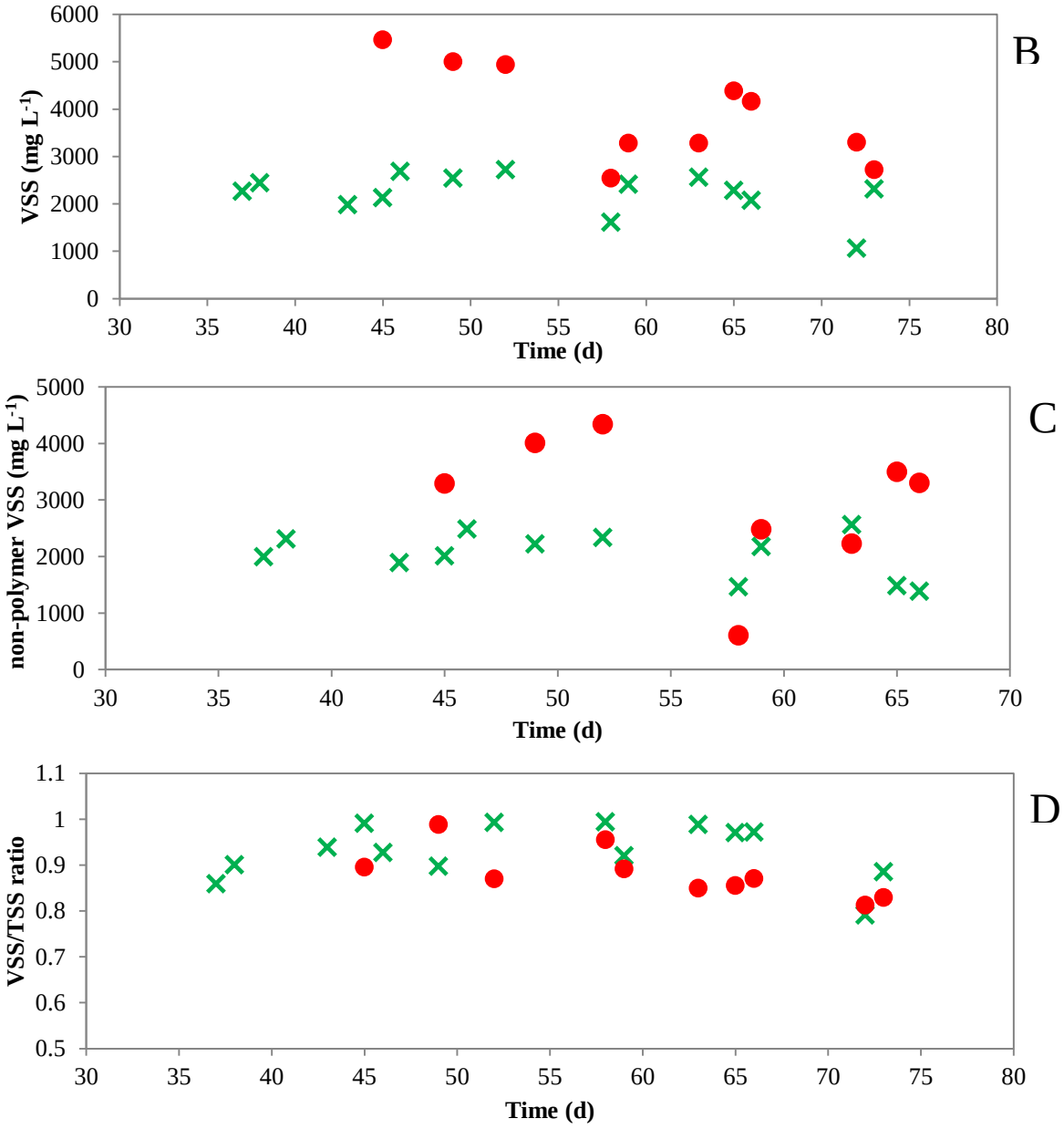


Figure 24: Suspended solids behavior during the accumulation stage performance in terms of TSS (A), VSS (B), non-polymer VSS (C) and VSS/TSS ratio (D).

4.4.2. Kinetic tests

In order to deeply analyze the microbial behavior in terms of PHA production, two kinetic tests were performed in the accumulation reactor. Each accumulation assay lasted 6 hours during which OMEs were fed through four pulses, every 90 minutes, corresponding to 6.78 gCOD L^{-1} or, in terms of VFA, to 4.05 gCOD L^{-1}. For the sake of example, the

main results obtained during one kinetic test (performed with the biomass withdrawn at the end of the cycle from the SBR on the 37th operation day), are discussed below.

The trend of both polymer and total VFAs concentrations during the kinetic test are shown in Figure 25. Generally, after each feed pulse microorganisms started to consume the substrate and to simultaneously produce PHAs. More in detail, after the two first feed pulses, it was observed a rapid and complete exhaustion of VFAs associated with PHA production, indicating a storage response of the microbial culture. Furthermore, during the 1st spike, VFA depletion was so quick that some PHA was consumed and the same occurred after the 3rd feed pulse in spite of residual VFA present.

The storage yield during the first 240 min of the kinetic test, during which no PHA degradation by microbial cells was noticed, was 0.81 COD COD_{VFA}^{-1} indicating the good ability of the culture to store the polymer. More in detail, the storage yield increased during the 3 first pulses, from 0.45 COD COD_{VFA}^{-1} to 0.84 COD COD_{VFA}^{-1}, achieving 2.00 COD COD_{VFA}^{-1} at 240 min from the beginning of the kinetic test. A great improvement in polymer storage was observed from the 1st to the 2nd feed pulse. The extremely high value observed after the 3rd feed pulse likely indicates that other COD, which may not related to VFAs, was converted into PHA. Indeed, in literature was previously reported the conversion of alcohols (e.g. ethanol) into PHAs, suggesting that probably other compounds played a major role among substrates other than VFAs (Dionisi *et al.* 2002). In a previous work, by using OMEs as substrate it was determined that 80% of the overall alcohols contained in the OME was ethanol (Beccari *et al.* 2009). So, further investigation is needed in terms of the characterization of the substrate to certainly conclude the conversion of compounds other than VFAs into PHA. It was also possible to observe that from the 3rd feed pulse the VFAs were no more completely depleted by microorganisms and, at the same time, they started to consume the polymer. This could be due to the high produced PHA concentration and consequent saturation of the cells which limited its storage ability. On the other hand, it could be also possible that the microbial culture partially lose its storage ability after three feed pulses, likely due to a decreased strength of the "feast and famine" regime (as imposed in the SBR), resulting in completely different conditions during the accumulation stage. However, after the last feed pulse, the microbial PHA production was resumed and at the end of the accumulation stage, the obtained PHA concentration was 2525 mgCOD L^{-1}, whereas the remaining VFAs were present in a

concentration of 1362 mgCOD L^{-1}. These results suggested the change in the feeding strategy at the 63rd operation day, by decreasing the duration between each pulse (from 90 to 72 minutes), in order to impose any feed pulse before microorganisms started to consume the intracellular polymer and to prevent the loss of storage capacity time, with the aim to increase the amount of final obtained PHA.

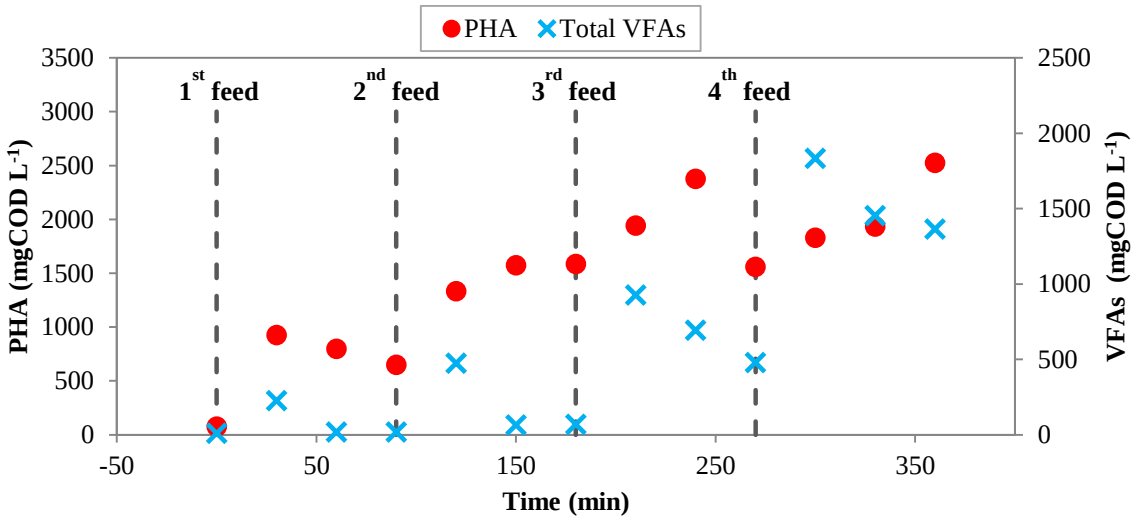


Figure 25: PHA and total VFA concentrations throughout a typical kinetic test in the accumulation reactor.

In Figure 26 is reported the HB and HV variation during the accumulation assay. As for PHA variation, both the HB and HV monomers concentration increased until after the 3rd feed pulse, when they started to be consumed. The HV content in the polymer showed a slight decreasing tendency, which varied between 20% (w/w) (at the beginning of the accumulation assay) and 14% (w/w) (at the end of the accumulation assay). This could be due to the propionic and valeric acids early consumption, not allowing for further HV production by the cells (Figure 27).

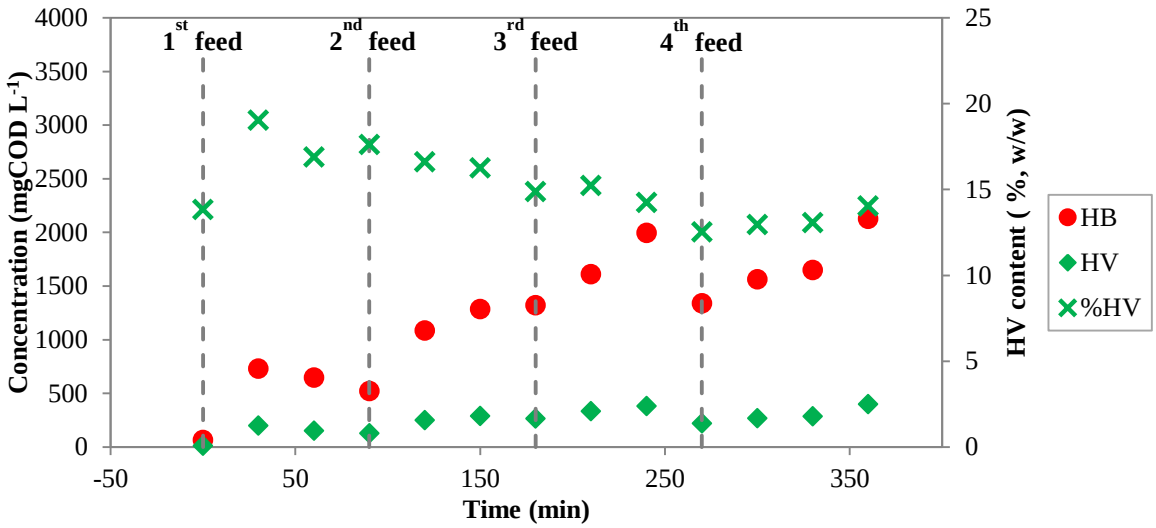


Figure 26: Trend of HB and HV monomers concentration and HV content in the polymer during a typical kinetic test in the accumulation reactor.

More in detail, in Figure 27A, are reported the trends of each VFA during the kinetic test in the accumulation reactor, which was similar to that already described for the total VFAs. In particular, all acids were completely consumed after the first and the second feed pulses, with the only exception for the isobutyric acid, whose concentration just before the third feeding was 45 mgCOD L^{-1}.

Interestingly, except for isobutyric acid, all the acids were completely depleted after the 2nd feed pulse. Instead, butyric acid was the only acid completely depleted after the 3rd feed pulse (Figure 27B). The high remaining content of VFAs in the medium, after the third and the fourth feed pulses, was mainly due to acetic acid, which is the main acid constituent of OMEs (accounting for about 40% of VFAs). As for propionic and valeric acids, they accounted for about 14 and 7% of total VFAs and were rapidly consumed during the kinetic test, indeed propionic acid peak was only detected after the 2nd pulse and valeric acid peak after the last feed pulse. Based on the 2nd and the 3rd feed pulses, it was calculated the consumption rate of each VFA, based on the average value of non-polymer VSS through the accumulation stage performance (since no VSS sampling was performed during this kinetic test). In fact, higher consumption rates were observed after the 2nd feed pulse (102.3 mgCOD $gCOD^{-1}$ h^{-1} for acetic acid, 35.1 mgCOD $gCOD^{-1}$ h^{-1} for propionic acid) than that observed after the 3rd feed pulse. In particular, butyric acid was consumed at the higher consumption rate during the first 30 min (164.3 mgCOD $gCOD^{-1}$ h^{-1}), being almost completely depleted, while no substantial isobutyric acid was consumed (7.4

mgCOD gCOD^{-1} h^{-1}). After the 3rd pulse feed, butyric acid was consumed at the higher consumption rate (59.4 mgCOD gCOD^{-1} h^{-1}), being completely depleted by the microbial culture. Valeric acid was the first to be totally depleted even though at a low consumption rate of 40.5 mgCOD gCOD^{-1} h^{-1}. Acetic and propionic acids were not completely degraded after the 3rd pulse feed, being consumed at a rate of 28.0 and 9.4 mgCOD gCOD^{-1} h^{-1}, respectively. Once more, isobutyric was the less preferable acid to be consumed by the microorganisms.

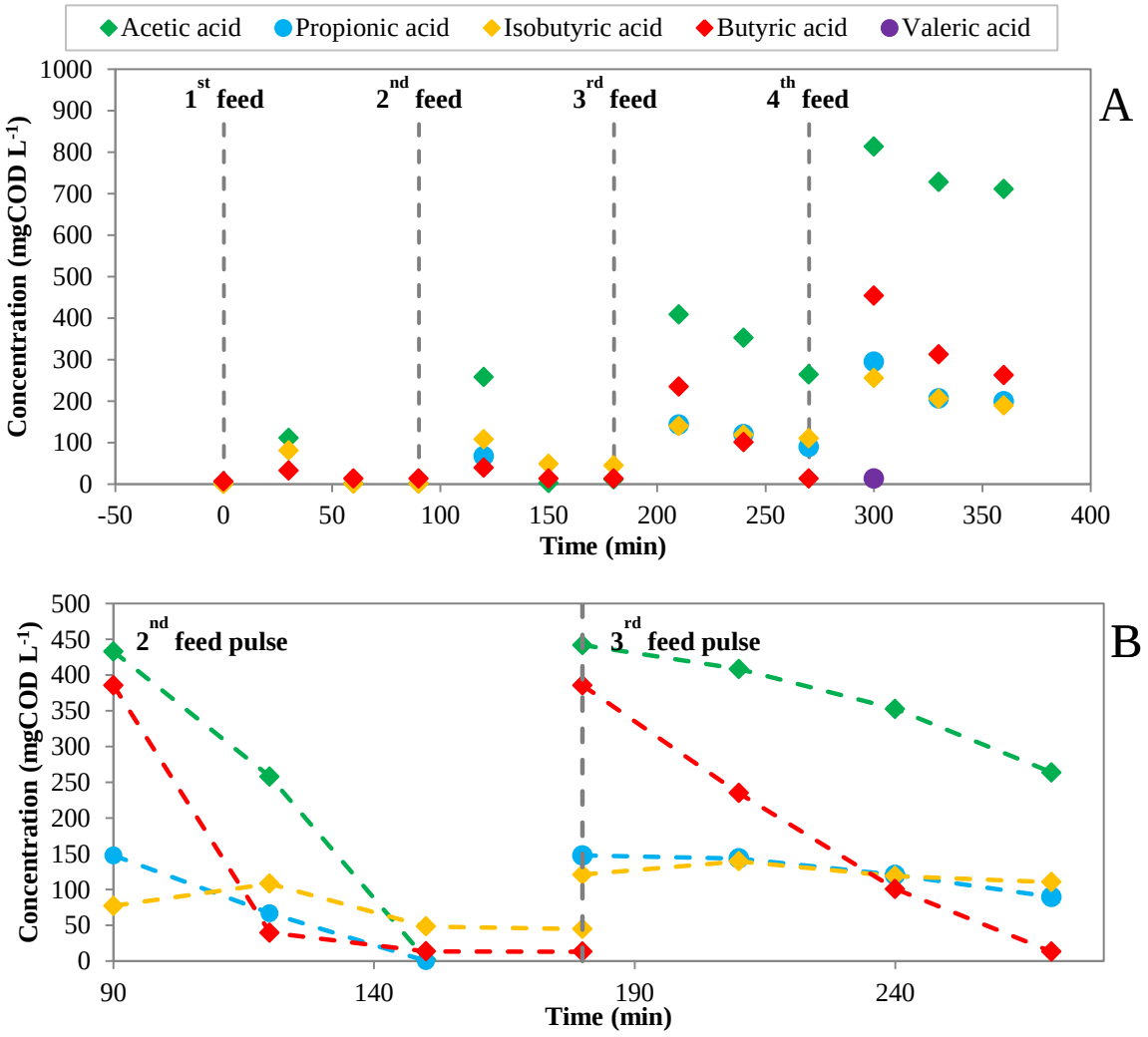


Figure 27: Trends of VFA concentrations during a typical kinetic test in the accumulation reactor (A) and VFAs consumption after the 2nd and the 3rd feed pulses.

In Figure 28 it is reported the ammonium concentration during the kinetic test. The OME is an N-poor substrate and N limiting conditions were imposed in the accumulation reactor. However, as soon as biomass was withdrawn from the SBR to the accumulation

reactor (0 min), it was observed an N concentration of about 55 mg L^{-1}, likely due to the residual N content in the SBR effluent. N was suddenly consumed by the biomass and, as consequence, its concentration decreased during the first 90 minutes, suggesting the occurrence of microbial growth together with PHA storage. Actually, the growth yield based on N consumption during the first 90 min of the kinetic test was 0.14 $COD_{biomass}$ COD_{VFA}^{-1}, which was lower than the correspondent storage yield (0.45 COD COD_{VFA}^{-1}) indicating that the substrate uptake was driven towards PHA storage more than microbial growth. During the remaining period of the test, after each pulse the N concentration slightly increased and accumulated in the medium. Therefore microorganisms were not consuming the ammonium, suggesting that microbial growth did not occur any more. The residual ammonium content from the SBR and consequent N content accumulated in the accumulation reactor, determined the diminution of N concentration in the mineral media used to prepare the SBR feed (starting from 63rd operation day, Figure 14).

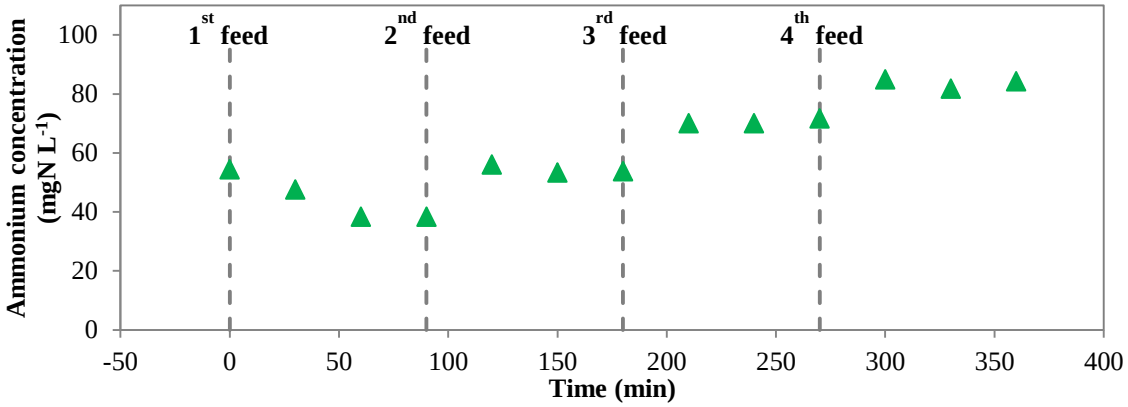


Figure 28: Ammonium concentration during a typical kinetic test in the accumulation reactor.

4.5. Performance of the overall reactors

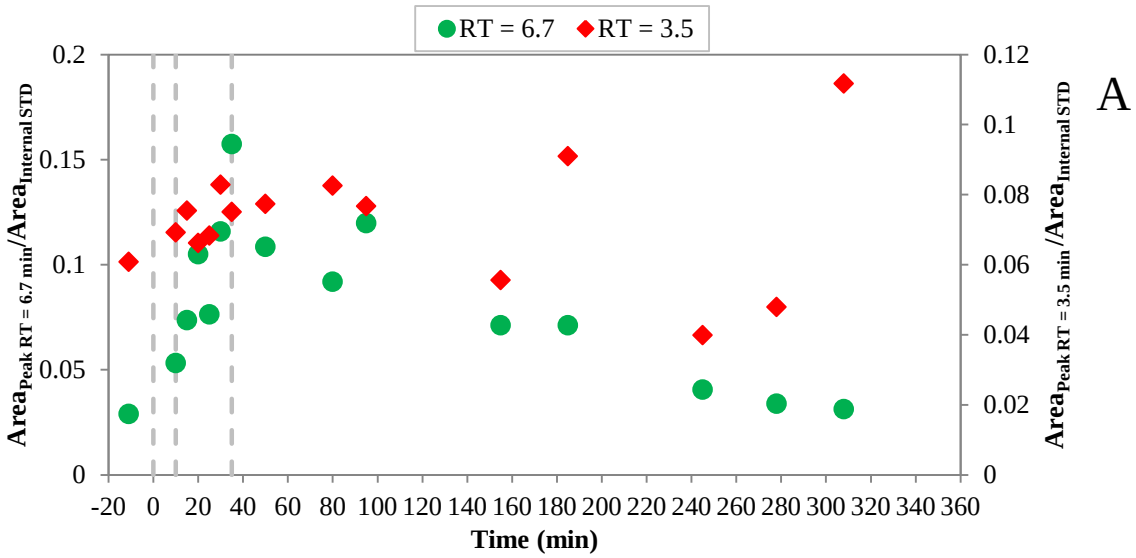
As a conclusion, in Table 9 are presented the already discussed results of the overall process operation. It was worth noting the great improvement in the production of PHA from the culture selection to the subsequent accumulation stage. During the reactor operation, the SBR performance strongly influenced the amount of stored polymer by the culture during the accumulation assays. No significant changes in terms of HV content in the polymer. However, further optimization of the reactors performance is needed, mainly due to the considerable biomass growth during the accumulation stage by storing the polymer, influencing the final amount of produced polymer.

Table 9: Performance of the overall operation of the process.

	Selection Stage SBR under FF regime				**Accumulation stage Pulse feeding regime**
	OLR = 2.37 gCOD L^{-1} d^{-1}		**OLR = 4.74 gCOD L^{-1} d^{-1}**		
	End of the cycle	**End of the feast phase**	**End of the cycle**	**End of the feast phase**	
F/F ratio	0.08		0.07		-
TSS (mg L^{-1})	1255 ± 47	-	2607 ± 96	2380 ± 123	4428 ± 346
VSS (mg L^{-1})	1197 ± 48	-	2430 ± 77	2217 ± 119	3906 ± 323
Non- polymer VSS (mg L^{-1})	1154 ± 46	-	2401 ± 83	2136 ± 101	2967 ± 395
PHA (mgCOD L^{-1})	74 ± 9	235 ± 42	53 ± 6	732 ± 176	2365 ± 288
Maximum PHA (mgCOD L^{-1})	-	445	-	654	4350
PHA content in the biomass (%, w/w)	4.0 ± 0.3	-	1.3 ± 0.2	14 ± 3	31 ± 8
HV content in the polymer (%, w/w)	10 ± 1	14 ± 1	13 ± 3	20 ± 4	10 ± 1
r_{PHA} (mgCOD $gCOD^{-1}$ h^{-1})	192 ± 42		155 ± 39		102 ± 17
r_s (mgCOD $gCOD^{-1}$ h^{-1}) referred to kinetic	-357.53		-648.05		-349.60 (2^{nd} pulse)
Y_{STO} (COD COD^{-1}) referred to kinetic	0.71		0.52		-
Y_{STO} (COD COD_{VFA}^{-1}) referred to kinetic	0.89		0.69		0.81 (referred to the first 240 min of the kinetic test)

Interestingly, the gas chromatograms obtained during the GC analysis of PHA revealed two unknown peaks that had different retention times from those of HB and HV monomers. One peak was observed at a retention time 3.5 min (just lower than HB monomer peak) and the other at a retention time 6.7 min (just higher than the HV monomer peak). Those peaks were visualized in the SBR, in the accumulation stage and in the lyophilized; hence, it is probable that they correspond to other HA monomers (Figure 29A, B and C). Indeed, results reported in literature evidenced the occurrence of HA monomers other than HB and HV when using agro-industrial wastes as feedstock for PHA production. Particularly, Bengtsson and coworkers, by using sugar cane molasses obtained a 5 monomer polymer containing 3HB, 3-hydroxy-2-methylbutyrate (3H2MB), 3HV, 3-hydroxy-2-methylvalerate (3H2MV) and 3-hydroxyhexanoate (3HHx) (Bengtsson *et al.* 2010b).

As the identification of those peaks is still on course, no effective quantification has been performed yet. However, by calculating the ratio between the area of unknown peaks (RT = 3.5 and 6.7 min) and internal standard, it is possible to give a preliminary description of their trends. The unknown peak revealed at 6.7 min presented a similar trend of that presented for PHA during the SBR kinetic tests (Figure 29A and B). On the other hand, the peak at 3.5 min showed no clear trend during SBR kinetic tests and was not usually present during the GC analysis of the lyophilized polymer (Figure 29D).



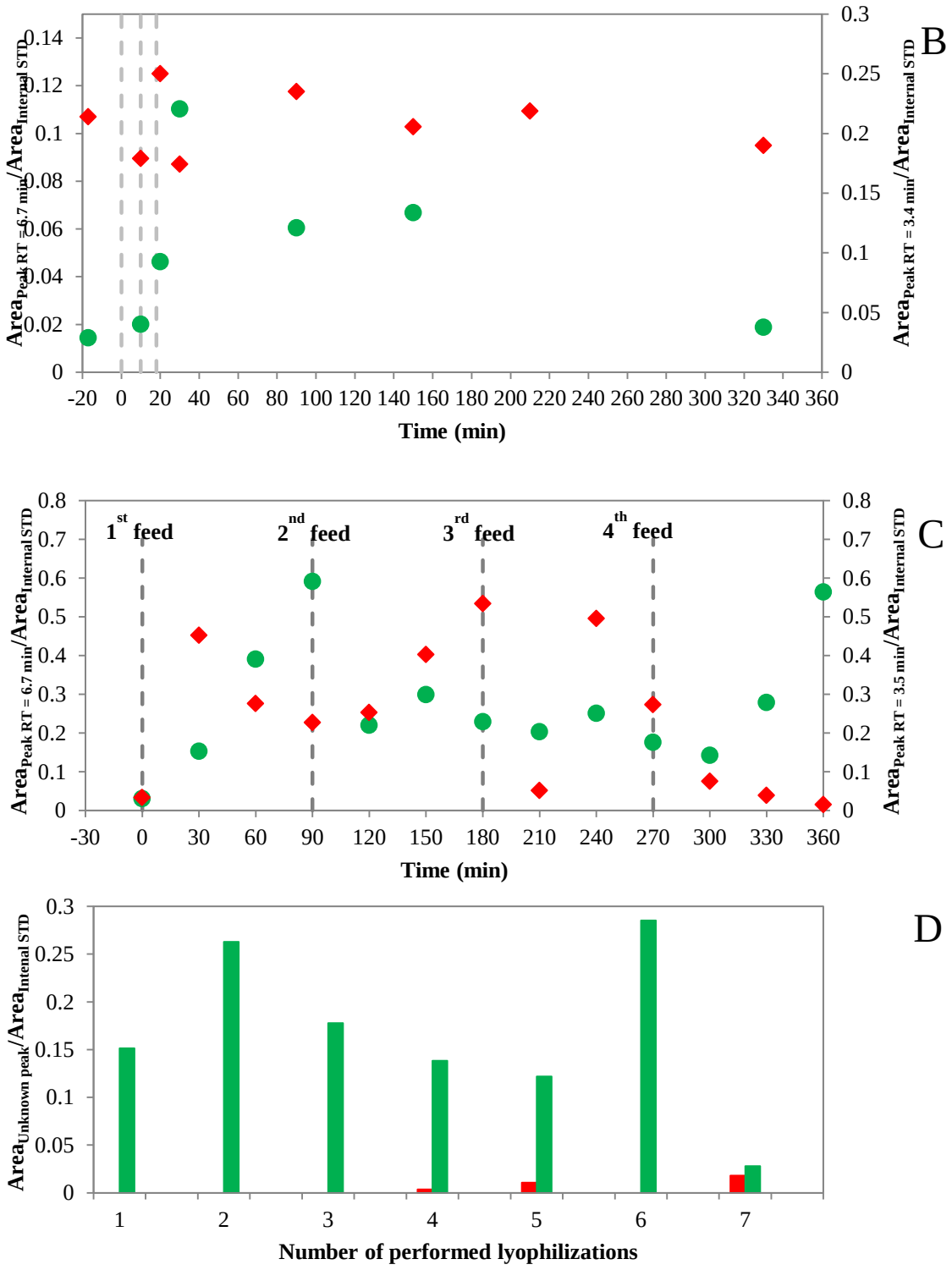


Figure 29: Profile of the ratio obtained through GC analysis between the area of unknown peaks (RT = 3.5 and 6.7 min) and internal standard during kinetic tests performed in the SBR at OLR = 2.37 gCOD L^{-1} d^{-1} (A) and OLR = 4.74 gCOD L^{-1} d^{-1} (B) and in the accumulation stage (C). The ratios revealed during lyophilized polymer analysis are also reported (D).

4.6. PHA recovery

The PHA produced in the accumulation stage of the process was recovered by means of a solid-liquid separation followed by chemical digestion with sodium hypochlorite performed after each accumulation assay. The first centrifugation step was performed to eliminate the residual COD contained in the mixed liquor, since a real OME was used as substrate and its complexity may contain or originate compounds that affect the purity of the obtained polymer.

Sodium hypochlorite enhances the polymer recovery by solubilizing its surrounding compounds (e.g. non-PHA cellular mass). The use of this chemical as digester was supported by its rather low environmental impact, when compared with other chemical solvents (e.g. chloroform) as well as its low cost, affecting positively this overall cost of PHA production. Besides that, the optimization of the extraction step passed through a strategy of washing with water the extracted polymer with successive solid-liquid separation steps aiming at eliminating the remained impurities (Koning and Witholt 1997). In this way, the addition of washing with water step was implemented from the 42nd operation day. In both cases, the recovered polymer was then lyophilized in order to remove the totality of its water content and obtain the PHA in the form of dry powder. The lyophilization step was performed on several samples that were collected together before to be processed.

The overall performance of the extraction step is reported in Table 10. From the 34 performed accumulation assays, it was possible to recover a total of 15.96 g of PHA, at an average value of 0.45 ± 0.07 g per each accumulation assay. The purity of extracted PHA was around 71.6 ± 3.7% and its HV content was on average 13.6 ± 0.4%. During the extraction assays performed between the 30th and 39th operations days, were recovered 0.61 ± 0.01 g of polymer per accumulation assay with high purity (81.3 ± 2.9%). When the polymer was washed with water, less amounts of polymer were recovered (0.37 ± 0.07 g of PHA per accumulation assay), as reported in literature (Koning and Witholt 1997), but the purity of the extracted PHA also decreased (66.7 ± 3.8%). Due to this decrease in the efficiency of the extraction step, washing the recovered polymer with water seemed to be unsuitable for the extraction step in the present scenario. However, it cannot be excluded that this behavior also reflects poorer performance of the accumulation stage (see paragraph 4.4.1, Figure 23A).

The HV content in the polymer was not affected during the extraction step; however, higher ratios of HV content in the polymer were obtained than that achieved at the final of accumulation stage (10%). In fact, in the literature was reported the degradation of P(3HB) by sodium hypochlorite (Jacquel *et al.* 2008). These results suggested that the chemical digester used in this investigation promoted more considerable losses in terms of the HB monomer, which presents a smaller carbon chain, than HV monomer in the polymer.

From the 58th operation day, it was observed a great drop in terms of the purity of the recovered polymer, reaching 38.5% at the last recovery assay. Also, the amount of recovered PHA per accumulation assay achieved 0.17 g (between the 70th and 74th operation days). These results are in accordance with the inefficient performance of the accumulation stage, during the last days of operation, likely due to the N lack in the SBR (see paragraphs 4.3.1 and 4.4.1). According to the literature (Reis *et al.* 2011), the efficiency of polymer recovery decreases with low PHA content in the biomass which was around 20% as well as low PHA concentration (about 1400 mgCOD L^{-1}) during the final period of operation (Figure 23A and B).

Table 10: Performance of PHA recovery step.

Operation days	**Number of accumulation assays**	**Mass of lyophilized PHA** (g)	**Mass of lyophilized PHA per assay** (g)	**Purity of recovered PHA** (%)	**HV content in the recovered polymer** (%, w/w)
30 – 32	4	2.41	0.60	78.4	13.9
35 – 39	6	3.73	0.62	84.2	12.2
42 – 46	8	4.65	0.58	70.3	14.7
49 – 53	4	1.00	0.25	75.7	13.4
58 – 60	4	1.02	0.26	60.6	14.7
63 – 67	8	3.15	0,39	60.3	13.0
70 – 74	5	0.83	0.17	38.5	9.3
Overall step (excluded 70 – 74)	34	15.96	0.45 ± 0.07	71.6 ± 3.7	13.6 ± 0.4

Chapter 5 | Conclusive remarks and future perspectives

The results obtained in this research demonstrated the feasibility of using olive mill effluents (OMEs) as no cost feedstock for polyhydroxyalkanoates (PHAs) production by mixed microbial cultures (MMCs) in a multi-stage process. Main attention has been paid at investigating the performance of the PHA production stages, from the microbial culture selection and enrichment to the intracellular PHA accumulation and recovery. As for MMC selection in a sequencing batch reactor (SBR) operated at a cycle length of 6 hours, two organic load rates (OLR), of 2.37 and 4.74 gCOD L^{-1} d^{-1}, have been applied by diluting the OMEs. The establishment of the "feast and famine" conditions required to select microorganisms with high storage ability was observed at both OLRs and, in particular, biomass production was more than doubled (from 1197 ± 48 to 2430 ± 77 mgVSS L^{-1}, measured at the end of the cycle) by increasing the applied OLR. The ability to store PHAs for the biomass selected at the higher OLR was tested in the further accumulation stage, carried out through successive 6 hours accumulation assays in a pulse-feeding regime with undiluted OMEs. As a main result, it was observed a considerable increase of the intracellular polymer content in the biomass (up to 31 ± 8 %, w/w) in the accumulation reactor. Finally, the developed PHA recovery procedure, consisting of a solid-liquid separation followed by a chemical digestion with sodium hypochlorite, allowed to obtain on average 0.45 ± 0.07 g of PHAs (having a purity of 71.6 ± 3.7 % and an HV content of 13.6 ± 0.4%, w/w) per each accumulation assay. It is worth noting that 4 accumulation assays could be daily performed and this would allow obtaining on average 1.8 g of PHAs per day.

Based on these results, a further process optimization would be required to increase the PHA production by operating the SBR at an OLR higher than those tested in this study. In this context, the research is currently going on investigating the process performance by operating the SBR stage at 8.5 gCOD L^{-1} d^{-1}. The investigation of different feeding strategies in the accumulation reactor in order to increase the VFA conversion into PHAs is also of great importance. Finally, the optimization of the process requires the improvement of PHA extraction and recovery procedure, to increase the polymer purity. Furthermore, the role of other non-VFA COD during the main stages of the process needs to be studied. Main attention is also being paid at performing the microbial analysis of the selected

microbial culture as well as at deeply characterizing the final polymer, in terms of physical-chemical properties. These improvements certainly will allow a better exploitation of the OME as feedstock of this process in order to obtain a more effective PHA production by the selected microbial culture as well as to adequate the polymer composition depending on its targeted final application.

Even though the overall stages of the process still require further optimization, the results obtained in this study pinpoint the great potential of the proposed multi-stage process to treat OMEs while simultaneously converting their organic content into valuable products.

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